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Evaluation of genetic variability of sorghum (*Sorghum bicolor* L. Moench) in northwestern Morocco by ISSR and RAPD markers

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Abstract

The study of the genetic variability of the Moroccan landraces of sorghum constitutes a necessary step that can be exploited in the programs of improvement and valorisation of this marginalized species. The aim of this investigation is to evaluate the variability of sorghum populations and to establish their phylogenetic relations using RAPD and ISSR markers. Sampling was taken in 33 fields of northern regions where this species is most cultivated. Individual plants (398) were collected in 13, 11, 5, and 4 fields of Larache, Tangier, Chefchaouen, and Tetouan, respectively. Thirty-eight RAPD primers and four ISSR primers were used. The percentage of polymorphic fragments revealed with ISSR (98%) is higher than the one revealed with RAPD (85%). The level of the variability obtained through the two techniques is very high. Nevertheless, ISSR markers revealed more diversity than RAPD (0.995 ± 0.006 against 0.946 ± 0.031). The classification based on Jaccard's similarity index distinguished the totality of fields. Data analysis revealed a genetic structure that is closely related to the micro-geographical repartition of the different fields. ***To cite this article: L. Medraoui et al., C. R. Biologies 330 (2007).***
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Résumé

Évaluation de la variabilité génétique de sorgho grain (S*orghum bicolor* L. Moench) dans le Nord-Ouest du Maroc par les marqueurs RAPD et ISSR. L'étude de la variabilité génétique des variétés-populations de sorgho grain marocain constitue une étape nécessaire à exploiter dans les programmes d'amélioration et de valorisation de cette céréale marginalisée. L'objectif de cette étude est d'évaluer la variabilité des populations de sorgho et d'établir leurs relations phylogénétiques via les marqueurs RAPD et ISSR. L'échantillonnage a été réalisé dans 33 champs au niveau de la région nord du Maroc, où cette espèce est largement cultivée. Il concerne 398 individus collectés respectivement dans 13, 11, 5 et 4 champs de Larache, Tanger, Chefchaouen et Tétouan. Trente-huit amorces RAPD et quatre amorces ISSR ont été utilisées. Le pourcentage de polymorphisme révélé avec ISSR

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(98%) est supérieur à celui révélé avec RAPD (85%). Le niveau de diversité obtenu par les deux techniques est élevé. Néanmoins, l'ISSR a montré plus de diversité que le RAPD ($0{,}995 \pm 0{,}006$, contre $0{,}946 \pm 0{,}031$). La classification selon l'indice de similarité de Jaccard a permis la distinction de la totalité des champs étudiés. L'analyse des données a révélé une structure génétique qui est étroitement liée à la répartition micro-géographique des différents champs. ***Pour citer cet article : L. Medraoui et al., C. R. Biologies 330 (2007).***

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Keywords: Sorghum bicolor L. Moench; Moroccan landraces; Genetic variability; RAPD; ISSR

Mots-clés : Sorghum bicolor L. Moench ; Variétés-populations ; Variabilité génétique ; RAPD ; ISSR

1. Introduction

The sorghum (*Sorghum bicolor* L. Moench) is one of the major cultivated cereals in the world.

It has been domesticated for approximately 3000 years before J.-C. in the Ethiopian regions of Africa [1–3]. The cultivated sorghum is subdivided into five races (*guinea*, *kafir*, *durra*, *bicolor*, and *caudatum*) and ten inter-races hybrids [4,5]. The sorghum is largely cultivated in Africa, where it is mostly the first human food. In fact, this spring cereal is known for its ability to survive the severe environmental conditions [6] and for the nutritious value of its grains (71% of starch, 10% of proteins, and 3% of lipids), which is similar to the case of the other cereals, justifying its importance in the arid and semi-arid regions. However, the production rates in Africa still remain weak compared to the rates of improved lineages grown in the USA with high yields (3 to 5 t/ha), because cultivated sorghums in Africa consist of local varieties with low yields (0.5 to 0.7 t/ha) [7]. The improvement of the productivity of this species constitutes a priority to overcome the demographic explosion of the African population [7].

The sorghum was introduced into Morocco with Islamisation, approximately ten centuries ago, and it was extended to the occidental Mediterranean area by the Arabs [8]. Moroccan sorghum is essentially represented by the *durra* race [9], which is related for its expansion toward Asia and Mediterranean to the history of Islamisation [2,10].

In Morocco, sorghum is considered a minor cereal and cultivated on 29,000 ha, corresponding to 0.3% of the Moroccan arable surface [9]. Nowadays, this species is a culture regularly practiced in the northwestern regions of Morocco at altitudes of 20 to 800 m. In these regions, sorghum constitutes one of the most important cereals by the extent of its surfaces in comparison to corn, and by its food-producing character [9]. The Moroccan production fluctuates around 6 q/ha and never exceeds 9 q/ha [9]. These low yields are assigned to the traditional agricultural techniques based on old methods, lands parcelled out, and to the use of non-improved seeds. These seeds were not exchanged between regions, and they generate the local value of the 'ecotype' [9]. Thus, the knowledge of the genetic structure of these ecotypes is important, since these are populations of weak dimensions and in progressive regression. The phenotypic selection exerted by farmers has engendered landraces or locally adapted varieties. This selection has begun with the introduction of sorghum in Morocco and carried on about 900 to 1000 generations. Landraces are characterized by morphological differences (panicle, grain, awn, colour ...) that have been confirmed by several studies. A high degree of morphological variation among landraces, which were assigned to the race *durra*, *bicolor* and their intermediates, was noted [9]. Morphological characters and allozymes could account for 63% and 20%, respectively, of the variability between fields [11]. Morphological and enzymatic markers have been typically used to study the genetic variation of sorghum cultivars (e.g., [10–15]). Molecular markers constitute preferential tools to assess the genetic diversity and the phylogenetic relationships in sorghum; several studies have used the following analytical methods: RFLP markers [16–18], mitochondrial DNA markers [19], RAPD markers [20], and microsatellites markers [21,22].

In this investigation, we analyzed DNA bulks using RAPD and ISSR markers, which have never been used on the Moroccan sorghum. This approach allows us to get more genetic information on the studied populations. Our objective was to evaluate the genetic variability of northwestern fields, and to estimate the level of the variation between the studied regions. The obtained data were compared to those obtained with other markers (morphological traits, allozymes, and SSR) in the same regions.

2. Materials and methods

2.1. Plant materials

Seed samples were directly collected within fields (in situ sampling varying from 6 to 17 individuals) in four regions of northwestern Morocco: Larache, Tangier, Chefchaouen, and Tetouan (Fig. 1). Table 1 shows the plant material used for this study (accessions Lb1 to Teb5) and its geographical origin by region.

2.2. DNA extraction

DNA was extracted from a bulk of a totality of individuals for each field (population) as described by [23], with major modifications. Three grams of fresh leaves, stored at −80 °C, were ground in liquid nitrogen, mixed with 10 ml of CTAB extraction buffer [Tris

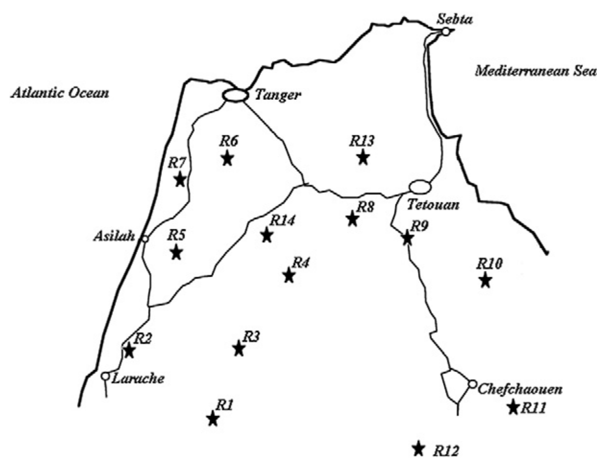

Fig. 1. Geographical location of the studied sorghum cultivation regions in northwestern Morocco.

Table 1
Accessions list and geographical origin of the *Sorghum* plant samples

Accession (field)	Province	Region	Locality	Number of individuals per field
Lb1	Larache	R1	Oulad ben Lahcen	12
Lb2			Naaimiine	12
Lb3			Boujediane	10
Lb4			Maâizate	10
Lb5		R2	Oulad Soltan	12
Lb6			Arrbaâ Ayacha	10
Lb7			Douar hjrat el Garni	10
Lb8			Douar Ain Mouloud	10
Lb9			Mghazlienne	10
Lb10			J'miaât 1	11
Lb11			J'miaât 2	11
Lb12		R3	Luxus	12
Lb13			Khmis sahel/Arrihiyine	13
Tab1	Tanger	R4	Douar oulad larbi/Boutil	13
Tab2			Takrii	11
Tab3			M'nazla	11
Tab4		R5	Had Bghaghaz Dar chaoui	10
Tab5		R6	Route Tanger-Tetouan1	10
Tab6			Route Tanger-Tetouan2	10
Tab7			Zaînate	15
Tab8		R7	Route Asilah-Tanger	4
Tab9			Forêt diplomatique	16
Tab10			Melloussa douar el Mekhfi	10
Tab11			Douar Ain Homra	12
Chb1	Chefchaouen	R8	Ouad laou	19
Chb2		R9	Talembote	6
Chb3		R10	Taghzoute	15
Chb4		R11	Dardara1	16
Chb5			Dardara2	17
Teb2	Tetouan	R12	Beni Maaden	11
Teb3			Douar Lhbar	13
Teb4		R13	Khemis Anjra	17
Teb5		R14	Beni Harchin	12

0.22 M pH 8.0, EDTA 0.22 M, Sorbitol 0.14 M, NaCl 0.8 M, *N*-laurylsarcosine 1% (w/v), CTAB 1.5% (w/v), and β-mercaptoethanol 1% (v/v), incubated at 55 °C for 1 h, and then cooled on ice and mixed with 5 ml of a phenol/chloroform/isoamylic alcohol (v/v, 25/24/1) mixture. The tube was centrifuged at 10,000 *g* for 5 min and the supernatant transferred into a new tube. The DNA was precipitated by cool isopropanol, washed with 75% ethanol, treated by the RNAse A and then by proteinase K before being reprocessed again to the phenol/chloroform/isoamylic alcohol mixture, and reprecipitated with NaCl and absolute ethanol. Centrifugation was performed at 10,000 *g*. The pellet was dried, resuspended in TE buffer [Tris-HCl 10 mM; EDTA 0.1 mM, pH 8.0]. DNA concentration and quality were determined through 0.8% agarose gel electrophoresis in 1X TBE buffer [Tris-HCl pH 8.0, 0.89 M; voric acid 0.89 M; EDTA 25 mM, pH 8.0] [24] with ethidium bromide staining, and by spectrophotometric measurements at 260 and 280 nm.

2.3. DNA amplification by RAPD and ISSR

The polymerase chain reaction (PCR) was run according to the modified protocol of [25] for RAPD and of [26] for ISSR, in a final volume of 25 µl containing 10 mM Tris-HCl pH 8.3 (at 25 °C), 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM aliquots of dATP, dCTP, dGTP, and dTTP (Promega), 0.2 µM RAPD primer or 4 µM ISSR primer, 100 ng of genomic DNA, and 1 unit of Ampli Taq DNA polymerase (Roche). The amplifications were performed on a GenAmp thermal cycler (Applied Biosystems, CA, USA) for RAPD following the program: at 95 °C for 5 min, 45 cycles (1 min at 95 °C, at 35 °C for 1 min, at 72 °C for 2 min) and final elongation of 6 min at 72 °C, and for ISSR: at 94 °C for 7 min, 32 cycles of 30 s at 94 °C, 45 s at 45 °C, 2 min at 72 °C, and final extension at 72 °C for 7 min. A total of 81 (10 bp) random primers (AD02, AK01-04, AM01-05, AN01-04, B10, C01-07, D01-05, E01-05, F01-05, G01-05, H01-05, I01-05, AF01-05, M01, R01-03, S01-08, T01-08, U02-03, U09 et Y15) (Operon Technologies, Almeda, CA, USA) were used in RAPD, and 4 primers F01: $(CA)_6AT$, F02: $(CA)_6GC$, F03: $(CA)_6AG$ and F04: $(AGC)_4T$ from (Operon Technologies) were used in ISSR. RAPD and ISSR amplification products were analysed by electrophoresis onto 1.2% and 2.8% agarose gels, respectively, run in 1XTBE and visualized by staining with ethidium bromide and illumination with UV light.

2.4. Data analysis

The parameter 'polymorphism information content' (PIC) was calculated according to reference [27]: $\mathrm{PIC}_{ij} = n\ (1 - \sum P_{ij}^2)/(n-1)$, where n is the sample size, and P_{ij} is the frequency of the ith pattern revealed by the RAPD or ISSR primer j summed across all patterns revealed by the primer j.

The electrophoretic gel images were processed with the aid of the Gel Compar II software (version 2.5; Applied Maths, Kortrijk, Belgium). Using the same program, RAPD and ISSR bands were scored as 0 for absence and 1 for presence in each field. Jaccard distances [28], based on similarity index, were calculated, dendrograms were generated using Ward algorithm [29] and multidimensional scaling (MDS) was configured to facilitate the graphical visualization of the relationship between the formed groups [30].

3. Results

3.1. RAPD analysis

The number of polymorphic fragments per profile varied per primer from 11 (OPU2) to 27 (OPI2), and ranged from 138 to 3992 bp (Table 2). The minimum size difference between any of the two polymorphic products generated by a primer was approximately 20 bp. Overall, 812 (85%) polymorphic fragments were generated, and 140 (15%) fragments were common to all accessions studied. Among polymorphic fragments, 25 unique markers were detected (3%): six fragments were specific for Tangier fields (AF-2, 1559 bp; E-3, 804 bp; H-3, 966 bp; T-1, 1742 bp; T-6, 762 bp and T-6, 656 bp), three for Larache fields (AM-2, 1054 bp; T-1, 489 bp and T-6, 1566 bp), four for Tetouan fields (C-6, 823 bp; R-1, 761 bp; S-7, 234 bp and S-7, 138 bp) and twelve for Chefchaouen fields (D-3, 381 bp; H-5, 2208 bp; I-2, 2459 bp; I-2, 2008 bp; R-1, 1905 bp; R-2, 2321 bp; R-2, 1998 bp; S- 6, 2795 bp; S-7, 1540 bp; T-4, 2829 bp; T-4, 2004 bp and T-6, 1672 bp). The diversity (D) values varied from 0.86 to 0.97, with an average of 0.946 ± 0.031 (Table 2). In addition, high heterogeneity among fields was observed (Fig. 2). The genetic distances calculated from these data, and represented in a dendrogram, allow the probable relationships among the fields to be evaluated (Fig. 3). Six principal clusters were obtained. The first cluster was composed by two sub-groups: the first regrouped three fields of Larache and the second contained two fields of Tetouan and one field of Larache. The second cluster comprised two sub-groups: the first contained two

Table 2
RAPD and ISSR markers information and diversity in 33 sorghum fields

	Primer	Molecular weight range, bp	Total number of markers	Number of markers		
				M	P	D
RAPD	OPAF2	476–3535	20	3	17	0.9200
	OPAF3	300–2455	22	3	19	0.8674
	OPAF4	395–3269	18	2	16	0.9438
	OPAK4	234–2420	25	4	21	0.9627
	OPAM1	341–2010	20	3	17	0.8785
	OPAM2	212–1624	18	2	16	0.8741
	OPAM4	304–2451	27	5	22	0.8996
	OPAN3	355–2939	29	4	25	0.9730
	OPB10	491–1851	16	3	13	0.8985
	OPC1	194–1733	25	4	21	0.9526
	OPC3	185–2232	31	5	26	0.9790
	OPC6	541–3369	24	3	21	0.9021
	OPD1	361–2304	30	4	26	0.9731
	OPD3	381–2361	23	3	20	0.9524
	OPD5	327–2523	28	5	23	0.9635
	OPE1	361–3255	21	2	19	0.9569
	OPE3	309–2323	27	4	23	0.9577
	OPE4	494–2014	18	3	15	0.9276
	OPF1	185–3104	25	4	21	0.9402
	OPF3	419–3174	25	5	20	0.9525
	OPF4	212–2951	24	2	22	0.9671
	OPF5	333–3520	24	4	20	0.9329
	OPG2	182–1920	26	3	23	0.9449
	OPG3	447–3992	29	4	25	0.9618
	OPH3	411–2100	26	3	23	0.9714
	OPH5	241–2208	29	4	25	0.9767
	OPI2	267–2752	33	6	27	0.9768
	OPI3	189–2421	30	4	26	0.9629
	OPR1	330–3100	28	5	23	0.9658
	OPR2	358–2600	28	4	24	0.9633
	OPS6	342–2795	23	3	20	0.9451
	OPS7	138–1847	29	4	25	0.9717
	OPT1	199–2380	28	3	25	0.9726
	OPT4	195–2829	31	5	26	0.9744
	OPT5	300–3293	28	3	25	0.9708
	OPT6	415–2638	24	3	21	0.9518
	OPU2	583–1940	15	4	11	0.9190
	OPU3	233–1639	25	5	20	0.9539
	Total		952	140	812	**0.946 ± 0.031**
Polymorphism percentage					**85%**	–
ISSR	$(CA)_6AT$	314–2562	36	1	35	0.9855
	$(CA)_6GC$	183–1931	44	0	44	0.9989
	$(CA)_6AG$	122–2317	50	1	49	0.9984
	$(AGC)_4T$	160–1638	47	2	45	0.9964
	Total		177	4	173	**0.995 ± 0.006**
Polymorphism percentage					**98%**	–

fields of Larache, one field of Chefchaouen, and one of Tetouan. The second sub-group was represented by two fields of Chefchaouen. The third cluster included four fields of Larache. The fourth cluster was represented by three fields of Tangier, one of Larache, and one of Tetouan. The fifth one was composed by two subgroups: the first one regrouped two fields of Larache and two fields of Chefchaouen, whereas the second subgroup comprised three fields of Tangier. Five fields of Tangier were found in the sixth cluster.

The spatial configuration of multidimensional scaling elaborated based on RAPD data allowed the distribution of the fields on three sectors according to the X-, Y-, and Z-axes to be established.

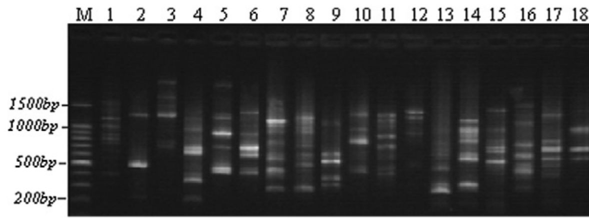

Fig. 2. Example of an RAPD polymorphism obtained with the OPAN3 primer through TBE-agarose gel electrophoresis. M: 100-bp ladders; 1–5: Lb1, Lb3, Lb6, Lb8, and Lb10; 6–10: Tab1, Tab4, Tab6, Tab9, and Tab11; 11–14: Chb1, Chb2, Chb3, and Chb5; 15–18: Teb2, Teb3, Teb4, and Teb5.

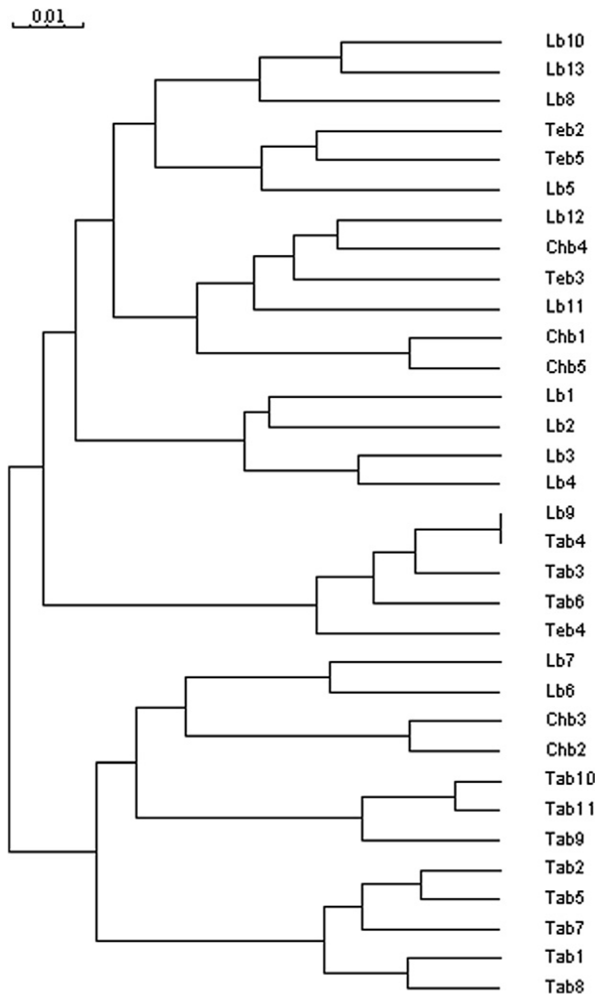

Fig. 3. Dendrogram obtained with Jaccard's similarity coefficients using RAPD data of 33 fields of *Sorghum bicolor* L. Moench (Lb1–13; Tab1–11; Chb1–5; Teb2–5). The tree was constructed using the Ward algorithm.

The (A) sector contained eight fields of Tangier and two fields of Larache, the (B) sector grouped six fields of Larache, three fields of Tangier, and two fields of

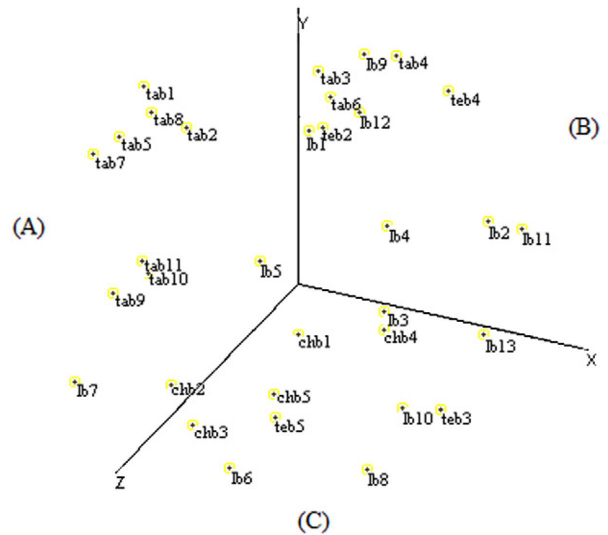

Fig. 4. Scatter diagram of the MDS analysis of Moroccan sorghum accessions based on RAPD data.

Tetouan, and the (C) sector was represented by the entirety of Chefchaouen fields, five fields of Larache, and two fields of Tetouan (Fig. 4).

3.2. ISSR analysis

The number of polymorphic fragments was important for the four primers, with an average of 43 bands, and ranged in size from 122 to 2562 bp (Table 2). The minimum size difference between any two polymorphic products generated by a primer was approximately 10 bp. Generally, 173 (98%) polymorphic fragments were generated, and 6 (2%) fragments were common to all accessions. Among polymorphic fragments, four different classes of markers were distinguished: (1) markers that were found in the four studied regions – Tangier, Larache, Chefchaouen and Tetouan (39%) –; (2) markers that were common only between the fields of Larache and some fields of Tangier (33%); (3) markers that were common between both fields of Tetouan and Chefchaouen and some fields of Tangier (10%); (4) a class of 31 unique markers (18%). Fourteen fragments were specific to the Larache fields (F-1, 715 bp; 534 bp; 320 bp; F-2, 715 bp; 534 bp; 183 bp; F-3, 1459 bp; 675 bp; 568 bp; 217 bp; 174 bp; 167 bp; F-4, 1638 bp; 161 bp), 13 to the Tangier fields (F-1, 2562 bp; 1170 bp; 919 bp; 359 bp; F-2, 1458 bp; 1369 bp; 989 bp; 376 bp; 330 bp; F-3, 2317 bp; 209 bp; F-4, 1638 bp; 161 bp), two to the Chefchaouen fields (F-1, 631 bp and F-4, 209 bp), and two to the Tetouan fields (F-3, 141 bp and F-4, 466 bp). The diversity (D) values varied from 0.98 and 0.99, with an average of 0.99 ± 0.006.

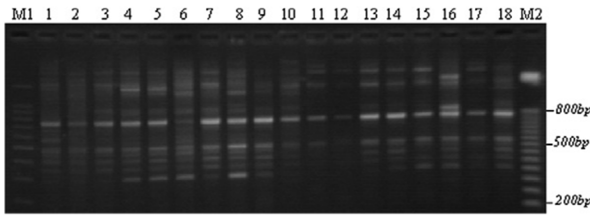

Fig. 5. Example of an ISSR polymorphism obtained with the F1 primer through TBE-agarose gel electrophoresis. M: 100-bp DNA ladders; 1–5: Lb1, Lb3, Lb6, Lb8, and Lb10; 6–10: Tab1, Tab4, Tab6, Tab9, and Tab11; 11–14: Chb1, Chb2, Chb3, and Chb5; 15–18: Teb2, Teb3, Teb4, and Teb5; M2: 50-bp DNA ladders.

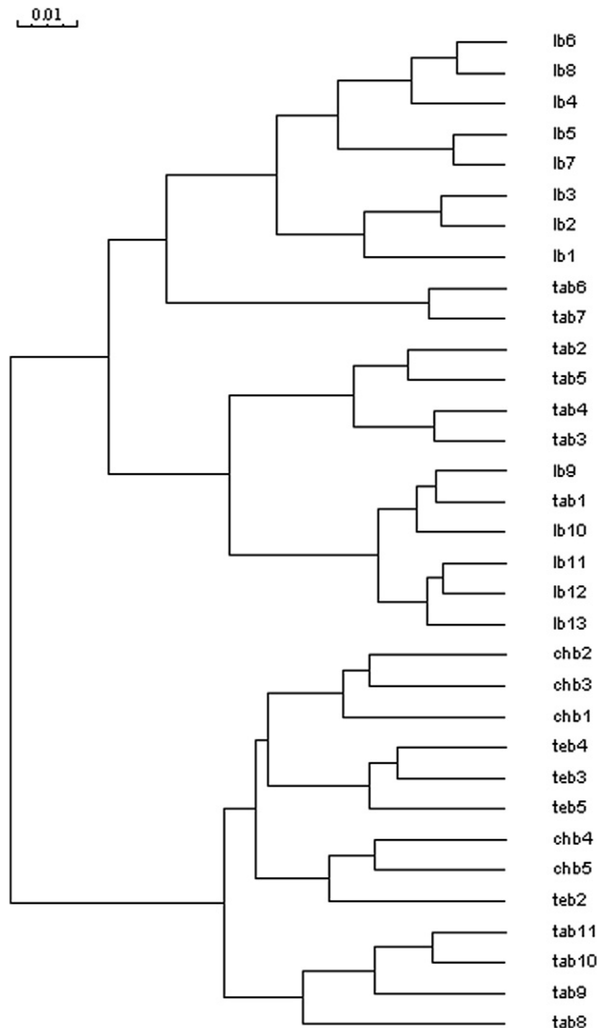

Fig. 6. Dendrogram obtained with Jaccard's similarity coefficients using ISSR data of 33 fields of *Sorghum bicolor* L. Moench (Lb1–13; Tab1–11; Chb1–5; Teb2–5). The tree was constructed using the Ward algorithm.

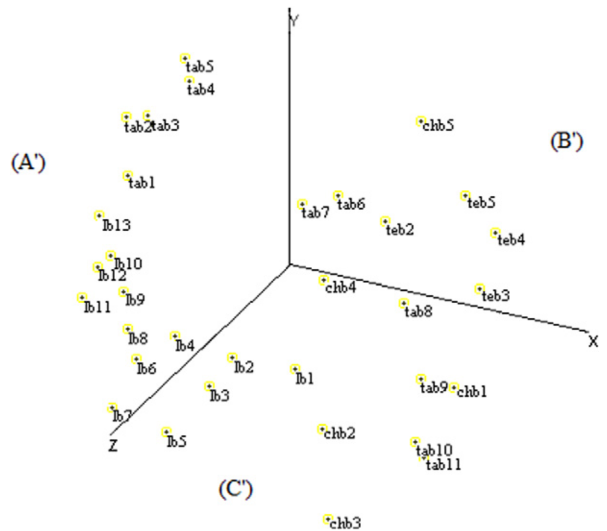

Fig. 7. Scatter diagram of the MDS analysis of Moroccan sorghum accessions based on ISSR data.

High heterogeneity among fields was observed and each field was distinguished (Fig. 5). The genetic distances were calculated from the obtained data, allowing the probable relationships among the fields to be evaluated (Fig. 6). Two principal clusters were obtained. The first cluster was composed of two sub-groups: the first one regrouped eight fields of Larache and two fields of Tangier, whereas the second sub-group was divided into two parts, the first regrouping four fields of Tangier and the second five fields of Larache associated with one field of Tangier. The second cluster was composed by two sub-groups; the first one was divided into three parts: the first part included three fields of Chefchaouen, the second part contained three fields of Tetouan, and the third part regrouped two fields of Chefchaouen and one field of Tetouan; the second sub-group was represented by four fields of Tangier.

The distribution of fields by MDS based on ISSR data determined three sectors according to the X-, Y-, and Z-axes. The (A′) sector contained nine fields of Larache and five fields of Tangier, the (B′) sector grouped five fields of Tetouan, two fields of Tangier and one field of Chefchaouen, and the (C′) sector was represented by four fields of Chefchaouen, four fields of Larache, and four fields of Tangier (Fig. 7).

4. Discussion

DNA analysis using RAPD and ISSR markers on seed bulks was a useful tool to assess the genetic diversity of the Moroccan populations of *Sorghum bicolor* L. Moench. A great number of bands were obtained

with each primer and for the two types of markers. The totality of RAPD and ISSR primers were highly informative, PIC values were close to 1 [27]. The variability level between the different fields revealed by both RAPD and ISSR is very important. Our results are in keeping with the works of Djè et al. [11], who showed a large proportion of morphological variability between fields, while allozymes and microsatellites revealed more intra-field than inter-field variability [21].

Additional region-specific polymorphic fragments were found in this study using the RAPD and ISSR techniques. RAPD revealed 13 specific markers for Larache, 6 for Tangier, 4 for Tetouan and 12 for Chefchaouen. ISSR revealed 14 specific markers for Larache, 13 for Tangier, 2 for Tetouan, and 2 for Chefchaouen. These results suppose the probable presence of specific genomic zones that are common to the totality of the studied populations belonging to the same region. These methods revealed more diversity than the use of morphological traits, allozymes, or microsatellites previously used to distinguish between regions [9,19]. The specificity of the ISSR allowed us to divide studied fields into two groups according to two classes of markers: the first ones are common to the fields belonging to the west of Tangier and all fields of Larache, and the second ones are common to the fields belonging to the east of Tangier and to all fields of Tetouan and Chefchaouen. These data show that there exists an influence of the micro-geographical distribution of sorghum fields on the structuring of their variability, while Djè et al. [11] have also demonstrated that there was no correlation between geographical distances and micro-geographical and allozymic data. Our results confirm those found by Djè et al. [12], who showed that quantitative characters, in contrast to qualitative traits, are influenced by location, topography, and soil fertility.

Compared to RAPD, the clustering and multidimensional scaling obtained by ISSR groups more clearly defines fields according to their geographic distribution. The flexible reproduction system of the Moroccan sorghum combining autogamy and allogamy supposes genes flow between fields to be in favour of a similarity between nearby fields and therefore between fields belonging to the same region [31]. Our results are confirmed by data obtained with the morphological analysis carried out by Kadiri and Ater [9], who found that Moroccan sorghum would belong to two cultivars of the race *durra*, *durra*, and *cernuum*, which occupied two domains of distribution that are geographically different. The first domain is internal and the second domain is coastal external of the Rifian chain. Moreover, the first field of Larache (lb1, Ouled ben Lahcen) was isolated in both ISSR and RAPD clustering. This can be explained by the introduction of seeds of American cultivars during trials [9].

The local seeds of the sorghum constitute veritable populations qualified as ecotypes that have resulted from a long process of selection carried out by the farmer in order to choose adapted plants. The polymorphism was important; therefore, each population represents a conservation entity and constitutes an important reserve of genes to be used for the improvement of this cereal. These ecotypes have very interesting potentialities for their hardiness and their capacity to give constant yields despite the environmental conditions. Thus, all efforts to evaluate this potential would be important and urgent.

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