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Full Length Research Paper

Study of genetic polymorphism of *ARTEMISIA HERBA-ALBA* from Tunisia using ISSR markers

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ARTEMISIA HERBA-ALBA **is an herbaceous aromatic and therapeutic plant widely distributed in semi-arid regions of Tunisia and is potentially usable to restore degraded ecosystems. A study of genetic variation among 216 accessions was conducted using ISSR (Inter Simple Sequence Repeat) markers to assess the polymorphism at the species level. A total of 60 polymorphic loci were scored using four primers revealing a high level of genetic polymorphism among *A. HERBA-ALBA* accessions. Correlation analysis revealed no direct relation between morphological traits, geographic distance and genetic distance. Correlogram analysis showed a patchy distribution of the genetic variability of *A. HERBA-ALBA* accessions revealing the contribution of local ecological and geographic conditions on variability.**

Key words: *Artemisia herba-alba*, DNA extraction, genetic polymorphism, ISSR markers.

INTRODUCTION

The genus *Artemisia* L. (Asteraceae) comprises a variable number of species (from 200 to over 400, depending on the authors) found throughout the northern half of the world (Marco and Barbera, 1990). According to Tutin et al. (1976), the genus may be divided into sections *Artemisia* and *Dracunculus*, the former including the species *Artemisia herba-alba* which also is a medicinal and aromatic dwarf shrub that grows wild in arid areas of the Mediterranean basin, extending into northern Himalayas (Vernin et al., 1995). This species has a vegetative growth in autumn (large leaves) and then at the end of winter to spring (small leaves). It is traditionally known for its essential oils. It has a very pronounced purgative effect and playing a major role in the control of intestinal worms (Idris et al., 1982). Extracts from *A. herba-alba* have antidiabetic effect (al-Shamaony et al., 1994; Al-Waili, 1986; al-Khazraji et al., 1993; Jouad et al., 2001) and strong antibacterial activities (Hatimi et al., 2001; Neerman, 2003). It also shows an allelopathic role against some other plants (Escudero et al., 2000). Flavonoids from this plant have a neurological action (Medhat Salah and Jäger, 2005).

In Tunisia this plant is found from the semi arid climatic stage to the Saharan stage (between 400 and 90 mm of annual precipitations). It is widely distributed in pre-Saharan regions (Le Floc'h et al., 1989; Le Houérou, 1969). A karyosystematic analysis of different populations of this plant from pre Saharan Tunisia revealed the existence of two cytotypes (Ferchichi, 1997), a diploid one with n = 2x = 9 and a tetraploid one with n = 4x = 18. The differences between these two cytotypes allow the classification of both races as being different taxons (Ferchichi, 1997). A study of morphological traits variability conducted on 226 accessions of *A. herba-alba* from different bioclimatic stages of Tunisia revealed a high level of polymorphism (Chaïeb, 2000). Some accessions showed many adaptations to local ecological conditions. Some other studies pinpointed a high chemical polymorphism of essential oil from *A. herba-alba* defining different chemotypes for this plant (Salido et al., 2004; Lamiri et al., 1997).

The morphological and chemical polymorphism observed for *A. herba-alba* may be due to a genetic variability. To investigate this variability, we studied the genetic polymorphism of this plant on the DNA level using inter simple sequence repeats (ISSR) markers. ISSR markers involve amplification of DNA by a single primer 16 - 18 bp long composed of a repeated sequence anchored at the

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Table 1. Accessions names and origins.

Name	Number	Origin	Climatic stage	Name	Number	Origin
AIN	5	Ain Inba (Medenine)	A*	HS	3	Hassi Amor (Medenine)
AL	2	Alamet (Medenine)	A	JB	5	Jbel Brighith (Gabes)
AM	3	Amaymia (Gafsa)	S-A**	K	5	Krikria (Medenine)
AMR	4	Amra (Medenine)	A	KA	3	Khanguet Aïcha (Gabes)
B	5	Bir Lahmar (Tataouine)	A	KB	5	Kebili
B3	5	Bir Thaline (Tataouine)	A	KH	5	Ksar Hallouf (Medenine)
BA	4	Bir Amir (Tataouine)	A	KM	7	Ksar Mahadha (Tataouine)
BG	2	Boughrara (Medenine)	A	KMR	1	Kamour (Tataouine)
BH	5	Bou Hedma (Gafsa)	S-A	KR	5	Kirchaou (Tataouine)
BK	5	Beni Khdech (Medenine)	A	M	3	Matmata (Gabes)
BLK	4	Belkhir (Gafsa)	S-A	MB	2	Mazraet Ben Salem (Gabes)
BO	5	Bou Omrane (Gafsa)	S-A	MD	5	Medenine
DH	2	Dhiba (Tataouine)	A	MK	4	Meknessy (Sidi Bouzid)
DR	4	Darghoulia (Medenine)	A	MZ	5	Mezzouna (Sidi Bouzid)
DT	4	Toujaine (Gabes)	S-A	OI	5	Ouargigen (Tataouine)
EL	2	Ellabba (Medenine)	A	OM	5	Oued Mansour (Gafsa)
ELM	3	El Meguecem (Gafsa)	S-A	OMA	5	Oum Ali (Gabes)
ES	2	Essnam (Medenine)	A	OZ	5	Oum Zouggar (Tataouine)
F	5	Ferch (Tataouine)	A	RF	4	Route Feriana (Gafsa)
FA	2	Faïdh (Sidi Bouzid)	S-A	S	3	Smar (Tataouine)
G	5	Ghomrassen (Tataouine)	A	SA	3	Sâadane (Medenine)
GA	4	Gattoufa (Tataouine)	A	SM	1	Smar (Medenine)
GB	3	Gabes	S-A	SN	3	Sned (Gafsa)
GF	5	Gafsa	S-A	ST	3	Sidi Toui (Medenine)
GH	5	Chenini (Tataouine)	A	SU	2	Suitir (Medenine)
GT	4	Guettar (Gafsa)	S-A	T	3	Tejra (Medenine)
H	2	Haddaj (Gafsa)	S-A	TT	5	Tataouine
HCH	3	Hichria (Sidi Bouzid)	S-A	Z	2	Zmerten (Gabes)
HM	5	Hamma (Gabes)	S-A			

A: arid, **S-A: semi arid.

3' or 5' ends by 2 - 4 arbitrary nucleotides (Zietkiewicz et al., 1994). They are easy to handle, highly informative and reproducible. Since repeated sequences are abundant throughout the genome, SSR primers anneal in several regions typically giving a complex amplification pattern in which fragments are often polymorphic between different individuals.

ISSR primers anneal directly to simple sequence repeats and thus, unlike SSR markers, no prior knowledge of target sequence is required for ISSRs (Godwin et al., 1997). Also, the sequences targeted by ISSRs primers are abundant throughout the eukaryotic genome and evolve rapidly. Consequently ISSRs may reveal a much higher number of polymorphic fragments per primer than RAPDs (Fang and Roose, 1997; Esselman et al., 1999). In addition, studies have indicated that ISSR produce more reliable and reproducible bands compared with RAPDs because of the higher annealing temperature and longer sequence of ISSR primers (Tsumura et al., 1996; Nagaoka and Ogihara, 1997). The principal limitation with the use of such molecular markers in *A. herba-alba* is its excessive richness in secondary metabolites (El-Kar, 2003). The presence of those metabolites can hamper the DNA isolation procedures and reactions such us DNA restriction, amplification and cloning.

MATERIAL AND METHODS

Plant origins

Two hundred and sixteen (216) accessions of *A. herba-alba* were used in this study. These accessions were collected and conserved since several years *ex situ* at the Institut des Régions Arides of Medenine. These accessions were originated from different regions of Tunisia including semi-arid and arid stages (Table 1).

PCR reaction

DNA extraction was conducted using the protocol of Linder et al.

Table 2. Summary of ISSR primers characteristics and genetic diversity indexes.

Primer	Sequence	Optimal Ta	Loci number	Bands size	PIC mean values	PI values
A830241	$(ACTG)_5$	54°C	15	530 - 2300	0.29548575	4.29781232
HB10	$(GA)_6CC$	43°C	17	240 - 1800	0.34665906	5.76258996
HB12	$(CAC)_3GC$	43°C	12	200 - 1000	0.36861625	4.42339504
17899B	$(CA)_6GG$	43°C	16	350 - 1600	0.41698291	6.59180436

(2000) with few modifications. Twenty four primers were tested for their ability to amplify ISSR markers. They are composed of di-, tri-, or tetra-nucleotide repetitions and are anchored or not in their 3' end. These primers were assessed for their ability to give clear, polymorphic and reproducible patterns of amplification. For ISSR assay, reaction conditions were optimized to get reproducible, unambiguous patterns. Optimization concerned $MgCl_2$ concentration, dNTP concentration, primer concentration, and annealing temperature. Amplification was performed in a total volume of 25 µl containing 2.5 µl PCR buffer (10x, Qbiogene), 1.5 µl $MgCl_2$ (20 mM, Sigma), 1 µl dNTP (10 mM, Qbiogene), 0.5 µl primer (40 µM, Eurogentec), 1 µl template DNA, 0.2 µl Taq polymerase (5 U/µl, Qbiogene) and 18.3 µl H_2O. PCR reactions were performed on a DNA thermal cycler with heated cover (Genius, Techne) with initial denaturation at 94°C for 2 min followed by 35 cycles of a. denaturation at 94°C for 45 s, annealing (Ta) at optimal temperature (Table 2) for 45 s and extension at 72°C for 2 min. A final extension at 72°C for 5 min was included.

Amplification products were resolved in 2% agarose gels (Sigma) with ethidium bromide (0.5 µg/ml) in 1X TBE (100 mM Tris-Borate pH 8.0, 2 mM EDTA) buffer at 100 V. Molecular weights were estimated using 100 bp DNA ladder. The ISSR profiles were visualized under ultra violet light, photographed with a video camera, and stored for further analysis.

Data analysis

Only intensely stained, unambiguous and reproducible bands were scored for presence (1) or absence (0). Pair-wise comparisons of accessions, based on the presence or absence of unique and shared fragments produced by ISSR markers, were used to generate similarity matrix based on the simple matching (SM) coefficient (Sokal and Michener, 1958) as Sij = a + d/a + b + c + d, where Sij is the similarity between two individuals, i and j, a is the number of bands present in both i and j, b is the number of bands present in i and absent in j, c is the number of bands present in j and absent in i and d is the number of bands absent in both i and j. The SM coefficient was calculated using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System for personal computers) software, version 2.11 (Rolf, 2000).

The information content of ISSR markers system was calculated for each locus using the polymorphism information content (PIC) (Smith et al., 1997) which provides an estimate of the discriminating power of a locus by taking into account not only the number of alleles expressed but also their relative frequencies. Calculations were made using following formula:

$$PIC = 1 - \sum_{I=1}^{N} P_I^2$$

Where p_i is the frequency of the i^{th} allele. In the case of ISSR, the PIC was considered to be $1-p^2-q^2$, where p is band frequency and q is no band frequency (Ghislain et al., 1999). PIC values were then used to calculate a primer index (PI), which is generated by adding up the PIC values of all the markers amplified by the same primer (Raina et al., 2001).

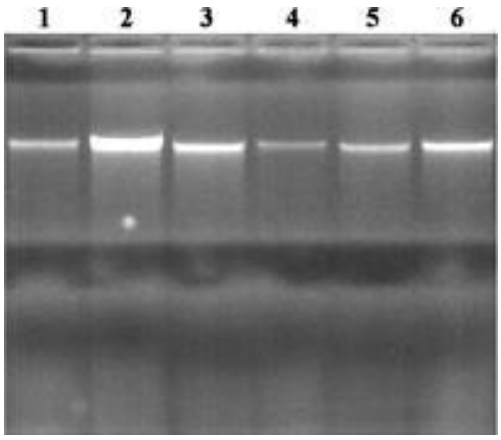

Figure 1. Total DNA extracted from different accessions of *Artemisia herba-alba*.

Non metric multidimensional scaling (NMDS) analysis was applied to the similarity matrix in order to reveal possible grouping of individuals using the program NTSYS-pc. This analysis was a function minimization algorithm that evaluates different configurations with the goal of maximizing the goodness of fit. Stress index measures reliability of the NMDS plot: the smaller the stress index, the better the fit of the reproduced distance matrix to the relative distances on the plot. To find the relationship between genetic composition and geographic distribution of the accessions we used the Mantel test (1967). Mantel test was realized using the NTSYS-pc software. To study the spatial structure of the genetic diversity distribution, we used a spatial autocorrelation analysis (Sokal and Oden, 1978). This technique overcomes the limitations of Mantel test which is only sensitive to linear relationships of spatial autocorrelation (Heywood, 1991). We used Moran's *I* statistic (1950) included in the PASSAGE software (Rosenberg, 2001) to construct a correlogram to analyze this autocorrelation.

RESULTS

DNA extraction

Isolation of good quality DNA from *A. herba-alba* proved difficult because leaves are very rich in polyphenolic compounds. These compounds can link covalently to DNA which becomes useless in enzymatic based reactions (PCR, restriction). By trying a set of protocols developed by Linder et al. (2000) to extract DNA from difficult plant material (old roots) we developed a suitable protocol for DNA extraction from *A. herba-alba* leaves. This protocol gave a DNA with a sufficient quality to PCR reaction (Figure 1).

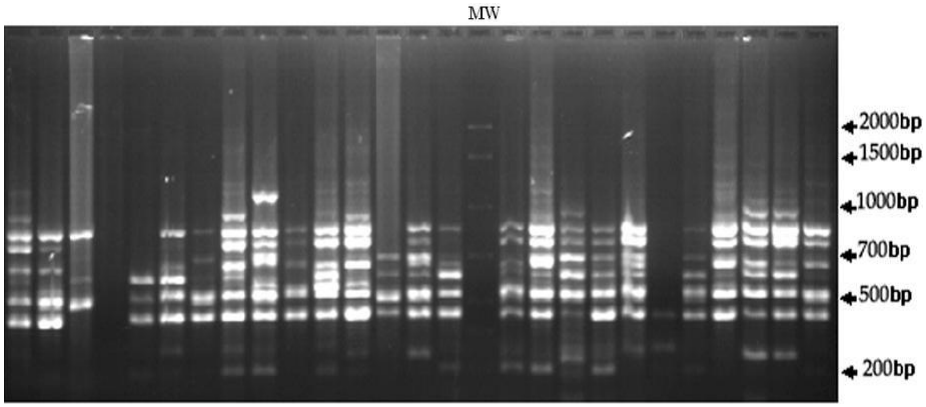

Figure 2. Example of ISSR amplification profile (primer HB10).

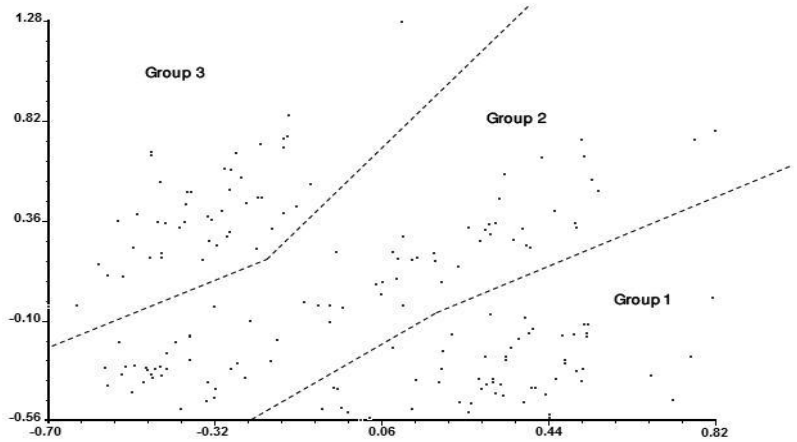

Figure 3. NMDS distribution of the genetic diversity generated by ISSR markers.

Genetic diversity

Twenty four primers were evaluated for their ability to produce polymorphic bands with a subset of *A. herba-alba* samples. Twenty primers were discarded because they gave no amplification or the patterns were monomorphic. The four remaining primers were selected. They produce interpretable and variable banding patterns. An example of the polymorphism detected among some accessions by primer 17899B is shown in Figure 2. For the 216 accessions, these four primers produced 60 scorable polymorphic bands with an average frequency of 15 bands per primer. The primer HB10, composed of dinucleotide (GA) repetitions (Table 2) gave the highest number of bands (17 bands) and the primer HB12 composed of a trinucleotide repetitions, gave the lowest number of bands (12 bands). The size of the bands amplified by the ISSR primers ranged from 200 bp, with primer HB12, to 2300 bp, with primer A830241. The percentage of polymorphic bands is 100%.

To characterize the capacity of each ISSR marker to reveal polymorphic loci in *A. herba-alba*, we analyzed the PIC content. The calculated PIC values for ISSR markers ranged from 0.1 to 0.49. The highest mean PIC value (0.42) is given by primer 17899B (Table 2) indicating that this primer are the most polymorphic. The primer A830241, which give the lowest mean PIC value of 0.29, is the less polymorphic. The primer index (PI) ranged from 4.29 for the primer A830241 to 6.59 for the primer 17899B (Table 2). These PI values confirm that primer 17899B is the most efficient in detecting polymorphisms among *A. herba-alba* accessions.

The similarity between *A. herba-alba* accessions was estimated by the SM coefficient. Pair-wise values of SM coefficient of similarity ranged from 0.38 to 0.88. The lowest coefficient is observed between accessions KA2 and SA1 and the highest similarity coefficient between Z1 and Z2. The SM similarity coefficient matrix was used for NMDS analysis. The minimal stress coefficient obtained is 0.036. The NMDS distribution shows a disposition of *A. herba-alba* accessions in three groups (Figure 3) composed respectively of 70, 90 and 56 accessions. The first group is essentially composed of accessions from the regions of Medenine and Tataouine which are the

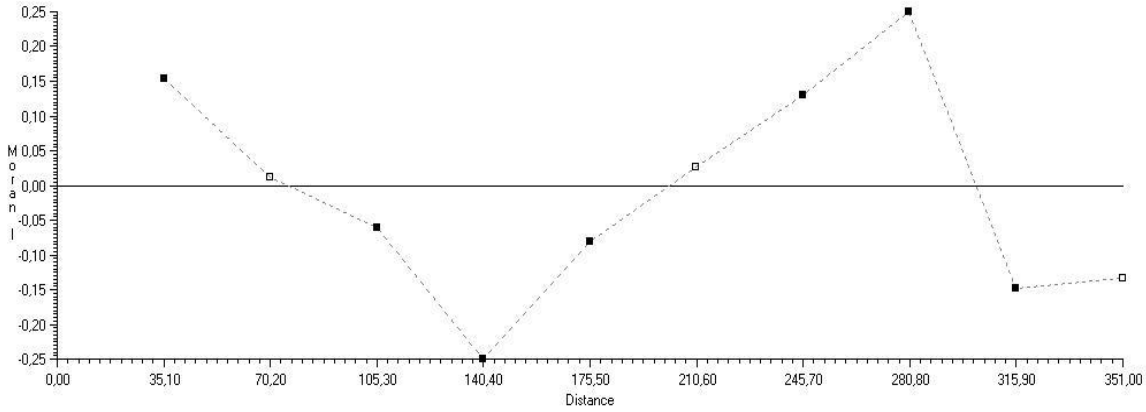

Figure 4. Moran's *I* correlogram showing the correlation between classes of geographic distances and genetic distances. (□ non significant value; ■ significant value).

most arid regions. So it seems that accessions from this group are the most tolerant to drought. The third group is composed of accessions originated from regions with the highest level of hygrometry so they are the less tolerant to drought. The second group is an intermediate group with accessions from regions located between the two regions.

Correlation analysis

Mantel test gave week correlation (r = 0.05910) between genetic distances matrix and geographic distance matrix indicating that the isolation by distance (IBD) process is not the principal actor in the generation of the diversity. Because Mantel test is only effective in detecting linear correlations, we analyzed Moran's *I* correlogram to verify the correlation at a lower level by dividing accessions in classes of geographic distances and testing the correlation for each distance class. We get a globally significant correlogram ($p < 0.005$) as shown by the Bonferroni criterion. The correlogram has a sinusoidal shape with alternation between positive and negative significant Moran's *I* values (Figure 4). This particular shape of the correlogram indicates a patchy distribution of the variation (Radeloff et al., 2000).

DISCUSSION

A. herba-alba is a source of natural products. It produces a large amount of secondary metabolites of aromatic and medicinal importance. Thus, while working with this plant it is common to encounter problems arising from the presence of essential oils, polyphenols and other secondary metabolites in the lysate and the DNA preparations. The secondary compounds may hamper DNA isolation as well as any further reaction to be carried out on DNA preparations for example: restriction enzymes and PCR enzymes my be inhibited because of the presence of unusual substances (Sangwan et al., 1998; Khanuja et al., 1999).

Following the procedures described by Doyle and Doyle (1990), Dellaporta et al. (1983), and Porebski et al. (1997) we encountered several problems when trying DNA extraction from *A. herba-alba* leaves. Major encountered problems were poor quality DNA (as shown by dark colored suspension), very low yield, and total lack of PCR amplification. To get a DNA with an acceptable quality we made some modifications to the extraction buffer as suggested by Linder et al. (2000). These modifications concern: i. The use of CTAB (instead of SDS) to get less oxidized polyphenols. ii. Replacing TRIS by boric acid thus lowering buffer pH and minimizing polyphenols oxidization. iii. Increasing the concentration of the antioxidant agent. By applying these modifications, a good quality DNA useable in PCR amplification was obtained.

Like RAPD, ISSR uses arbitrary primers thus; the choice of efficient primers is of primary importance (Ruas et al., 2003; Pharmawati et al., 2004). By beginning with a large set of arbitrary primers and selecting those giving the better (polymorphic and reproducible) patterns we could avoid problems generally encountered when using these primers. Problems are essentially lack of amplification due to the absence of priming sites in target DNA, monomorphic patterns because no mutations are located in target sites, or false amplifications due to non optimized amplification protocols.

Out of 24 ISSR primers evaluated for their ability to amplify DNA from *A. herba-alba*, twenty primers were discarded because they gave no amplification or patterns were monomorphic. After PCR reaction optimization, the remaining four primers gave 60 polymorphic bands confirming that ISSR markers are valuable tools in the study of genetic polymorphism at species level. Primers with dinucleotide repetitions gave the highest number of bands and are the highest polymorphic with PI>5. These

high PI indicate that *A. herba-alba* DNA has a high mutation probability (Raina et al., 2001). This high mutation probability could be due to wide geographic distribution and considerable ecologic variation. These conditions imply an adaptation of *A. herba-alba* at morphological and physiological levels with accessions being highly adapted to local conditions. This adaptation is shown by NMDS distribution in three groups of *A. herba-alba* accessions. The first group corresponds to the tetraploid cytotype which is characterized by its high drought tolerance (Ferchichi, 1997) and the third group to the diploid cytotype less tolerant to drought. Thus this distribution pinpoints the disposition of *A. herba-alba* in Tunisia in three domains: a sub-Saharan domain, a semi-arid domain and between these two domains. Inside each domain this species seems to have acquired genetic adaptations to local conditions.

The correlation between genetic distance and geographic distance was non significant indicating the very limited effect of the geographic distance on variability distribution. Some authors (Chapman et al., 2003) obtained a strong correlation between the genetic diversity, as shown by ISSR markers, and geographic distance indicating the importance of IBD (Isolation By Distance) process in the generation of the diversity but in most of the studies (Camacho and Liston, 2001; Bahulikar et al., 2004) IBD process has a lower importance because correlation values were very weak.

Non significant correlation values could be due to the existence of geographic barriers (González-Astorga and Núñez-Farfán, 2001; Epperson, 2000) that lead to a non linear relation between geographic and genetic distances. This non linearity could not be evident when tested by the Mantel test (Epperson, 2000; Epperson and Li, 1997; Epperson, 1995). To explain this non linear distribution we constructed a correlogram of Moran's *I* index. The significance of this correlogram indicates that the distribution of the variability is not random. The shape of the correlogram indicates that *A. herba-alba* genetic variability is patchy distributed confirming the results of NMDS analysis. The generation of polymorphism seems to act locally as a result of the adaptation to local ecological and geographic conditions which are extremely variable in the south of Tunisia.

Conclusions

In this study we developed a protocol for DNA extraction from leaves of *A. herba-alba*. This protocol could be used for other species from the genus *Artemisia* or for other species with a high content of secondary metabolites. By using ISSR markers we demonstrated the potential use of these markers as genetic tools in the study of the genetic variability of *A. herba-alba* at the species level. These markers revealed a high level of genetic polymor-phism for this species. Genetic distances vary in a large interval indicating a high level of intra specific heterogeneity. This heterogeneity reflects the ecologic differences between accessions as shown by NMDS distribution in three groups as a function of the bioclimatic stage. The IBD process model failed to explain the origin of this polymorphism as Mantel test values are non significant indicating that differentiation process is not linear. By introducing non linear models, using correlograms, we could explain that variability is patchy distributed, thus adaptation to micro ecological and environmental conditions play a major role in the generation of polymorphism in *A. herba-alba* populations.

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