

Amplified Fragment Length Polymorphism of *Puccinia graminis* f. sp. *tritici* populations in Ethiopia

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Abstract

The genetic structure of forty eight Ethiopian *Puccinia graminis* f. sp. *tritici* (Pgt) isolates, representing three major wheat growing regions was investigated using 15 AFLP primer combinations. AFLP analysis generated large number of polymorphic bands (markers) and allowed easy identification of the different genotypes. The study showed a high level of genetic diversity within the isolates. There was no population subdivision based on origin of isolates as reflected by a low coefficient of genetic differentiation (0.107), and a single dendrogram cluster consisting of all isolates except three. Gene flow among populations was estimated to be high. The AFLP analysis characterized the isolates to have high genetic diversity and homogeneity across regions. The developed AFLP fingerprints for the Ethiopian Pgt isolates reported herein could support the breeding program to develop strategies for the deployment of resistance genes in its continued effort to minimize the impact of stem rust on wheat in Ethiopia.

Keywords: AFLP, wheat, stem rust, genetic diversity, population differentiation

Introduction

Bread wheat (*Triticum aestivum* L.) is one of the most widely grown cereal crops worldwide. Diseases are seen as the key limitation on crop yield since mankind started to cultivate plants (Oerke *et al.*, 1994), and are the major focus of breeding programs. Fungal diseases have been considered as the main causes of crop losses in wheat. Of these, stem rust caused by *Puccinia graminis* f. sp. *tritici* (Pgt) is the main limiting factor for wheat production throughout the world, where the environment is warm. Estimates of yield losses in wheat due to stem rust range from 15 - 35% in Canada and the United States, respectively (Dubin and Brennan, 2009). Ethiopia is one of the hot spot areas of the world for stem rust. It is widespread in the major wheat rowing regions of the country at varying levels (Admassu and Fekadu, 2005; Admassu *et al.*, 2009). The appearance and spread of Ug99, which is virulent to most of Sr-genes that were effective against all known Pgt races (Pretorius *et al.*, 2000; Singh *et al.*, 2006) has aggravated the problem in Ethiopia as well as elsewhere. Since the first report of Ug99 in Ethiopia it has gone to become the dominant race throughout the

country (Admassu *et al.*, 2009). Although there are effective fungicides available to manage stem rust, they are not recommended to farmers in the developing countries due to their affordability and environmental safety. Hence, the use of disease resistant cultivars is the best option from the economic and environmental point of view. However, effective resistance breeding requires extensive information on the pathogen genetics.

The development of molecular genetics technology has greatly improved research in many disciplines such as Taxonomy, Phylogeny, Ecology, Genetics and Evolutionary biology (Tingey and Del tufo, 1993). Molecular markers can also facilitate quantification of existing genetic diversity within and among organisms. One of these molecular techniques used to study the genetic diversity of organisms is Amplified Fragment Length Polymorphism (AFLP), which is a PCR-based molecular marker that is being used for the characterization and evaluation of organisms (Vos *et al.*, 1995). In the case of plant pathology molecular techniques such as AFLP have been successfully employed for the characterization and assessment of the genetic relatedness of plant pathogens (Brake *et al.*, 2001; Kieper *et al.*, 2006; Mebrate *et al.*, 2006; Bouajila *et al.*, 2007; Szabo, 2007). In addition, they have been utilized to develop molecular markers linked to disease resistance gene (Nachtigall *et al.*, 2000) and mapping of resistance genes (Chelkowski *et al.*, 2003). Although there is scattered information regarding the virulence diversity of *Pgt* in Ethiopia, there is scarcity of information on the genetic diversity of the pathogen population. Therefore, the objective of this study was to study the genetic structure of *Pgt* populations in Ethiopia using AFLP.

Materials and Methods

***Pgt* isolates**

Puccinia graminis f. sp. tritici samples were collected from farmers' wheat fields in Ethiopia. Monopustule isolates were developed and multiplied according to Fetch and Dunsmore (2004). For the sake of a better insight in to the molecular diversity of *Pgt* populations, 48 isolates were selected and differentiated into three population groups based on their geographic origin. These three populations were: southeast Ethiopia (Arsi and Bale), central Ethiopia (east, west, southwest, northwest and north Shewa) and northwest Ethiopia (Gojjam and Gondar). The three regions had 16, 21 and 11 isolates, respectively.

Genomic DNA isolation

The total genomic DNA was extracted from 20 mg urediospores using the Nucleoplex™ Plant DNA kit (Tepnel Life Sciences, Manchester, UK) according to the manufacturer's instruction with minor modification, i. e., urediospores were first grounded by shaking each sample with beads for 90 s at a frequency of 27/s twice in a Mixer Mill MM 301 shaker (Retsch, Hannover, Germany). The concentration and quality of DNA were estimated using the NanoDrop ND-1000 spectrophotometer (PeQLab, Erlangen, Germany) and gel electrophoresis.

AFLP analysis

50 *EcoRI*+2/*MseI*+2 primer pair combinations were screened for polymorphism using a subset of nine isolates, three from each region. Out of these, 15 primer combinations were selected (Table 1) based on their polymorphism and un-ambiguity of bands they produced, and used for the AFLP analysis. The AFLP analysis was conducted using the method described by Vos *et al.* (1995), with little modification, i.e., 1 μ l genomic DNA (150 ng) was digested for one hour at 37°C with 2 μ l each of *EcoRI*/*MseI* restriction enzymes. The pre-amplification PCR was performed in a reaction volume of 50 μ l consisting of 5 μ l buffer (10x), 1.5 μ l of each *EcoRI*+1/*MseI*+1 primers (50 ng). The selective amplification PCR was performed using the *EcoRI*+2 (50 ng) and *MseI*+2 (10 ng) primerpairs. PCR was run in GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, USA) according to the programme described by Vos *et al.* (1995). Two randomly selected isolates were run in adjacent lanes in all PCR assays as an internal control to check the reproducibility of the tests. Finally, fragments were separated on Li-COR 4300 genetic analyzer (Li-COR Biosciences, Lincoln, USA).

Data analyses

Only clearly distinguishable AFLP bands ranging from 50 to 600 bp were scored and used for the statistical analyses. In the AFLP analysis, the absence or presence of allele(s) at a particular locus was recorded as 0 or 1, respectively. Distance matrices were calculated using the Neighbour Joining method, and were later used to generate the dendrogram using the software NTSYSpc, v2.01e (Rohlf, 1998).

Population genetic analyses were done using the computer software POPGENE (Yeh *et al.*, 1999). Accordingly, the genetic distance between populations (D) was estimated from allele frequencies using Nei's (1978) unbiased genetic distance for small samples. The genetic identity is $1 - D$. Gene diversity at a given locus (h) was estimated using the formula: $h = 1 - \sum X_i^2$, where X_i is the frequency of the i^{th} allele of a given locus. Population differentiation was calculated using the equation: $G_{st} = (H_t - H_s)/H_t$, where H_t refers to the total gene diversity, and H_s refers to the gene diversity within sub-populations. Gene flow between populations (N_m) was estimated using the formula: $N_m = 0.5 (1 - G_{st})/G_{st}$. Other population genetic parameters that were estimated using POPGENE software included: number of observed and effective alleles, and percentage polymorphic loci. Multilocus, v1.3 (Agapow and Burt, 2001) computer software was used to estimate the genotypic diversity of the populations.

Results

Of the 48 *Pgt* isolates, DNA from one isolate was not amplified during the AFLP analysis. Hence, data generated from 47 isolates in the AFLP assays was used for the final analyses. The internal controls utilized in the AFLP fingerprinting were analysed as separate isolates, and the duplicates were identical (100% genetic similarity), showing the reliability of the method. 631 fragments were scored over the 15 AFLP

primer pairs on the set of 47 isolates. Of these, 335 (53.1%) of the AFLP bands were polymorphic (Table 1). The average number of AFLP bands per primer pair was 42.1.

Table 1. Selected *EcoRI/MseI* primer combinations and the corresponding polymorphic fragments produced using 47 isolates from Ethiopia

Primer combination	Total number of amplicons	Number of polymorphic amplicons	Polymorphism (%)
E-CT/M-AG	48	27	56.3
E-AT/M-CT	45	26	57.8
E-AG/M-AG	26	13	50.0
E-AA/M-AG	35	16	45.7
E-AC/M-AG	35	21	60.0
E-AT/M-CG	40	26	65.0
E-CT/M-TT	36	21	58.3
E-TT/M-AG	30	12	40.0
E-TT/M-TG	31	8	25.8
E-AC/M-AA	47	16	34.0
E-AT/M-AT	28	11	39.3
E-TT/M-AT	33	11	33.3
E-AG/M-AA	35	24	68.6
E-TT/M-CG	86	55	64.0
E-CT/M-CG	71	48	67.6
Total	631	335	
Average	42.1	22.3	53.1

The genetic structure of the three populations is summarized in Table 2. The AFLP analysis showed higher genotypic diversity 'within' the northwest Ethiopia (0.231) and central Ethiopia (0.236) populations than in the southeast Ethiopia (0.186) population. In a similar trend low gene diversity was observed within the southeast Ethiopia population (0.165) compared to northwest (0.210) and central Ethiopia (0.213) populations. The analyses also revealed that all the isolates studied were distinct from one another.

Pair-wise population comparisons for genetic distance and identity indicated a high degree of similarity among populations (Table 3). The highest similarity was observed between northwest Ethiopian and central Ethiopian populations with estimated identities of 0.966. On the other hand, a relatively low degree of genetic identity (0.832) was observed between northwest and southeast Ethiopia populations. A further indication to a high genetic similarity among populations was provided by the coefficient of genetic differentiation (G_{ST}), which was estimated at 0.107 (Table 4). G_{ST} values of less than one are an indicator to a high degree of genetic similarity among populations and absence of population differentiation. The high level of gene flow among populations might have contributed for the absence of genetic differentiation among populations. The present study revealed high estimates of gene flow among populations of the three regions. The estimated gene flow per season was 4.2 when analysed using the AFLP method. The other genetic parameters described in this study were the number of observed and effective alleles. Effective alleles represent the

number of genetically distinct individuals that contribute gametes to the next generation. Out of 1.53 average observed alleles across populations 1.40 (91.5%) alleles were effective in the AFLP analyses. Looking at individual regions, central and northwest Ethiopia populations had the highest number of observed and effective alleles respectively (Table 2).

Clustering of isolates by the unweighted pair group method with arithmetic averages (UPGMA) using the software NTSYSpc, v2.01e (Rohlf 1998) placed 44 (93.6%) of the isolates in one cluster while the remaining three (6.4%) were grouped in a second cluster at about 60% genetic similarity (Fig 1).

Table 2. Genetic structure of Pgt populations in Ethiopia analysed by AFLP

Region	Sample size	N _a	N _e	PL (%)	GnD	NG	GtD	G _{st}	N _m
Total	47	1.53	1.40	53.1		47		0.107	4.2
SEE	15	1.49	1.28	48.71	0.165	15	0.186		
NWE	11	1.52	1.38	52.16	0.210	11	0.231		
CEE	21	1.53	1.38	52.59	0.213	21	0.236		

N_a = Observed number of alleles

PL = Polymorphic loci

NG = Number of genotypes

G_{st} = Genetic differentiation between populations

N_e = Effective number of alleles

GnD = Gene diversity

GtD = Genotypic diversity

N_m = Estimate of gene flow

Discussion

The AFLP markers proved to be informative with the exception of a few, like E-TT/M-TG that produced a small number of fragments and revealed a low percentage polymorphism. On the other hand, AFLP markers E-AG/M-AA, E-TT/M-CG and E-CT/M-CG yielded the largest numbers of fragments and revealed a high percentage polymorphism (Table 1). Visser et al. (2008) utilized four different AFLP markers, and obtained an average of 91 bands per primer, which was higher than in the present study (42.1 per primer); but their average polymorphism (41.1%) was lower than in the present study (53.1%). They suggested that primers that produce a high number of bands and polymorphism like E-AG/M-AA and E-TT/M-CG could be used to develop SCARs for a rapid detection of isolates.

Table 3. Genetic identity (below) and distance (above) between Pgt populations in Ethiopia analysed by AFLP

	NEW	CEE	SEE
NWE	-	0.034	0.168
CEE	0.966	-	0.092
SEE	0.832	0.908	-

*Nei's (1978) unbiased measure of genetic distance and identity

The present study showed that AFLP analyses revealed the genetic diversity of Pgt isolates in Ethiopia. The level of genetic diversity is reflected by the genotypic diversity, the large number of genotypes and the gene diversity within populations.

The genetic diversity revealed in this study is in line with the high virulence diversity among *Pgt* populations previously reported (van Ginkel *et al.* 1989; Admassu and Fekadu 2005; Admassu *et al.* 2009). However, microsatellite based analysis of *Pgt* isolates revealed a significantly higher level of genetic diversity than the AFLP analysis (Admassu *et al.* 2010). This might be due to the lower sensitivity of AFLPs to fine genetic variations than the microsatellite markers. Comparison of the AFLP analyses to previous studies indicated that the Ethiopian isolates have a higher genetic diversity than isolates from South Africa (Visser *et al.* 2008). The other potential sources of variation that can contribute to genetic diversity in *Pgt* as reported by various authors (McDonald 2004; Bouajila *et al.* 2007; Anderson and Pryor 1992; Visser *et al.* 2008) include sexual reproduction, accumulated mutation, somatic hybridization and introduction of genetically distinct exotic isolates like Ug99.

The isolates from the three regions were closely related to one another as reflected by the high genetic similarity among populations ranging between 0.85 – 0.97. The clusters generated using the AFLP data (Fig. 1) also supported the absence of genetic differentiation in the Ethiopian *Pgt* populations. This was in contrast to the hypothesis that *Pgt* populations in Ethiopia are differentiated based on geographic origin of isolates. The AFLP analysis, however, is in line with the SSR tests that confirmed absence of population differentiation based on geographic separation (Admassu *et al.* 2010). Low level of genetic differentiation based on geographic separation is associated with the dispersal mechanism of the pathogen, which is easily carried by wind long distances, and is not unique to Ethiopian isolates. McCallum *et al.* (1999) and Ordonez and Kolmer (2007) have also reported similar results for the different wheat rust pathogens. On the other hand, various authors (Brake *et al.* 2001; Kieper *et al.* 2006; Visser *et al.* 2008) reported clonal lineage in wheat rusts. The high estimate of gene flow among Ethiopian *Pgt* populations (4 per generation), suggests that there have been only few restrictions to gene flow among regions.

The present results as well as previous study (Admassu *et al.*, 2010) showed that *Pgt* populations in Ethiopia are characterized by a high genetic diversity and homogeneity across regions. Such phenomena can easily make deployed resistance genes ineffective in a very short period of time. Hence, the agricultural research system needs to develop an anticipatory breeding programme as well as deploy cultivars carrying multiple resistance genes to attain durable stem rust control in the country.

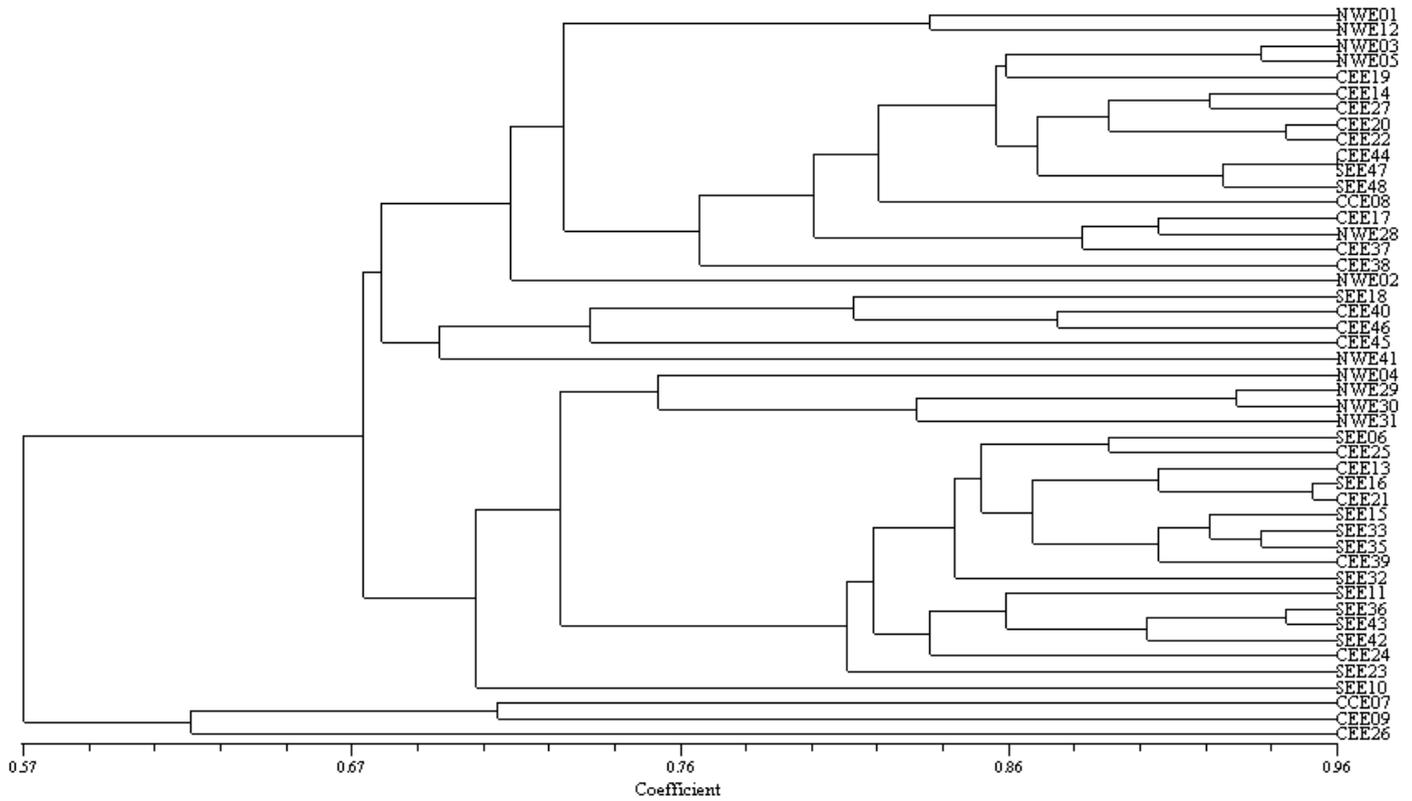


Fig 1. Cluster of 47 Pgt isolates from Ethiopia based on data generated from 15 *EcoRI/MseI* primer combinations.

Acknowledgement

The first author was supported by a scholarship from the Katholischer Akademischer Ausländer-Dienst (KAAD), Germany to conduct this research. We would like to thank the Ethiopian Institute of Agricultural Research for providing leave of absence for the first author to carry out the research.

References

- Admassu B. & Fekadu E. 2005. Physiological races and virulence diversity of *Puccinia graminis* f. sp. *tritici* wheat in Ethiopia. *Phytopathologia Mediterranea*, 44, 313-318.
- Admassu B., Lind V., Friedt W. and Ordon F. 2009. Virulence analysis of *Puccinia graminis* f. sp. *tritici* populations in Ethiopia with special consideration of Ug99. *Plant Pathology* 58: 362 – 369.
- Agapow, P. M. & Burt, A. 2001. Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes*, 1, 101-102.
- Anderson, P. A. & Pryor, A. J. 1992. DNA restriction fragment length polymorphisms in the wheat rust fungus, *Puccinia graminis* f. sp. *tritici*, *Theor Appl Genet* 83, 715-719.
- Brake, V. M., Irwin, J. A. G. & Park, R. F. 2001. Genetic variability in Australian isolates of *Puccinia coronata* f. sp. *avenae* assessed with molecular and pathogenicity markers. *Australasian Plant Pathology*, 30, 259-266.
- Bouajila, A., Abang, M. M., Haouasa, S., Udupa, S., Rezgui S., Baum M. et al. 2007. Genetic diversity of *Rhynchosporium secalis* in Tunisia as revealed by pathotype, AFLP, and microsatellite analyses. *Mycopathologia*, 163, 281-294.
- Chelkowski J, Tyrka M, Sobkiewicz A. 2003. Resistance genes in barley (*Hordeum vulgare* L.) and their identification with molecular markers. *J Appl Genet* 44:291-309.
- Dubin, H. J. and Brennan, J. P. 2009. Combating Stem and Leaf Rust of Wheat: Historical Perspective, Impacts, and Lessons Learned. IFPRI Discussion Paper 00910.
- Fetch, T. G. & Dunsmore, K. M. 2004. Physiological specialization of *Puccinia graminis* on wheat, barley, and oat in Canada in 2001. *Canadian Journal of Plant Pathology*, 26, 148-155.
- Keiper, F. J., Haque, M. S., Hayden, M. J. and Park, R. F. 2006. Genetic diversity in Australian populations of *Puccinia graminis* f. sp. *avenae*. *Phytopathology*, 96, 96-104.
- McCallum, B. D.; Roelfs, A. P., Szabo, L. J. and Groth, J. V. 1999. Comparison of *Puccinia graminis* f. sp. *tritici* from South America and Europe. *Plant Pathology*, 48, 574-581.
- McDonald, B.A. (2004). Population Genetics of Plant Pathogens. *The Plant Health Instructor*. DOI:10.1094/PHI-A-2004-0524-01.
- Mebrate, S. A., Dehne, H. W., Pillen, K. and Oerke, E. C. 2006. Molecular diversity in *Puccinia triticina* isolates from Ethiopia and Germany. *J. Phytopathology*, 154, 701-710
- Nachtigall M, Saker M, Kopahnke D, Walther U. 2000. Development of molecular markers in barley against fungal resistance. *Ber Arbeitstag Vereinigung Osterreichischer Pflanzenzüchter* 51.
- Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 89, 583-590
- Oerke E, Dehne H, Schonbeck WF, Weber A. 1994. Crop production and crop protection : estimated losses in major food and cash crops. Amsterdam: Elsevier, 808 p.
- Ordóñez, M. E. and Kolmer, J. A. 2007. Simple sequence diversity of a worldwide collection of *Puccinia triticina* from durum wheat. *Phytopathology*, 97, 574-583.

- Pretorius ZA, Singh RP, Wagoire WW, Payne TS. 2000. Detection of virulence to wheat stem rust resistance gene Sr31 in *Puccinia graminis* f. sp. *tritici* in Uganda. *Phytopathology* 84:203
- Rohlf, F. J. (1998). NTSYSpc Version 2.01e: User Guide. (New York: Applied Biostatistics Inc.)
- Singh, R. P., Hodson, D. P., Jin, Y., Huerta-Espino, J., Kinyua, M. G., Wanyera, R., Njau, P. & Ward, R. W. 2006. Current status, likely migration and strategies to mitigate the threat to wheat production from race Ug99 (TTKS) of stem rust pathogen. *Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, 1, 1- 13.
- Szabo L. J. 2007. Development of simple sequence repeat markers for the plant pathogenic rust fungus, *Puccinia graminis*. *Molecular Ecology Notes*, 7, 92-94.
- Tingey V. and Del Tufo, P. 1993. Genetic analysis with random amplified polymorphic DNA markers. *Plant Physiol* 101:349-352.
- Van Ginkel, M. G., Getnet, G. and Tessema, T. 1989. Stripe, stem and leaf rust races in major wheat producing areas in Ethiopia. *IAR Newsletter of Agricultural Research*, 3, 6-8.
- Visser, B., Herselman, L. and Pretorius, Z. A. 2008. Genetic comparison of Ug99 with selected South African races of *Puccinia graminis* f. sp. *tritici*. *Molecular Plant Pathology*. DOI: 10.1111/j.1364-3703.2008.00525.x
- Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., et al. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23: 4407- 4414
- Yeh, F. C., Yang, R. C., Boyle, T. B. J., Ye, Z. H. & Mao, J. X. (1999). POPGENE: The user-friendly shareware for population genetic analysis. (Edmonton: University of Alberta).