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Full Length Research Paper

Characterization of *Medicago* populations under cold acclimation by morphological traits and microsatellite (SSR) markers

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The study was carried on 16 accessions of annual *Medicago* species (*M. truncatula* Gaertn. *M. ciliaris* Krocker., *M. aculeata* Wild. and *M. polymorpha* L.). Seedlings of different accessions collected from sites of contrasting altitudes (10 to 1170 m) were subjected to different durations of low temperature regimes. Root to shoot ratios of acclimated and non acclimated plants were compared. Among the 16 accessions studied, 12 were used to assess the degree of genetic polymorphism by SSR microsatellites. Results show that accessions that originated from high altitude had a better root to shoot ratios and so had better ability to cold acclimation than accessions that originated from low altitude (lower ability to cold acclimation). Tests differentiation between species by fisher pair indicates that all species were different from each other. Results show the highest level of homozygosity for all species (> 80 %). Moreover, there were differences between populations of the same species of cold acclimation, which will encourage for a study of association between cold acclimation and molecular polymorphism.

Key words: Cold acclimation, root: shoot ratios, molecular polymorphism, annuals populations, *Medicago*

INTRODUCTION

The genus *Medicago* has emerged as an important experimental species and its species are important forage sources of the world. Species of *Medicago* are interesting because of their adaptability to different soil and climates, their good winter growth, self reseeding, their possible use in rotation with cereal and their low input requirements (Bullita et al., 1994). Annual species adapted to cold, can guarantee consistent production grazing replacing unproductive fallow. A sufficient level of cold tolerance might be needed to extend area of utilization of annual species of *Medicago* in environments with low winter temperatures. Environmental constraints

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Abbreviations: PL, Seedling length; **SL,** shoot length; **RL,** root length; **CA,** cold acclimated; **NA,** non-acclimated; **LG,** linkage group; **Tm,** melting temperature; **SSR,** single sequence repeat; **Rt/Tt,** root: shoot ratio.

are limiting factors for agricultural productivity and play an important role in the distribution of plant species across different types of environments (Singh et al., 2002;Dita et al., 2005; Zhang et al., 2008; Saibo et al., 2009). Cold is one of the most important constraints limiting and controlling seed germination, development of seedlings (post germination growth), the growth and development of the adult plant (Boyer, 1982; Mohapatra et al., 1989; Janda et al., 2007; Baruah et al., 2009; Kurt, 2010; Dias et al., 2010). Up to 15% of the world's agricultural production was lost to frost (Zou et al., 2007). During crop establishment, extreme temperatures can decrease plant emergence and lead to drastic losses in crop yield and quality (Kim and Tai, 2011; Avia et al., 2013).

Germination and growth of seedling were rarely targeted by breeders. Increased knowledge on mechanism of cold stress tolerance, at early stages, can lead to possible genetic improvements (Brunel et al., 2009; Dias et al., 2010). Many plants increase in freezing tolerance in response to low, non-freezing temperature, a process known as cold acclimation (Levitt, 1980; Guy, 1990; Thomashow, 1999; Oullet, 2007; Baruah et al., 2011; Pirzadah et al., 2014). During the progressive lowering of temperature (chilling temperatures), plants that tolerated frost (freezing temperature) were those able to adjust their metabolism and basic cell functioning biophysical constraints imposed by the transition to low positive temperatures (Houde et al., 2006). If the plant can adjust its cellular processes for a long time of exposure to low positive temperatures, it unlikely and highly become tolerant to freezing (Sakai and Larcher, 1987; Guy, 1990; Thomashow, 2010). Many morphological changes had been documented during the acquisition of cold tolerance in different species (Meyer and Badarudin, 2001; Castonguay et al., 2009; Castonguay et al., 2010; Baruah et al., 2011; Iraba et al., 2013). Colds tolerance can be evaluated by changes in morphological indices such root: shoot ratios (Hekneby et al., 2001; Thapa et al., 2008; Hund et al., 2008; Janska et al., 2010). Thapa et al. (2008) showed that its ability to adapt cold acclimates was evident by significant increase in freezing tolerance of two genotypes of *M. truncatula* with the exposure to specific cold acclimation regimes. Sutka and Galiba (2003) showed that plants acclimating at a temperature below 10°C can tolerate temperatures as low as -30°C. The organs of plants differed in their tolerance to cold. In general, cold-tolerant species (grasses, shrubs and grasses) had small size, low leaf area and root: shoot ratio significant. The root stem ratio was much higher in acclimated plants than in controls (Hekneby et al., 2001). Other authors, however, had stipulated that the roots were more sensitive than the collars (Mackersie and Leshem, 1994).

Phenotypic assessment can provide a direct and easy estimation of variability for cold stress adaptations. However, they were often affected by environment. Microsatellite markers, free from this constraint, were often used in combination with phenological traits to characterize populations and their adaptation to constraint environments (Dias et al., 2008; Badri et al., 2008; Touil et al., 2008; Lazerek et al., 2009; Li et al., 2009; Avia et al., 2013). Cui et al. (2013) reported that there was a positive association between morphological characters and SSR markers in relation to the origin of Japonica rice improved populations and their cold tolerance. Lazerek et al. (2009) investigated the genetic diversity of a collection of *M. truncatula* using a set of 18 microsatellites and found that seven had a correlation with altitude, rainfall and environmental salinity of origin of these populations. These authors also suggested that these markers loci linked to genes are involved in the adaptation to the altitude. Dias et al. (2008) highlighted some concordance between morphological characters and SSR markers in red clover (*Trifolium pratense* L.). Badri et al. (2008), studying the genetic variability in natural populations of *M. laciniata* Mill. (Fabaceae) found that the morphological diversity and molecular diversity were significantly associated with eco-geographical factors. Touil et al. (2008), studying the genetic diversity in cultivated populations of *M. sativa* L., highlighted that there was no correlation between SSR markers and the geographical origin of populations. However, these authors had emphasized that SSR markers are very informative and appropriate approaches in characterization and molecular polymorphism in populations of *Medicago*. In Algeria, annual populations of *Medicago* species were often used in rotation cereal-forage areas; growth of this species in these areas was seriously limited by the ability of each species to grow during cold winters. A sufficient level of cold tolerance might be needed and specific responses to these conditions are required to extend the area of utilization of this annual species. The aims of this project are therefore: to determine the ability and evaluate cold acclimation (CA) in range of annual accessions of *Medicago* representing broad geographic origins. CA was evaluated by measuring root to shoot ratios of treated plants at the different durations in comparison with the non-acclimated (control) and to estimate genetic diversity of this natural *Medicago* accessions using SSR markers and determine if there was a relationship between cold acclimation of populations and SSR markers used.

MATERIALS AND METHODS

Plant material, growth conditions and cold stress treatment

The study was carried on four annuals *Medicago* species (*M. truncatula* Gaertn. *M. ciliaris* Krocker., *M. aculeata* Wild. and *M. polymorpha* L.). A set of 16 accessions was tested for their ability of cold acclimation (Table 1). For growth conditions, ten seeds for each accession and repetition were germinated, after scarification, at room temperature in Petri dishes (85 mm) containing universal compost moistened with distilled water at the same conditions (16/8 (h) photoperiod (day/night), light intensity (6700 Lux)). A total of

4800 plants were treated. At three days growth stage, seedlings of each accession were divided into two lots; 150 in cold acclimation (CA) lot at 4°C for three durations 5, 8 and 11 days (T1, T2 and T3) and 150 in lot non-acclimated (NA) (control) kept at 23°C (T01, T02 and T03). The experimental design was a randomized plot with five replications. The total length of the seedlings (LP), the shoot (LT) and the root (LR) were measured at the end of each treatment on both the treated and control lots. LR and LT were used to calculate root: shoot ratios of cold acclimated and non-acclimated plants. Measuring root to shoot ratios at different durations in comparison with the control, were used to assess degree of cold acclimation at low temperature of the different accessions.

Statistical analysis

Statistical analyses were conducted using the Statistical Analysis System Statistica 6.1 version (Stat Soft, Inc. France). Data were analyzed by calculating mean and standard deviations values, for different settings and different treatments. Differences between the treatments means for each treat were performed using two-way ANOVA.

SSR analysis

Among 16 accessions studied, 12 populations were characterized using microsatellites markers (SSR). SSR analysis was carried out using 14 primer pairs originating from *M. truncatula*; selected based on their position on the genetic linkage map (Julier et al., 2003) and provided by the research unit URP3F/genetics INRA, Lusignan, France (Table 2).

DNA extraction and PCR

DNA was extracted from 200 mg samples of fresh young leaves from individual plants, in liquid nitrogen and 3 ml of 2% cetyltri-methylammonium bromide (CTAB) (BIOCHEM-CHEMOPHARMA. CANADA), according the technique of Doyle and Doyle (1990). PCR amplification was performed with 14 SSR microsatellites. The DNA from cultivars Magali, Mercedes, Jemalong and Gabes was extracted according to the method described by Cheung et al. (1993). PCR analysis was carried out at INRA, Lusignan France. PCR was performed for 10 µl total volume containing 1 × buffer, 02 mM dNTP, 0.2 µM SSR primers, 1.5 mM $MgCl_2$, 50 ng genomic DNA 0.3 units *Taq* polymerase (SIGMA-ALDRICH). Reactions were performed in a PTC-100 thermocycler (MJ Research, USA) programmed for an initial melting at 94°C for 4 min followed by 35 cycles at 94°C for 30 s, at melting temperature (Tm) for 1 min, a 72°C for 1 min, and then a final extension step at 72°C for 10 min. PCR products (2 µL/lane) were separated in 6.5% polyacrylamide gels in the LI-COR IR2 automated DNA sequence (LI-COR Inc.). Of the 14 SSR used, only nine of them were selected for which there was no missing data. For each primer pairs selected, alleles specifically detected in tolerant accessions were scored as (+). The different parameters were calculated using the GENETIX software (ver. 5.04) and GENEPOP (ver. 4.2).

RESULTS AND DISCUSSION

Growth evaluation among species of acclimated and non-acclimated seedlings

The results indicate that, among the four *Medicago* species, development for all traits measured at non-acclimated (control) and cold acclimated regimes varied both seedlings, shoots and roots lengths. ANOVA revealed highly significant differences ($p \leq 0.001$) among the populations studied between acclimated and non-acclimated lots (Table 4). These differences were more pronounced with treatment durations (Table 3). Under control generally, development of *M. ciliaris* was better than for the remaining three species (*M. truncatula, M. polymorpha* and *M. aculeata*). For example, for seedlings length, it varied on average from 9.57 to 13.83 cm (*M. ciliaris*) and from 5.88 cm to 6.89 cm (*M. polymorpha*) under control. Even under a low temperature regime, these species had a better development in comparison with the other three species. It varied from 6.20 to 3.32 cm (*M. ciliaris*) and from 3.51 to 4.48 cm (*M. polymorpha*). Compared to the control, radical length decreased significantly. This deceased was greater in *M. polymorpha* than others specified at all treatments. As stress persists over time, *M. aculeata* displays better root development.

Thus, the species *M. aculeata* and these populations were good candidates for improving cultivars tolerant to cold. Janska et al. (2010) had emphasized that species adapted by natural selection to cold environments have a good development root. Development of shoot was also affected by low temperature at the different durations. The decreases of this development were different from one species to another and from one treatment to another. *M. ciliaris* and *M. aculeata* had a rapid development (2.93 and 2.50 cm respectively) in comparison with M. *polymorpha* (1.80 cm) after five days under cold treatment. This trend seemed to be reversed after 11 days of cold treatment; *M. polymorpha* showed better development (2.55 cm) than *M. ciliaris* (1.73 cm).

Root to shoot ratio cold acclimation ability

In the literature, it was reported that there was a close relationship between tolerance to cold and frost and the ability of acclimation to low non-freezing temperatures. The degree of acclimatization was estimated by the root to stem ratio of treated plants to sub-zero temperature. Plants that displayed the most important ratios were considered cold-tolerant.

In this study, all accessions of the four *Medicago* species maintained at cold acclimated and non-acclimated (control) regimes varied for root to shoot ratio at the tree durations of treatment time (Figures 1, 2 and 3). In general, root to shoot ratio was higher in cold acclimated than in control. These results are in accordance with those that had been demonstrated by Thapa et al. (2008), which showed that root to shoot ratio was higher in cold acclimated than control plants in *Medicago truncatula.* For example, in *Medicago aculeata* genotypes, Ac 80 had a better ratio at the different

Table 1. Accessions analyzed for cold acclimation with their origin and ecological description. Cultivars, Mercedes, Gabes, Jemalong and Magali were used only as control in SSRs characterization by PCR.

Species	Population	Origin	Latitude	Longitude	Altitude (m)
M. aculeata Willd.	cv*. Ac 15678	Australia	-	-	-
	cv. Ac 15679	Australia	-	-	-
	cv. Ac 14821	Australia	-	-	-
	cv. Ac 80	Syria	-	-	-
M. ciliaris Krocker.	Cil 123	Algeria	36°46'02''N	8° 18' 9.57'' E	16
	Cil 124	Algeria	36°17'15'' N	7° 57' 14.77'' E	565
	Cil 125	Algeria	36°17'15''N	7° 57' 14.77'' E	565
	Cil 126	Algeria	36° 28' 0'' N	7° 26' 0'' E	290
M. polymorpha L.	Poly 57	Algeria	36°17'15''N	7° 57' 14.77'' E	565
	Poly 54	Algeria	36°17'15''N	7° 57' 14.77'' E	565
	Poly 136	Algeria	36°49'0'' N	5° 46' 0'' E	10
	Poly 213	Algeria	35°23'17''N	1° 19' 22'' E	1170
	Poly 42†	Algeria	36°54'15''N	7°45'07''E	200
M. truncatula Gaertn.	Tru 210	Algeria	34°6' 50'' N	2° 5' 50.14'' E	1150
	Tru 216	Algeria	34° 6' 50'' N	2° 5' 50.14'' E	1150
	Tru 62	Algeria	36° 28' 0'' N	7° 26' 0'' E	290
	Tru 26	Algeria	35° 23' 17'' N	1° 19' 22.16'' E	1170
M. sativa	cv. Magali	France	-	-	-
	cv. Mercedes	France	-	-	-
	cv. Gabes	France	-	-	-
	cv. Jemalong	France	-	-	-

*, Cultivars; †, accession having been only in the molecular characterization.

duration of cold acclimation (1.63, 2.31 and 1.40) than Ac 15679 (0.82, 1.10 and 1.30) respectively for T1, T2 and T3 (Figures 1, 2 and 3). Moreover, it seemed that populations from high altitudes were the best ratios under cold treatment compared to native lowland and T3 regime was most effective in distinguishing cold acclimation ability of accessions studied, especially for *M. truncatula* and *M. polymorpha* accessions. In *M. truncatula*, Tru 216, originating in high altitude (1150 m) (Table 1), showed the best ratio (1.20) in comparison with Tru 62 (0.69). The similar trend was observed in *M. polymorpha*, Poly 213, originating from 1170 m, which showed a ratio of 0.93 while Poly 57 originating from a low altitude (565 m) exhibited lower ratio (0.27).

Janska et al. (2010) showed that cold tolerant species - herbs, grasses and ground shrubs - had low leaf surface area and a high root: shoot ratio and cold-adapted plants tend to be slow growing. Thapa et al. (2008), in order to understanding cold acclimation of two contrasting genotypes of *M. truncatula* growing under low temperature regimes at different durations, observed that growing under low temperature regimes, comparatively to control conditions, resulted in a global growth reduction and root to shoot ratio was higher in cold acclimated than in control plants. Moreover, these authors related that tolerant genotypes had a higher root: shoot ratio and the behavior to the higher frost tolerance exhibited by tolerant ecotypes of *M. truncatula* after a low temperature period, highlight their greatest cold acclimation ability. In pea, Lejeune-Hénaut et al. (2010) reported that root: shoot ratio was higher under low temperature, particularly for the frost tolerant genotype Champagne. Bounejmate et al. (1994) revealed that there was a relationship between frost tolerance and winter temperature at site of collection for *M. aculeata*, with the most frost tolerant genotypes coming from high altitudes.

It appears that the populations from high altitudes areas presented superior ratio and thus had a great capacity for cold acclimation better than those of populations originating from lower geographical areas. The influence of the geographical origin of plants on their level of cold acclimation ability was often highlighted. Studying the growth of *M. truncatula* and *M. aculeata* genotypes collected from sites of contrasting altitudes and winter temperatures, Bounejmate et al. (1994) reported that there was a relationship between frost tolerance and winter temperature at site of collection, with the most frost tolerant genotypes coming from high altitudes and genotypes from high altitudes represent a promising source for breeding for first tolerance with greater variation in *M. aculeata* than *M. truncatula.* Avia et al. (2013) analyzed the genetic variability freezing associated to cold acclimation in range of accessions of *M. truncatula* representing broad geographic origins, and

Table 2. SSR primers used for annuals accessions of *Medicago* DNA amplification (INRA, Lusignan. France).

Marker	LG	Primer	*Tm* (°C)	Allele size in alfalfa (bp)
ATP456	3	L-GGGTTTTTGATCCAGATCTT R-AAGGTGGTCATACGAGCTCC	55	125
FMT-13	1	L-GATGAGAAAATGAAAAGAAC R-CAAAAACTCACTCTAACACAC	50	132
MTIC-79	5	L-AAAATCCAAAGCCCTATCACA R-AGCGTGAGATTTTTCCATCG	55	117
MTIC- 332	4	L-CCCTGGGTTTTTGATCCAG R-GGTCATACGAGCTCCTCCAT	55	124
MTIC- 338	3	L-TCCCCTTAAGCTTCACTCTTTTC R-CATTGGTGGACGAGGTCTCT	55	146
MTIC- 134	6	L-GCAGTTCGCTGAGGACTTG R-CAATTAGAGTCTACAGCAGCCAAAAACT	60	176
MTIC- 365	2	L-ATCGGCGTCTCAGATTGATT R-CGCCATATCCAAATCCAAAT	55	123
MTIC- 082	7	L-CACTTTCCACACTCAAACCA R-GAGAGGATTTCGGTGATGT	55	132
MTIC- 451	2	L-GGACAAAATTGGAAGAAAAA R-AATTACGTTTGTTTGGATGC	55	129
MTIC- 135	8	L-GCTGACTGGACGGATCTGAG R-CCAAAGCATAAGCATTCATTCA	55	123
MTIC- 343	6	L-TCCGATCTTGCGTCCTAACT R-CCATTGCGGTGGCTACTCT	55	134
MTIC- 131	3	L-AAGCTGTATTTCTGATACC R-CGGGTATTCCTCTTCTTCCTCCA	55	163
MTIC- 432	7	L-TGGAATTTGGGATATAGGAA R-GGCCATAAGAACTTCCACTT	55	163
B14B03	5	L-GCTTGTTCTTCTTCAAGCTC R-ACCTGACTTGTGTTTTATGC	55	151

Tm, melting temperature; LG, Linkage Groupe

Table 3. Average of the different traits measured under different treatments for all species.

Species	Treatment	PL(cm)	SL(cm)	RL(cm)
		Mean ± Std. Dev.	Mean ± Std. Dev.	Mean ± Std. Dev.
M. truncatula	T01	6.89 ± 2.69	3.52 ± 1.47	3.37 ± 1.68
	T1	4.84 ± 2.49	2.35 ± 1.33	2.48 ± 1.67
	T02	7.70 ± 7.70	4.08 ±2.02	3.61 ± 1.82
	T2	4.32 ± 2.58	2.19 ± 1.44	2.07 ± 1.47
	T03	8.15 ± 2.51	4.50 ± 1.73	3.64 ± 1.28
	T3	3.46 ± 3.13	1.79 ± 1.50	1.67 ± 1.85
M. ciliaris	T01	9.57 ± 3.87	4.62 ± 2.08	4.94 ± 2.41
	T1	6.20 ± 4.74	2.93 ± 2.45	3.26 ± 2.68
	T02	11.69 ± 3.45	5.92 ± 1.98	5.76 ± 2.15
	T2	4.92 ± 3.49	2.25 ± 1.54	2.67 ± 2.19
	T03	13.83 ± 2.57	7.20 ± 1.32	6.62 ± 2.02
	T3	3.32 ± 3.57	1.73 ± 1.83	1.58 ± 1.95
M. polymorpha	T01	5.88 ± 2.97	3.25 ± 1.88	2.62 ± 1.40
	T1	3.51 ± 2.16	1.80 ± 1.13	1.71 ± 1.45
	T02	6.52 ± 2.46	3.63 ± 1.54	2.88 ± 1.41
	T2	4.35 ± 2.30	2.30 ± 2.39	1.95 ± 1.23
	T03	6.89 ± 1.81	4.12 ± 1.26	4.12 ± 1.26
	T3	4.48 ± 2.40	2.55 ± 1.38	2.55 ± 1.38
M. aculeata	T01	7.42 ± 3.43	3.71 ± 1.92	3.71 ± 1.99
	T1	5.81 ± 8.04	2.58 ± 1.72	2.74 ± 2.10
	T02	8.85 ± 3.55	4.20 ± 1.88	4.64 ± 2.16
	T2	6.18 ± 3.43	2.69 ± 1.69	3.48 ± 2.38
	T03	9.90 ± 3.54	4.73 ± 1.83	5.16 ± 2.10
	T3	6.04 ± 3.78	2.60 ± 1.75	3.43 ± 2.26

PL, plant length; SL, shoot length; RL, root length; Std. Dev., standard deviation.

Table 4. Analysis of variance of different morphological parameters studied for cold acclimated and non-acclimated ecotypes.

Source of variation	ddl	Seedling length	Shoot length	Root length
		F	F	F
Ecotypes	15	12.95***	8.15***	18.96***
Treatment	5	70.14***	73.83***	56.62***
Ecotype* Treatment	75	1.99***	1.86***	2.38***
Error	4705			

*P ≤ 0.05; ** P ≤ 0.01; ***P ≤ 0.001; ns, non significant; F, coefficient of Fisher-Snedecor (test at level 5 %).

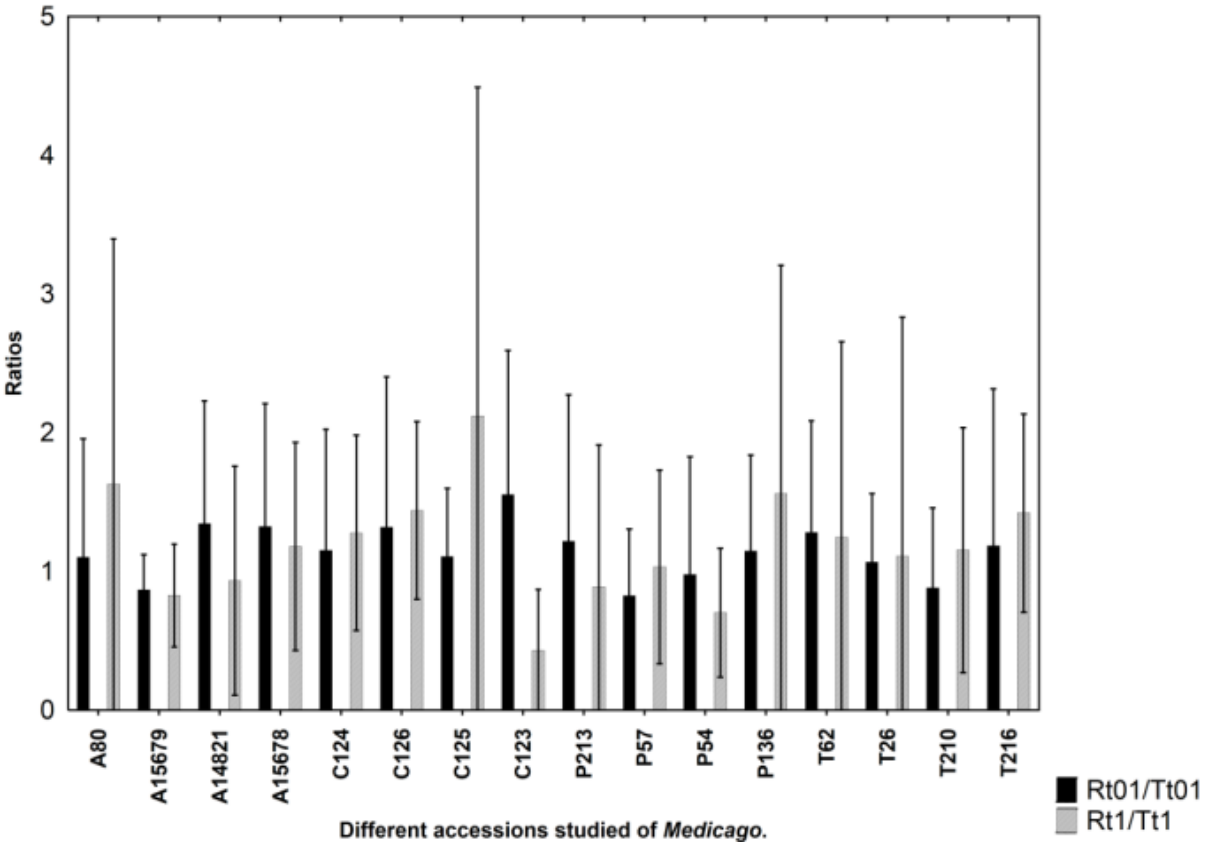

Figure 1. Ratios root to shoot after 5 days under 4 °C regime (Rt1/Tt1) and control (Rt01/Tt01), for different *Medicago* accessions studied.

revealed that accessions originating from higher latitudes were globally the most tolerant; which may reflect the adaptation tendency of accessions having evolved in geographical areas characterized by more frequent frost events.

Genetic diversity

Of the 14 SSR markers used, nine were selected (not missing data). The SSR loci used in our study were polymorphic. An example of SSR variation detected in populations studied is given in Figure 4. A total of 81 alleles were detected at the nine SSR loci for all species. The number of alleles detected per locus ranged from 6 (for ATPase456) to 13 (for Mtic432) (Table 5). Fisher tests differentiation between species pair showed that all species are different from each other. Results show the high level of homozygosity for all species (> 80%). The homozygosity percentage varied from 50 to 100% (Table

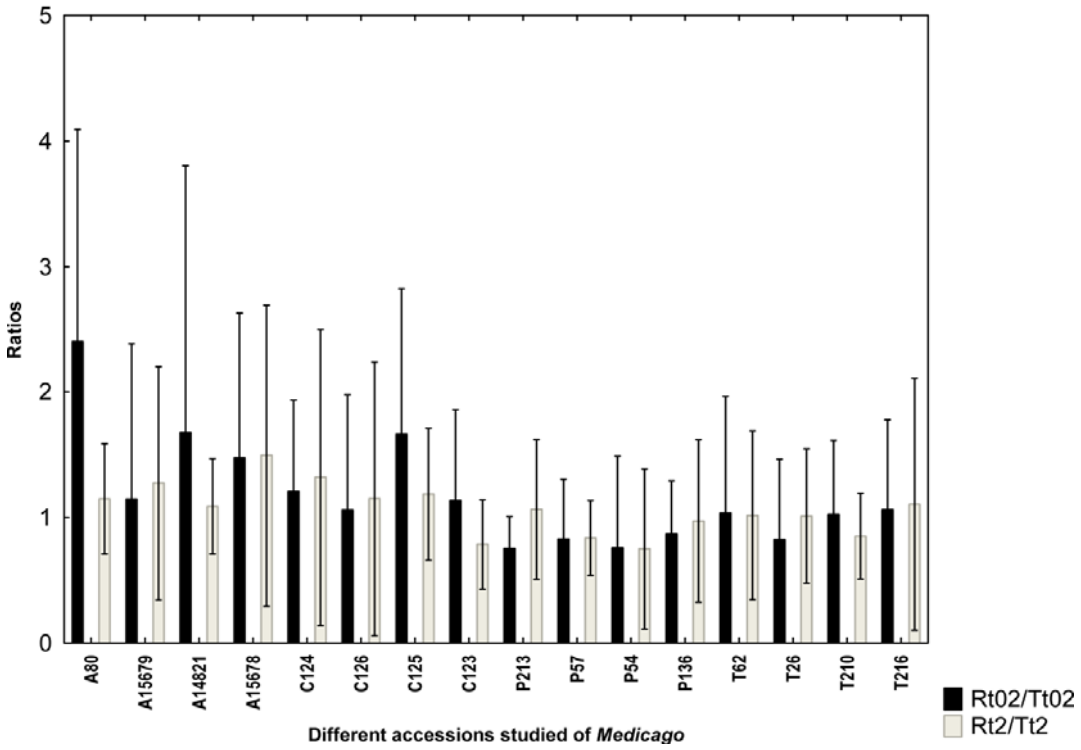

Figure 2. Ratios root to shoot after 8 days under 4 °C regime (Rt2/Tt2) and control (Rt02/Tt02), for different accessions studied of *Medicago*.

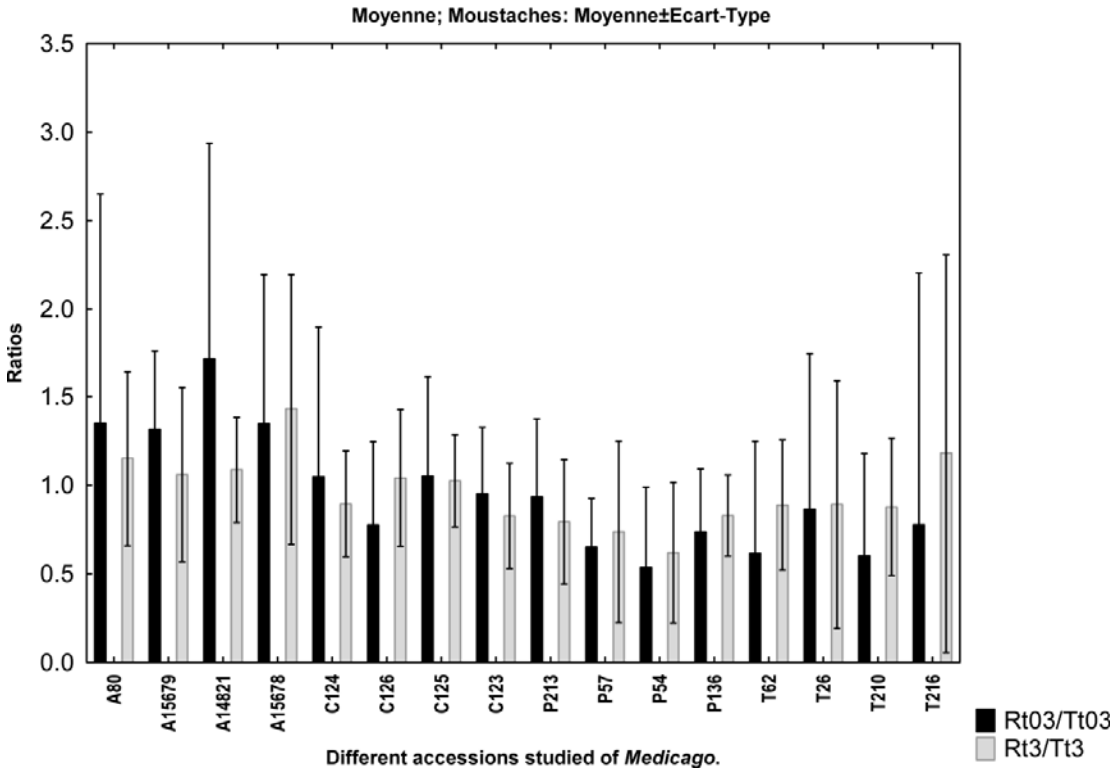

Figure 3. Ratios root to shoot after 11 days under 4 °C regime (Rt3/Tt3) and control (Rt03/Tt03), for different accessions studied of *Medicago*.

Figure 4. Example of SSR variation at MTIC-451 locus on twelve accessions of *Medicago* on five individuals. Black arrows indicate different alleles. M. marker leader (bp); 1-12: accessions, Cil 126, Cil 123, Cil 124, Tru 26, Ac 15679, Ac 80, Ac 15678, Poly57, Poly 213, Poly 42, Poly 136 and Poly 54 respectively and T : control, Mercedes, Gabes, Jemalong and Magali, respectively.

Table 5. Number of alleles detected among 09 markers loci selected and percentage of homozygosity.

Loci	*M. ciliaris*	*M. truncatula*	*M. aculeata*	*M. polymorpha*	All species
ATPase456	1(100%)*	1(100%)	5 (77%)	3 (50%)	6 (79%)
Mtic338	3 (85%)	1 (100%)	4 (92%)	5 (100%)	7(93%)
Mtic082	1(100%)	1 (100%)	2 (100%)	4 (67%)	7 (91%)
mtic451	5 (100%)	3 (100%)	5 (85%)	2 (75%)	12 (88%)
B14B03	1 (100%)	1 (100 %)	3 (100%)	5 (92%)	10 (98%)
mtic135	3 (92%)	2 (100%)	3 (100%)	2 (100%)	8 (98%)
mtic343	2 (100%)	2 (100%)	6 (92%)	6 (83%)	12 (93%)
mtic131	2 (92%)	2 (100%)	4 (100%)	4 (83%)	06 (93%)
Mtic432	1 (100%)	2 (80%)	2 (100%)	6 (83%)	13 (93%)
Total	19 (97%)	15 (98%)	34 (94%)	37 (81%)	81 (92%)

*The percentage of homozygosity is indicated in parenthesis

5). The overall level of polymorphism of *M. ciliaris* was lower than for *M. polymorpha* and *M. aculeata*. Moreover, it was possible to differentiate the four species with nine microsatellite markers and it was possible to differentiate between populations for *M. aculeata.* The population structure analysis showed that differences between populations of *M. aculeata* were similar to those between species and that the individual 12-1 is atypical; it seemed that it is an interspesific hybrid (Figure 5). In all alleles detected, allele 135 detected at markers loci Mtic131, alleles (187 and 190) detected at Mtic432 and alleles (133 to 154) detected at Mtic079 seemed to have a relationship with cold tolerance and the geographical origin of accessions.

Particularly, the alleles detected on the level of Mtic131 and Mtic-079 seemed to be specific to cold tolerant populations at both *M. aculeata* (Ac 15678, Ac 80 and Ac 15679) and *M. polymorpha* (Poly 57 and Poly 213) species (Table 6). Recently, Avia et al. (2013) found that these markers loci (Mtic131, Mtic432 and Mtic079) were linked to a QTL for cold tolerance in recombinant strains of *M. truncatula* from tolerant parents. They showed also in this recombinant, that strains of *M. truncatula* derived from crosses between two accessions acclimated to low

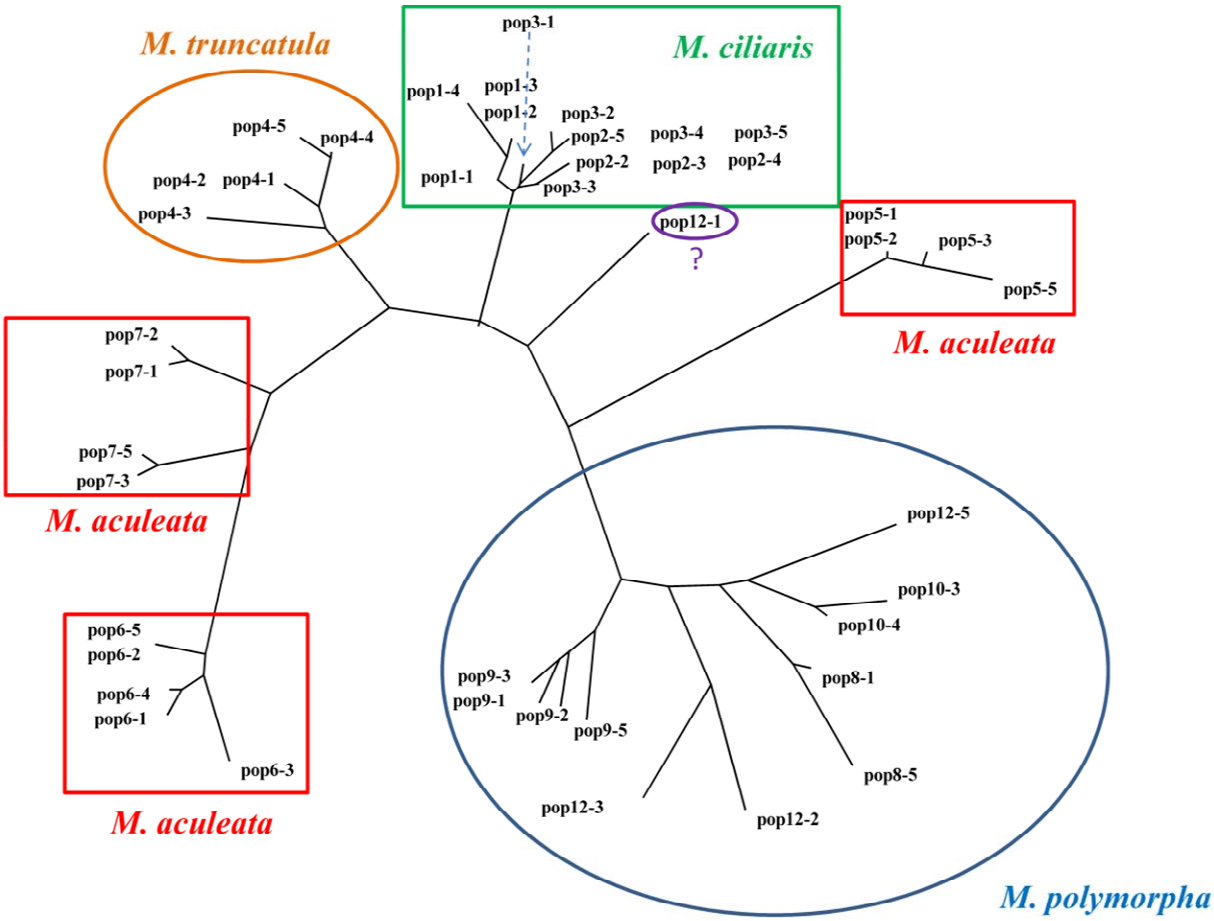

Figure 5. Nei standard Distance (1972), Neighbourg Joining, 1000 bootstraps, (no missing data). Pop1, Cil126; Pop 2, Cil 123; Pop 3, Cil 124; Pop 4, Tru 26; Pop 5, Ac 15679; Pop 6, Ac 80; Pop 7, Ac 15678; Pop 8, Poly 57; Pop 9, Poly 213; Pop 10, Poly 42; Pop 11, Poly 136 and Pop 12, Poly 54.

temperature and evaluation for cold tolerance by morphological characters showed

that the QTL associated with these markers (MTIC-131-432 MTIC, MTIC-079) had an additive effect and located at the liaison groups LG1, LG4 and LG6. These favorable effects of these additives alleles for cold tolerance were carried by both parents, suggesting that these ecotypes with a good ability to cold acclimation, effectively contributed to a good frost tolerance. These authors indicated that a relationship existed between the geographical origin of populations of *M. truncatula* and cold tolerance. Dias et al. (2008) highlighted some concordance between morphological characters and SSR markers in red clover (*Trifolium pratense* L.).

Bagavathiannan et al. (2010) investigating the genetic diversity of natural populations and cultivated alfalfa, find that there was correlation between phenotypic variables and SSR markers used. Badri et al. (2007) demonstrated in a study of genetic diversity among *Medicago ciliaris*, using morphological markers and locus SSR markers that the divergence of populations was due to local adaptation via genotype interactions / eco-geographical factors and these populations had a high plasticity to adapt too many different environments.

Conclusion

In conclusion, the investigation of cold acclimation ability at an early stage of development showed a significant variation between different populations for cold acclimation ability and cold tolerance. Moreover, this study demonstrated that accessions from high latitude areas had a high rate of root to shoot ratio. Ac 80, Ac and 15678 of *M. aculeata,* two populations of *M. polymorpha* Poly 136 and Poly 57, Cil 125 and Cil 126 of *Medicago ciliaris* and Tru 62 and Tru 216 of *M. truncatula* had been found that their degree of acclimation is more efficient for tree durations of treatment. This permitted to conclude clearly that Cold acclimation ability, at an early stage, was a good marker for cold stress tolerance. In total, 03 markers were identified that were associated with cold

Table 6. Alleles detected in tolerant accessions at marker loci Mtic432, Mtic079 and Mtic131, in parenthesis (size in bp).

Marker (allèles)	Accessions											
	Cil126	Cil123	Cil124	Ac15679 (T)	Ac80 (T)	Ac15678 (T)	Poly57 (T)	Poly 213 (T)	Poly 42	Poly136	Poly54	Tru 26
Mtic432												
(187)					+	+						
(190)				+		+						
Mtic079												
(133)				+	+		+					
(140)						+	+					
(154)				+	+							
Mtic131												
(135)				+		+	+	+				

(T), cold tolerant, **+**, allele detected only in the tolerant accessions

tolerance in two species, *M. polymorpha* and *M. aculeata*. Particularly, the microsatellite marker, Mtic-432, was identified only in *M. aculeata*. The fact that there was no structure between populations for *M. polymorpha* and *M. ciliaris* (to be confirmed with more individuals and markers) while there were differences between populations of the same species (*M. aculeata*) for cold acclimation was encouraging from the perspective of an association study between cold acclimation and molecular polymorphism.

Conflicts of interest

The authors declare that they have no conflict of interest.

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