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Genetic structure, differentiation, and slight evidence for no selective neutrality of some SSR markers in populations of *Medicago ciliaris* (L.) Krock and *Medicago intertexta* (L.) Mill species

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Abstract: *Medicago ciliaris* (L.) Krock and *Medicago intertexta* (L.) Mill are most often found in saline regions and are considered as potential resources for genes related to salinity resistance. To explore the genetic diversity of these two species by mean of nrSSR (simple-sequence repeats) markers, 11 accessions originated from different regions in north of Africa and Middle East were chosen. Ten among 14 markers turned out to be polymorphic and gave 72 alleles. Eight markers were very informative with PIC values > 0.5, and three of them were found to be nonneutral. Allelic richness was limited with an average number of alleles per locus of 2.7. All populations have a significant heterozygous deficit evidenced by both observed heterozygosity rates (null for most populations) and multilocus fixation index estimates. A strong differentiation between populations was highlighted by F_{ST} (0.805) and G_{ST} (0.919) values. Low values of gene flow (0.01–0.38) revealed a very weak gene exchange between populations regardless of the proximity of their sites of origin. The total variability breakdown by analysis of molecular variance (AMOVA test) showed a preponderance of interpopulation component (73%–72%) with respect to intrapopulation one (18%), mainly due to the mating system. Relationship analysis performed by NJ tree representation trends to place *M. ciliaris* as an ancestor and *M. intertexta* as a derivative. The two populations from the Sebkha site, characterized by the highest variability and more private alleles, constitute an ideal reservoir of genes for tolerance to salinity. Significant genetic diversity in the two aforementioned species is encouraging for their conservation.

Key words: Genetic diversity, genetic structure, *M. ciliaris*, *M. intertexta*, salinity tolerance, simple-sequence repeats

1. Introduction

Belonging to the intertextae section of the genus Medicago, *Medicago ciliaris* (L.) Krock and *Medicago intertexta* (L.) Mill are two annual species. These two taxa are characterized by a good quality of forage and exceed all other annual Medicago in vigor of growth (Lesins and Lesins, 1979). According to the literature, this fairly close pair of species can be distinguished by a single character, the presence of gland-tipped trichomes, which can be found on the fruits of *M. ciliaris* (Small et al., 1999). *M. ciliaris* is predominantly autogamous, while *M. intertexta* shows an allogamous tendency and is considerably more variable than *M. ciliaris* (Small et al., 1999; Abdelkefi et al., 2001). *M. intertexta* could be hybridized easily with *M. ciliaris* (Lesins et al., 1971); it was suggested that introgression between them is predominantly unidirectional, from *M. ciliaris* into *M. intertexta* (Small et al., 1999).

Agriculture is increasingly impacted by rising soil salinity. Therefore local adaptation of wild populations is of great importance since creating new crops tolerant to salt and drought stress became essential in agriculture production to face future climate change (Friesen et al., 2014). *M. ciliaris* and *M. intertexta* are most often found in sites classified as salty and are known to be resistant to salinity (Laouar and Abdelguerfi, 2014). Saline origin genotypes are less impacted by salt than those of nonsaline origin; these populations thus likely contain adaptively diverged alleles. Better knowledge of genetic resources is indeed necessary for their optimized management and their appropriate use in new breeding programs. The favored way to quantify variation among and within species and populations is the molecular characterization using molecular tools. Single sequence repeats (SSR) markers are routinely used for genetic diversity studies because of their proven usefulness for population genetics inferences, their higher degree of polymorphism, and their codominance (Lazrek et al., 2008). SSRs have been used by several authors in different studies on species of the Medicago genus. A growing number of studies have used SSR markers for estimating the genetic diversity

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in alfalfa (Flajoulot et al., 2005; Bagavathiannan et al., 2010; Cholastova and Knotova, 2012;) and *M. truncatula* (Ellwood et al., 2006; Ronfort et al., 2006; Lazrek et al., 2008). Available information on the genetic diversity of *M. ciliaris* and *M. intertexta* is scarce and no molecular characterization has been published on *M. intertexta*. The objective in this study is to assess, using SSR markers, the genetic diversity of the populations of these two highly productive halophyte species subjected to significant selective environmental pressure from the Sebkha of Oran, a very salty Ramsar site. These populations will be compared with populations from other sites with different qualifications.

2. Materials and methods

2.1. Plant material

A total of 11 populations of *M. ciliaris* and *M. intertexta*, from different regions in north of Africa and Middle East, were chosen for this study (Table 1). The seeds of eight populations were supplied by the International Center for Agricultural Research in Dry Areas (ICARDA), those of the three remaining populations were chosen from our own prospection in two regions of western Algeria, Mascara and Sebkha of Oran (a highly salted area). The populations chosen for this study come from low, medium or high-altitude regions. From the geographic distance point of view, these populations come from the same site, distant sites, or neighboring sites (Figure 1). Two additional genotypes, *Medicago truncatula* (line 0008, INRA Montpellier) and *Medicago sativa* (Var Orca, INRA Lusignan station), were used as references.

2.2. DNA isolation

DNA extraction was performed using the Cetyl trimethylammonium bromide (CTAB) method following the Doyle and Doyle (1987) protocol with some modifications. Young leaves are crushed directly in small tubes using liquid nitrogen, the extraction buffer is added and the tubes incubated at 60 °C for 60 min. Ammonium acetate solution (10 mM) with isopropanol is added to precipitate the DNA, the tubes are kept at -20 °C overnight. After centrifugation and washing of the DNA pellet with 70% ethanol, the DNA is suspended in a TE solution (Tris: 10 mM, EDTA: 1 mM, PH 8), and RNase 10 mg/µL is added at the end. DNA quality test was performed by electrophoresis on a 0.8% agarose gel stained with ethidium bromide (BET). The migration was made in a 0.5×TBE buffer prepared from a 10× stock solution (Tris: 89 mM, Boric acid: 89 mM, disodium EDTA: 2 mM, pH 8.3), for 20 min at 100 V. Finally, the DNA extracts were diluted to 1/5 in a sterile TE buffer for carrying out the amplifications.

2.3. In vitro DNA amplification

A total of 20 markers that have already been mapped in *M. truncatula* were selected to test their amplification, 14 of them (Table 2) were retained for the rest of the study. The PCR reaction was carried out according to Baquerizo-Audiot et al. (2001) with some modifications. The reaction mixture consists of: 2 µL genomic DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 25 ng/µL of each primer and 0.2 µL of Taq-Polymerase (5 u/µL), for a total of 20 µL. The PCR amplifications were performed on a 2720 Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific Corp., California, CA, USA) and carried out according to the following program: a denaturation phase of 8 min at 94 °C, followed by 25 cycles each comprising a denaturation step at 94 °C for 1 min, then hybridization for 45 sec at a temperature between 50 and 58 °C, an extension step at 72 °C for 1 min, and finally an elongation phase of 10 min at 72 °C. The PCR products were separated by electrophoresis on a 6.5% polyacrylamide gel under denaturing conditions with Urea (3.5 M) and with 50 bp or 100 bp DNA ladder. The running conditions were 4 h at 120 V in a SE 600 Hoeffer electrophoresis apparatus. The gels were visualized under UV rays after staining for 20 min with BET.

2.4. Data analysis

RF values of the size marker bands (DNA ladder) were plotted against the logarithm of their respective MW. The regression line (least squares) obtained, allowed the reading of MW of an unknown band from its RF value. The genotyping results of the 110 individuals for all markers were used to make a genetic diversity analysis. Allelic frequencies, number of alleles per locus, allelic richness (A), observed and expected heterozygosity (H_O, H_E) and F parameters were calculated using the GENETIX software version 4.02 (Belkhir et al., 2004)[1]. Allelic richness refers to the number of variants in a sample. Heterozygosity is the proportion of heterozygous individuals for a locus and H_E is the theoretical frequency of heterozygotes calculated under the Hardy-Weinberg equilibrium assumption. Statistics F are considered as measure of the correlation between genes from different levels of a subdivided population (hierarchically). Fixation index Fis reflects the differentiation of individuals within populations, the Fst represents the degree of differentiation between populations and Fit is the deficit or excess of average heterozygotes in a group of populations. Nei's distance (1978) (Ds) and that of Cavalli-Sforza and Edwards (1967) (Dc) and the gene flow (Nm) between population pairs according to Wright's formula (1969) were estimated by GENETIX software. Bennet's formula was used for self-fertilization rate estimation. Polymorphic information content (PIC) was calculated according to the formula described by (Botstein et al., 1980). FreeNa software

[1] Belkhir KP, Borsa P, Chikhi L, Raufaste N, Bonhomme F et al. (2004). GENETIX 4.05, Windows TM software for population genetics. Montpellier, France: Genome, populations, interaction laboratory, CNRS UMR 5000, University of Montpellier II.

Table 1. Geographical data of collection sites and SSR markers diversity of 11 natural populations of *M. ciliaris* and *M. intertexta.*

Species	Ac	Origin	Province	Long	Lat	Alt	Geno	A
Medicago ciliaris	Seb	Algeria	Sebkha of Oran	W0 52 52	N35 33 33	110	6	2
	1587	Lebanon	Zahle	E36 01	N33 52	1000	3	1.14
	1588	Lebanon	Zahle	E36 01	N33 52	1000	2	1.07
	1576	Tunisia	Tunis	E10 13	N36 51	50	4	1.14
Medicago intertexta	Mas	Algeria	Mascara	E0 08 23	N35 23 40	492	5	1.57
	seb	Algeria	Sebkha of Oran	W0 52 52	N35 33 33	110	9	2.07
	1157	Egypt	Kafr el Sheikh	E30 56	N31 07	18	4	1.21
	1158	Egypt	Kafr el Sheikh	E30 56	N31 07	18	5	1.21
	2939	Morocco	Center	W07 13	N33 47	20	3	1.14
	3084	Morocco	Tensift	W07 40	N31 36	500	4	1.07
	1600	Morocco	North center	W04 00	N34 12	500	5	1.21

Accession (Ac), average number of alleles per locus (A) and number of multilocus genotypes (Geno).

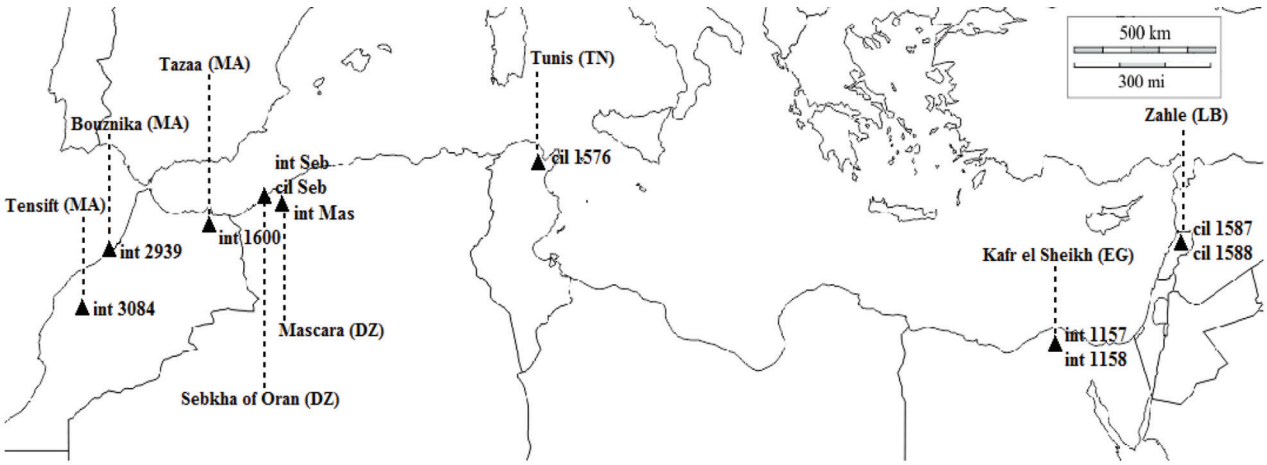

Figure 1. Geographical localization of collection sites of *Medicago ciliaris* and *Medicago intertexta* populations. int: *M. intertexta population*; cil: *M. ciliaris* population; Morocco (MA) sites: Bouznika, TenSift, and Tazaa; Algeria (DZ) sites: Sebkha of Oran and Mascara; Tunisia (TN) site: Tunis; Egypt (EG) site: Kafr el Shiekh; Lebanon (LB) site: Zahle.

(Chapuis and Estoup, 2007) was used to estimate null alleles frequencies and to compare between global F_{ST} values and between F_{ST} values in pairs, before and after exclusion of the null allele procedure (ENA) and that with 1000 bootstrap repetitions. The neutrality of the SSR markers has been tested with PopGene software Version 1.32 (Yeh et al., 1999)[2].

2.5. Population structure

Molecular variance analysis (AMOVA) was carried out with Arlequin software version 3.1 (Excoffier et al., 2006)[3]. Different groups of populations were tested. This analysis was carried out five times: according to the species, the altitude (two test), the origin site, and finally the salinity of the site. A factorial correspondence analysis (FCA) was performed using the Genetix software. The Bayesian population structure was therefore estimated using STRUCTURE 2.3.4 software (Pritchard et al., 2000). The number of groups or populations k was set between 1 and 11, three iterations were made. An admixture model with 1 000 Markov Chain Monte Carlo repetitions has been

[2] Yeh FC, Yang RC, Boyle T, Ye ZH, Mao JX (1997). POPGENE, Version 1.32: The user friendly software for population genetic analysis [online]. Website https://www.scienceopen.com/document?vid=c020f786-97ac-4fc4-b81f-48b13b62d56a [accessed 3 November 2019].

[3] Excoffier L, Laval G, Schneider S (2006). Arlequin Ver 3.1, An integrated software package for population genetics data analysis [online]. Website http://Cmpg.Unibe.Ch/Software/Arlequin3 [accessed 23 June 2019].

Table 2. Sequences of microsatellite primer pairs for each locus and linkage group (LG) in *M. truncatula,* number of alleles per locus and estimated PIC of all SSR markers.

Locus	LG	Primer sequences F: (5'-3') and R: (3'-5')	Core sequence	Nb alleles	PIC	References
TP36B (=FMT11)	1	F: ggcccaaccacaatttc R: cataacttccaataactgcca	$(GA)_{16}$	2	0.487	Badri et al., 2007
MTIC-19	2	F: tctagaaaaagcaatgatgtgaga R: tgcaacagaagaagcaaaaca	-	3	0.231	Julier et al., 2003
MTIC-451	2	F: ggacaaaattggaagaaaaa R: aattacgtttgtttggatgc	$(TC)_{11}$	11	0.875	Julier et al., 2003
MTIC-452	2	F: ctagtgccaacacaaaaaca R: tcacaaaaactgcataaagc	$(TC)_{15}$	5	0.611	Julier et al., 2003
MTIC-169	3	F: tcaaaaccctaaaaccctttctc R: gcgtgctaggtttgagagga	$(TC)_{7}$	8	0.818	Julier et al., 2003
MTIC-297	4	F: ctaagctttggccatgtatc R: tgaaatgagtttgactgagg	$(TAC)_{5}$	1	0	Lazreg et al., 2008
MTIC-339	4	F: ccacacaaaacacgcactct R: ggtaggattgccacgactgt	-	1	0	Julier et al., 2003
MTIC-126	6	F: cccagttgcagactctctca R: gaagggtttaccggaggaag	$(AC)_{8}$	1	0	Lazreg et al., 2008
MTIC-153	6	F: tcacaactatgcaacaaaagtgg R: tgggtcggtgaattttmmctgt	$(AG)_{5}$	5	0.732	Julier et al., 2003
TPG20C (=FMT08)	7	F: caatcactggaagcaaggt R: agcctgctcatttgtattgc	$(CT)_{16}$, $(CA)_{7}$	12	0.767	Badri et al., 2007
MTIC-86	8	F: atggcagctgcttcaacttt R: cctcccccaaataacacaaa	$(TC)_{13}$	10	0.808	Badri et al., 2007
MTIC-635 (mt122)	7	F: ccccaaatcaaacaacacatc R: gggccaccactataaacacc	-	6	0.73	Foroozanfar et al., 2014
MTIC-183 (mt124)	7	F: aaatggaagaaagtgtcacg R: ttctcttcaagtgggaggta	$(AG)_{8}$	1	0	Foroozanfar et al., 2014
MTIC-232 (mt120)	7	F: taagaaagcaggtcaggatg R: tccacaaatgtctaaaacca	$(ATT)_{7}$	6	0.716	Foroozanfar et al., 2014

adapted. The efficient (k) value was adapted to the highest index of Δk according to Evanno et al. (2005); it was estimated using Structure Harvester (Earl and VonHoldt, 2012). Finally the neighbor joining (NJ) analysis was performed with Darwin software version 6.0.21 (Perrier and Jacquemoud-Collet, 2006)[4] to represent the kinship between the populations studied and between the two species.

3. Results

3.1. Genetic diversity

The transferability of SSR loci from *M. truncatula* to *M. ciliaris* and *M. intertexta* was approximately 75% since 15/20 of the SSRs tested are amplified with these two species. Among the 15 markers amplified, one of them gave profiles containing many bands. This marker was not retained for further study. The 14 remaining markers were used to analyze molecular genetics differentiation of the natural populations. Ten of them turn out to be polymorphic (e.g. Figure 2 (A)), whereas MTIC-126, MTIC-297, MTIC-339, and MTIC-183 were monomorphic. TGPC20 was the most polymorphic marker with an allele number of 12. A total of 72 alleles were detected for all studied loci and 30 of them were private alleles. The PIC value of polymorphic loci was between 0.231 and 0.875 (Table 2). The highest value was observed with MTIC-451. Among

[4] Perrier X, Jacquemoud-Collet JP (2006). DARwin software version 5.0.148 [online]. Website http://Darwin.Cirad.Fr/Darwin [accessed 24 November 2019].

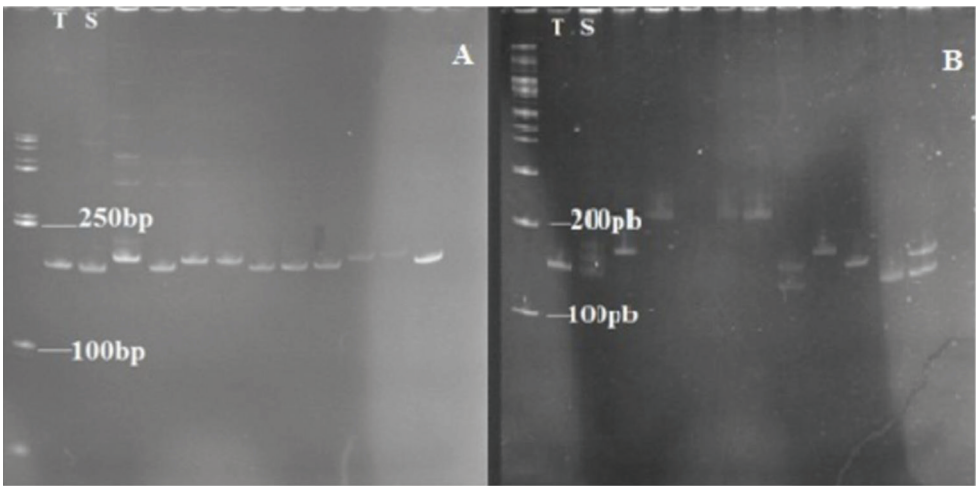

Figure 2. Inter and intrapopulation electrophoretic profile of SSR markers amplifications. A: amplification profile of (FMT-11) for different populations, B: amplification profile of TGPC-20 in *M. intertexta* sebkha population, lane 1: DNA leader, T: *M. truncatula*, S: *M. sativa*.

Table 3. The Ewens-Watterson test for locus neutrality performed with PopGene software version 1.32.

Locus	n	k	Obs. F	Min F	Max F	Mean*	SE*	L95*	U95*
Neutral SSRs									
MTIC-86	218	10	0.1713	0.1000	0.9208	0.3157	0.0129	0.1698	0.6131
FMT08	216	12	0.2413	0.0833	0.9033	0.2679	0.0095	0.1419	0.5144
MTIC-635	212	6	0.2704	0.1667	0.9539	0.4809	0.0288	0.2445	0.8651
MTIC-232	206	6	0.3201	0.1667	0.9526	0.4731	0.0253	0.2496	0.8365
MTIC-452	220	5	0.3891	0.2000	0.9643	0.5264	0.0300	0.2755	0.9118
FMT11	220	2	0.5134	0.5000	0.9910	0.8319	0.0274	0.5015	0.9910
MTIC-19	218	3	0.7694	0.3333	0.9818	0.7022	0.0345	0.3764	0.9728
MTIC-297	220	1	1.0000	1.0000	1.0000	***	****	****	****
MTIC-339	220	1	1.0000	1.0000	1.0000	***	****	****	****
MTIC-126	220	1	1.0000	1.0000	1.0000	***	****	****	****
MTIC-183	220	1	1.0000	1.0000	1.0000	***	****	****	****
Nonneutral SSRs									
MTIC-451	218	11	0.1221	0.0909	0.9125	0.2921	0.0114	0.1558	0.5839
MTIC-169	220	8	0.1820	0.250	0.9384	0.3856	0.0196	0.1990	0.7466
MTIC-153	220	5	0.2681	0.2000	0.9643	0.5338	0.0314	0.2804	0.8950

n: total number of alleles in all populations, k: number of alleles's versions.

all polymorphic loci analyzed, eight of them were found to be very informative (PI C> 0.5). According to the Ewens-Watterson test for locus neutrality, MTIC-451, MTIC-169, and MTIC-153 were found to be nonneutral (Table 3).

3.2. Intrapopulation genetic diversity

The number of alleles per locus ranged from 1 to 12 (Table 2). The number of alleles per locus per population ranged from 1 for most loci in most populations to 4 for the TGPC-20 locus in the two populations originating from the Sebkha of Oran. TGPC-20 appears to be the most polymorphic marker in *M. ciliaris* and *M. intertexta*. The allelic frequencies calculated for each locus in each population ranged from 1 to 0.1 (data not shown). The frequency of null alleles per locus and per population ranged from 0.001 to 0.40. This last value was obtained in the *M. intertexta* Sebkha population with the MTIC-169

locus. The number of private alleles per population and per locus varied from 1 to 2 alleles. Populations characterized by a number of private alleles equal to 2 are: *M. ciliaris* Sebkha (for MTIC-86 and TGP-20), *M. intertexta* Sebkha (TGP-20), and *M. ciliaris* 1576 (MTIC-169). The total number of private alleles per population for all the studied loci varied from 1 (*M. intertexta* 3084 and 2939) to 5 and 6 (*M. ciliaris* Sebkha and *M. intertexta* Sebkha), respectively. The average number of alleles per locus ranged from 1.0714 in *M. ciliaris* 1588 and *M. intertexta* 3084 to 2.0714 in *M. intertexta* Sebkha. The expected heterozygosity was slightly higher than the observed one for all populations (data not shown). H_O was null in all populations excluding *M. intertexta* Sebkha which presented a value of 0.0159 and *M. intertexta* 1158 with H_O equal to (0. 0071). The studied populations all had a fixation index equal to one, with the exception of *M. intertexta* populations (1158 and Sebkha). Values of F_{IS} (0.7229 and 0.654) were found in *M. intertexta* Sebkha and *M. intertexta* 1158 populations with TGP-20 and MTIC-232 loci, respectively. Indeed, certain individuals have proved to be heterozygous for these loci (Figure 2 B). We notice a complete absence of heterozygotes in *M. ciliaris*.

3.3. Interpopulation genetic diversity

The F_{IS} and F_{IT} values were equal to one for all markers, apart from TGP-20 marker (F_{IS} = 0.99201, F_{IT} = 0.99851) and MTIC-232 (F_{IS} = 0.98445, F_{IT} = 0.99718). These values remain high and close to that of the other markers. The multilocus estimations according to Weir & Cockerham (1984) have F_{IS} and F_{IT} values equal to 0.98025 and 0.99615, respectively. This corresponds to an overall deficit of heterozygotes of 99.61%. As for the F_{ST} value, genetic differentiation was relatively high and equal to 0.80523. According to Wright (1978), a value of F_{ST} > 0.25 indicates a significant differentiation. Such value of F_{ST} means that 80.52% of genetic variability is due to differences between populations, and only 19.48% has as origin an intrapopulation variation. The value of the global F_{ST} with and without ENA correction was 0.799 and 0.805, respectively. The G_{ST}, used to measure the degree of differentiation between populations, showed high values with most of the studied loci, e.g., the value (G_{ST} = 0.9196) of MTIC-452 highlights a strong differentiation between these populations.

Self-fertilization rate estimations are close or equal to 1 for all populations. Moreover, the highest values of gene flow between population pairs did not exceed 0.38. These very low values between pairs of populations denote low gene exchange between populations regardless of the proximity of the populations' origin sites. The smallest genetic distance value was the one among the two populations that are originated from the Sebkha of Oran *M. ciliaris* and *M. intertexta* (0.315). The next smallest values were followed by the values of 0.332, 0.370, 0.386 between *M. intertexta* 1157 and *M. intertexta* 1158, *M. ciliaris* 1588 and *M. ciliaris* 1576, *M. intertexta* Sebkha and *M. intertexta* 1600, respectively. The first two populations are from the same site (Kafr el Sheikh, Egypt), the seconds are from different countries (Lebanon and Tunisia), and the last two ones come from different sites but geographically close to each other (West of Algeria and Morocco). These rather low values indicate that the populations have a genetic similitude and belong to the same genetic groups. While the highest values were 1.151 between *M. ciliaris* 1587 and *M. intertexta* 3084, it was 1.019 between *M. ciliaris* 1587 and *M. intertexta* 1157, and 1.015 between *M. ciliaris* 1587 and *M. intertexta* 1158. We notice that these higher values occurred each time among two populations belonging to the two different species (*M. ciliaris* and *M. intertexta*) explains the presence of some genetic divergence between them. These divergences are much stronger than those observed between populations of the same species. The graphic mapping of populations showed the same structuration using the Dc and Ds distances in spite of the fact that Dc is lower than Ds for all populations. AMOVA analyzes showed that about 73%-79% of variation occurred at the interpopulation level. For the five analyzes performed, a great level of variation was detected at the interpopulation level within the groups. The intrapopulation variation level was low and the percentage variation between groups did not exceed 7.38%. The effects of species, altitude, and the origin site were 7.38%, 6.62%, and 5.2% respectively. The two axes of the FCA analysis (10.42% on the first axis and 8.84% on the second one) explain 19.26% of the genetic variability (Figure 3). The projection of individuals showed a structuration of genetic diversity within and between populations.

With an estimate of K = 2, we noticed that the STRUCTURE software has classified populations into two clusters that was not formed by the populations species or by populations' site of origin (Figure 4). Indeed, each cluster grouped two species in a population randomly. According to the Evanno method, the Δk reached its maximum value at K = 6. The sampled individuals were therefore grouped into 6 clusters. There is no systematic correlation between the geographical position of the site of origin and the grouping of populations, given by a series of analysis. However, we see that the populations of Egypt are always grouped together regardless of the analysis. The majority of individuals from the int Seb, cil Seb and Mas populations are also always classified in the same group but sometimes with certain individuals from these populations are associated either with those of a population of Morocco or with a population of Lebanon. Finally, an ensemble made up of the cil 1576 population of Tunisia, one of Lebanon cil 1588 and a pop of Morocco

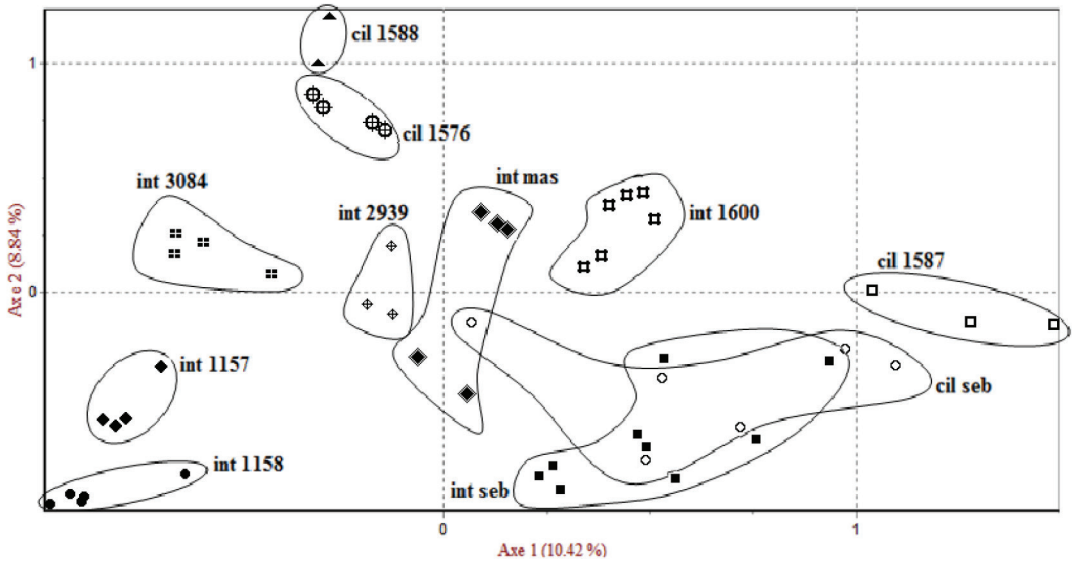

Figure 3. Association among populations of *M. ciliaris* and *M. intertexta* distributed revealed by factorial correspondence analysis (FCA). cil: *M. ciliaris*, int: *M. intertexta*. Each population is made up of 10 individuals, the superposed individuals in the graph are represented by the same point.

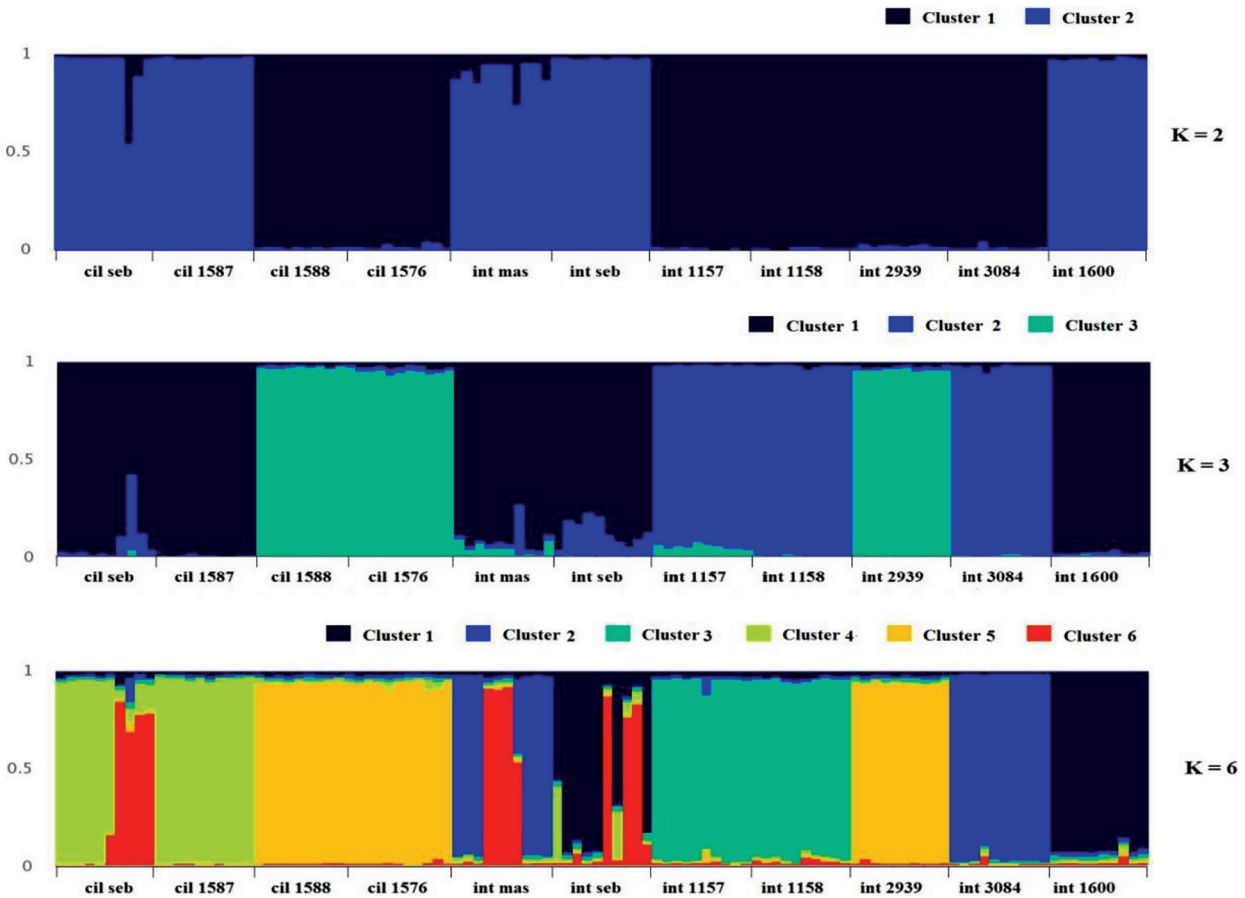

Figure 4. Probability of belonging of each individual to each of the clusters defined by the STRUCTURE software (Pritchard and Coll., 2000) with K = 2, K = 3 and K = 6.

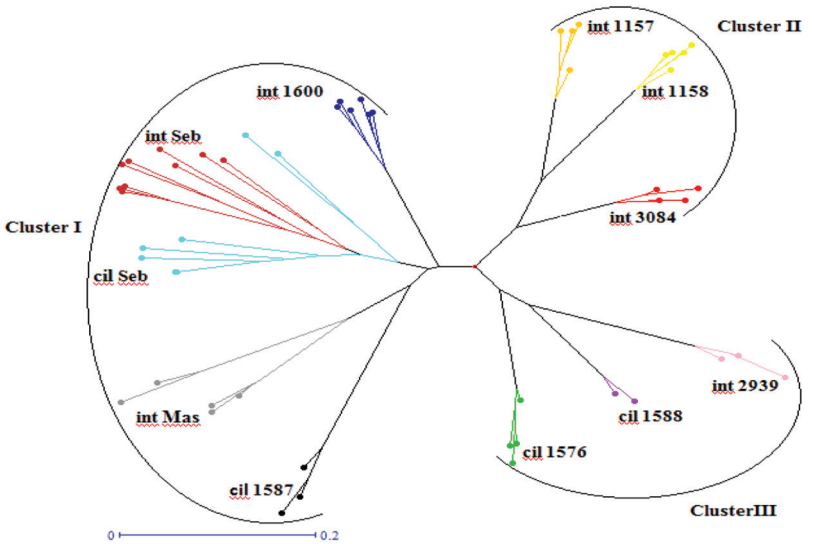

Figure 5. Neighbour joining tree constructed for the 11 populations of *M. ciliaris* and *M. intertexta* based on SSR allele sizes.

int 2939 always form a separate group. The neighbor joining tree allowed grouping the populations into three distinct clusters (Figure 5). The outer nodes of each cluster represent the populations evaluated as potential neighbors. The aforementioned three clusters are similar to those generated by the STRUCTURE software with K = 3 and group the same populations. We note that cluster 2 groups three populations of the *M. intertexta* species, whereas clusters 1 and 3 contain populations of both species.

4. Discussion

In this study, we present the first report of molecular characterization of the *M. intertexta* populations and a comparison of their genetic diversity with *M. ciliaris* populations using SSR markers. Available information on the investigation of genetic diversity using molecular markers in *M. ciliaris* and *M. intertexta* is scarce; only one study has been published on the assessment and analysis of genetic diversity in *M. ciliaris* using microsatellite markers (Badri et al., 2007), another study analyzed the genetic diversity in *M. ciliaris* using AFLP markers (Jabri et al., 2016), and no molecular characterization was done on *M. intertexta* so far. The transferability rate of the studied SSR markers coming from the genome of *M. truncatula* to *M. ciliaris* and *M. intertexta* in this study is higher than the one that is found by Badri et al. (2007). We assume that this is either due to the manipulation or to the hybridization temperature of primers. Our results suggest that SSR markers developed from the genome of *M. truncatula* may be effective genetic markers for *M. ciliaris* and *M. intertexta*. The number of polymorphic loci found in our study was higher than that obtained by Badri et al. (2007). This is probably due to the technique used for genotyping individuals, since the use of a polyacrylamide gel is much better and more definitive than using agarose gel (Mishra Kundan et al., 2014). Moreover, this technique allowed us to highlight 72 alleles for 14 loci compared to only 28 alleles for 17 loci in *M. ciliaris* found by Badri and his collaborators (2007). Among the monomorphic markers found in our study, MTIC-126 appears to be less polymorphic also in other studies (Ronfort et al., 2006; Badri et al., 2007; Lazrek et al., 2008). On the other hand, MTIC-297 proves to be very informative in studies done on other species of the Medicago genus (Lazrek et al., 2008; Touil et al., 2008; Cholastova and Knotova, 2012; Iqbal et al., 2017). This suggests that the alleles of these loci were probably fixed during the evolution any process of these species. The genetic drift and/or the reproductive pattern of these species are probably responsible.

The three largest values of calculated allelic richness found in the populations originating from the Sebkha of Oran and from Mascara indicate that these populations of both species constitute a rich source of genetic variation. This estimation cannot be compared between studies because it is dependent on the sample size, and can increase each time a new individual is observed (Foulley and Ollivier, 2006). The potential for adaptation and persistence of populations to future environmental conditions may be reduced if allelic richness decreases. However, the exploitation of allelic richness is a fundamental, crucial, and decisive criterion, among many others in population management and conservation programs (Greenbaum et al., 2014). The highest values of gene flow between population pairs indicate very little gene exchange between populations and the low level of

heterozygosis observed indicates that the two studied species are preferentially autogamous. The finding of a low degree of heterozygosity cannot be underestimated by the number of individuals studied. Indeed, Nei (1978) noted that the number of individuals to be examined depends on the degree of heterozygosis; he pointed out that more individuals should be examined when heterozygosis is high (higher than 0.1) than when it is low. In addition, H_O and H_{NB} measurements are more sensitive to allelic frequencies in the population than they are to the number of alleles. The two populations of the Sebkha of Oran are those that have the highest total number of private alleles (6 and 5, respectively). These alleles could be the ones of the native populations that have been selected by local ecological factors. In that case they can be suitable markers for salinity tolerance. Another assumption is that these alleles could be related to the average number of migrants exchanged per generation between populations (Barton and Slatkin, 1986). However, in self-pollinated species, migrating individuals in other populations may not effectively incorporate their private alleles into local populations by cross-pollination. As a result, this can lead to the loss of migratory alleles by drifting annual species (Yan et al., 2009).

The selective neutrality test allowed us to prove that SSR markers are not always neutral. But assumptions about the neutrality of SSRs should be cautious because this type of marker is often assumed to be neutral (Hodel et al., 2016). Researchers often afraid that these markers may be subject to selective pressures when they occur in coding regions or nearby (Morgante et al., 2002). Indeed, the three nonneutral markers revealed by the selective neutrality test are located in an expressed sequence tags (ESTs). Such markers are sometimes subjected to positive and negative selection for various reasons such as genetic hitchhiking. These reasons are undetectable for a researcher without extensive genomic resources available (Haasl and Payseur, 2011).

According to the F_{IS} values, self-fertilization appears to be the dominant reproduction regime in *M. ciliaris*, whereas in *M. intertexta* a very low heterozygosis observed in natural populations indicates its ability to allopollinate; similar results were found by some authors (Lesins et al., 1971; Lesins and Lesins, 1979; Cherifi, 1996; Abdelkefi et al., 2001). In addition, Small et al., 1999 noted that *M. intertexta* is the least widespread but considerably more variable than *M. ciliaris*. In addition to an autogamous reproductive system, a significant deficit of heterozygotes may also be due to a Wahlund effect. For some authors (Chapuis and Estoup, 2007), null allels can contribute as well to reduce the heterozygosity rate in large size populations producing an increase in both F_{IS} and F_{ST} statistics, which was observed in our study. While the Wahlund effect changes both F_{IS} and F_{ST}, it increases F_{IS}, and in most situations, decreases F_{ST} (De Meeûs, 2018). Overall, the F_{ST} values for all loci and F_{ST} in pairs were slightly higher for some populations and somewhat lower for others with and without ENA correction. The differences were not statistically significant. Then, it seems that null alleles have no influence on results in this study. Rico et al. (2017) also demonstrated that the high frequencies of null alleles do not always have a significant effect on the parameters of genetic diversity of the populations evaluated for the SSR loci. In addition, the populations we studied have very low levels of gene flow; in this case, the F_{ST} bias is more important after correction (Chapuis and Estoup, 2007). Therefore, subsequent tests were performed with uncorrected allelic frequencies.

The genetic diversity assessed by AMOVA test made between populations from different altitudes showed a slight altitude effect. This test made it possible to estimate a rate of variation of 6.61% with an altitude effect between the 4 groups and 3.08% between two population groups. This intergroup variation rate remains low compared to that of interpopulation within groups. The high F_{ST} values found in the results of this study indicate that genetic variation is much important between populations and much less within them. Also, a greater molecular differentiation has been recorded at the interpopulation level than at the intergroup level with the AMOVA results. Thus, these results revealed that there was not enough allele sharing between populations. This suggests either a high degree of isolation between populations, or isolation reinforced by local adaptation. In autogamous species, the genetic variation is more frequent between populations than within them (Mateu-Andre´s and De Paco, 2006). A strong differentiation between populations can be explained by the fact that they cannot exchange their genetic material. Each population keeps its own mutations, which will not spread in other populations. The genetic structure of autogamous populations will therefore be heterogeneous with homozygous individuals compared to a homogeneous structure with heterozygous individuals in a cross-pollinated population. Inbreeding, therefore, greatly affects levels of diversity as it increases isolation between individuals and populations (Charlesworth, 2003). According to Chapuis and Estoup (2007), the Dc distance gives better results than the standard Ds distance, because it is less affected by the null alleles. After comparing the results of the two distances, we noticed that structuration of populations does not change. The highest values of distances are specific to *M. ciliaris* 1587. These high values occur each time between this population and another one of *M. intertexta* (1157, 1158 and 3084). Originating site

of *M. ciliaris* 1587 population is geographically far from the others. These highest values explain the presence of an important genetic divergence between them. *M. ciliaris* 1587 has also a distance value close to 1 with *M. ciliaris* 1588 belonging to the same species and originating from the same site. At the intraspecific level, in Jabri's work (2016) using AFLP markers, the lowest value of the Nei distance between populations of *M. ciliaris* was 0.034, while the highest was of 0.48. In this work, populations of this species are much more elongated between them, the lowest value is 0.37 and the highest is 0.95. Also, in *M. intertexta* species, the lowest value is 0.33 and the highest is 0.93. The degree of isolation of populations is proportional to the distance between them. The great geographical distance separating the populations makes them very different genetically; they probably have had to be separated for a very long time, and each has evolved differently.

In this study, the two axes of FCA analysis explain only 19.26% of the total genetic variability. Also, a low variance explained by the FCA axes was observed in other studies carried out in other Medicago species (Lazrek et al., 2008; Touil et al., 2008). The distribution of the populations according to the FCA result showed geographical structuring of the genetic diversity, with the exception of the two populations of Lebanon which appear clearly separated. These two populations presented also high values of Ds and Dc distances. The divergence between the two populations of *M. ciliaris* (1587 and 1588) may reflect a recent migration event. One of two populations or both sets would originate from another place, since in such plant a single seed can give rise to a new population (founder effect). This leads to major changes in the genetic structure of the founder population through random changes in allele frequencies, including the loss and fixation, which lead to genetic divergence from the ancestral population, and reduction of genetic variability (Templeton, 1980). In fact, these two populations represent the lowest number of genotypes, 3 for the 1587 population and only 2 for the 1588 population (Table 1). In the newly established population, genetic variation is reduced to that carried by the few colonizing individuals involved in the founding event.

With K = 2 the STRUCTURE software classified the populations into two clusters including a mixed population of the two species. With the highest value of Δk, the different populations of this study were structured in 6 groups. The level of genetic differentiation between populations does not show a geographical pattern. Colocated populations are not always more similar than those more distant. We conclude that much of the observed genetic variation between and within populations is neutral and reflects various genetic processes including mating system, founding effect, and genetic drift. It should be noted that consanguinity modifies the distribution of genetic variation between populations, thereby increasing genetic structure and differentiation disproportionately (Wright, 1965). Genetic drift can bring a new population that can be distinguished genetically from its original population, thus playing a vital role in the evolution of the species.

According to the representation of the neighbor joining tree, the genetic differentiation between populations is very high. Moreover, this method allowed us to note that, at the level of the molecular markers, there are no significant differences between the two taxa, since some populations of the two species are genetically closer to each other than to other populations of the same species. The external nodes that represent them make it possible to evaluate them as being more close to each other. We also find that the nodes that represent all individuals of *M. intertexta* population of Sebkha without exception derive from the internal root that represents the *M. ciliaris* population. Therefore, according to this cladogram, *M. ciliaris* represents the primitive state and *M. intertexta* represents the derived one. With this observation, it may be suggested that it is *M. intertexta* that derives from *M. ciliaris* and not the other way around; the same hypothesis was proposed by Jauzein (1995). This observation supports the idea that more in-depth studies of these two taxa should be done to classify them more accurately.

5. Conclusion

At the end of this study, we note that some populations are geographically isolated and this isolation is confirmed by the result of the genetic analysis. This is the case of the populations of Egypt and to a lesser extent the cases of the populations of Sebkha and Mascara. On the other hand, the other populations seem to have been probably subjected to migratory events which explain the difficulty of classifying them in a particular geographical group. In addition, it appears that the populations of the Sebkha site differ markedly from other populations regardless of the analysis to which they were subjected. The estimated genetic diversity assessment parameters show that they contain significantly more variability than the rest of the populations with a superiority of the *M. intertexta* population. They contain more specific alleles than the rest of the populations which may suggest that they have been shaped by the selective pressure exerted by the particular environment of the Sebkha site. They, therefore, constitute potentially interesting populations to conserve in situ and/or ex situ for future use in breeding programs to create varieties or to provide tolerance factors to cultivated species.

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