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ORIGINAL ARTICLE

# Genetic differentiation in *Pyrenophora teres* f. *teres* populations from Syria and Tunisia as assessed by AFLP markers

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**Significance and Impact of the study**: The study represents a comparative analysis of the genetic diversity in *P. teres* isolates from two countries spanning two continents and also shows that several distinct *P. teres* genotypes may be found in a given environment. The implications of these findings for *Pyrenophora teres* f. *teres* evolutionary potential and net blotch-resistance breeding in Syria and Tunisia were also discussed.

**Keywords**
AFLP, Barley, genetic diversity, net blotch, *Pyrenophora teres* f. *teres*, Syria, Tunisia.

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## Abstract

To investigate the level of genetic differentiation and diversity among *Pyrenophora teres* isolate populations originating from different agro-ecological areas of Syria and Tunisia and to determine the potential of AFLP profiling in genotyping *Pyrenophora teres* f. *teres*. In this study, AFLP markers have been employed to identify patterns of population structure in 20 *Pyrenophora teres* f. *teres* populations from Syria and Tunisia. Ninety-four isolates were studied by the use of a protocol that involved stringent PCR amplification of fragments derived from digestion of genomic DNA with restriction enzymes *EcoRI* and *MesI*. Based on 401 amplified polymorphic DNA markers (AFLP), variance analyses indicated that most of the variation was partitioned within rather than between populations. Genotypic diversity (GD) was high for populations from Rihane, local landraces and different agro-ecological zones (GD = 0·75–0·86). There was high genetic differentiation among pathogen populations from different host populations in Syria ($G_{st}$ = 0·31, ht = 0·190) and Tunisia ($G_{st}$ = 0·39, ht = 0·263), which may be partly explained by the low gene flow around the areas sampled. A phenetic tree revealed three groups with high bootstrap values (55, 68, 76) and reflected the grouping of isolates based on host, or agro-ecological areas. AFLP profiling is an effective method for typing the genetically diverse pathogen *Pyrenophora teres* f. *teres*.

## Introduction

*Pyrenophora teres* Drechs. [anamorph: Drechslera teres (Sacc.) Shoemaker], is a haploid ascomycete that causes net blotch in barley (Smedegaard-Petersen 1971). This persistent disease is common in all barley-growing regions of the world and occurs typically in cool and humid areas. However, it is a serious disease in the dry areas of North Africa, the Middle East and Australia, and may cause up to 40% loss in grain yield (Hovmoller *et al.* 2000; Jayasena 2007; Jebbouj and El Yousfi 2010) in susceptible barley cultivars under epidemic conditions (McLean *et al.* 2009). Two forms of the disease, the net form (*P. teres* f. *teres*) and the spot form (*P. teres* f. *maculata*), were described based on the symptoms in barley (Smedegaard-Petersen 1971; McLean *et al.* 2009). The net form (*P. teres* f. *teres*) is more common in North Africa and the Middle East (Harrabi and Kamel 1990; Arabi *et al.* 2003).

Croissant fertile and North Africa are primary centres of barley diversity; however, a few improved varieties are currently replacing a wide range of genetically heterogeneous barley landraces across the region. Some of these varieties, for example Rihane, are susceptible to net blotch, and high infection levels have been observed across the region (Yahyaoui 2004). Reduction in yield due to barley net blotch can be as high as 40%, and the disease is currently one of the major constraints to barley production in Syria and Tunisia (Yahyaoui 2004). This can be attributed to the increasingly intensive production, large monocultures of a few varieties and the environmental conditions that are conducive for disease development (Yahyaoui 2004). Resistant barley cultivars could potentially form the basis of sustainable management strategies for net blotch; however, knowledge of *P. teres* population genetics is needed to understand disease epidemiology, and to effectively breed and use resistant cultivars (McDonald and Linde 2002; McLean *et al.* 2009).

*Pyrenophora teres* is known for its high level of pathogenic variation in several countries (Afanasenko and Levitin 1979; Tekauz 1990; Steffenson and Webster 1992; Gupta and Loughman 2001; Cromey and Parkes 2003; Wu *et al.* 2003; Serenius 2006; Grewal *et al.* 2008; Silvar *et al.* 2009), including Syria and Tunisia (Harrabi and Kamel 1990; Arabi *et al.* 2003). Studies of *P. teres* populations using colony colour, isozymes, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) revealed high genetic diversity within field populations of the pathogen (Rau *et al.* 2003, 2005; Wu *et al.* 2003; Serenius 2006; Keiper *et al.* 2007).

Information on the dispersal (gene flow) and mode of reproduction of *P. teres* could influence current management strategies for barley net blotch in Syria and Tunisia. AFLP markers are well suited for studies that focus on the role of selectively neutral evolutionary processes such as mating system, genetic drift and gene flow (McDonald and Linde 2002). A more complete understanding of the genetic structure of *P. teres* f. *teres* populations in Syria and Tunisia necessitates hierarchical sampling of a relatively large number of isolates on different geographical and spatial scales (Wu *et al.* 2003).

Genetic differentiation of geographically separated pathogen populations has important implications for the identification and deployment of disease resistance genes. Barley is cultivated in Syria and Tunisia in widely varying agro-ecological zones ranging from subhumid to semiarid, and one hypothesis is that *P. teres* f. *teres* populations in these areas are genetically differentiated. The objectives of this study were, to (i) characterize the amount and distribution of genetic variation within and among populations of *P. teres* f. *teres* sampled in different barley-growing areas of Syria and Tunisia and (ii) use this information to infer the role of different evolutionary forces on the pathogen population in the two countries.

## Results

### AFLP analysis

*Combined analysis of AFLP data.* A total of 401 discernible and polymorphic AFLP bands were generated with six primer combinations selected across the 94 isolates of 20 Syrian and Tunisian populations of *Pyrenophora teres* f. *teres* (Table 2). Primers varied in their ability to detect variation at both within and between populations. The within-populations $H_o$ varied from 0·127 for E11XM50 primer to 0·195 for E11XM47 primer (Table 2).

On average over all primers, the *Pyrenophora teres* population from Attayet (Siliana, Tunisia) was the most diverse based on several indices (Table 4). While the level of within-population genetic diversity was lower among the samples of population 5 (mean $H_o = 0{\cdot}104$). Even the genetic diversity between Syrian populations ranged from 0·128 to 0·220 (Tables 1 and 4).

Genotypic diversity ranged from 0·75 (population 3, population 7, population 8, population 14, population 15) to a maximum of 0·86 that was recorded at seven of the twenty sites surveyed, indicating that at these sites, every strain represented a distinct genotype.

The variance components of within and between populations detected with AMOVA were 72·76 and 27·24% of the total variance, respectively, which were both significant at $P < 0{\cdot}001$ (Table 3). This was in approximate agreement with results derived from $H_o$ index, in which within- and between-population variations were 56·61 and 43·39%, respectively. It seems clear that while most of the variation is partitioned within populations, there is still considerable variation between populations.

Across all 20 populations, the number of polymorphic loci at each population ranged from 63 (population 5) to 239 (population 12) and with a percentage of polymorphic loci 15·71–59·60%, respectively (Table 4). Average gene diversity across Syrian and Tunisian populations ranged from 0·104 (population 5) to 0·261 (population 8) (Table 4).

Touiref (population 5) samples were the least variable based on differences between isolates and Shannon's information index (Tables 1 and 4).

A matrix of pairwise $F_{st}$ values is presented in Table 5. Values of $F_{st}$ ranged from −0·0053 (between populations 16 and 17) to 0·637 (between populations 5 and 15). Genetic differentiation was not significant only between Tunisian populations: Barrage Meleg, Touiref and Hamrounia samples (−0·021, −0·0031, −0·0053, respectively)

**Table 1** List of *Pyrenophora teres* f. *teres* 'isolates' used on this study collected from Syria and Tunisia

| Code | Reference/site | Populations code/collection site(district)/Geographic position/Rainfall | Host/row type/year collection |
|---|---|---|---|
| 1 | T1B1/TUN1 | Popl/Testour 1 (Beja) HR, NW | Rihane/6R/2009 |
| 2 | T1D1/TUN2 | Popl/Testour 1 (Beja) HR, NW | Rihane/6R/2009 |
| 3 | T1F1/TUN3 | Popl/Testour 1 (Beja) HR, NW | Rihane/6R/2009 |
| 4 | T1C1/TUN71 | Popl/Testour 1 (Beja) HR, NW | Rihane/6R/2009 |
| 5 | T1C2/TUN72 | Popl/Testour 1 (Beja) HR, NW | Rihane/6R/2009 |
| 6 | T3C1/TUN4 | Pop2/Teboursek 1 (Beja) HR, NW | Rihane/6R/2009 |
| 7 | T3F1/TUN5 | Pop2/Teboursek 1 (Beja) HR, NW | Rihane/6R/2009 |
| 8 | T3H1/TUN6 | Pop2/Teboursek 1 (Beja) HR, NW | Rihane/6R/2009 |
| 9 | T6A1/TUN7 | Pop3/Bahra (Kef) MR, NW | Local barley landrace/6R/2009 |
| 10 | T6E1/TUN8 | Pop3/Bahra (Kef) MR, NW | Local barley landrace/6R/2009 |
| 11 | T6G1/TUN9 | Pop3/Bahra (Kef) MR, NW | Local barley landrace/6R/2009 |
| 12 | T6H1/TUN10 | Pop3/Bahra (Kef) MR, NW | Local barley landrace/6R/2009 |
| 13 | T9A1/TUN11 | Pop4/Barrage Maleg (Kef) MR, NW | Local barley landrace/6R/2009 |
| 14 | T9C1/TUN12 | Pop4/Barrage Maleg (Kef) MR, NW | Local barley landrace/6R/2009 |
| 15 | T9D1/TUN13 | Pop4/Barrage Maleg (Kef) MR, NW | Local barley landrace/6R/2009 |
| 16 | T9E1/TUN14 | Pop4/Barrage Maleg (Kef) MR, NW | Local barley landrace/6R/2009 |
| 17 | T9H1/TUN15 | Pop4/Barrage Maleg (Kef) MR, NW | Local barley landrace/6R/2009 |
| 18 | T14B1/TUN16 | Pop5/Touiref (Kef) MR, NW | Local barley landrace/6R/2009 |
| 19 | T14B2/TUN17 | Pop5/Touiref (Kef) MR, NW | Local barley landrace/6R/2009 |
| 20 | T14G1/TUN18 | Pop5/Touiref (Kef) MR, NW | Local barley landrace/6R/2009 |
| 21 | T15F1/TUN19 | Pop6/Gboulat (Siliana) MR, NW | Local barley landrace/6R/2009 |
| 22 | T15G1/TUN20 | Pop6/Gboulat (Siliana) MR, NW | Local barley landrace/6R/2009 |
| 23 | T15G2/TUN21 | Pop6/Gboulat (Siliana) MR, NW | Local barley landrace/6R/2009 |
| 24 | T15B1/TUN59 | Pop6/Gboulat (Siliana) MR, NW | Local barley landrace/6R/2009 |
| 25 | T15D1/TUN60 | Pop6/Gboulat (Siliana) MR, NW | Local barley landrace/6R/2009 |
| 26 | T15C1/TUN61 | Pop6/Gboulat (Siliana) MR, NW | Local barley landrace/6R/2009 |
| 27 | T16A1/TUN22 | Pop7/Mosrata (Siliana) MR, NW | Rihane/6R/2009 |
| 28 | T16B1/TUN23 | Pop7/Mosrata (Siliana) MR, NW | Rihane/6R/2009 |
| 29 | T16E1/TUN24 | Pop7/Mosrata (Siliana) MR, NW | Rihane/6R/2009 |
| 30 | T16H1/TUN25 | Pop7/Mosrata (Siliana) MR, NW | Rihane/6R/2009 |
| 31 | T17C1/TUN26 | Pop8/Attayet (Siliana) MR, NW | Rihane/6R/2009 |
| 32 | T17E1/TUN27 | Pop8/Attayet (Siliana) MR, NW | Rihane/6R/2009 |
| 33 | T17F3/TUN28 | Pop8/Attayet (Siliana) MR, NW | Rihane/6R/2009 |
| 34 | T17H1/TUN29 | Pop8/Attayet (Siliana) MR, NW | Rihane/6R/2009 |
| 35 | T19A1/TUN30 | Pop9/Sers (Kef) MR, NW | Local barley landrace/6R/2009 |
| 36 | T19B1/TUN31 | Pop9/Sers (Kef) MR, NW | Local barley landrace/6R/2009 |
| 37 | T19E1/TUN32 | Pop9/Sers (Kef) MR, NW | Local barley landrace/6R/2009 |
| 38 | T25A1/TUN33 | PoplO/Sidi Mtir, Bouficha (Sousse) LR, C | Local barley landrace/6R/2009 |
| 39 | T25B2/TUN34 | PoplO/Sidi Mtir, Bouficha (Sousse) LR, C | Local barley landrace/6R/2009 |
| 40 | T25C3/TUN62 | PoplO/Sidi Mtir, Bouficha (Sousse) LR, C | Local barley landrace/6R/2009 |
| 41 | T28G1/TUN35 | Popl 1/Hincha (Sfax) LR, S | Local barley landrace/6R/2009 |
| 42 | T28H1/TUN36 | Popl 1/Hincha (Sfax) LR, S | Local barley landrace/6R/2009 |
| 43 | T28A1/TUN73 | Popl 1/Hincha (Sfax) LR, S | Local barley landrace/6R/2009 |
| 44 | T28D1/TUN74 | Popl 1/Hincha (Sfax) LR, S | Local barley landrace/6R/2009 |
| 45 | T28D2/TUN75 | Popl 1/Hincha (Sfax) LR, S | Local barley landrace/6R/2009 |
| 46 | T28F1/TUN76 | Popl 1/Hincha (Sfax) LR, S | Local barley landrace/6R/2009 |
| 47 | T51E2/TUN37 | Popl2/Bir Mcharga (Zaghouan) MR,NE | Rihane/6R/2009 |
| 48 | T51C2/TUN63 | Popl2/Bir Mcharga (Zaghouan) MR, NE | Rihane/6R/2009 |
| 49 | T51A2/TUN65 | Popl2/Bir Mcharga (Zaghouan) MR, NE | Rihane/6R/2009 |
| 50 | T51F2/TUN66 | Popl2/Bir Mcharga (Zaghouan) MR, NE | Rihane/6R/2009 |
| 51 | T51C1/TUN67 | Popl2/Bir Mcharga (Zaghouan) MR, NE | Rihane/6R/2009 |
| 52 | T51D2//TUN68 | Popl2/Bir Mcharga (Zaghouan) MR, NE | Rihane/6R/2009 |
| 53 | T51H1/TUN69 | Popl2/Bir Mcharga (Zaghouan) MR, NE | Rihane/6R/2009 |
| 54 | T51H2/TUN70 | Popl2/Bir Mcharga (Zaghouan) MR, NE | Rihane/6R/2009 |

**Table 1** (*continued*)

| Code | Reference/site | Populations code/collection site(district)/Geographic position/Rainfall | Host/row type/year collection |
|---|---|---|---|
| 55 | T52A1/TUN38 | Popl3/El fahs (Zagouhan) MR, NE | Local barley landrace/6R/2009 |
| 56 | T52B1/TUN39 | Popl3/El fahs (Zagouhan) MR, NE | Local barley landrace/6R/2009 |
| 57 | T52C1/TUN40 | Popl3/El fahs (Zagouhan) MR, NE | Local barley landrace/6R/2009 |
| 58 | T52D1/TUN41 | Popl3/El fahs (Zagouhan) MR, NE | Local barley landrace/6R/2009 |
| 59 | T52D2/TUN42 | Popl3/El fahs (Zagouhan) MR, NE | Local barley landrace/6R/2009 |
| 60 | T52E1/TUN43 | Popl3/El fahs (Zagouhan) MR, NE | Local barley landrace/6R/2009 |
| 61 | T52H2/TUN44 | Popl3/El fahs (Zagouhan) MR, NE | Local barley landrace/6R/2009 |
| 62 | T53B2/TUN45 | Popl4/Saoif (Zagouhan) MR, NE | Rihane/6R/2009 |
| 63 | T53D1/TUN46 | Pop14/Saoif (Zagouhan) MR, NE | Rihane/6R/2009 |
| 64 | T53F2/TUN47 | Pop14/Saoif (Zagouhan) MR, NE | Rihane/6R/2009 |
| 65 | T53G3/TUN48 | Pop14/Saoif (Zagouhan) MR, NE | Rihane/6R/2009 |
| 66 | T55A3/TUN49 | Pop15/Mograne (Zagouhan) MR, NE | Rihane/6R/2009 |
| 67 | T55C2/TUN50 | Pop15/Mograne (Zagouhan) MR, NE | Rihane/6R/2009 |
| 68 | T55D1/TUN51 | Pop15/Mograne (Zagouhan) MR, NE | Rihane/6R/2009 |
| 69 | T55G1/TUN52 | Pop15/Mograne (Zagouhan) MR, NE | Rihane/6R/2009 |
| 70 | T57B1/TUN53 | Pop16/Souidia (Bizerte) HR, NN | Rihane/6R/2009 |
| 71 | T57B2/TUN54 | Pop16/Souidia (Bizerte) HR, NN | Rihane/6R/2009 |
| 72 | T57C1/TUN55 | Pop16/Souidia (Bizerte) HR, NN | Rihane/6R/2009 |
| 73 | T57A1/TUN77 | Pop16/Souidia (Bizerte) HR, NN | Rihane/6R/2009 |
| 74 | T57H1/TUN78 | Pop16/Souidia (Bizerte) HR, NN | Rihane/6R/2009 |
| 75 | T59B1/TUN56 | Pop17/Hamrounia (Bizerte) HR,NN | Rihane/6R/2009 |
| 76 | T59H1/TUN57 | Pop17/Hamrounia (Bizerte) HR,NN | Rihane/6R/2009 |
| 77 | T59E1/TUN64 | Pop17/Hamrounia (Bizerte) HR,NN | Rihane/6R/2009 |
| 78 | T59G1/TUN79 | Pop17/Hamrounia (Bizerte) HR, NN | Rihane/6R/2009 |
| 79 | S1/ICAPtr1 | Pop18/Al Bab (ALEPPO) MR, N | Local barley landrace/2R/1998 |
| 80 | S2/ICAPtr2 | Pop18/Al Bab (ALEPPO) MR, N | Local barley landrace/2R/1998 |
| 81 | S7/ICAPtr5 | Pop18/Al Bab (ALEPPO) MR, N | Local barley landrace/2R/1998 |
| 82 | S3/ICAPtr3 | Pop19/Mossaf (HAMA) MR, W | Local barley landrace/2R/2003 |
| 83 | S8/ICAPtr6 | Pop19/Souran (HAMA) MR, W | Local barley landrace/2R/2006 |
| 84 | S10/ICAPtr7 | Pop19/Al Gab (HAMA) MR, W | Local barley landrace/2R/2009 |
| 85 | S11/ICAPtr8 | Pop19/Al Gab (HAMA) MR, W | Local barley landrace/2R/2009 |
| 86 | S12/ICAPtr9 | Pop19/Al Gab (HAMA) MR, W | Local barley landrace/2R/2009 |
| 87 | S13/ICAPtr10 | Pop19/Al Gab (HAMA) MR, W | Local barley landrace/2R/2009 |
| 88 | S14/ICAPtr11 | Pop19/Al Gab (HAMA) MR, W | Local barley landrace/2R/2009 |
| 89 | S16/ICAPtr12 | Pop20/ICR-Station (TARTOUS) HR, W | Local barley landrace/?/2007 |
| 90 | S17/ICAPtr13 | Pop20/ICR-Station (TARTOUS) HR, W | Local barley landrace/?/2007 |
| 91 | S18/ICAPtr14 | Pop20/ICR-Station (TARTOUS) HR, W | Local barley landrace/?/2007 |
| 92 | S19/ICAPtr15 | Pop20/ICR-Station (TARTOUS) HR, W | Local barley landrace/?/2007 |
| 93 | S21/ICAPtr16 | Pop20/ICR-Station (TARTOUS) HR, W | Local barley landrace/?/2007 |
| 94 | S23/ICAPtr17 | Pop20/ICR-Station (TARTOUS) HR, W | Local barley landrace/?/2007 |

(Table 5). The rest of values were all significant at $P < 0{\cdot}001$. This indicates that all populations may be considered different from each other, with population 5 being the most different from the others and populations 1, 4, 9 and 16 being the most similar. The overall $F_{st}$ across all the populations was 0·27.

The significant differentiation between populations was also revealed in the clustering analysis (Fig. 1) and reflected in the estimates of gene flow ($N_m$) (Table 2).

Values of $N_m$ ranged from a moderate value of 0·84 to a high value of 1·18, averaging 0·653, which indicated that *Pyrenophora teres* populations tend to differentiate between the twenty studied populations.

The total variation among *Pyrenophora teres* was further tested based on geographic countries (Syria, Tunisia) (Table 2). Most of the total variation (68·55%) was identified within Syrian populations, 40% was identified among Tunisian populations. Although, genetic differentiation was significant between countries ($F_{st}$ ranging from 0·126 to 0·572), results show that the isolates of *P. teres* are genetically more distinct across a wide geographical area. Moderate gene flow was found in

**Table 2** Genetic diversity identified by six AFLP primers combinations in 94 isolates from Syria and Tunisia

| AFLP primer combinations | $H_t$ | $H_s$ | $G_{st}$ | $N_m$ |
|---|---|---|---|---|
| E11XM47 | 0·295 | 0·194 | 0·404 | 0·985 |
| E11XM50 | 0·218 | 0·127 | 0·340 | 1·180 |
| E17XM47 | 0·320 | 0·172 | 0·441 | 0·866 |
| E17XM50 | 0·262 | 0·147 | 0·399 | 0·845 |
| E15XM47 | 0·296 | 0·169 | 0·371 | 1·085 |
| E15 X M50 | 0·266 | 0·157 | 0·382 | 1·052 |
| TUN pop (17pop, 78 isolates) | 0·263 | 0·158 | 0·398 | 0·756 |
| Syrian pop(3pop, 16 isolates) | 0·190 | 0·130 | 0·314 | 1·092 |
| All populations (20pop, 94 isolates | 0·272 | 0·154 | 0·433 | 0·653 |

$H_t$, Gene diversity totalled among populations; $H_s$, Gene diversity within populations; $G_{st}$, Genetic differentiation between populations; $N_m$, Number of migrants.

**Table 3** AMOVA analysis for the *Pyrenophora teres* f. *teres* populations using 401 AFLP bands

| Source of variation | df | Sum of squares | Variance components | Variation (%) |
|---|---|---|---|---|
| Among populations | 19 | 2134·373 | 15·290*** | 27·24 |
| Within populations | 74 | 3022·808 | 40·848*** | 72·76 |
| Total | 93 | 5157·181 | 56·139 | |

***$P < 0·001$.

Tunisian populations $N_m < 0·756$, while $N_m = 1$ in Syrian populations.

*Cluster polymorphism*. The genetic distance among field populations was estimated using Nei's unbiased measure of genetic distance (Nei 1978).

Cluster analysis based on UPGMA allowed a graphical representation of genetic similarity among 94 isolates from different sampling sites of Syria and Tunisia. *Pyrenophora teres* populations were grouped onto three main clusters that were genetically very distant from each other with bootstrap 55, 68, 76 (Fig. 1). Bootstrap values were generally high, in 47 cases were the values >50 (Fig. 1). There were two main clusters at the linkage distance level of 0·37 that were well supported by bootstrap values of 100%. The first cluster contained two isolates ICAPtr 16 and ICAPtr 17 from Syrian populations Hama and Tartous with bootstrap 76. The second cluster comprised 13 isolates from Aleppo, Hama and Tartous in Syria with only three isolates from two areas of Tunisia namely TUNPtr 59, 60, 76 (Gboulat, Siliana, NW), (Hincha, Sfax, SE) (Table 1). The third cluster comprised most of the Tunisian isolates (76 isolates) with only two isolates from Syria (Hama) ICAPtr 12 and 13 (Fig. 1, Table 1).

Conversely, the obtained dendrogram revealed no apparent association between geographical distance or

**Table 4** Molecular diversity estimates and its significance for *Pyrenophora teres* f. *teres* populations based on all isolates sampled from Syria and Tunisia

| TUN/Syr pop | Average of gene diversity overall loci | Average of pairwise differences | $F_{ST}$ indices | Number of polymorphic loci | % of polymorphic loci | Shannon's index |
|---|---|---|---|---|---|---|
| TUN pop 1 | 0·196 ± 0·119 | 78·600 ± 41·064 | 0·275 | 160 | 39·9 | 0·231 |
| TUN pop 2 | 0·221 ± 0·165 | 88·666 ± 53·345 | 0·284 | 133 | 33·17 | 0·211 |
| TUN pop 3 | 0·225 ± 0·148 | 90·333 ± 49·725 | 0·264 | 161 | 40·14 | 0·245 |
| TUN pop 4 | 0·198 ± 0·120 | 79·400 ± 41·478 | 0·274 | 171 | 42·64 | 0·237 |
| TUN pop 5 | 0·104 ± 0·079 | 42·000 ± 25·455 | 0·36 | 63 | 15·71 | 0·100 |
| TUN pop 6 | 0·234 ± 0·136 | 94·000 ± 47·348 | 0·238 | 206 | 51·37 | 0·289 |
| TUN pop 7 | 0·258 ± 0·170 | 103·833157·103 | 0·239 | 189 | 47·13 | 0·283 |
| TUN pop 8 | 0·261 ± 0·171 | 104·833 ± 57·650 | 0·237 | 195 | 48·62 | 0·287 |
| TUN pop 9 | 0·2391 ± 0·179 | 96·000 ± 57·728 | 0·273 | 144 | 35·91 | 0·228 |
| TUN pop 10 | 0·22210·167 | 89·333 ± 53·744 | 0·283 | 134 | 33·41 | 0·212 |
| TUN pop 11 | 0·217 ± 0·126 | 87·333 ± 44·015 | 0·251 | 199 | 49·62 | 0·272 |
| TUN pop 12 | 0·217 ± 0·119 | 87·321 ± 42·162 | 0·243 | 239 | 59·60 | 0·294 |
| TUN pop 13 | 0·2421 ± 0·136 | 97·238 ± 47·774 | 0·226 | 233 | 58·10 | 0·312 |
| TUN pop 14 | 0·143 ± 0·094 | 57·666 ± 31·871 | 0·323 | 109 | 27·18 | 0·159 |
| TUN pop 15 | 0·139 ± 0·092 | 56·000 ± 30·960 | 0·326 | 101 | 25·18 | 0·152 |
| TUN pop 16 | 0·199 ± 0·121 | 79·800 ± 41·685 | 0·273 | 168 | 41·89 | 0·236 |
| TUN pop 17 | 0·194 ± 0·127 | 77·833 ± 42·893 | 0·286 | 144 | 35·91 | 0·213 |
| Syr pop 18 | 0·128 ± 0·096 | 51·333 ± 31·034 | 0·345 | 77 | 19·20 | 0·122 |
| Syr pop 19 | 0·143 ± 0·081 | 57·428 ± 28·356 | 0·308 | 142 | 35·41 | 0·186 |
| Syr pop20 | 0·220 ± 0·128 | 88·466 ± 44·581 | 0·249 | 197 | 49·12 | 0·273 |
| All populations | 0·227 ± 0·199 | 70·347 ± 39·857 | 0·286 | 158 | 39·46 | 0·227 |

$F_{st}$, genetic differentiation between *P. teres* f. *teres* populations Shannon's index, measures populations' diversity in a community.

**Table 5** Genetic differentiation between *Pyrenophora teres* f. *teres* populations from Syria and Tunisia

| TUN/Syr pop | TUN popl | TUN pop2 | TUN pop3 | TUN pop4 | TUN pop5 | TUN pop6 | TUN pop7 | TUN pop8 | TUN pop9 | TUN pop10 | TUN pop11 | TUN pop12 | TUN pop13 | TUN pop14 | TUN pop15 | TUN pop16 | TUN pop17 | Syr pop18 | Syr pop19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TUN pop2 | 0·152 | | | | | | | | | | | | | | | | | | |
| TUN pop3 | 0·316 | 0·260 | | | | | | | | | | | | | | | | | |
| TUN pop4 | 0·380 | 0·333 | (−0·003 | | | | | | | | | | | | | | | | |
| TUN pop5 | 0·539 | 0·511 | 0·115 | (−0·003 | | | | | | | | | | | | | | | |
| TUN pop6 | 0·150 | 0·158 | 0·145 | 0·194 | 0·325 | | | | | | | | | | | | | | |
| TUN pop7 | 0·155 | 0·077 | 0·074 | 0·153 | 0·286 | 0·021 | | | | | | | | | | | | | |
| TUN pop8 | 0·084 | 0·073 | 0·239 | 0·314 | 0·447 | 0·102 | 0·062 | | | | | | | | | | | | |
| TUN pop9 | 0·146 | 0·136 | 0·188 | 0·249 | 0·420 | 0·095 | 0·052 | 0·003 | | | | | | | | | | | |
| TUN popl0 | 0·206 | 0·202 | 0·084 | 0·109 | 0·302 | 0·040 | 0·022 | 0·106 | 0·004 | | | | | | | | | | |
| TUN pop11 | 0·206 | 0·144 | 0·278 | 0·330 | 0·468 | 0·170 | 0·129 | 0·105 | 0·201 | 0·175 | | | | | | | | | |
| TUN pop12 | 0·078 | 0·201 | 0·320 | 0·384 | 0·501 | 0·164 | 0·188 | 0·116 | 0·177 | 0·217 | 0·177 | | | | | | | | |
| TUN pop13 | 0·152 | 0·124 | 0·280 | 0·340 | 0·461 | 0·166 | 0·160 | 0·087 | 0·114 | 0·179 | 0·145 | 0·156 | | | | | | | |
| TUN pop14 | 0·158 | 0·225 | 0·374 | 0·443 | 0·629 | 0·195 | 0·209 | 0·155 | 0·153 | 0·274 | 0·213 | 0·175 | −0·005 | | | | | | |
| TUN pop15 | 0·213 | 0·304 | 0·392 | 0·362 | 0·637 | 0·200 | 0·249 | 0·162 | 0·227 | 0·310 | 0·252 | 0·155 | 0·190 | 0·244 | | | | | |
| TUN pop16 | 0·109 | 0·198 | 0·325 | 0·396 | 0·538 | 0·156 | 0·171 | 0·119 | 0·150 | 0·221 | 0·197 | 0·046 | 0·156 | 0·179 | 0·019 | | | | |
| TUN pop17 | 0·121 | 0·220 | 0·394 | 0·423 | 0·575 | 0·175 | 0·191 | 0·115 | 0·198 | 0·249 | 0·212 | 0·044 | 0·187 | 0·215 | 0·178 | 0·005 | | | |
| TUN pop18 | 0·484 | 0·458 | 0·351 | 0·416 | 0·590 | 0·335 | 0·324 | 0·353 | 0·436 | 0·413 | 0·397 | 0·436 | 0·412 | 0·564 | 0·552 | 0·466 | 0·496 | | |
| TUN pop19 | 0·510 | 0·503 | 0·383 | 0·411 | 0·512 | 0·365 | 0·355 | 0·462 | 0·470 | 0·416 | 0·451 | 0·484 | 0·477 | 0·569 | 0·572 | 0·519 | 0·534 | 0·263 | |
| TUN pop20 | 0·262 | 0·212 | 0·203 | 0·269 | 0·380 | 0·126 | 0·090 | 0·192 | 0·198 | 0·160 | 0·251 | 0·251 | 0·222 | 0·298 | 0·288 | 0·255 | 0·278 | 0·241 | 0·211 |

Significant pairwise ($P < 0·05$), Abbreviations as in Table 1.

regional affiliation and the genetic distance between the populations. Indeed, *P. teres* f. *teres* isolates obtained from different sampling sites in the same area or country did not cluster together (Fig. 2). The mean genetic distance of each of the 20 populations from all others ranged from 0·027 to 0·325. The least genetic distance (0·027) was recorded between populations Bahra (Kef, NW Tunisia) and Barrage Meleg (Kef, NW Tunisia), while populations Touiref (Kef, NW Tunisia) and Hamrounia (Bizerte, NN Tunisia) were the most genetically distant (0·325). The 20 *P. teres* populations were grouped into two main clusters that were genetically very distant from each other. The cluster I was composed only by the Tunisian populations from 14 locations corresponding to different agro-ecological zones (Béja, Siliana, Kef, Zaghouan, Bizerte, Sousse, Sfax). The cluster II contained, however, apart from region Kef (Northwest Tunisia) that was represented by a three field populations (Bahra, Barrage Meleg and Touiref) and all Syrian populations (Fig. 2). None of the individualized subclusters comprised field populations from a single area, indicating considerable gene flow between regional populations (e.g. populations T8, T11,

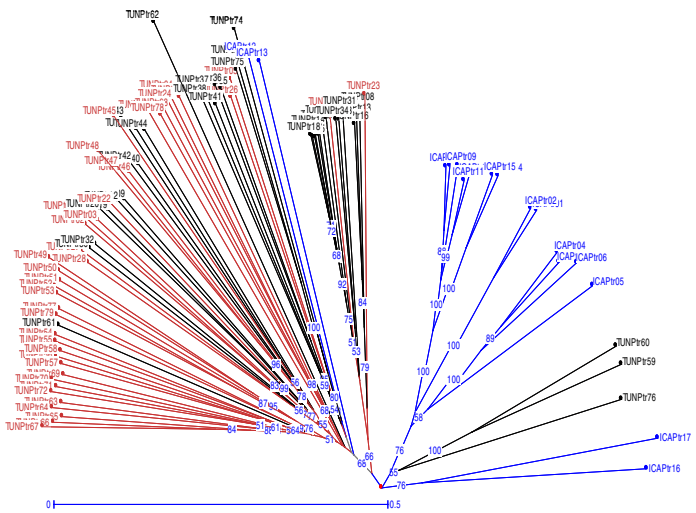


**Figure 1** UPGMA phenogram of *Pyrenophora teres* f. *teres* based on the AFLP data. Numbers on branches are bootstrap values. The isolates are designated by alphanumerics represent the origin (ICAPtr blue color represent isolates collected from Syria; TUNPtr: represent isolates sampled from Tunisia), followed by the host (the red color, for cv. Rihane and black one for a landrace cultivar).

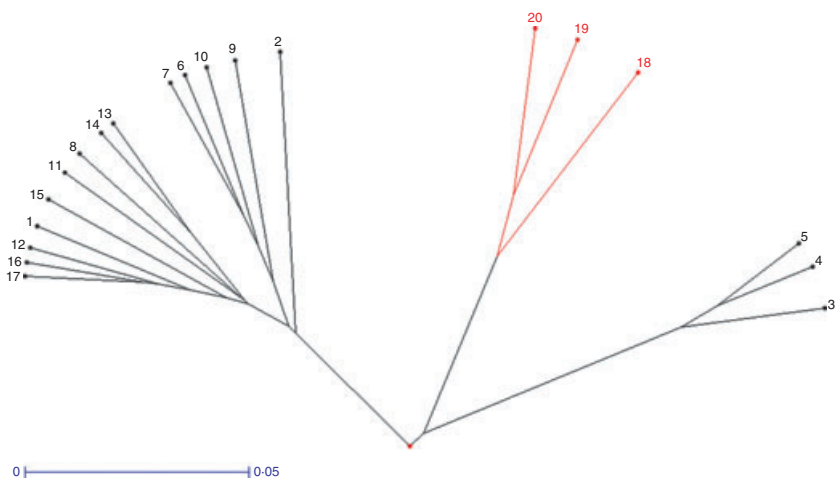


**Figure 2** UPGMA dendrogram of twenty *Pyrenophora teres* f. *teres* populations from Syria and Tunisia based on the AFLP data. The populations are designated by numerics (see Table 1). From P1 to P20: Tunisian and Syrian net blotch populations were represented on black and red, respectively. Cluster I Tunisian populations 1(P1), 2(P2), 6(P6), 7(P7), 8(P8), 9(P9), 10(P10), 11(P11), 12(P12), 13(P13), 14(P14), 15(P15), 16(P16), and Cluster II Syrian and Tunisian populations: 17(P17), 18(P18), 19(P19), 20(P20), 3(P3), 4(P4), 5(P5).

T13 and T14; T6, T7, T9 and T10; T15 and T1) (Fig. 2, Table 1).

## Discussion

Croissant fertile and North Africa are primary centres of barley diversity, and many barley growers in Syria and Tunisia still rely on genetically heterogeneous landraces, some of which are derived from the seeds that were transferred from generation to generation (Ceccarelli *et al.* 1987; Zoghlami *et al.* 2011). If pathogenicity on barley was acquired by a large number of genetically distinct strains, then *P. teres* populations in Syria and Tunisia have coevolved with many barley genotypes over a long period of time, resulting in a diverse pathogen population. It is, therefore, not surprising that all populations exhibited a high degree of genotype diversity (0·8) within a spatial scale of 1 $m^2$.

In this study, the marker technology has been employed to detect genetic variation and population structure of *Pyrenophora teres* f. *teres* from Syria and Tunisia and has once again demonstrated its usefulness in gaining information quickly and usefully in breeding programmes.

AFLP markers, along with appropriate statistical procedures are suitable for genetic variation analyses at both intra- and interpopulation levels (Serenius *et al.* 2007; Bentata *et al.* 2011).

The level of genetic diversity at AFLP markers detected here (*H*sp = 0·276) is comparable to or higher than, diversity levels reported for pathogen when using biochemical and molecular markers (Jonsson *et al.* 2000; Zaki and Al-Masry 2008).

The genetic variation was maintained within rather than between the studied populations (58·33%) as detected with AMOVA. Similarly, Serenius *et al.* (2007) found that in Russian *P. teres* f. *teres* 20 and 80% of the total variation was partitioned among and within populations, respectively. Leisova *et al.* (2005a,b) showed that 98·1% of genetic variance occurred within local populations and 1·9% was found among populations. However, Bentata *et al.* (2011) revealed a high genetic variability between the studied isolates in Morocco. The Syrian and Tunisian isolates were highly variable and contributed 27·24% of the total variation observed among all isolates using AMOVA analysis. AMOVA analysis revealed significant differences among populations and among samples within population ($P < 0·001$) (Table 3).

Conversely, high genetic variability at population level was detected. Indeed, a high number of polymorphic markers was produced by the Tunisian population no. 12 (239 markers) and the Syrian population no. 20 (197 markers), respectively, with a percentage of polymorphic loci 52 and 49% (Table 4). Also, 87 different haplotypes were identified among the 94 isolates studied. In others words, 87 isolates have specific genotypes, suggesting that there is more genetic recombination, which may have played a major role in genetic diversity of Syrian and Tunisian *Pyrenophora teres* f. *teres*. In addition, the high diversity might be either a result of a high mutation rate, large population size or retrotransposons, which can all contribute to diversity in asexually reproducing populations (McDonald and Linde 2002; Taylor *et al.* 2004).

These findings also indicated significant genetic subdivision of the Syrian and Tunisian *P. teres* populations according to $F_{st}$ values ($F_{st} = 0·27$, $P < 0·001$). The $F_{st}$ estimate is considered to be more biased than $\Phi_{ST}$ for the evaluation of differentiation coefficient for dominant marker data and may suggest that the *P. teres* populations analysed are moderately differentiated according to Wright's interpretation of $F_{st}$ values (Wright 1978).

As Syria is the primary centre of origin of *Hordeum* species, it can therefore shelter old pathogen populations, where mutation events occurring over a long period of time have increased pathogen population diversity. This was supported by the $F_{st}$ value (Table 4) that identified Syria to have most of the variation identified elsewhere. High diversity has also been observed in *Fusarium graminearum* Schwabe [teleomorph (*Gibberella zeae*) (*Schwein.*)] in West Asia and Southern Russia, which was suggested to be the origin of *F. graminearum*, whereas the Finnish *F. graminearum* isolates were significantly less variable (Gagkaeva and Yli-Mattila 2004).

In the present study, genetic differentiation among the 20 field populations (sample size of 3–8 isolates) was high ($G_{st} = 0·398$) but dropped to 0·31 when Syrian field populations were grouped together, and increase to 0·40 in Tunisian field populations (Table 2). In fact, cereal pathogen populations are relatively old in Syria (Burdon and Silk 1997; Khan and Tekauz 1982), and were likely established by relatively few isolates. Migration via seed to intra- and interareas in Syria, and therefore the diversity, has remained comparatively high.

A moderate level of genetic differentiation was found between the pathogen population from Tunisia and Syria based on Nei's genetic distances, high $G_{st}$ values with $N_m = 0·653$. Although, evidence for differentiation was found in pairwise comparisons involving populations from all other areas ($F_{st} = 0·11–0·63$), suggesting that the 'geographic populations' in this study are evolving independently and hence may be considered not a part of the same 'genetic population'. The moderate differentiation observed may have resulted from the rather small number of isolates sampled in populations (8, 20) although bootstrap tests of significance enabled us to estimate indices of population differentiation with a reasonable degree of

confidence (Grünwald *et al.* 2003). This differentiation may indicate local adaptation of *P. teres* to the high rainfall conditions of different areas in Syria and Tunisia compared with the moderate or low rainfall conditions in the more arid regions. Assuming that our AFLP data accurately reflect the differentiation between these populations in Syria and Tunisia, they indicate that the identification and deployment of net blotch-resistant varieties would need to take into consideration these two different regional populations. However, detailed analysis of gene flow between areas using larger sample sizes and codominant markers is warranted to corroborate the moderate population differentiation observed in this study. Knowledge of pathotype distribution will also help determine whether *P. teres* pathotypes in the country are geographically circumscribed (Bouajila *et al.* 2011).

The most probable explanation for the high genotype diversity, moderate gene flow and high genetic differentiation of the pathogen population in Syria and Tunisia is sexual reproduction and air dispersal of ascospores. Forcibly discharged ascospores become airborne and may serve as primary inoculum over considerable distances (Trail *et al.*, 2002). Wind dispersal of ascospores enables the migration of new genotypes into barley fields and may facilitate the rapid dissemination of virulent mutants across entire areas. Many studies showed the identification of the telemorph and detection of sexual recombination in natural populations of *P. teres* (McLean *et al.* 2009).

The importance of genetic diversity and the potential consequences associated with narrowing the genetic base of cultivated barley has long been recognized by plant pathologists and plant breeders. The potential for severe net blotch epidemics has increased with the production of modern homogenous, genetically uniform barley varieties with specific genes for resistance to barley net blotch. The high genetic diversity, the high gene flow and the potential for sexual recombination in Syria and Tunisian populations of *P. teres* mean that reliance on major gene resistance is unlikely to be an appropriate breeding strategy (McDonald and Linde 2002).

In conclusion, the existence of genetic variability observed, can be explained by inter-regional and intraregional gene flow between the Syrian and Tunisia isolates. However, the durability of specific resistance genes may be increased by the use of multiline cultivars, by combining ('pyramiding') different resistance genes. Syria includes in Middle East, which is the centre of origin and earliest knowledge of domestication for many cereal crops. Increased knowledge of the population biology of *P. teres* is likely to lead to better management of disease in agricultural ecosystems. Recently, *P. teres* f. *teres* isolates were differentially pathogenic. CI09214 and CI05401 cultivars were released as the most effective sources of resistance in Syria and Tunisia (Bouajila *et al.* 2011) and combined (pyramided) into elite varieties and by deploying many cultivars with different resistance genes in space or time.

## Materials and methods

### Collection of *P. teres* isolates

In this study, 94 *P. teres* f. *teres* isolates were sampled from barley-infected leaves from 20 locations (17 populations from Tunisia and three populations from Syria) throughout the major barley-growing areas in Syria and Tunisia (Table 1).

For the Tunisian isolates, leaf samples were collected from naturally infected fields during the 2009 season using the hierarchical sampling method described by McDonald *et al.* (1999): one field population was sampled at each location, drawing a composite sample from eight circular spots of 1 m diameter each located along two parallel transects (with four spots per transect). The two transects and the collection sites (spots) along the transects were separated from the adjacent members by 10 m. This field design allowed a total sampling area of 408 $m^2$ (12 × 34 m). Ten infected leaves were sampled from different plants or tillers in a circular sweep of each sampling spot. Most collections were made from the northern subhumid to semi-arid region (69 isolates), which is the largest barley-growing area in Tunisia, and the rest from the central (three isolates) and the southern regions (six isolates) of the country.

Sixteen Syrian net blotch infected leaf samples were collected from three locations covering the major barley-growing areas in the northern subhumid to the semi-arid region in North Western (three isolates), and Western (13 isolates) Syria (Table 1) in springs of 1998, 2003, 2006, 2007 and 2009. Fields were sampled randomly along major roads in the principal barley productions areas in Syria (three populations).

Leaf samples were placed in paper envelopes, air-dried at room temperature for 48 h and stored at 6°C until required.

### Single-spore isolation and inoculum production

Leaves showing typical net blotch symptoms were cut into discs 5–10 mm in diameter, surface-sterilized in 90% ethanol for 10 s and in 1% NaCl for 60 s, rinsed twice in sterile deionized water for 1 min each time, blotted, dried and aseptically transferred to Potato dextrose agar (PDA) plates. The plates were incubated at 20°C for several days under alternating cycles of 12 h of near-ultraviolet (NUV)

light and 12 h of darkness. After 3–5 days, single conidia were transferred with a needle, while looking through a microscope, to fresh PDA plates and incubated for 2 weeks to induce growth of mycelia.

### DNA extraction

Approximately 0·05 g of the freeze-dried fungal tissue was ground into a fine powder in a 2-ml microfuge tube using a mixer-mill. Total DNA from each fungal isolate was extracted using a modified hexadecyltrimethylammonium bromide (CTAB) extraction procedure (Bouajila *et al.* 2007). Extracted DNA was resuspended in Tris–EDTA buffer (10 mmol $l^{-1}$) and stored at −20°C. DNA concentrations were estimated by spectrophotometry and/or agarose gel electrophoresis. DNAs were run in a 0·8% agarose gel to verify the quality and the concentration.

### AFLP analysis

The AFLP procedure was performed with minor modifications according to the protocol of Vos *et al.* (1995). Approximately 40-ng DNA was digested simultaneously with *EcoRI* and *MseI* at 37°C for 4 h. The digested samples were incubated at 70°C for 15 min to inactivate the restriction endonucleases. *EcoRI* and *MseI* adapters were ligated to the digested samples at 20°C for 4 h. This was performed to generate template DNA for amplification. Pre-amplification was carried out with + 1- primers each carrying one selective nucleotide (*EcoRI* + A and *MseI* + C) in a thermocycler for 30 cycles (94, 56 and 72°C/30 s). The amplification products were stored at −20°C. Selective amplification was carried out with *EcoRI* + 1 and 2- primers and *MseI* + 3- primers and 5 $\mu$l of the diluted PCR products from the pre-amplification. Six primer pair combinations were employed in this study (Table 2). The PCR amplification was performed as follows: 12 cycles at 94°C for 30 s, 65°C for 30 s and 72°C for 60 s, with annealing temperature lowered by 0·7°C every cycle. This was followed by 23 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 60 s.

### Gel analysis

The reaction products were mixed with equal volumes of formamide loading buffer (95% formamide, 10 mmol $l^{-1}$ EDTA, bromophenol blue and xylene cyanol), denaturated by incubating at 95°C for 5 min and quickly cooled on ice. The products were analysed on 6% denaturing polyacrylamide gels. The gel was run at constant power (50–55 W) until the dye was about 2/3 down the length of the gel. AFLP bands were visualized by silver staining.

### Data analysis

For AFLP analysis, bands were scored as 1 denoting presence or 0 denoting absence, and a matrix of AFLP phenotypes was then assembled across all individuals and populations. For each primer, the total number of bands and the polymorphic ones were calculated.

The index of phenotypic diversity ($H_o$), the average diversity over all populations ($H_{pop}$) and the mean diversity at species level ($H_{sp}$) were estimated as described by Yeh and Boyle (1997). The component of within-population diversity was estimated as $H_{pop}/H_{sp}$, and that of between-population diversity as $1 - H_{pop}/H_{sp}$. All the above calculations were undertaken by POPGENE 1·32 (Yeh and Boyle 1997).

A pairwise Euclidean distance matrix was generated with the computer package AMOVA-PREP 1·01 (Miller 1998) and was then used as input for WINAMOVA 1·55 for AMOVA analysis (Excoffier *et al.* 1992) to test whether populations had differing amounts of AFLP variation.

The gene flow (*Nm*, number of migrants per generation) (Whitlock and McCauley 1999) was approximated as: $N_m = (1/4)\ [(1\ /F_{st}) - 1]$, where $F_{st}$ (inbreeding index) values were available from a matrix of pairwise combinations produced by WINAMOVA.

The Shannon information index (*H*), which is commonly used to characterize populations' diversity in a community and accounts for both abundance and evenness of the populations that are present. It was calculated using Popgene v1·31 software (Yeh *et al.* 1999) according to the formula:

$$H = -\sum p_i \ln p_i$$

where ($p_i$) represents the proportion of a population *i* relative to the total number of the populations analysed at AFLP markers.

A dendrogram among the populations was constructed with software Darwin (Version 5·0·148) (http://darwin.cirad.fr/darwin) using the matrix of pairwise $F_{st}$ values and the unweighted pair-group method with arithmetical averages (UPGMA) (Sneath and Sokal 1973) algorithm.

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