VIRULENCE AND MOLECULAR POLYMORPHISM OF *PUCCINIA TRITICINA* PATHOTYPES IN EGYPT

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ABSTRACT

Forty wheat leaf rust (*Puccinia triticina*) pathotypes were collected from eleven Egyptian governorates during the two growing seasons 2016/2017 and 2017/2018 were analyzed based on both virulence and molecular marker analysis. Virulence analysis was carried out based on infection type of the tested pathotypes on 20 differential monogenic lines, each carrying single leaf rust resistance genes (Lr). Six simple sequence repeats (SSR) markers were used for molecular characterization of *P. triticina* to detect the genotypic variation among pathotypes. Almost all of the tested pathotypes were phenotypically and genetically varied that confirms a high diversity within Egyptian leaf rust populations. Cluster analysis based on both virulence analysis and molecular patterns classified the tested pathotypes into three main groups. A relatively weak correlation was found between virulence and molecular analysis (r = 0.03). High similarity was found between leaf rust populations in the three governorates; Sohag, Bani Swear, and Fayoum. Also, the high similarity was found between leaf rust populations in the five; Egyptian governorates; Minufiya, Kafr-Elsheikh, Gharbiya, Alexandria, and Qalyubia, while, wide variation was found between leaf rust populations of the three governorates; Beheira, Sharqiya and Dakahlia. The results of this study support using molecular markers analysis to estimate genetic diversity between *P. triticina* pathotypes.

Keywords: *Puccinia triticina*, virulence, molecular markers, genetic diversity, leaf rust resistance genes.

INTRODUCTION

Leaf rust caused by *P. triticina* is the most common and widespread rust disease as it was annually found everywhere wheat (*Triticum aestivum* L.) is grown in Egypt and worldwide. Severe yield losses in the susceptible wheat cultivars due to leaf rust infection may be reached to approximately 50% under favorable environmental conditions for disease incidence and development (Germán *et al.*, 2007). In Egypt, experimentally grain yield losses as a result of artificial leaf rust infection reached up to 32% on the highly susceptible wheat cultivars (Shahin and El-Orabey, 2016; El-Orabey *et al.*, 2017).

Breeding for resistance or growing resistant cultivars is still the most effective and preferred control method for minimizing the annual grain yield losses due to rust diseases, especially leaf rust (Kolmer *et al.*, 2012). So, a successful breeding program for leaf rust resistance requires specific information about the evolution and virulence of the new *P. triticina* pathotypes. Due to the high evolutionary potential of leaf rust populations and the large population sizes that consisted of a large number of pathotypes, it is essential to understand and measure the diversity of *P. triticina* pathotypes within its populations. However, wide variations and high diversity of virulence in *P. triticina* populations in Egypt, mainly due to high selection pressure and migration, are the main two evolutionary forces for the emergence of many new leaf rust pathotypes (McVey *et al.*, 2004). Recently, a total of 226 leaf rust pathotypes were detected during the 2016/17 and 2017/18 growing seasons from 12 Egyptian governorates (El-Orabey *et al.*, 2018). Diversity characteristics and analysis of the genetic structure of pathogen populations for rust fungi, in general, are very important to make a proper and
correct decision for the planning and direction of the successful breeding programs for rust resistance especially leaf rust. However, knowledge on the diversity of leaf rust populations in Egypt is very limited; thus, little information is available about the diversity of P. triticina pathotypes occurring within its pathogen population in the country. Also, few studies were conducted on the molecular characterization of P. triticina pathotypes in Egypt. Out of these studies, El-Orabey et al. (2018) studied phenotypic diversity on P. triticina populations in Egypt using the three main and widely used indexes i.e. Shannon, Gleason, and Simpson. This study revealed that, there was a high diversity among leaf rust populations in Egypt due to the selection pressure and migration. Molecular marker analysis has been used to assess and measure genetic variations among P. triticina populations using simple sequence repeat (SSR) markers (Szabo and Kolmer, 2007). Several different molecular markers were used as the most effective method to estimate the genetic variation in P. triticina populations which provide direct information concerning the effects of host selection in the potential effectiveness of leaf rust resistance genes (Kolmer, 1999).

The main objectives of this study were to determine the polymorphism and diversity among 40 selected leaf rust pathotypes by using virulence analysis as well as DNA markers; simple sequence repeat marker (SSR). Also, to study the relationship among the tested pathotypes using virulence analysis and molecular marker.

MATERIALS AND METHODS

P. triticina pathotypes: Forty pathotypes of P. triticina were selected from the Egyptian leaf rust populations during the 2016/17 and 2017/18 growing seasons (El-Orabey et al., 2018). Some of these chosen pathotypes were dominant in its population, as they represented by more than one isolate, and the other was less frequent and represented by only one isolate during these two growing seasons.

Determination of virulence phenotypes: Urediniospores of each of the forty selected leaf rust pathotypes were used to inoculate seven-day-old seedlings of the highly susceptible wheat variety; Morocco as described by Kolmer et al. (2009) to multiply and increase the urediniospores that used for virulence analysis and DNA isolation. To detect virulence phenotypes of leaf rust pathotypes under study, five sets of four Thatcher monogenic lines of wheat leaf rust; set 1 (Lr 1, Lr 2a, Lr 2c, and Lr 3a); set 2 (Lr 9, Lr 16, Lr 24 and Lr 26); set 3 (Lr 3ka, Lr 11, Lr 17, and Lr 30); set 4 (Lr 10, Lr 18, Lr 21, and Lr 2b); set 5 (Lr 14b, Lr 15, Lr 36, and Lr 42) were used (Long and Kolmer, 1989; McVey et al., 2004). The inoculation and disease assessment were done according to Long and Kolmer (1989) and Kolmer et al. (2009). Virulence phenotypes were determined after 10 - 12 days of inoculation for each pathotype on the 20 differentials monogenic lines using the 0-4 scale (Long and Kolmer, 1989). Pathotypes with infection types (ITs) 0, 0; 1 and 2 were classified as avirulent pathotypes, while those with infection types 3 and 4 were classified as virulent pathotypes. Each pathotype was given a five-letter code based on virulence or avirulence to the 20 differential monogenic lines (Long and Kolmer, 1989; McVey et al., 2004).

Molecular marker characterization of P. triticina pathotypes: DNA isolation method was carried out according to the procedure of Niazmand et al. (2013) from the urediniospores of each of the 40 pathotypes under study using 20 mg of the stored urediniospores. Cell walls of the spores were crushed using 20 mg autoclaved carborundum powder by adding the carborundum powder to the urediniospores for each of the tested pathotypes to 1.5 ml micro-centrifuge tubes. The tubes were placed into a medium-sized mortar and liquid nitrogen was added. Frozen urediniospores were ground using plastic mini-pestles mounted in an electric drill under a low speed for 20 sec. After grinding, 500 µl of extraction buffer (200 m mol Tris-HCl (pH 7.5), 250 m mol NaCl, 25 m mol EDTA, 0.5% SDS) was added to the cracked spores, which were then homogenized for 5 min at maximum speed using Vortex-Genie 2. Then, 350 µl phenol was added and the tubes inverted gently four times. Subsequently, 250 µl chloroform was added and the tubes inverted gently 40 times. The tubes were centrifuged at 4 °C for 30 min. After centrifugation, the aqueous supernatants were decanted into new tubes. The samples were treated with 2 µl RNase (20 µg ml TE) and incubated at 37 °C for 10 min. An equal volume of chloroform was added and mixed gently. The tubes were centrifuged at 4 °C for 10 min. The aqueous layer was transferred to a new Eppendorf tube, to which a 0.54 volume of cold isopropanol was added for precipitation of the DNA. The tubes were centrifuged at 4°C for 5 min. The supernatants were poured into a sink gently and 100 µl cold 70% ethanol was added to pellets, which were
then centrifuged at 4 °C for 5 min; the ethanol was then removed from tubes. Each pellet was dried in an incubator at 37 °C for 30 min and dissolved in (50 μl) sterile double-deionized water. The subsequent DNA yields and quality were assessed by standard electrophoresis through a 1% (w/v) ethidium bromide-stained agarose gel. DNA for each of the tested pathotype was amplified according to the protocol of Niazmand et al. (2013) using five SSR primers (Table 1). Amplification products were separated on 7% polyacrylamide gel.

Table 1. Sequences of six SSR primers used for molecular analysis of 40 P. triticina pathotypes.

| Marker | Primer sequence (5′-3′) | No. of allele | No. of polymorphic allele | Product (bp) |
|--------|-------------------------|---------------|---------------------------|--------------|
| PtSSR68 | F: GACTCAGCCCACTGCTAA R: GATGGCGACGTATTGGTCT | 5 | 5 | 362, 360, 356, 347, 337 |
| PtSSR154 | F: AGGTCAACAGGCAACTACC R: CCTGTCATCCTGGTGTAGT | 5 | 5 | 287, 281, 279, 276, 260 |
| PtSSR164 | F: GTGGAAGTGAGCGGAAGAAG R: GGAGATGGGCAGATGAGGTA | 3 | 2 | 218, 220 |
| PtSSR173 | F: CTACGCGACCTCAAAGAACC R: GAGACGCGAGTCTACAAC | 4 | 4 | 220, 217, 212, 210 |
| RB10 | F: AAGATTGTTGATATGGTGTTGGA R: TTGTCTTTCTACCTCACAGCC | 1 | 1 | 218 |
| RB29 | F: CTACCAAAACATCAAGGCACC R: GAGCCTAGCATCAGCATC | 9 | 2 | 118-129 |

**Data analysis:** The infection type data (0-4, scale) were converted into a binary code of 0 for avirulence and 1 for virulence of the tested pathotypes on the differential monogenic lines. Moreover, a binary data matrix was generated for all SSR markers based on the presence (1) or absence (0) of amplification products. A matrix cluster of both virulence and molecular data were derived with GenAlex 6 (Peakall and Smouse, 2006). Correlation between the SSR marker data and virulence data was determined by a comparison of the two similarity matrices using the MXCOMP, program of NTSYS-pc software.

**RESULTS**

**Geographical distribution of the tested leaf rust pathotypes:** A total of eight pathotypes were detected in Beheira population, which showed 20.00% of the whole population, followed by Fayoum, which has six pathotypes and showed 15.00% of the entire population. While, the five governorates; Alexandria, Kafr-Elsheikh, Gharbiya, Sharqiya and Sohag showed the lowest number of pathotypes i.e. 1 (2.50%), 2 (5.00%), 2 (5.00%), 2 (5.00%) and 2 (5.00%), respectively. The other tested governorates showed a moderate number of pathotypes; ranged from 3 (7.50%) to 5 (12.50%) (Table 2).

**Frequency (%) of the tested leaf rust pathotypes:** The two leaf rust pathotypes; STTTK and TTTTT were the most common with relatively high frequency, as they showed 9.81% and 5.10% frequency within the pathogen population during the two growing seasons of the study. While, twenty of the tested pathotypes showed the lowest frequency (%), i.e. 0.47%, each represented by only one isolate in leaf rust population. The frequency (%) of the other pathotypes ranged from 0.93% to 2.80% frequency (Table 2).

**Virulence analysis:** A total of 40 leaf rust pathotypes were collected during the 2016/17 and 2017/18 growing seasons survey in Egypt. Virulence analysis of the tested pathotypes was conducted based on the reaction of each pathotype to 20 differentials leaf rust monogenic lines. Out of the forty tested pathotypes, only pathotype TTTTT was the most aggressive, as it proved to be virulent to all 20 wheat leaf rust monogenic lines; Lr1, Lr2a, Lr2b, Lr2c, Lr3a, Lr3ka, Lr9, Lr10, Lr11, Lr14b, Lr15, Lr16, Lr17, Lr18, Lr21, Lr24, Lr26, Lr30, Lr36 and Lr42, while, pathotype GBHLD was virulent to only five monogenic lines; Lr2a, Lr10, Lr11, Lr30 and Lr36. The other most aggressive pathotype; STTTST was virulent to 19 leaf rust monogenic lines; Lr1, Lr2a, Lr2c, Lr3a, Lr3ka, Lr9, Lr10, Lr11, Lr14b, Lr15, Lr16, Lr17, Lr18, Lr21, Lr24, Lr26, Lr30, Lr36 and Lr42. The other tested pathotypes were virulent to 11-18 leaf rust monogenic lines (Table 3).
Table 2. Geographical distribution and frequency (%) of leaf rust pathotypes identified in 11 Egyptian governorates during the 2016/17 and 2017/18 growing seasons.

| No. | Governorate    | Identified Pathotype and Frequency (%) | No. of Pathotypes and Percentage (%) |
|-----|----------------|----------------------------------------|--------------------------------------|
| 1   | Alexandria     | STTTK (9.81)                           | 1 (2.50 %)                           |
| 2   | Kafr-Elsheikh  | PHTTT (1.87), PTKTH (0.47)              | 2 (5.00 %)                           |
| 3   | Beheira        | CTTTT (1.40), GBTMT (0.47), NJTPK (0.47), NTTSR (0.47), PPKTT (0.93), PKTPR (0.47), PPPT (0.47), PRSTT (1.40) | 8 (20.00 %) |
| 4   | Gharbiya       | PSTST (0.93), TTTTT (5.10)              | 2 (5.00 %)                           |
| 5   | Minufiya       | MTTTT (1.18), PHTTT (0.47), PTCTT (0.47), PKKTT (0.93), PKTPR (0.47), PPPT (0.47), PRSTT (1.40) | 5 (12.50 %) |
| 6   | Qalyubia       | NTKTS (1.40), PTKTS (0.47), PTTS (0.47), TTTST (1.87) | 4 (10.00 %) |
| 7   | Sharqiya       | DHTTT (0.47), FTTNS (0.47), PJSFT (0.47), PTSS (1.87), PTTTP (0.47) | 5 (12.50 %) |
| 8   | Dakahlia       | DHTTT (0.47), FTTNS (0.47), PJSFT (0.47), PTSS (1.87), PTTTP (0.47) | 5 (12.50 %) |
| 9   | Fayoum         | KTSPT (0.47), NPTNK (0.93), PTKST (1.87), PTJNP (0.47), PTTNT (0.47), TTTMS (1.40) | 6 (15.00 %) |
| 10  | Bani Sweif     | NTKTS (1.40), PTKGT (0.93), PTSNS (1.40), | 3 (7.50 %) |
| 11  | Sohag          | GBHLD (0.47), PTTNS (2.80)              | 2 (5.00 %)                           |
|     | **Total**      | **40 (100.00 %)**                      |                                      |

Table 3. Virulence phenotypes of forty *Puccinia triticina* pathotypes isolated from Egypt during 2016/17 and 2017/18 growing seasons and used in this study.

| No. | Pathotypea | Virulence (*Lr* genes)                                                                 | No. of ineffective genes |
|-----|------------|---------------------------------------------------------------------------------------|--------------------------|
| 1   | CTTTT      | 3a, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 18, 21, 2b, 14b, 15, 36, 42                    | 17                       |
| 2   | DFTTPS     | 2c, 24, 26, 3ka, 11, 17, 30, 10, 21, 2b, 14b, 15, 36                                 | 13                       |
| 3   | DHTTT      | 2c, 16, 26, 3ka, 11, 17, 30, 10, 18, 21, 2b, 14b, 15, 36, 42                        | 15                       |
| 4   | FTTNS      | 2c, 3a, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 21, 14b, 15, 36, 42                    | 15                       |
| 5   | GBHLD      | 2a, 11, 30, 10, 36                                                                 | 5                        |
| 6   | GBTMT      | 2a, 3ka, 11, 17, 30, 10, 2b, 14b, 15, 36, 42                                      | 11                       |
| 7   | KTSPT      | 2a, 2c, 3a, 9, 16, 24, 26, 3ka, 11, 17, 10, 21, 2b, 14b, 15, 36, 42                | 17                       |
| 8   | MTTTT      | 1, 3a, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 18, 21, 2b, 14b, 15, 36, 42             | 18                       |
| 9   | NJTKP      | 1, 2c, 16, 24, 3ka, 11, 17, 30, 10, 21, 2b, 15, 36, 42                             | 14                       |
| 10  | NPTNK      | 1, 2c, 9, 24, 26, 3ka, 11, 17, 30, 10, 21, 14b, 15, 36, 42                         | 14                       |
| 11  | NRKDS      | 1, 2c, 9, 16, 26, 11, 17, 30, 21, 14b, 15, 36                                     | 12                       |
| 12  | NTKTS      | 1, 2c, 9, 16, 24, 26, 17, 30, 10, 18, 21, 2b, 14b, 15, 36                           | 15                       |
| 13  | NTTSR      | 1, 2c, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 18, 21, 14b, 15, 42                    | 16                       |
| 14  | PHTTT      | 1, 2c, 3a, 16, 26, 3ka, 11, 17, 30, 10, 18, 21, 2b, 14b, 15, 36, 42                | 17                       |
| 15  | PJSFT      | 1, 2c, 3a, 16, 26, 3ka, 11, 17, 21, 2b, 14b, 15, 36, 42                          | 14                       |
| 16  | PKKTT      | 1, 2c, 3a, 16, 26, 24, 3ka, 11, 17, 30, 10, 18, 21, 2b, 14b, 15, 36 42           | 17                       |
| 17  | PKTPR      | 1, 2c, 3a, 16, 24, 26, 3ka, 11, 17, 30, 10, 21, 2b, 14b, 15, 42                   | 16                       |
| 18  | PKTST      | 1, 2c, 3a, 16, 24, 26, 3ka, 11, 17, 30, 10, 18, 21, 14b, 15, 36, 42               | 17                       |
| 19  | PKTFF      | 1, 2c, 3a, 16, 24, 26, 3ka, 11, 17, 30, 10, 18, 21, 2b, 36, 42                   | 16                       |
| 20  | PKTTT      | 1, 2c, 3a, 16, 24, 26, 3ka, 11, 17, 30, 10, 18, 21, 2b, 14b, 15, 36, 42          | 18                       |
| No. | Pathotype | Genotypes | Virulence | Molecular Analysis |
|-----|-----------|------------|-----------|--------------------|
| 21  | PPTPT     | 1, 2c, 3a, 9, 24, 26, 3ka, 11, 17, 30, 10, 21, 2b, 14b, 15, 36, 42 | 17 |
| 22  | PRSTT     | 1, 2c, 3a, 9, 16, 26, 3ka, 11, 17, 10, 18, 21, 2b, 14b, 15, 36, 42 | 17 |
| 23  | PSTST     | 1, 2c, 3a, 9, 16, 24, 3ka, 11, 17, 30, 10, 18, 21, 14b, 15, 36, 42 | 17 |
| 24  | PTJNP     | 1, 2c, 3a, 9, 16, 24, 26, 11, 17, 10, 21, 14b, 36, 42 | 14 |
| 25  | PTKGT     | 1, 2c, 3a, 9, 16, 24, 26, 11, 17, 30, 18, 14b, 15, 36, 42 | 15 |
| 26  | PTKTH     | 1, 2c, 3a, 9, 16, 24, 26, 11, 17, 30, 10, 18, 21, 2b, 15, 42 | 16 |
| 27  | PTKTS     | 1, 2c, 3a, 9, 16, 24, 26, 11, 17, 30, 10, 18, 21, 2b, 14b, 15, 36 | 17 |
| 28  | PTSNS     | 1, 2c, 3a, 9, 16, 24, 26, 3ka, 11, 17, 10, 21, 14b, 15, 36 | 15 |
| 29  | PTTNS     | 1, 2c, 3a, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 21, 14b, 15, 36 | 16 |
| 30  | PTTNT     | 1, 2c, 3a, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 21, 14b, 15, 36, 42 | 17 |
| 31  | PTTNP     | 1, 2c, 3a, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 21, 2b, 14b, 15 | 16 |
| 32  | PTTPR     | 1, 2c, 3a, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 21, 2b, 14b, 15, 42 | 17 |
| 33  | PTTPS     | 1, 2c, 3a, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 21, 2b, 14b, 15, 36 | 17 |
| 34  | PTTSS     | 1, 2c, 3a, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 18, 21, 14b, 15, 36, 42 | 17 |
| 35  | STTTK     | 1, 2a, 2c, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 18, 21, 15, 36, 42 | 18 |
| 36  | TPTMP     | 1, 2a, 2c, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 18, 21, 2b, 15, 36, 42 | 17 |
| 37  | TTTMS     | 1, 2a, 2c, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 2b, 14b, 15, 36, 42 | 16 |
| 38  | TTTST     | 1, 2a, 2c, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 18, 21, 14b, 15, 36, 42 | 19 |
| 39  | TTTTT     | 1, 2a, 2c, 9, 16, 24, 26, 3ka, 11, 17, 30, 10, 21, 2b, 14b, 15, 36, 42 | 20 |

* Nomenclature of leaf rust pathotypes according to Long and Kolmer (1989) and McVey et al. (2004).

**Virulence polymorphism:** Based on the virulence/avirulence formula to 20 leaf rust monogenic lines, the tested pathotypes could be divided into three main groups, belonging to three main clusters (Figure 1). The first cluster consisted of only one pathotype; NTTSR which was avirulent to the four leaf rust resistance genes; *Lr* 2a, *Lr* 2b, *Lr* 3a and *Lr* 36. The second cluster also contained only one pathotype; PSTST that was avirulent to the three leaf rust resistance genes; *Lr* 2a, *Lr* 2b and *Lr* 26. The third cluster consists of 38 pathotypes and contained two sub-clusters. The first sub-cluster contained 28 pathotypes and separated into two sub-sub-clusters. The first sub-sub-cluster contained 25 pathotypes i.e. PTKTS, KTSPT, PSFT, NJTPK, PRSTT, PPTPT, NPTNK, GBTMT, GBHLD, TPTMP, TTTMS, DFTPS, PTTPQ, PKTPR, PTTTS, MTSS, CTTTT, PTKTH, PPKTT, PKTF, PHTTT, DHTTT, PTKTT and PTKST. The second sub-sub-cluster contained only three pathotypes, i.e. TTTTT, TTTSST and STTTT, which were the most aggressive pathotypes and were virulent to 20, 19 and 18, respectively of the 20 differential monogenic lines. The second sub-cluster contained 10 pathotypes and separated into two sub-sub-clusters. The first one sub-sub-cluster contained seven pathotype i.e. PTTNS, FTTNS, PSNS, PTSS, PTKGT, NRKDS, and NTKTS, and these pathotypes were avirulent to *Lr* 2a. The second sub-sub-cluster contained only three pathotypes, i.e. PTTS, PTJNP and PTTNT which were avirulent to the two leaf rust resistance genes *Lr* 2a and *Lr* 2b.

**Molecular analysis:** Based on the six SSR primers, the genetic similarities between the 40 tested pathotypes and the Jaccard similarity coefficient, cluster analysis was done using software NT sys (Ver. 2.02), three main groups were obtained (Figure 2). Group 1 and group 2 each included four pathotypes, i.e. PKKTT, NTTSR, FTTNS, DHTTT and PTJNP, PRTT and PTPTT, respectively. Group 3 included two sub-clusters, sub-cluster 1 included 30 pathotypes i.e. NJTPK, MTTTT, TTTTT, PTKGT, PTKST, PSTST, NRKDS, PTTTP, PTTNT, KTSPT, CTTTT, PTSS, PTTPQ, PJST, PKTPR, TTTMS, PTKTH, GBHLD, TTTTT, PKTTT, PTKTS, PTKS, FTTTT, DFTPS, NTKTS, GBTMT, NPTNK, PTSS and TPTMP. Sub-cluster 2 included only two pathotypes, i.e. STTTT and PTTPS.
Figure 1. Dendogram of 40 *P. triticina* pathotypes based on virulence to 20 Thatcher isogenic lines with different leaf rust resistance genes of wheat.
Figure 2. Dendogram of 40 *P. triticina* pathotypes based on simple sequence (SSR) repeat using Nei's genetic distance between groups collected from 11 Egyptian governorates.
Diversity among the tested leaf rust pathotypes:
Diversity based on virulence analysis: Principal coordinate analysis in Figure 3 grouped the tested leaf rust pathotypes depending on their virulence to 20 leaf rust monogenic lines. The X-axis is principal coordinates (PC1), and Y-axis is the second principal coordinates (PC2) which were accounted for 27.9 and 11.8% of the total variation, respectively in the tested leaf rust pathotypes.

Diversity based on molecular marker pattern: Diversity and genetic distance among the tested populations based on molecular marker pattern data were calculated by Power Marker software (Table 4). Results of molecular analysis of variance showed that the genetic variation among populations is 97% and difference among populations (from the different governorates) is 3% and very high gene flows among populations exist.

Figure 3. Principal coordinate analysis (PCA) plot of 40 leaf rust pathotypes isolated from Egypt during 2016/17 and 2017/18 growing seasons based on virulence differences to 20 leaf rust differential lines.

Table 4. The calculated values for genetic diversity between and within leaf rust populations.

| Source         | Df | SS    | MS    | Est. Var. | %  |
|----------------|----|-------|-------|-----------|----|
| Among Pops     | 9  | 27.641| 3.071 | 0.076     | 3% |
| Within Pops    | 29 | 80.667| 2.782 | 2.782     | 97%|
| Total          | 38 | 108.308| 2.858 |           | 100%|

Geographical distribution of the tested pathotypes:
The cluster analysis of the distribution of the tested 40 leaf rust pathotypes in the 11 locations were carried out based on similarities and dissimilarities of the tested pathotypes and illustrated in Figure 4. Three main clusters were formed; the first and second clusters contain only one location for each Beheira and Sharqiya, respectively. The second cluster included nine locations and divided into two sub-clusters. The first sub-clusters divided into two sub-sub-clusters. The first sub-sub-clutter consists of three locations i.e. Sohag, Bani Sweif and Fayoum. The second sub-sub-clutter included five locations Minufiya, Kafr-Elsheikh, Gharbiya, Alexandria and Qalyubia. On the other hand, the second sub-cluster contains only one location i.e. Dakahlia.

Correlation between the tested pathotypes based on virulence analysis and molecular characterization: The relationship between virulence analysis data and molecular characterization data to detect variations between 40 *Puccinia triticina* pathotypes was illustrated in Figure 5. The correlation between virulence analysis and molecular characterization data was very low ($R^2 = 0.03$).

**DISCUSSION**
Leaf rust disease occurred annually under the Egyptian field conditions in the second half of February, while it was recorded in most of the commercial wheat cultivars, nationwide. This disease causes significant yield losses under Egyptian field conditions (Shahin and El-Orabey, 2016; El-Orabey et al., 2017). Host-genetic resistance or using resistance cultivars for
controlling leaf rust in wheat is the most effective and common control method for reducing yield losses (Kolmer et al., 2012). Breakdown and/or overcome host resistance especially race-specific resistance, found to be mainly due to the emergence and evolution of new leaf rust pathotypes (McVey et al., 2004).

Figure 4. Dendogram of 40 *P. triticina* pathotypes based on the presence and absence of the tested pathotypes in each of the 11 locations.

Figure 5. Association between virulence analysis data and molecular characterization data to detect variations between 40 *Puccinia triticina* pathotypes.

An annual survey of leaf rust pathotypes in wheat is essential to be carried out to identify and detect virulence pathotypes that may be introduced to different regions in the country. Also, it provides useful information about the effectiveness of leaf rust resistance gene (s) that were widely used in the
commercial wheat cultivars and the new resistance genes which not yet deployed in these cultivars (Park et al., 2011). The dynamic nature of the causal pathogen and variation or diversity of genetic structure of leaf rust populations was mainly due to some evolutionary potential forces such as; migration, sexual recombination and mutation (McVey et al., 2004; El-Orabey et al., 2015). Moreover, a selection pressure, cultivation of wheat cultivars having a high level of resistance promoted the selection of virulent pathotypes (Burdon and Silk, 1997). All of these evolutionary forces usually are occurred, except sexual reproduction that did not occurred in Egypt, because of the absence of the alternative hosts, volunteer plants and stubs. The primary inoculum for leaf rust comes from external sources each year from the neighboring countries and the spores of leaf rust pathotypes did not survive summers in Egypt (McVey et al., 2004). El-Orabey et al. (2018) estimated the diversity of leaf rust pathotypes using the three indexes; Shannon, Gleason and Simpson. They found that, highest diversity values for the Egyptian leaf rust population during 2016/2017 and 2017/2018. Analysis of diversity of leaf rust pathotypes was carried out by virulence analysis or using molecular techniques i.e. SSR markers (Agarwal et al., 2018). Analysis of the diversity of plant pathogens based on molecular markers enables understanding the taxonomy and structure of populations. Also, it helps in knowing the genetic structure and relationship among plant pathogens pathotypes (Agarwal et al., 2018).

Two methods are commonly used for the study of genetic diversity within and among wheat rust populations. First, study virulence and avirulence analysis of pathotypes on the differential lines and the second is using molecular markers (Kolmer, 2001). In this study, the analysis of the tested leaf rust pathotypes using virulence analysis on the 20 leaf rust resistance genes and molecular markers confirmed the presence of high variation in pathogenicity and genetic diversity among these pathotypes. Moreover, virulence characteristics on resistance genes are more applicable and more useful in race analysis but because of virulence properties under strong selection provide incorrect estimations. Using DNA markers to study characteristics of leaf rust populations is very important because these markers are highly informative and more accurate tool (McDonald, 1997). Virulence analysis data revealed that, all tested pathotypes which were similar in virulence behavior were present in the same group except for some pathotypes which were in the same cluster but not identical in the same virulence behavior. This may be due to the Egyptian differential sets used for the nomenclature of P. triticina pathotypes should be changed especially for the last two subsets. The first three subsets are the same all over the world (Kolmer, 1991) and the last two subsets (4 and 5) are changed worldwide according to the effectiveness of the other leaf rust monogenic lines in each country. So, leaf rust monogenic lines in set four contain Lr 10, Lr 18, Lr 21 and Lr 2b and set five include Lr 14 b, Lr 15, Lr 36 and Lr 42 should be changed and adapted according to the efficacy of leaf rust resistance genes under Egyptian conditions. Moreover, the two leaf rust resistance genes Lr 11 in subset 3 and Lr 18 in subset 4 of nomenclature of P. triticina pathotypes in Egypt are sensitive to temperature and must be tested below 18 °C and temperature in the greenhouse is higher than 18 °C especially at the end of February and during March (Dyck and Johnson, 1983).

Molecular marker data showed that, all the tested pathotypes were genetically different and produced a unique SSR allele. Similar results were found by Gultyaeva et al. (2018) studied 46 isolates of P. triticina using 12 SSR markers and they found that, a high variation among the tested P. triticina pathotypes in Russia. Mantovani et al. (2010) tested 24 isolates of P. triticina from Italy using 15 SSR markers. They found that, the tested isolates were found into three groups. The first group, included isolates which were virulent and collected from durum wheat. The second group, included isolates that had virulence similar to the isolates from common wheat but were distinct for SSR genotypes compared to the isolates from durum wheat and common wheat. Isolates in the third group had virulence phenotypes and SSR genotypes closely related to the isolates from common wheat. Also, they found that virulence phenotypes and molecular genotypes were highly correlated with r = 0.74.

CONCLUSION

Based on the results of this study, leaf rust pathotypes in Egypt is very diverse and had very high evolutionary potential. Thus, the ability of single gene resistance probably is short. The strategy for breeding for resistance to leaf rust disease should be established based on the use of quantitative genes (partial resistance genes; race-nonspecific resistance genes) with other
race specific resistance genes resistance. In Egypt, effective leaf rust resistance genes such as \( Lr \) 17, \( Lr \) 18, \( Lr \) 21 and \( Lr \) 28 should be used in combination with non-specific genes such as \( Lr \) 34, \( Lr \) 46, \( Lr \) 67 and \( Lr \) 68 could create a more effective and stability of resistance. Moreover, a more powerful technique for detecting molecular polymorphism such as amplified fragment length polymorphism, may allow more discrimination between and within leaf rust pathotypes. Correlation between molecular variation and diversity in pathotypes based on virulence analysis was low and this correlation may be improved by using large numbers of markers.

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