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Genetic diversity characterization of genus *Atriplex* using RAPD markers

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Abstract

The genus *Atriplex* is spread out in all continents, except Antarctica, and includes many species useful for rehabilitation of degraded soil, revegetation and animal production. *Atriplex* exhibits a high morphological and physiological variability that can be used to establish genetic relationships between species and populations. Variability is an important feature to select *Atriplex* genotypes adapted to harsh conditions, but it also strongly affects the data reproducibility of genetic analysis.

Molecular markers offer an effective tool for estimation of genetic diversity. Random Amplified Polymorphic DNAs (RAPDs) have being extensively used as highly polymorphic molecular markers. We used thirteen primers (1) to assess genetic variation of forty individual plants of eight Moroccan *Atriplex* species. To assure RAPD pattern reproducibility some reactions were repeated three times.

Amplification rendered 220 intense bands that were scored as present or absent across all species, with 11 to 21 bands for each primer. 95% of the loci revealed to be highly polymorphic, indicating that RAPDs are an abundant source of polymorphic markers in *Atriplex* species.

The results obtained show that RAPD variability was mostly at inter-specific level, being the intra-specific variability much lower.

The RAPD analysis reported here confirms previous studies made using morphological and isozyme markers. It also proved to be a reliable and fast tool for assessing the genetic diversity of *Atriplex*.

Key words: species, *Atriplex*, RAPDs, markers, genetic diversity.

Introduction

Arid and semi arid lands constitute approximately one third of the world's land surface (Archibold, 1995). The surface of these areas is in continuous increasing, mainly, due to high frequency of drought. Consequently livestock productivity has continuously suffered from feed shortage and many degraded lands have been generated.

The plants belonging to genus *Atriplex* species are the most geographically widespread (Cibils et al., 1998) and growing naturally in arid and semi arid regions of the world. More than 400 *Atriplex* species have been recognised in all continents except Antarctica (Le Houerou, 1992). These plants occur in the arid zone with annual rainfall average ranging from 100 to 250 mm and even in Saharan areas that receive less than 100mm rain (Tazi et al., 1996). Many species of *Atriplex* are considered useful as forage for livestock and wildlife, and for revegetation of saline and degraded soils.

Atriplex are annuals, perennials or shrubs. *Atriplex spp.* originated from Australia and have spread to arid and semi arid parts of the world (Osman et Ghassaeli, 1997). It is often grown as fodder plants in drier areas because of its great resistance to drought and tolerance to salt (Abou El Nasr et al, 1996), which qualified it as saltbushes.

Most saltbushes have been successfully used for rehabilitation of rangelands, and of fauna and flora (Boulanouar et al., 1996). Since the mid of 1980s a program of forage shrub planting have been established in several pastoral zones of Morocco. By the end of 1995, an area of 48,284 ha had been planted (MAMVA, 1995).

Atriplex species are erect or prostrate shrubs, grow up to 2m high and spread to 2.4m wide, with white branches. These species have been classified as monoecious and sometimes diecious, but recently Talamali et al (2001) have pointed that it could be also trimonoecious. Albeit, the genus *Atriplex* has been described as relatively sex labile (Freeman and McArthur, 1984; Rennerand and Ricklefs, 1995; Walsh, 2005). Some studies demonstrated that genus *Atriplex* contain a high genetic diversity. However this latter have been found on the basis of morphological, physiological and biochemical characters (Hdaddou, 1996; Haddioui and Baaziz, 2001). Nevertheless, these characteristics are often affected by environment and consequently, many traits become difficult to analyse. DNA-based markers offer an effective alternative to study genetic diversity and relationships between species (Gepts, 1993). Random Amplified Polymorphic DNA (RAPD) has proved useful in many genetic studies (Oiki et al., 2001; Jorgensen et al., 2003; Trindade and Chaves, 2005). In the current study we report the use of RAPD markers for assessing the genetic diversity structure and relationships of eight *Atriplex* species.

Materials and methods

Plant material

Eight species of *Atriplex* were used in this study (Table 1). The trees of theses shrubs are growing and maintaining, since 1985, at the CPSP (Centre de Production des Semences Pastorals) orchard located at Kmiss M'touh, El Jadida, Maroc. The seeds of these materials were obtained from this centre. It had been sown in pots on a 2/3:1/3 mixture of peat and vermiculite under greenhouse.

Table 1. List of Atriplex species used in the study, with their vernacular name and their origin.

Species	Vernacular name	Origin	Abbreviation
Atriplex amnicola	River saltbush or swamp saltbush	USA	A
Atriplex canescens	Four-Wing salt bush, Chamisa, Cenizo	USA	C
Atriplex halimus MAR	Saltbush	Morocco	HM
Atriplex halimus USA	Saltbush	USA	HU
Atriplex lentioformis	Quail bush	USA	L
Atriplex nummularia	Old Man saltbush, Giant saltbush	Morocco	N
Atriplex semibaccata	Australian saltbush, Creeping saltbush	USA	S
Atriplex undulata	Wavy leaf saltbush	USA	U

DNA extraction and PCR reactions

Five individuals from each species were chosen at random for DNA extraction. Total DNA from young leaves was extracted with nucleon Phytopure DNA Extraction Kits (Amersham Biosciences) after grinding the material in the liquid nitrogen. DNA concentrations were measured using spectrophotometer. RAPD-PCR was carried out according to the protocol of Williams et al. (1990) with some modifications. Thirteen primers (Operon Technologies) of sets A, B, C, D and E, displaying reliable banding patterns, were used for PCR amplification (Dorda et al., 2005). Amplifications were performed in Primus 96 Plus thermal cycler 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).

Amplifications products were electrophoresed on 1% agarose gels in TAE buffer stained with ethidium bromide and photographed under UV light with red filter. Some RAPD-PCR reactions were repeated three times to ascertain the reproducibility of banding pattern.

Data scoring and analysis

RAPD bands were scored as present (1) or absent (0) to compile a binary matrix for cluster analysis. Dendrograms were constructed using UPGMA (Unweighted Pair-Group Method with Arithmetical Average) method and genetic similarity percentage between individuals and species. All the analysis were performed by the statistical software STATISTICA (StatSoft, Inc.).

Results

The thirteen primer used in this study rendered 220 bands across forty *Atriplex* individuals, with 11 to 21 bands per primer, 16.92 as average (Table 2) and their size range from 260 to 2340 bp. Of the 220 bands, 209 (95%) were polymorphic and eleven (5%) were monomorphic among the *Atriplex* individuals. This indicates that RAPDs are an abundance source of polymorphic markers in genus *Atriplex*. The optimised RAPD-PCR protocol resulted in highly reproducible banding patterns. The banding patterns obtained showed a high differences between species and individuals of some species (Fig. 1). The dendrogram reveals considerable inter-specific diversity, with a clear separation of individual plants (Fig. 2). In fact, by analysing forty plants we have obtained 40 RAPD-PCR haplotypes. The dendrogram also shows the grouping of species into eight separate clusters; each species alone form a cluster. This indicates that there is a significant structuring and separation of *Atriplex* species, hence a high genetic diversity between species. The UPGMA phenogram shows that *Atriplex undulata* and *Atriplex semibaccata* to be the most divergent from the remaining groups, with only a 58% genetic similarity to the remaining six species. It is notable that both species are 64% similar. Whereas, the American *Atriplex halimus* population (HU) exhibited a 77.5% genetic similarity with *Atriplex lentiformis*, and both are 72.5% similar to Moroccan *Atriplex halimus* population (HM), and these three species joined a group formed by *Atriplex canescens* at a genetic similarity of 62.5%. *The Atriplex nummularia* has revealed to be related to *Atriplex amnicola* by 63.75% of genetic similarity. The UPGMA phenogram shows that the species clustered following a pattern tentatively related in part with morphology and in other part with taxonomy. In the phenogram, five individuals from the same species clustered together except for one individual of *Atriplex nummularia* (N4), which clustered with the individuals of *Atriplex lentiformis*. The number of primers utilized and /or the number of RAPD polymorphism scored bands can determine the informativeness and reliability of the data collected for genomic similarity studies (Bhat and Jarret, 1995). It was recommended that at least 50 loci should be studied (Nei, 1978). The number of individuals subsets clustered by using successive combinations of primers was found similar between all dendrograms (Table 3).

Table 2. Sequences of the thirteen primers, with the number of scorable amplified and polymorphic bands.

Primer	Sequence	No. of amplified bands	No. of polymorphic bands
OPA-02	TGCCGAGCTG	17	17
OPA-05	AGGGGTCTTG	17	16
OPA-09	GGGTAACGCC	14	13
OPB-01	GTTTCGCTCC	19	18
OPB-03	CATCCCCCTG	18	17
OPB-06	TGCTCTGCCC	15	15
OPC-07	GTCCCGACGA	20	20
OPC-08	TGGACCGGTG	18	16
OPC-15	GACGGATCAG	14	14
OPD-08	GTGTGCCCCA	17	15
OPD-11	AGCGCCATTG	19	18
OPD-15	CATCCGTGCT	21	19
OPE-12	TTATCGCCCC	11	11
Range		11-21	11-20
Mean		16.92	16.07
Total		220	209

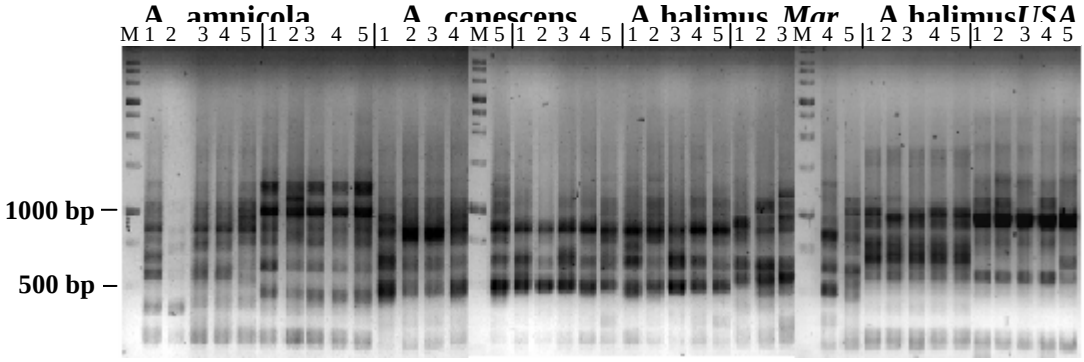

Figure 2. Example of an agarose gel showing the amplified DNA patterns obtained with a RAPD-PCR reaction with primer OPD-11 and five plants of eight *Atriplex* species. M, 1 kb ladder marker.

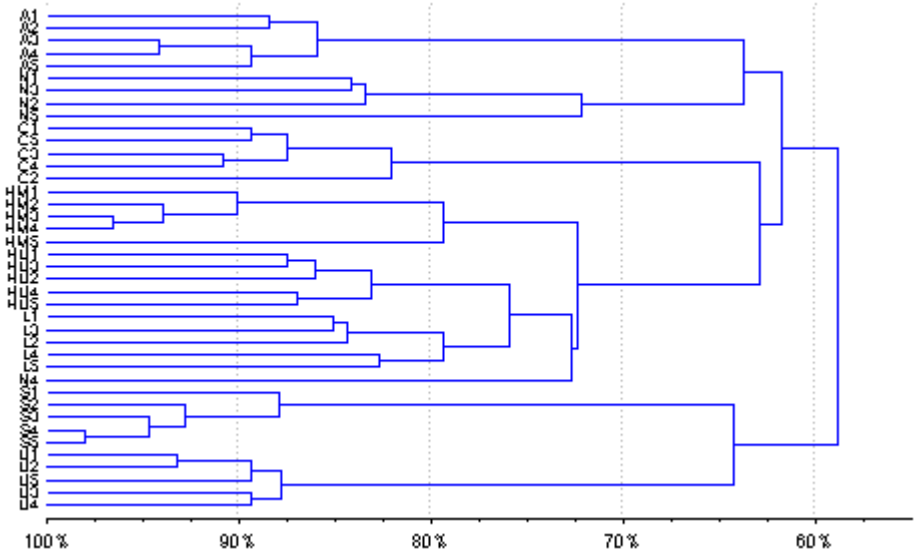

Fig. 3. Dendogram illustrating genetic relatedeness among 40 *Atriplex* individuals generated by the UPGMA cluster Analysis based on 209 RAPD-bands.

Table 3. Numbers of subsets distinguished by primer combinations

Dendrogram number	Primers combinations	No. of Polymorphics bands	No. of subsets distinguished
D1	OPA-02	17	40
D2	OPA-02, OPA-05	33	40
D3	OPA-02, OPA-05, OPA-09	46	40
D4	OPA-02, OPA-05, OPA-09, OPB-01	64	40
D5	OPA-02, OPA-05, OPA-09, OPB-01, OPB-03	81	40
D6	OPA-02, OPA-05, OPA-09, OPB-01, OPB-03, OPB-06	96	40
D7	OPA-02, OPA-05, OPA-09, OPB-01, OPB-03, OPB-06, OPC-07	116	40
D8	OPA-02, OPA-05, OPA-09, OPB-01, OPB-03, OPB-06, OPC-07, OPC-08	132	40
D9	OPA-02, OPA-05, OPA-09, OPB-01, OPB-03, OPB-06, OPC-07, OPC-08, OPC-15	146	40
D10	OPA-02, OPA-05, OPA-09, OPB-01, OPB-03, OPB-06, OPC-07, OPC-08, OPC-15, OPD-08	161	40
D11	OPA-02, OPA-05, OPA-09, OPB-01, OPB-03, OPB-06, OPC-07, OPC-08, OPC-15, OPD-08, OPD-11	179	40
D12	OPA-02, OPA-05, OPA-09, OPB-01, OPB-03, OPB-06, OPC-07, OPC-08, OPC-15, OPD-08, OPD-11, OPD-15	198	40
D13	OPA-02, OPA-05, OPA-09, OPB-01, OPB-03, OPB-06, OPC-07, OPC-08, OPC-15, OPD-08, OPD-11, OPD-15, OPE-12	209	40

Discussion

The results obtained show an average number of 16.07 polymorphic bands per primer in genus *Atriplex*, while Ortiz-Dorda et al. (2005) have found a mean of 9.5 in *Atriplex halimus*. Others studies show 16.7 in sweetpotato (He et al., 1995), and 18.6 in pea (Samec and Nasinec, 1996). Our study indicates that reproducibility of RAPD technique can be very high if experimental parameters were standardized.
In general, the more polymorphic fragments are amplified by primers, the more information about genetic diversity could be obtained. Nei (1978) stated that a minimum number of 50 different loci should be used for estimating genetic distances. Thence the number of primers used is a consideration in phenetic analysis. The present work, which used 209 polymorphic RAPD bands (loci) to study the genetic diversity and relatedness among *Atriplex* species, is accurate.
Different primers combinations were used in cluster analysis of *Atriplex* species to test the ability of primer combinations to discriminate between species and individual plants of *Atriplex*. The maximum number of 40 subsets clustered by UPGMA analysis could be obtained only by 17 bands (Table 3). The general topology of the dendrogram was conserved when the bands of the primers were added successively to generate dendrograms (from D1 to D13). These dendrograms retained their structure, with eight clusters.

Little was known about the genetic diversity and relationships within the genus *Atriplex*. Until now, one study DNA markers-based about genetic variability of *Atriplex*. This was made by Ortiz-Dorda et al. (2005), assessing genetic structure of 51 populations of *Atriplex halimus* from the Mediterranean Basin using RAPD. The authors found that there are a clear differentiation of *Atriplex halimus* populations and also a considerable separation of individual plants in the same population. These results are consistent in some parts with ours, which concerned eight species of genus *Atriplex*. Besides, the same authors have analysed the phylogeny of *Atriplex halimus* with four other *Atriplex* species as outgroups using internal transcribed spacer regions. They found that *Atriplex canesnens* are relatively close to *Atriplex halimus*, which corroborate our results.

The UPGMA phenogram (Fig. 2) suggests that gene flow is more restricted because individuals from the same species clustered together (Fig 2). This could be due to predominately monoecious character of genus *Atriplex* (Talamali et al, 2001). However, considering that an individual from *Atriplex nummularia* and five individuals from *Atriplex lentiformis* species form one cluster on the phenogram, gene flow may occasionally occur between species. The similar results were found by Oiki et al. (2001). In fact, *Atriplex lentiformis* can be confused morphologically with *Atriplex nummularia*. The species trees are growing nearly each to other in the orchard of CPSP, possibly permitting some gene flow between *Atriplex nummularia* and *Atriplex lentiformis*. Gene flow via seeds is likely because distance between both species is court and seeds could be dispersed by wind or water currents. Furthermore, the gene flow via pollen is also possible seeing that both species, *Atriplex nummularia* and *Atriplex lentiformis*, are predominately dioecious (Stanley, 2000).
The species concerned in this investigation have exhibited, in previous study, a large variability in morphological and forage traits. Thus, the present work confirms that these differences between species have a genetic basis and provides genetic markers to avoid sometimes morphology identification confusion between *Atriplex* species.
The RAPD analysis reported here showed that genetic diversity of genus *Atriplex* reside at inter-specific level, being the intra-specific lower. It may be said that RAPD analysis used correctly, have proved to be a reliable and fast tool for assessing the genetic diversity of Atriplex and hence sould be encouraged.
The present study is the first step in assessing the genetic structure of species belonging to genus *Atriplex* using RAPDs markers. Further studies using more species of Atriplex may provide a better understanding of genetic diversity and relationships in this genus.

Acknowledgements

We thank Dr Tazi M., Centre de Production des Semences Pastorales, El Jadiada, Maroc, for supplying the seeds of *Atriplex* species. This work has been funded by Moroccan-Spanish Interuniversity Cooperation Program (AECI) through project 36/04/P/E and supported by a fellowship (contrat grant number 03/019) to Bouda S. from CNRST, Morocco.

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