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## Inter Simple Sequence Repeat Fingerprints for Assess Genetic Diversity of Tunisian Garlic Populations

Naouel Jabbes (Corresponding author)

Institut Supérieur Agronomique, Département des Sciences Horticoles et du Paysage

4042 Chott Mariem, Tunisie.

Tel: 216-24-881-841 E-mail: naouelj@yahoo.fr

Emmanuel Geoffriau

Agrocampus Ouest- Centre d'Angers, IFR QUASAV 149, UMR 1259

F-49045 Angers, France

E-mail: emmanuel.geoffriau@inh.fr

Valérie Le Clerc

Agrocampus Ouest- Centre d'Angers, IFR QUASAV 149

UMR 1259, F-49045 Angers, France

E-mail: valerie.leclerc@agrocampus-ouest.fr

Boutheina Dridi

Institut Supérieur d'Agronomie, Département des Sciences Horticoles et du Paysage

4042 Chott Mariem, Tunisie

E-mail: bouthaina2@yahoo.fr

Chérif Hannechi

Institut Supérieur Agronomique, Département des Sciences Horticoles et du Paysage

4042 Chott Mariem, Tunisie

E-mail: hannachi\_tnfr@yahoo.fr

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### Abstract

Garlic (*Allium sativum* L.) that is cultivated in Tunisia is heterogeneous and unclassified with no registered local cultivars. At present, the level of genetic diversity in Tunisian garlic is almost unknown. Inter Simple Sequence Repeats (ISSR) genetic markers were therefore used to assess the genetic diversity and its distribution in 31 Tunisian garlic accessions with 4 French classified clones used as control. It was the first time that ISSR markers were used to detect diversity in garlic. Seventeen ISSR primers were screened; seven primers detected 73 polymorphic bands. A high level of polymorphic loci (p) was found in Tunisian populations (54%). Nei's total genetic diversity coefficient was 0.45 and 0.34 respectively for Tunisian and French garlic. Genetic distances observed between Tunisian accessions, ranged between 38.4 and 78.1%. Factor analysis of distances' table (AFTD) did not classify accessions on the base of geographical origin or morpho-physiological characters, particularly bolting ability, but confirmed the appurtenance of analyzed accessions to *sativum* botanical subspecies. There was sufficient diversity detected to start a national collection of garlic germplasm which is crucial for the conservation of genetic diversity and its valorization.

**Keywords:** *Allium sativum* L., ISSR markers, Genetic diversity, Tunisian garlic populations

## 1. Introduction

Garlic (*Allium sativum* L.) is a heterozygote (Satyesh, 1978) diploid specie ( $2n=16$ ) (McCollum, 1987). It is generally not fertile and, thus, propagated by cloves. Etoh and Simon (2002) summarized the current classification of the *Allium sativum* species based on morphological, isozyme and molecular markers into four informal subspecies: the rather diverse *longicuspis* group including most garlic from Central Asia, the *subtropical* group which developed under climatic conditions of South, South East and East Asia, the *ophioscorodon* group from Central and East Europe; and the Mediterranean *sativum* group.

The *sativum* group, probably derived from *longicuspis* forms in West Asia more than 3000 years ago, has been grown in the Mediterranean area since ancient times (Etoh and Simon 2002). It has spread from there throughout the world during the last 500 years (Maab and Klaas, 1995). In fact, Mediterranean region has been postulated as the secondary center of origin of *A. sativum* (Etoh and Simon 2002). However, the classification of *sativum* taxon is still ambiguous. Messiaen *et al.* (1993), Pooler and Simon (1993) and Maab and Klaas (1995) declare that *sativum* subspecies comprises bolting and non or incomplete-bolting cultivars; whereas Engeland (1995) excludes from *Allium sativum ssp sativum* bolting cultivars, he classifies *sativum* group into two types: artichoke and silverskin. Despite a long history of obligate apomixes, cultivated garlic exhibits a large amount of morphological, physiological and bio-chemical variations (Etoh and Simon, 2002; Lallemand *et al.*, 1997).

Genetic diversity in garlic has been studied on the basis of morphological, physiological (Messiaen *et al.*, 1993; Burba and Riera, 1997), and isozyme markers (Pooler and Simon 1993; Maab and Klaas, 1995; Lallemand *et al.*, 1997). However, few markers or properties are unequivocally restricted to certain types and, for most traits; interactions between genotype and environment complicate the cultivar evaluation. DNA polymorphisms are the markers of choice for identification and characterization of plants. They may be representative of the whole genome and they are not subject to environmental modification (Bachmann *et al.*, 2001). Different molecular techniques have been developed to study garlic diversity, mostly Randomly Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) (Maab and Klaas, 1995; Bradley *et al.*, 1996; Al-Zahim *et al.*, 1997; Ipek *et al.*, 2003; Volk *et al.*, 2004; Xu *et al.*, 2005).

Inter Simple Sequence Repeat (ISSR) primers target simple sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome and evolve rapidly, but they do not require prior knowledge of DNA sequence for primer design (Fang and Roose, 1997). ISSR can rapidly differentiate closely related individuals (Zietkiewicz *et al.*, 1994) and have been successfully used to assess genetic diversity among closely related cultivars which were difficult to distinguish with other molecular marker (Fang and Roose, 1997; Dagani *et al.*, 2003; Salhi-Hannachi *et al.*, 2004, Salhi-Hannachi *et al.*, 2005 and Okpul *et al.*, 2005).

Identification of Tunisian garlic germplasm is primordial. In fact, garlic cultivated in Tunisia and in most south Mediterranean countries is botanically unclassified. Growers use their self regenerated garlic cloves and there are no local commercialized cultivars with registered names. Tunisian garlic germplasm is threatened by genetic erosion, uncontrolled introduction of foreign germplasm and under-valorization. There is little interest in collecting and studying Tunisian garlic because most local agricultural research effort is dedicated to "strategic" i.e. wheat, potato and exported crops. Although, garlic is the most expensive local vegetable in Tunisia at mid season (5\$/kg) and occupies nearly 3% of the total vegetable area.

At present the variation in Tunisian garlic is almost unknown, it has not been submitted to molecular investigation. The objective of this study is to determine levels of genetic diversity and its possible structure in Tunisian garlic populations. To achieve this objective, ISSR markers were applied and their effectiveness in detecting infra population variability of garlic was also determined.

## 2. Materials and methods

### 2.1 Plant material

Thirty five garlic (*Allium sativum* L.) accessions were studied. Among these, 31 local landraces were collected from growers in 27 villages located in 12 provinces all over Tunisia and 4 French clones were provided by Institut National d'Horticulture et de Paysage (INHP, France). French clones were Goulurose, Messidrome, Jolimont and Germidour obtained by selection from their original populations Rose de Lautrec (RL), Blanc de la Drome (BD), Blanc de Lomagne (BL) and Violet de Cadour (VC) respectively. These selected French cultivars used as control for comparison, had registered commercial names and clear botanical classification. Tunisian collected materials were assigned accession codes. The convention adopted to assign the accession code was: letters of the original province name and range number. For example JND<sub>14</sub> means province of Jendouba and range number is 14. Names, origins and varietal groups (according to Messiaen *et al.*, 1993) to which the

accessions belong to are provided in table 1. From each accession, except one (JND<sub>13</sub>), four individuals were randomly chosen, giving a total sampling size of 137.

### 2.2 DNA extraction

DNA was extracted from the storage leaf (excluding the bud) of 4 different bulbs from each accession. The extraction protocol is the one described by Briard *et al.* (2000). Quality of extracted DNA was checked on a 1% agarose gel, then after by PCR reaction with universal primers EF1 $\alpha$ , amplification products were loaded on a 2% agarose gel. The two gels were buffered with 0.5x TBE, stained with ethidium bromide and visualized by UV light.

### 2.3 DNA amplification

PCR reactions were performed in a 12 $\mu$ l reaction mix containing 40ng genomic DNA. Two mixtures were used for the PCR reactions according to primers (Table 2). The first one was composed of 1.5mM MgCl<sub>2</sub> (Interchim, Montluçon, France), 100mM of buffer (Interchim, Montluçon, France), 0.2mM of each dNTPs (AMRESCO Inc, Solon, Ohio, USA), 0.5pmol primer (EuroGentec, Angers, France) and 0.06 unit of Taq polymerase (Interchim, Montluçon, France). The second mixture consisted of 3mM MgCl<sub>2</sub> (Interchim, Montluçon, France), 100mM of buffer (Interchim, Montluçon, France), 0.1mM of each dNTPs (AMRESCO Inc, Solon, Ohio, USA), 0.8pmol primer (EuroGentec, Angers, France) and 0.1 unit of Taq polymerase (Interchim, Montluçon, France).

The mixtures were centrifuged briefly before transferring to a Peltier thermal cycler -100 (MJ research, Watertown, Massachusetts, USA). The Polymerase Chain Reaction (PCR) programs consisted of 40 cycles of denaturing at 94°C for 30s; hybridization temperature according to the used primer (Table 2), polymerization for 2min at 72°C, and the last cycle was followed by final polymerization at 72°C for 10min. The PCR products were denaturated at 92°C for 3 min after adding 3.8  $\mu$ l of denaturing dye to each sample and 7 $\mu$ l of denaturated PCR products were separated by electrophoresis in 5% polyacrylamide gel with silver staining for detection.

Seventeen primers were screened using the DNA of 4 individuals resulting from 2 independent extractions and amplifications. Seven of these primers (Table 2) were selected for further testing on all 137 bulbs on the basis of clear, polymorphic and reproducible bands.

### 2.4 Data analysis

The ISSR data were recorded as the presence (1) or absence (0) of each band in each sample, so a binary data matrix was generated for all accessions. To investigate genetic diversity, proportions of ISSR loci that are polymorphic (P) were calculated, together with total collection diversity (Ht), diversity within accessions (Hs) and the coefficient of genetic differentiation between accessions (Gst). The gene diversity was analyzed by Nei's (1987) coefficients using POPGEN32 software (Yeh *et al.* 1999).

Genetic distances between all analyzed individuals were estimated using Sokal and Michener coefficient (1958). The mean genetic distances within (IAS) and among (IES) accessions were calculated using Excel software formulae:  $\Sigma d/N$ , where d is the genetic distance for each pair-wise comparison and N is the number of pair wise comparisons (Table 3). AFTD analyses performed using Darwin 4.0 software (Perrier and Jacquemoud-Collet, 2006) on the totality of accessions. Results of this analysis are represented in figure 2.

## 3. Results and discussion

### 3.1 Genetic diversity

The seven selected ISSR primers generated 84 clear polymorphic reproducible bands. From these, 73 were chosen to be scored on the bases of their discriminate power. Indeed, markers with allelic frequency of 99% and which did not specifically distinguish accessions were eliminated to improve the discrimination between the accessions. ISSR fragments ranged from 200pb to 1300pb. Typical example of the amplified ISSR banding patterns with DNA stretches ranging from 200 to 500pb are depicted in Figure 1 using ISHY4 oligonucleotide (Table 2). Depending on the primer, 7 to 21 polymorphic bands were generated with an average of 12, which is comparable to those detected by AFLP in garlic produced from true seeds (12.8-13.9, Ipek *et al.*, 2005) and in populations of leek (11, Smilde *et al.*, 1999). This number of ISSR markers per primer is higher than those generated by RAPD in Brazilian and Chilean garlic (Buso *et al.*, 2008; Paredes *et al.*, 2008). This difference can be due mainly to the method of detection and possibly to the fact that primers are longer, and hence PCR conditions are more stringent (Godwin *et al.*, 1997). The availability of a relatively high number of polymorphic ISSR markers reflects the heterozygous genome and that ISSR technique is able to detect as much polymorphism in a vegetative as in sexually propagated species.

Garlic production plot is expected to be genetically invariable; however, the molecular distances existing within Tunisian accessions are high enough to be explained by the multiclonal composition of the populations (De Vos, 1992). In fact, the ISSR amplification of garlic DNA revealed a rather high degree of genetic variability among and within accessions not expected in vegetatively propagated specie. This variability is consistent with the phenotypic variability observed confirming the multiclonal composition of the analyzed landraces. Most of the primers produced a different banding pattern for each individual belonging to the same accession. Cultivar specific bands were not observed. The variation within accessions makes it difficult to define unique ISSR profiles for the groups of individuals that belong to the same landrace. The diversity within garlic populations might also be due to mutations that occurred over time due to non-reduction in grower's selection pressure (Simon and Jenderek, 2003).

The proportion of ISSR polymorphic loci (P) provided estimates of genetic diversity within accessions (Table 3). The analyzed accessions were separated into two groups: Tunisian populations group and French clones group. The mean percentage of polymorphic bands was 54.3% for Tunisian accessions, ranging from 30.1% (BJ<sub>18</sub>) to 75.3% (NB<sub>25</sub>); and 46.7% for French clones ranging from 39.7% (BD) to 56.1% (RL), suggesting that Tunisian populations have larger genetic diversity at the DNA level. There is reduction of diversity or variability within the process of selection through which French fixed clones were obtained, although this selection did not reduce dramatically the total diversity and with the reserve that the number of tested French clones is less than Tunisian accessions. New genotypes of garlic have not been obtained through hybridization, but through the selection of spontaneous mutations expressing traits of horticultural interest (Messiaen *et al.*, 1993). In fact, Goulurose and Messidrome clones were isolated after multiple selections from populations of Rose de Lautrec (RL) and Blanc de la Drome (BD) respectively, whereas Jolimont and Germidour were obtained by regenerated clones of some examples from their respective initial populations Blanc de Lomagne (BL) and Violet de Cadour (VC) (Messiaen *et al.*, 1993). They are registered clones, having certainly high degree of morphological homogeneity. At the molecular level, our study confirmed that this selection resulted in more homogeneity within French clones (Hs=0.19) in comparison with the diversity observed within Tunisian populations (Hs=0.22).

Measures of genetic diversity (Table 4) indicate that the total diversity in Tunisian garlic group (Ht= 0.45) is higher than French one (Ht= 0.34). Population differentiation parameters such as diversity between accessions (Gst) and diversity within accessions (Hs) showed a similar pattern across Tunisian populations and French clones, in both cases, Gst is always higher than Hs. Because of its asexual reproduction, it was expected that diversity between accessions (populations or clones) gets higher than within accessions. In terms of population genetic parameters, garlic is rather closer to a self pollinating species (Clegg and Epperson, 1992). Tunisian accessions have a higher total diversity (Ht) than French clones; it is the result of selection activities that reduced diversity in comparison with Tunisian rough material.

### 3.2 Genetic distances

According to the obtained matrix, the French accession BD (Messidrome) have the lowest genetic distance between its individuals (IAS= 21.9%) and NB<sub>23</sub> the highest distance (IAS=42%) (Table 3). On the other hand, JND<sub>12</sub> is the most genetically distant from the other materials (average of IES=48.1%), while BZT<sub>26</sub> is the nearest (average of IES=44.1%). The smallest genetic distance among accessions is observed between SBZ<sub>3</sub> and SBZ<sub>4</sub> both from Sidi Bouzid with IES= 24.8% whereas the maximum distance of 61.6% is between SBZ<sub>2</sub> and BZT<sub>27</sub>. Genetic distances among accessions are always higher than the distances between individuals of one accession, concordant with the observed diversity between and within accessions. The results obtained are in agreement with those reported by Lampasona *et al.* (2003) who found variation in Argentinean garlic clones of 24 to 97% using AFLP markers. Tunisia being in the south coast of the Mediterranean basin, great diversity in garlic germplasm is expected. Buso *et al.* (2008) revealed that considerable genetic variation exists among Brazilian garlic cultivars, ranging between 16 and 98% using the RAPD markers, whereas Paredes *et al.* (2008) found little genetic diversity among 69 Chilean garlic clones analyzed by same markers, the percentage of the similarity ranged between 94 and 98%.

The AFTD analysis, represented by figure 2, distinguishes four groups of accessions. The first one is composed of Tunisian accessions SBZ<sub>3,4</sub>, G<sub>5</sub>, KB<sub>6,7,8</sub>, JN<sub>12</sub>, BJ<sub>19</sub>, and NB<sub>24</sub>, the second group comprises Tunisian and French accessions: BD, VC, KF<sub>31</sub> and MH<sub>32</sub>. The French and Tunisian accessions RL, BL, JND<sub>13</sub>, BJ<sub>18</sub>, NB<sub>21</sub> and GAB<sub>30</sub> form the third cluster. The remaining accessions are confined in a well limited quart of the AFTD plot. Accession BJ<sub>17</sub> does not belong to any of those groups, but seems closer to the first one. Neither our results nor the results of Ipek *et al.* (2003) or Paredes *et al.* (2008) noted an association between banding patterns and geographical origins. AFTD analysis reveals grouping that does not reflect morphological variation, or physiological characteristics, particularly the bolting character. It does not separate the bolting and not bolting accessions. This

result suggests a common genetic basis in the implied accessions, confirming their appurtenance to the botanical taxon *sativum*. This thesis is strongly supported by the great similarities at the DNA level, registered between the Tunisian accessions BJ<sub>18</sub>, NB<sub>20</sub> and JN<sub>13</sub> (do not bolt) and Goulurose clone (produces flower stalk regularly), in spite of their distinctive morphology and physiology. Maab and Klaas (1995) classified four Tunisian accessions as bolting types by isozymes. Our observation of 31 Tunisian garlic accessions in-situ and in field trials for four years does not fit with this classification. In Tunisian climatic conditions, none of the accessions bolts regularly; they rather should be classified in the intermediate or non-bolting types.

#### 4. Conclusion

The present results reveal that the 31 Tunisian garlic accessions analyzed are, in fact, heterogeneous but distinct populations with high level of variability within accessions that belong to *sativum* subspecies. The AFTD analysis did not group the Tunisian accessions according to the bolting character or the geographical origin. ISSR markers proved to be powerful tools to assess the genetic diversity among and between garlic populations and clones, but cannot define specific profile for each cultivar. It will be interesting to analyze the same accessions through the use of other molecular techniques such as AFLP to compare their effectiveness in generating cultivar-specific profiles.

The genetic variation detected in these landraces has significant implications for its characterization and classification. This study will help the start of a national collection of garlic germplasm which is crucial for the conservation of genetic diversity and its valorization.

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Table 1. Garlic accessions, origins and varietal groups

Code accessions*/Names	Province of origin	Source	Country of origin	Genetic structure	Varietal group**
K <sub>1</sub> ,	Kairouan	Growers	Tunisia	Population	5
SBZ <sub>2</sub> , SBZ <sub>3</sub> , SBZ <sub>4</sub> , SBZ <sub>9</sub> ,	Sidi Bouzid	Growers	Tunisia	Population	5
G <sub>5</sub> ,	Gafsa	Growers	Tunisia	Population	5
KB <sub>6</sub> , KB <sub>7</sub> , KB <sub>8</sub> ,	Kébili	Growers	Tunisia	Population	5
KS <sub>10</sub> , KS <sub>11</sub> ,	Kasserine	Growers	Tunisia	Population	5
JND <sub>12</sub> , JND <sub>13</sub> , JND <sub>14</sub> , JND <sub>15</sub> ,	Jendouba	Growers	Tunisia	Population	5
BJ <sub>16</sub> , BJ <sub>17</sub> , BJ <sub>18</sub> , BJ <sub>19</sub> , BJ <sub>28</sub> ,	Béja	Growers	Tunisia	Population	5
NB <sub>20</sub> , NB <sub>21</sub> , NB <sub>23</sub> , NB <sub>24</sub> , NB <sub>25</sub> ,	Nabeul	Growers	Tunisia	Population	5
BZT <sub>26</sub> , BZT <sub>27</sub> ,	Bizerte	Growers	Tunisia	Population	5
KF <sub>29</sub> , KF <sub>31</sub> ,	Kef	Growers	Tunisia	Population	5
GAB <sub>30</sub> ,	Gabès	Growers	Tunisia	Population	5
MH <sub>32</sub>	Mehdia	Growers	Tunisia	Population	5
Violet de Cadour "Germidour" (VC)		INHP	France	Clone	3
Blanc de la Drome "Messidrome" (BD)		INHP	France	Clone	3
Blanc de Lomagne "Jolimont" (BL)		INHP	France	Clone	3
Rose de Lautrec "Goulurose" (RL)		INHP	France	Clone	2

\*Codes of Tunisian accessions were composed of letters and range numbers. Letters in one code were the abbreviation of original province name.

\*\*Varietal groups according to Messiaen *et al.* (1993)

Table 2. ISSR primers characteristics

Primer name	Primer sequences (5'-3')	Annealing temperature (°C)	Primer mixture n°	Number of polymorphic bands
ISSR8US	G(AACA) <sub>4</sub>	50	1	21
ISSR9	HVH(TC) <sub>8</sub>	50	1	11
ISHY 1b	(GA) <sub>8</sub> YT	39	2	9
ISHY 2	(AC) <sub>8</sub> YG	43	2	8
ISHY 3	(AG) <sub>8</sub> YT	39	2	17
ISHY 4	(CA) <sub>8</sub> RG	44	2	11
ISSR a	GCV(TG) <sub>8</sub>	51	2	7
Total				84

V=G; A; C H=A; T; C D=G; A; T B=G; C; T Y=C; T R=A; G

Table 3. Genetic variability detected in Tunisian and French garlic

Accessions	P %	IES	IAS
K1	67.1	47	37.4
SBZ2	72.6	46.9	40.2
SBZ3	41.1	46.8	23.7
SBZ4	41.1	46.6	22.4
G5	59	47.7	34.2
KB6	73	47.4	40.4
KB7	61.6	47.2	34.2
KB8	49.3	47.1	28.3
SBZ9	52	46.4	30.1
KS10	45.2	46.9	26.1
KS11	65.7	47.7	37.2
JND12	50.7	48.1	29
JND13		45.8	
JND14	73	46.1	41.8
JND15	67	46.1	39.3
BJ16	71	46	39
BJ17	49.3	45.3	32.9
BJ18	30.1	48	30.1
BJ19	58.9	47.4	32.2
NB20	49.3	45.5	32.9
NB21	53.4	46	35.6
NB23	63	47.2	42
NB24	50.7	47.6	29.2
NB25	75.3	45.8	40.6
BZT26	43.8	44.1	25.1
BZT27	46.6	46.4	25.8
BJ28	45.2	46.1	26
KF29	39.7	47	39.7
GAB30	46.6	46.3	25.6
KF31	35.6	45.9	23.7
MH32	50.7	44.7	27.9
RL	56.1	46.7	31.1
BL	43.8	45.7	24.9
BD	39.7	45.5	21.9
VC	47.3	46.1	25.3

P%: polymorphic loci, IES: mean genetic distance between accessions, IAS: genetic distance within individuals of one accession

Table 4. Partitioning of genetic diversity in Tunisian accessions and French clones

Population	Ht	Hs	Gst
<b>Tunisien</b>	0,45 (0.24%)	0,22 (0.5%)	0.5
<b>French</b>	0,34 (2.2%)	0,19 (1.5%)	0.43

Ht: total diversity, Hs: diversity within accessions, Gst: diversity between accessions

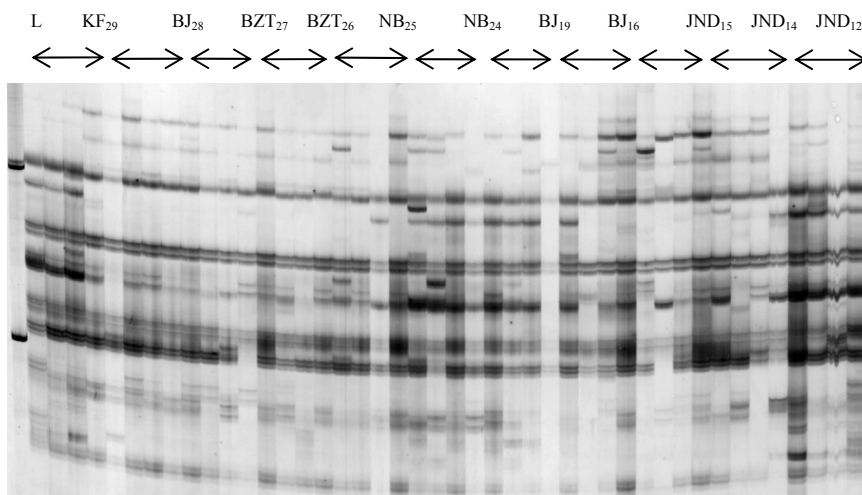


Figure 1. ISSR banding patterns of 11 garlic accessions generated by ISHY4 primer  
 Lanes from left to right: L: 100pb ladder, codes of the accessions with four replications for each one.

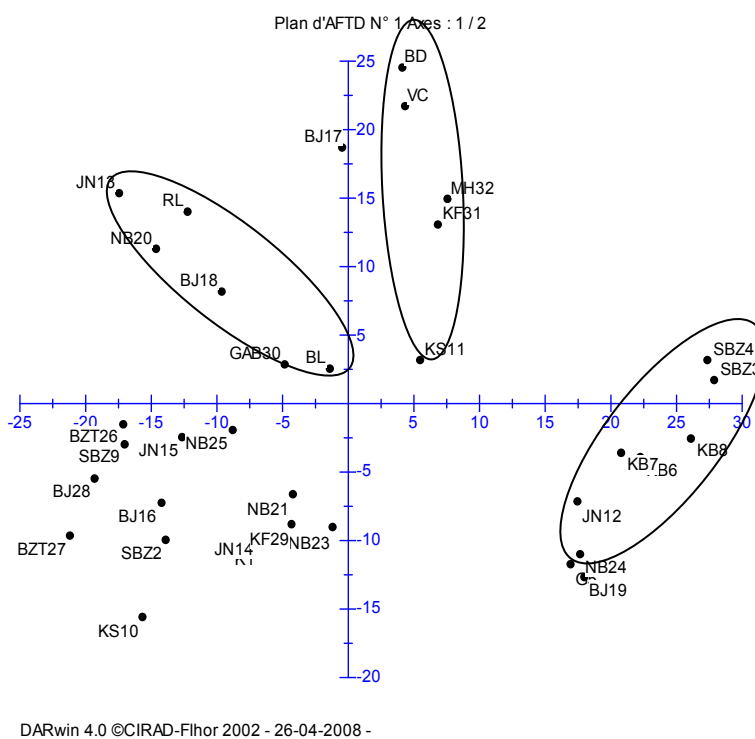


Figure 2. Ordination of the first two vectors of principal coordinates analysis on ISSR molecular banding patterns between 4 French garlic clones and 31 Tunisian garlic populations