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Full Length Research Paper

Analysis of diversity genetic of Moroccan net blotch populations using amplified fragment length polymorphism (AFLP) markers

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Net blotch caused by *Pyrenophora teres f. teres* is the most harmful foliar disease in barley generating significant economic losses in Morocco. Populations of *P. teres f. teres* were collected from different regions of Morocco. Thirty five (35) *P. teres f. teres* isolates, single conidial, were isolated and were subjected to molecular study using amplified fragment length polymorphism (AFLP) technique. Out of the fourteen primers combinations tested, four primers combinations were selected to disclose the polymorphism between the different *P. teres f. teres* isolates. The molecular characterization of these isolates showed high degree of polymorphism reaching 95% and identifying 25 specific genotypes. The genetic variability of the different isolates of *P. teres f. teres* within and between Moroccan regions was highlighted, disclosing no linkage between the isolates and their geographical origins. This result might be due to informal material flow between regions.

Key words: Barley, net blotch, *Pyrenophora teres f. teres*, amplified fragment length polymorphism (AFLP), genetic diversity.

INTRODUCTION

Barley net blotch caused by *Pyrenophora teres anam. Drechslera teres* is a foliar disease attacking all barley growing areas worldwide by affecting quality and yield potentials. In Morocco, as reported by Bentata (2009), it was the most prevalent foliar disease with 100% of severity. Thus, developing resistant or tolerant barley genotypes is the effective way to fight this disease.

Recently, researches on barley net blotch have been increasingly tackled to understand the population biology and the genetic structure of plant pathogens for control strategies improvement (Douhan et al., 2002; McDonald and Linde, 2002). Comprehending the evolutionary potential of pathogens may help in estimating the life expectancy of resistance genes and fungicides (McDonald and Linde, 2002) to prevent the random lost of effectiveness. To achieve durable resistance, a comprehensive body of information on pathogen biology is needed.

The development of molecular markers could offer a very fast and good technique to study genetic diversity of *Pyrenophora teres* (*PT*) inside the populations of various species (McDonald and Martinez, 1990; Linde et al., 2003). Molecular techniques such as random amplification of polymorphic DNA (RAPD) (Peever and Mil-groom, 1994; Peltonen et al., 1996; Frazzon, 2002; Weiland et

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Abbreviations: PTT, *Pyrenophora teres f. teres*; **PT,** *Pyrenophora teres*; **GS,** genetic similarity; **dd,** double distilled.

Table 1. Origin of isolates and their frequencies.

Region	Isolate	Frequency (%)
Doukkala	1-2-4-9-29-45	17
Chaouia	5-7-15-16-17-18-21-38-40-42-43-44-46-47-48-50-52	51.2
Abda	11-23-10	8.5
Zaer	41-20-51	8.5
Gharb	3-24-28-22	11.5
North of Tangier	27-36	5.7

al., 1999), restriction fragment length polymorphism (RFLP) (Wu et al., 2003) and amplified fragment length polymorphism (AFLP) (Rau et al., 2003; Kiros-Meles et al., 2005; Leisova et al., 2005a, b) were largely used. The genetic differentiation has been observed between *PT* populations across wide geographical areas with RAPD and AFLP markers (Peever and Milgroom 1994; Rau et al., 2003; Serenius et al., 2005).

In Morocco, seldom studies on the local net blotch pathotypes and the absence of resistant genes in barley germoplasm have resulted in implementing this study, to better understand the genetic diversity of *Pyrenophora teres f. teres (PTT)* using AFLP markers on a wide range of net blotch populations collected throughout the Moroccan barley areas.

MATERIALS AND METHODS

Isolates

A collection of *PTT* isolates were used in this study. It included thirty five (35) isolates collected from different geographic regions of Morocco: Doukkala, Abda, Chaouia, Zaer, Gharb and North of Tangier. It covers the whole barley growing areas in Morocco. Details concerning the origin and number of isolates are listed in Table 1.

Fungal culture and DNA isolation

All single conidial isolates were established from infected dry leaf material according to McDonald (1967) protocol. Single conidial isolates were first grown on V8 agar under near UV at 20°C for 10 days. The mycelium was transferred to liquid media malt 1% and incubated at same conditions as before but on a shaker at 130 rpm for 15 days. The mycelium was harvested, washed, filtrated, lyophilised and stored for DNA extraction as described by Williams et al. (2001).

Extraction of the DNA has been done by SDS (sodium dodecyl sulfate) method (Vos et al., 1995). In a mortar, 3 g of lyophilised isolates were ground to a fine powder in liquid nitrogen. To this powder, was added 10 ml of DNA extraction buffer (100 mM Tris.Cl; 100 mM NaCl; 50 mM EDTA; SDS 2%). After, 100 µl of proteinase k (1 mg/ml) was added to the suspension. This was incubated for 1 h at 20°C then the supernatant was extracted once with phenol-chloroform isoamylic alcohol (25/24/1) (v/v), once with chloroform-isoamylic alcohol (24/1) (v/v) after centrifugation at 4000 rpm for 10 min. The DNA was precipitated with cold (-20°C) pure ethanol (2v/v), washed with cold ethanol 80%, dried with speed vacuum and re-suspended in 500 µl of TE buffer (10 mM Tris; 1 mM EDTA) for one night at 4°C. The quality of DNA was performed by adding RNase (2 µl at 10 mg/ml) and incubated at 37°C in the water bath for 1 h. DNA suspension was extracted once with one volume of phenol-chloroform isoamylic alcohol (25/24/1) and once with chloroform-isoamylic alcohol (24/1) (v/v) after centrifugation at 4000 rpm for 10 min. The supernatant was collected and DNA was precipitated with 2 v/v of cold (-20°C) pure ethanol. The DNA was dried with speed vacuum and re-suspended in TE buffer. The quality and quantity of DNA was determined respectively by electrophoresis on agarose gel (1%) and with spectrophotometer.

Primers and amplification conditions

Molecular analysis was performed with AFLP technique using Pst I and Mse I restriction enzymes. This technique has included the following stages: Digestion, ligation, pre-amplification and selective amplification. For the restriction digest, 4.0 µl of 10x One-Phor Buffer, 0.25 µl of Pst I (40U/µl), 0.5 µl of Mse I (10U/µl), 25,25 µl of ddH_2O and 10 µl of *PTT* DNA (50 ng/µl) were used. The total restriction mixture was incubated at 37°C for 1 h. For the ligation, 1.0 µl of 10x One-Phor Buffer, 1.0 µl of 5 pM Pst I adapter (Table 2), 1.0 µl of 50 pM Mse I adapter (Table 2), 1.0 µl of 10 mM ATP, 4.5 µl of ddH_2O, 1.5 µl of T4 DNA ligase (1.5 U/µl) and 40 µl of restricted DNA mixture were combined and incubated for 3 h at 37°C. The resulting DNA solution was then diluted five times with $TE_{0.1}$ buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA) and stored at 4°C. For the pre-amplification, 2.0 µl of 10x PCR buffer, 2.5 µl of 2 mM dNTPs, 50 ng of Pst (0) and Mse (0) primer (Table 2), 5.0 µl of diluted DNA, 8.1 µl of ddH_2O and 2U (unit) of Roche Taq DNA polymerase were combined. This pre-amplification was carried out in an Eppendorf thermal cycler programmed for 1 cycle at 95°C for 5 min, followed by 23 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. The pre-amplification products were diluted five times in $TE_{0.1}$

For the selective amplification, 2.0 µl of 10x PCR buffer, 2.5 µl of 2 mM dNTPs, 50 ng of Mse I + 3 bp and 50 ng of Pst I + 2 bp or 3 bp (Table 2), 5.0 µl of diluted DNA, 8.1 µl of ddH_2O, and 2 U of Roche Taq DNA polymerase were combined. The program ran for 11 cycles at 94°C for 30 s, 65 to 56°C for 30 s and 72°C for 1 min, then 23 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min and then 1 cycle at 72°C for 10 min.

Final amplification products were separated on a 6% polyacrylamide denaturing gel for 1 h 40 min at constant power of 50 W. Short glasses was treated with binding silane and long one was treated with sigmacote (Repel Silane) so that after the run, the gel could be kept fixed on the first glass for staining.

The DNA bands in the gels were developed using a silver nitrate stain, after successive treatments with 2 x 1 L of 10% glacial acetic acid, 2 x 1 L of silver nitrate solution (1 g/l) with 1.5 ml/l of formaldehyde (37%). The DNA bands were developed with sodium carbonate solution (30 g/l, 1.5 ml/l of formaldehyde (37%) and 400 µl of sodium thiosulfate (10 mg/l) after short washing with cold ddH_2O (34°C).

Table 2. Primer adapter, non selective AFLP primers and selective AFLP primers.

Primer	Primer sequence
Mse adapter forward primer	5'-GACGATGAGTCCTGAG-3'
Mse adapter reverse primer	5'-TACTCAGGACTGAT-3'
M0 : MseI+0	5'-GATGAGTCCTGAGTAA-3'
M42 : MseI+AGT	5'-GATGAGTCCTGAGTAA-AGT-3'
M37 : MseI+ACG	5'-GATGAGTCCTGAGTAA-ACG-3'
M34 ; MseI+AAT	5'-GATGAGTCCTGAGTAA-AAT-3'
Pst adapter forword primer	5'-CTCGTAGACTGCGTACATGCA-3'
Pst adapter revers primer	5'-TGTACGCAGTCTAC-3'
P0 : PstI+0	5'-GACTGCGTACATGCAG-3'
P74 : PstI+GGT	5'-GACTGCGTACATGCAG-GGT-3'
P16 : PstI+CC	5'-GACTGCGTACATGCAG-CC-3'
P72 : PstI+GGC	5'-GACTGCGTACATGCAG-GGC-3'

Table 3. Polymorphic alleles produced by AFLP primers combinations and interval of molecular weight.

Molecular weight	Combination1: 16/M42	Combination2: 72/M37	Combination3: P74/M37	Combination4: P72/M34	Total
> 400	25	2	16	20	63
300-400	23	11	22	23	79
200-300	51	26	51	28	156
150-200	35	27	22	15	99
100-150	45	12	9	29	95
Total	179	78	120	115	492

Statistical analysis

Since AFLP are usually dominant markers, only two character states are scored: presence (1) or absence (0) of bands and entered into a binary matrix, only polymorphisms that could be scored unequivocally for presence or absence of bands were included in the analysis.

The matrix of similarity was calculated using statistical software NTSYS-PC 2.0 (Rohlf, 1997) and the coefficient of Jaccard (Jaccard, 1908). While the dendrogram was establish by the method of regrouping "unweighted pair group method" using arithmetic means "UPGMA" (Sneath et Sokal, 1973 ; Swofford & Olsen, 1990).

RESULTS AND DISCUSSION

Several AFLP primers' combinations PST/MSE were used to seek the best possible combinations for the genetic diversity analysis of Moroccan *PTT* populations. Combinations of primers P16 (Cc) - M42 (AGT); P72 (GGC) - M37 (ACG); P72 (GGC) - M34 (AAT) and P74 (GGT) - M37 (ACG) gave a high number of markers AFLP (Table 2).

Theses combinations used for the selective amplification of the DNA of the 35 isolates of *PTT* gave a total of 521 bands. Only 29 bands are monomorphic, whereas 492 were polymorphic (94.4%). It revealed a high genetic variability between the studied isolates. Indeed, 179 polymorphic markers were produced by combination 1; 78 by combination 2; 115 by combination 3 and 120 by combination 4. Theses AFLP markers have a variable molecular weight between 100 and 800 bp with domination of the fragments (73%) with 100 to 300 bp (Table 3). Also, 25 different haplotypes were identified for the 35 isolates studied. In other words, 25 isolates have specific genotypes, suggesting that there is more genetic recombination which plays a major role in genetic diversity of Moroccan *PTT*.

Figure 1 represents an example of AFLP bands of the *PTT* isolates using the primers combination P74 (GGT) - M37 (ACG). Number 1 to 35 represents the 35 isolates collected from Moroccan barley fields. M is a molecular weight marker and the numbers in the left indicate its molecular weight (bp).

On the other side, a dendrogram was conceived using the matrix of similarity based on the coefficient of Jaccard and UPGMA method. Tree principal groups were distinguished with a coefficient of similarity of 0.10 (Figure 2), each principal group contains several subgroups of isolates that have close genetic similarities.

As displayed in Table 4, 69% of the isolates were gathered in group I, 17% in group II and 5.7% in group III with genetic similarities (GS) equal to 13, 10 and 12%,

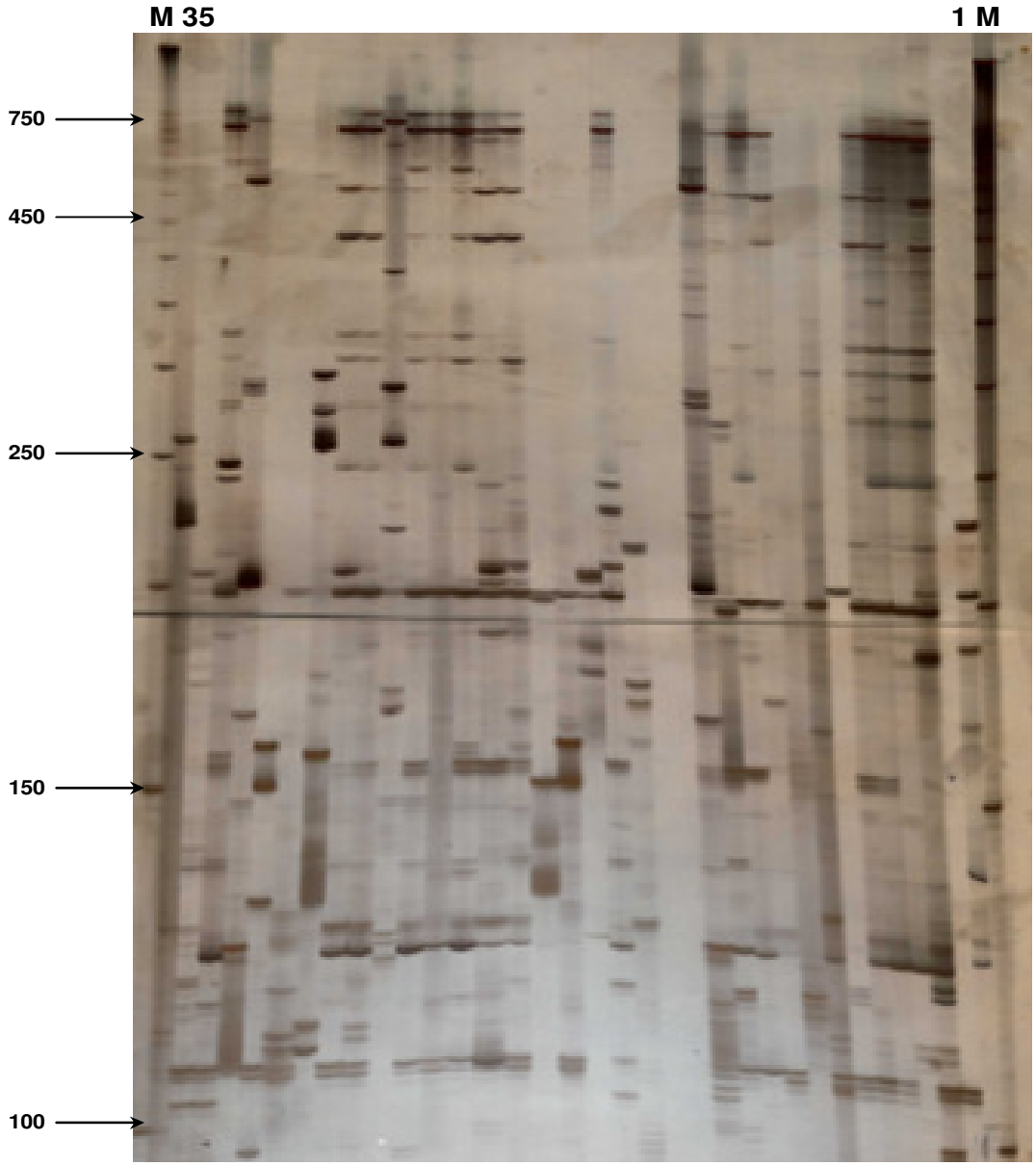

Figure 1. Gel showing amplification product obtained by AFLP primers P74 (GGT)- M37 (ACG) combination P74 of *PTT* isolates.

respectively. The group I was divided into 5 subgroups (with well defined GS) and it is representing most of Moroccan barley regions. The isolates from group II and III are belonging to Chaouia region except for the isolate 9 from Doukkala. These two groups are specific to Chaouia region showing that geographical environment has also an impact.

As depicted in the dendrogram (Figure 2), we noticed that 3 isolates 1, 45 and 2 constitute a group each one apart. Indeed, the isolate 1 (Group IV) has a GS = 9%, the isolate 45 (Group V) has a GS = 7% and isolate 2 (Group VI) has a GS = 4%. These isolates are from the same region (Doukkala). Also, among the 17 isolates from Chaouia, 58% were in the group I, 29% in the group II and 12% in group III. The geographical distribution of these isolates is not preserved. This result suggests that there is a higher genetic diversity in the inter and intra area (Doukkala- Chaouia).

Gene flow and population structure

The group I contains isolates representing all areas prospected (Table 4). This suggests that theses isolates are probably originated from the same ancestor. This hypothesis was proposed by Goodwin et al. (1993), who

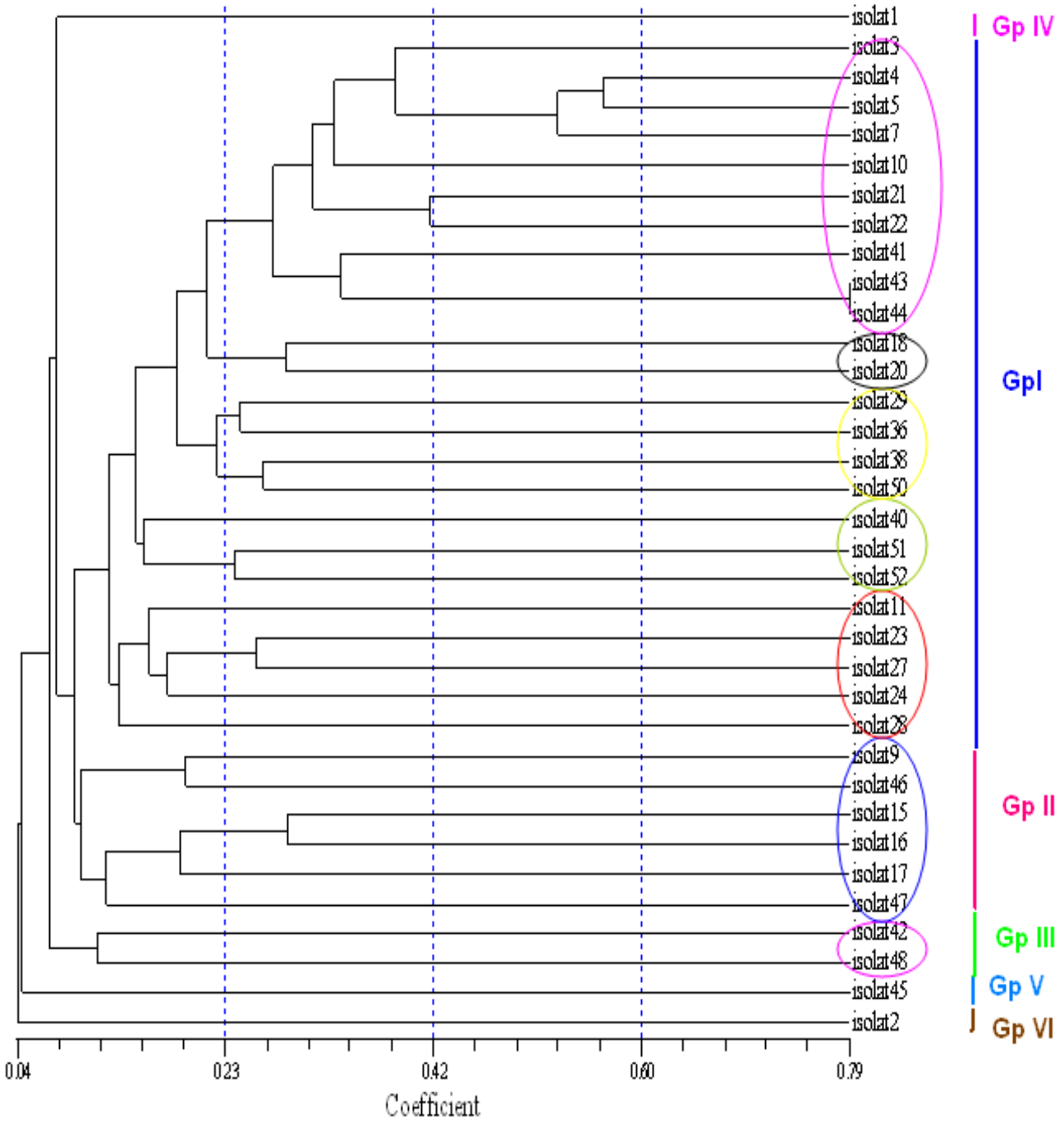

Figure 2. Dendrogram of the relationships between 35 isolates of *PTT* based on AFLP analysis and UPGM method.

explained the moderate level of variation seen in *Rynchosporium secalis* isolates from Europe, Australia and the USA.

As we know, the areas of Chaouia and Doukkala represent more than 60% of the cereal production in Morocco (MAPM, 2008) and new varieties of barley were been introduced to increase the annual production. However, the environmental conditions and the interaction between the host and pathogen act in a considerable way on the sexual reproduction, consequently on the genetic variation of the pathogenic population by gene flow. Indeed, *PT* like the majority of the ascomycetes, is able to reproduce by sexually and asexually mode. This relative contribution of each one of these modes of reproduction can affect the genetic structure of the populations of the pathogen (Peever and Milgroom, 1994). The sexual phase of *PT* was reported in several areas of the world (Shipton et al., 1973) and can

Table 4. Groups of isolates, degree of similarity and their areas.

Groups	I																								II						III	
Under groups	A										B		C				D			E												
Isolates	3	4	5	7	10	21	22	41	43	44	18	20	29	36	38	50	40	51	52	11	23	27	24	28	9	46	15	16	17	47	42	48
GS %	27										28		22				16			14					10						12	
Areas	Gharb	Doukkala	Chaouia	Abda	Chaouia	Gharb	Zaer	Chaouia			Zaer		Doukkala	North	Chaouia		Zaer	Chaouia		Abda	North	Gharb			Doukkala	Chaouia						

(1994), starting from the structure of multilocus of the populations of *PT* that the sexual reproduction is frequent in four of the five analyzed populations. In accordance with these results, the sexual recombination can be the primary source of the great genetic variability of the Moroccan population of *PTT,* which agrees with the result of Wu et al. (2003). These results are also in agreement with those described by Peltonen et al. (1996) who has used RAPD markers and deduced a high genetic variability between 48 Finnish isolates of *PTT* with a coefficient of similarity of 68%. In addition, the absence of the strict genetic correlation with the geographical origin of our isolates is the consequent of the high level of genetic variation. zThis confirms the result reported by Leisova et al. (2005b) in the study of Czech Republic population of *PT* using AFLP analysis.

Conclusions

The molecular characterization of the 35 Moroccan isolates by AFLP technique showed a high level of polymorphism (94.4%) by applying four combinations selected primers (P16-M42; P72-M37; P72-M34 and P74-M37) starting from 14 combinations tested. Thus, we could notice that 73% of markers AFLP have a molecular weight going from 100 to 300 bp. Also, 25 specific genotypes were identified and six principal groups were distinguished based on the matrix of similarity of Jaccard and the UPGMA method, with a coefficient of similarity equal to 0.10. Finally, the existence of genetic variability showed, can be explained by interregional and intra-regional gene flow between the Moroccan isolates.

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