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# RAPD and ITS-based variability revealed in *Atriplex* species introduced to semi-arid zones of Morocco

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ABSTRACT

Variability related to RAPD markers and sequences of nrDNA ITS region has been studied in seven species of the genus *Atriplex* (*A. amnicola*, *A. canescens*, *A. halimus* from Morocco (MAR) and from USA, *A. lentiformis*, *A. nummularia*, *A. semibaccata* and *A. undulate*). As a whole, the results show a high variability among the species. The biggest diversity was obtained by RAPD data, followed by ITSs. According to RAPD markers, two major groups can be distinguished, one formed by *A. semibaccata* and *A. undulata*, the least similar to the rest of species. *A. lentiformis* was closer to *A. halimus*. Phylogenetic analysis confirmed the divergence of *A. semibaccata* from all species and the closeness of *A. lentiformis* to *A. halimus*. The intraspecies variability was also high, as 39 individual RAPD haplotypes were obtained by analyzing 40 plants. Genetic diversity was bigger among species (60.23%) than among individuals (39.77%). The amplification of ITS region leads to three well-defined clades. The heterogeneous and larger clade includes all *A. amnicola* individuals and some individuals from *A. nummularia*, *A. lentiformis*, *A. halimus* USA, *A. halilmus* MAR and *A. undulata*. Both RAPDs and ITS analyses revealed with some exceptions, that all individuals from one species grouped together. RAPDS turn out to be more appropriate than ITS to differentiate *Atriplex* species. The two markers gave rise to the same species relationships, but to a different structure of the *Atriplex* genetic diversity. AMOVA analysis estimated interspecies differences for 11.20% of the total ITS variation.

Both the great variability and the apparently restrictive gene flow among the species are discussed considering the life history and characteristics of the plants studied.



## 1. Introduction

*Atriplex* is one of the most widespread plant genus, having colonized many arid and semi-arid regions of the world (Osman and Ghassali, 1997; Cibils et al., 1998). At present, ca. 330 *Atriplex* species, erect and prostrate shrubs and herbs, have been recognised in all continents except in Antarctica (Flores Olvera et al., 2006). Many of them exhibit a great resistance to drought and tolerance to salt (Abou El Nasr et al., 1996), hence their connote name of saltbushes. They usually colonize arid zones with an annual rainfall average from 100 to 250 mm, and occur even in Saharan areas, with less than 100 mm rain (Tazi et al., 1996). Arid and semi-arid lands constitute approximately one third of the World's land surface (Archibold, 1995), that is continuously expanding, probably as another landmark of the Global Weather Change. Consequently, many productive lands are degraded, and livestock productivity is increasingly suffering from feed shortage. To alleviate this problem, about 15 species of browse saltbushes have been introduced into arid and semiarid zones of West Asia and North Africa, to be added to the native saltbush, *A. halimus* L. (Le Houérou, 1996). After two decades of screening trials, only seven species are still considered worthwhile for the region: *A. nummularia* Lindl., *A. amnicola* PG. Wilson, *A. canescens* (Pursh) Nuttall. subsp. *canescens*, *A. canescens* subsp. *Linearis* (S. Wats.) Hall. and Clem., *A. undulata* (Moq.) De Dietr., *A. lentiformis* S. Wats. and *A. semibaccata* R. Br. (Le Houérou, 1996, 2000). They are being successfully used in Morocco as livestock forage, and for restoration of saline and degraded soils (Boulanouar et al., 1996). Since the mid 1980s a program of forage shrub planting has been established in several pastoral zones of Morocco. By the end of 1995, an area of 48,284 ha has been covered by *Atriplex* species (MAMVA, 1995).

*Atriplex* taxonomy is still equivocal (Welsh et al., 2003), mainly due to the lack of consistent morphological features. Nevertheless,

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it is generally recognized as having three subgenera. Welsh et al. (2003) recognized 3 subgenera, 10 sections and 9 subsections.

To date, few studies have been done on the genetic diversity of *Atriplex*. Hdadou (1996) analyzed physiological and biochemical aspects of peroxydase isozymes of nine *Atriplex* species. On the other hand, genetic diversity between several populations of the species *A. halimus* was carried out by using isozymes polymorphism (Haddioui and Baaziz, 2001; Mandàk et al., 2005). More recently, analysis of random amplified polymorphic DNA (RAPD) and internal transcribed spacer (ITS) region of 18–26S ribosomal DNA markers (Ortíz-Dorda et al., 2005) were also used to study population diversity within *A. halimus*. Therefore, to date there is still lacking data about the interspecies analysis of *Atriplex*.

Analyses of genetic variability of plants by studying different types of molecular markers offer more consistent as well as better-supported results (Morton et al., 1997). In many studies, RAPDs were used with other type of molecular markers (ITS, SSR, ISSR and AFLPs). On the other hand, ITS have proven to be useful to establish phylogenetic relationships among closely related genera and between species within genera in many angiosperm families (e.g., Baldwin, 1992).

Analysis of molecular variance (AMOVA), which is not influenced by the dominance problem, has become an important tool to investigate the partitioning of the RAPD (Huff et al., 1993) and ITS variation (Jorgensen et al., 2003). The main objective of our study was to analyze the genetic diversity and relationships of *Atriplex* species by considering RAPD markers and nucleotide sequences of ITS region. The apportionment of the variation among and within *Atriplex* species was estimated by the AMOVA statistical approach.

## 2. Materials and methods

### 2.1. Plant material

Eight taxa of *Atriplex* were used: *A. amnicola* (A), *A. canescens* (C), *A. halimus* from Morocco (HM), *A. halimus* from USA (HU), *A. lentioformis* (L), *A. nummularia* (N), *A. semibaccata* (S) and *A. undulata* (U). Each taxon was represented by one accession. Table 1 shows a summary of their geographical origin and the related molecular information (ITS). The seeds were obtained from the Centre de Production des Semences Pastorales (CPSP) orchard in Kmiss M'touh, El Jadida, Maroc, where the stock plants has been maintained on soil since 1985. The plants utilized throughout this work were grown in pots, in a mixture of sand, peat and vermiculite as substrate, and maintained in a greenhouse. The experiment was set up as a completely randomized design, with 10 replicates, each 1 of 5 plants.

### 2.2. DNA extraction and PCR reactions

Five individuals from each species were taken at random for DNA extraction. Genomic DNA from fresh young leaves was extracted with Nucleon Phytopure DNA extraction kits (Amersham Biosciences, UK Ltd.), following the instructions given by the supplier. DNA concentration was determined spectrophotometrically.

RAPD-PCR was performed after the protocol of Williams et al. (1990), slightly modified for optimization. The reaction mixture, in a final volume of 25 μl, contained: 40 ng of template DNA, 2 mM $MgCl_2$, 10× reaction buffer, 2 μM of each primer, 0.4 mM dNTPs, and 0.5 U of DNA polymerase (Biotools, Spain). Thirteen primers (OPA-02, OPA-05, OPA-09, OPB-01, OPB-03, OPB-06, OPC-07, OPC-08, OPC-15, OPD-08, OPD-11, OPD-15 and OPE-12, OPERON USA), displaying reliable banding patterns (Ortíz-Dorda et al., 2005) were used. PCRs were run in Primus 96 Plus (MWAG Biotech, Ebersberg, Germany) thermocycler through 45 cycles, each consisting of: 94 °C for denaturation step (1 min), 36 °C annealing step (2 min), and a 72 °C extension step (1 min), using the fastest available transitions between each temperature. Amplification products were electrophoresed on 1% agarose gels in TAE buffer, stained with ethidium bromide, and photographed under UV light by using KODAK 1D Analysis Software. Some RAPD-PCR reactions were repeated three times to ascertain the reproducibility of the banding pattern.

The ITS region was amplified using the primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCG-TAACAAGG-3′) described by White et al. (1990) in a single reaction. The region comprises the two spacers ITS1 (approximately 300 bp) and ITS2 (approximately 220 bp), along with the 5.8S gene (approximately 160 bp). The PCR reactions were performed in a volume of 50 μl containing: 40 ng of template DNA, 10 mM 10× PCR buffer (supplied with Fideli™ Taq DNA polymerase), 0.8 μM of each primer, 0.4 mM dNTPs, 1 mM $MgCl_2$, and 1 U of Fideli™ Taq DNA polymerase (USB Corporation, USA). Amplifications were conducted in Techgene (TECHNE, Cambridge, UK) thermal cycler through 45 cycles of 94 °C for 2 min, 50 °C for 30 s and 68 °C for 1 min. Successful PCR amplification produced a single band of approximately 700 bp. The amplicons were purified using the GFX PCR DNA purification kit (GE-Healthcare). DNA concentration of purified PCR products was estimated on agarose gel by comparison with a 1 kb DNA ladder (Sambrook et al., 1989). The purified products were sequenced, using the same primers utilized for PCR (ITS4 and ITS5), although for some individuals extra reactions were performed with the internal primers ITS2 and ITS3 (White et al., 1990) to eliminate ambiguities. Sequencing was carried out in the Genomic Service of *Universidad Autónoma de Madrid* (Spain) by using multicapillary Sequencer ABI Prism 3730 DNA Analyzer (Applied Biosystems, USA). Nucleotide sequences obtained were proofread, and contiguous sequences generated using the SeqMan v. 4.03, and then compiled into EditSeq v. 4.03, being both programs parts of the DANASTAR software package (LASERGEN, USA). Preliminary Blast searches confirmed that our products belonged to the *Atriplex* genus, based on their strong identity with other members of this genus. The ITS sequences obtained have been deposited in the EMBL database with the accessions numbers from AM420667 to AM420705 (Table 1).

### 2.3. Data analyses

The RAPD gel images were analyzed using the KODAK 1D image Analysis Software for finding and calculating the band size. RAPD bands were scored as present (1) or absent (0) to compile a binary matrix for cluster analysis using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) version 2.02 g (Rholf, 1998). Genetic similarity among individual plants was calculated according to Dice's similarity coefficients (Dice, 1945) using the SIMQUAL (Similarity for Qualitative Data) program. The similarity matrix obtained was then used to construct a dendrogram, using the UPGMA through the SHAN (Sequential, Hierarchical, Agglomerative and Nested clustering) routine of the NTSYS-pc package.

It was carried out the analysis of molecular variance (AMOVA; Excoffier et al., 1992), taking untransformed RAPD and ITS sequences data, to partition the total genetic variance into two hierarchical levels (among species and within species). The AMOVA was based on genetic distance calculated by the number of pairwise differences between haplotypes for RAPD data and the Kimura's (1980) 2-parameter method for the ITS sequences to correct the nucleotide multiple substitutions per site between each pair of sequences. The pairwise genetic differentiations (FST)