

Genetic Diversity of *Gaeumannomyces graminis* var. *tritici* Populations Using RAPD and ERIC Markers

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Abstract

Take-all disease which is caused by *Gaeumannomyces graminis* var. *tritici* (*Ggt*) is the most damaging root disease of wheat. Twenty four isolates of pathogen were recovered from diseased samples collected from different provinces of Iran including Fars, Markazi, and Mazandaran. All isolates were specifically identified as *Ggt* and simultaneously divided into two subpopulations of A and B types. Five primers of RAPD and ERIC primer pair were used to determine the DNA polymorphisms. Cluster analysis was noticed that with the exception of OPC-08 primer, the all remaining RAPD primers and ERIC separated T-28 (having both simple and lobed hyphopodia) from other *Ggt* isolates with simple hyphopodia. Additionally, these primers identified and separated the two varieties of the pathogen, *Gaeumannomyces graminis* var. *graminis* (*Ggg*) and *Ggt*. Polymorphism Information Content (PIC) value was estimated between 0.23-0.3. The tested isolates were divided into three groups including a single member group housed T-28 isolate at 39.5% similarity. All *Ggt* isolates with simple hyphopodia can be divided into two groups, the first group from Markazi province with a 12.5% frequency consisting of three isolates including two B-types (T-1, T-9) that also separated by Principle Component Analysis (PCA). The second group (A-type) contained 83.3% of the remaining isolates. The Iranian population of *Ggt* shows a high level of genetic diversity dominated by A-type. The dominant population of *Ggt* isolates provides the pathogen with the ability to fight or overcome any commonly used control measures such as chemicals or resistant cultivars.

Keywords: *Gaeumannomyces graminis* var. *tritici*; Genetic diversity; RAPD; ERIC markers

Introduction

The fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*) is the most important root disease of wheat worldwide tests [1]. Isolates of *G. graminis* var. *tritici* vary in phenotypic features (colony morphology and pathogenicity) and on the DNA level have been reported previously [2,3]. However, very little research on genetic diversity within populations of *G. graminis* has been stated [4]. Molecular tools have played a meaningful role in the recent development of specific and rapid identification genome, and considerable polymorphisms were observed among studied isolates. Augustin et al. [5] used RAPD marker to differentiate *G. graminis* at the intravarietal and intervarietal levels. Lebreton et al. [4] divided 120 *Ggt* isolates into two groups, G₁ and G₂, using RAPD and AFLP markers. The aggressiveness of isolates from group G₂ was significantly greater than those from G₁ group. Resulting dendrograms from analysis of RAPD data also grouped *G. graminis* isolates according to their variety identity [6]. In addition, isolates of *Ggt* were classified as rye-adapted (R) or non-rye-adapted (N) forming distinct groups in dendrograms [7]. On the basis of rDNA analysis using PCR-RFLP method, 144 isolates of *Ggt* divided into two major groups A and B [8]. Families of short intergenic repeated sequences have been determined in enteric bacteria, primarily *Escherichia coli* and *Salmonella typhimurium*. Traditionally REP and ERIC (enterobacterial repetitive intergenic consensus) primers have been used in the analysis of bacterial DNA from a variety of sources including those bacteria that cause plant diseases [9,10]. Crozier et al. [11] for the first time used Repetitive Extragenic Palindrome (REP) in fingerprinting *Ggt* genome, and considerable polymorphisms were observed among studied isolates. Prior to present study, ERIC marker had not been used in evaluating genetic diversity in *Ggt* population. Knowledge of the genetic structure of pathogen population has important implications for plant breeding and also for fungicide screening programs [12].

Actual damage of take-all disease has not been estimated correctly in Iran, however this disease has been reported as an important wheat disease [13]. Furthermore, this disease has reported as important root disease in the farms of Markazi province and also its dispersion was estimated 12.5, 4.5 and 9.11% for Shazand, Arak and Khomain, respectively. Despite the importance of this pathogen and economic damage caused annually by the disease on wheat farms, no research on the genetic diversity of its population and identification of *Ggt* has been taken. The aim of this study is to estimate genetic diversity within Iranian population of *Ggt* fungus using RAPD and ERIC markers in Iran.

Material and Methods

Taking sample and fungal isolation

In the survey conducted in wheat growing seasons, 24 isolates of pathogen were recovered from diseased samples collected from three province of Iran including Fars, Markazi, and Mazandaran. Isolates of *Ggt* were obtained from diseased pieces root, crown and stem. These pieces were surface-sterilized for 3 min in sodium hypochlorite, rinsed twice in sterile distilled water, and dried on filter paper under a sterile air flow. They were then placed on Potato Dextrose Agar (PDA) containing streptomycin sulphate (100 mgL⁻¹) and rifampicin (50 mgL⁻¹),

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and incubated at 20°C for 3-6 days. Cultures resembling *G. graminis* were then transferred to fresh PDA containing the same antibiotics [1]. Table 1 lists the morphological and pathogenicity characteristics of *Ggt* isolates used in this study, according to Sadeghi et al. [14].

DNA extraction and marker assay

Mycelia from 9-day-old cultures grown on liquid medium (5 g of glucose, 1 g of yeast extract and 1000 ml distilled water) were collected by vacuum filtration. DNA was extracted according to Safaie et al. [15] DNA concentration and purity was examined using spectrophotometer and by running on 0.8% agarose gel.

Ggt-specific identification

Primers *Ggt* fwd (5'-AAG AAC ATC GGC GGT CTC GCC), *Ggt* Arev (5'-TAG CGG CTG GAG CCC GCC G) and *Ggt* Brev2 (5'-CTA CCT GAT CCG AGG TCA ACC TAA GG) were designed by Freeman et al. [8], using 1x reaction Taq buffer, 1.5 mM MgCl₂, 0.4 mM dNTPs and DNA 50 ng. Cycling conditions were 35 cycles of 94°C for 30 s, annealing 72°C for 1 min and extension 72°C for 1min, followed by a final extension of 72°C for 10 min.

RAPD and ERIC assays

The RAPD and ERIC-PCR reactions were carried out in 20 µl reaction volumes containing 1x reaction Taq buffer, 1.5 mM MgCl₂ for RAPD primers and 2 mM MgCl₂ for ERIC primer, 0.2 mM dNTPs, 1 unit Taq DNA polymerase, 10 µM primer (All PCR materials were provided by CinnaGen Co.) and 50 ng fungal DNA. Program PCR for RAPD reactions: an initial denaturation for 2 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 36°C for 1 min and extension at 72°C for 2 min. The amplification was ended with an additional extension at 72°C for 5 min, and program PCR

No.	Isolate	Province	Hyphopodia	Pathogenicity level (Sadeghi et al. [14])
1	T-1	Markazi	Simple	Weak
2	T-3	Markazi	Simple	Strong
3	T-4	Markazi	Simple	Weak
4	T-7	Markazi	Simple	Weak
5	T-9	Markazi	Simple	Weak
6	T-10	Markazi	Simple	Strong
7	T-11	Markazi	Simple	Strong
8	T-19	Markazi	Simple	Strong
9	T-22	Fars	Simple	Strong
10	T-23	Fars	Simple	Strong
11	T-25	Fars	Simple	Strong
12	T-26	Fars	Simple	Strong
13	T-27	Fars	Simple	Strong
14	T-28	Fars	Lobed & simple	Weak
15	T-34	Mazandaran	Simple	Strong
16	T-37	Mazandaran	Simple	Strong
17	T-38	Mazandaran	Simple	Weak
18	T-39	Mazandaran	Simple	Strong
19	T-41	Mazandaran	Simple	Strong
20	T-42	Mazandaran	Simple	Strong
21	T-45	Mazandaran	Simple	Strong
22	T-46	Markazi	Simple	Weak
23	T-49	Markazi	Simple	Weak
24	T-51	Markazi	Simple	Strong
25	Ggg	Mazandaran	Lobed	

Table 1: Characteristics of *Gaeumannomyces graminis* var. *tritici* isolates recovered from wheat in different provinces of Iran.

Factor	Levels	Sample size	Φ _{st}		
Pathogenicity	Weak	7	0.06*		
	Strong	17			
Province	Markazi	11	Markazi	Fars	
			Fars	0.073*	
	Mazandaran	7	Mazandaran	0.031ns	0.033 ^{ns}

*: Significant at 0.05 level; ns: non-significant

Table 2: Analysis of molecular variance (AMOVA) of two factors on molecular data matrix. Φ_{st} values correspond to the fraction of the molecular diversity accounted by the factor. The significance of the values was calculated using 999 permutations on data sets.

for ERIC reaction: the first cycle at 95°C for 2 min was followed by 35 cycles at 94°C for 1 min, at 45.5°C for 1min, and at 72°C for 1:30 min; 1 cycle at 72°C for 8 min. [11,12,16]. Amplification reactions of specific PCR, RAPD and ERIC were performed using an Eppendorf thermocycler (Eppendorf Mastercycler Gradient, Germany). In order to check the reproducibility of all markers, PCR was repeated twice for all 24 samples. PCR products for RAPD, ERIC and specific primers were separated by electrophoresis in 1.5% agarose gels with 1x TBE buffer and visualized under UV light following ethidium bromide staining and photographed. A 1 Kb and 100 bp for DNA ladder were used as size marker in gels.

Statistical analysis

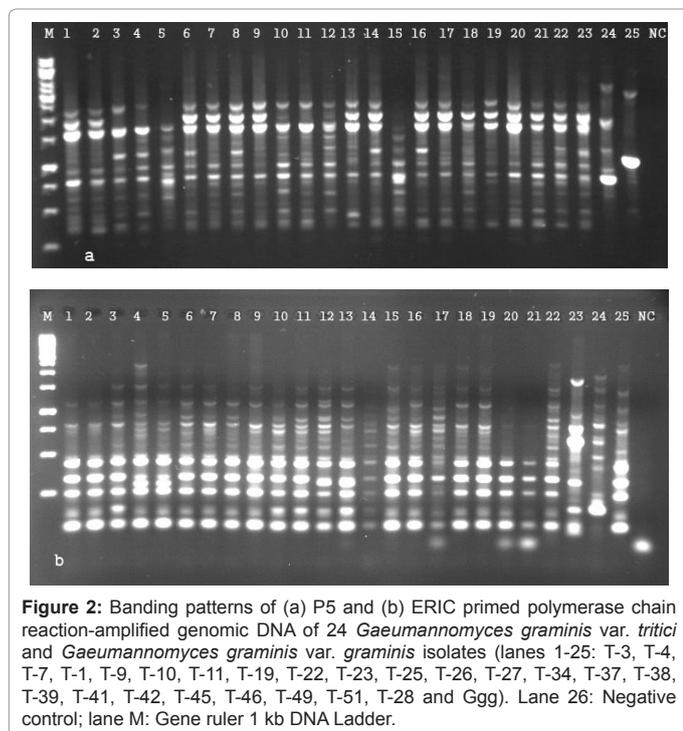
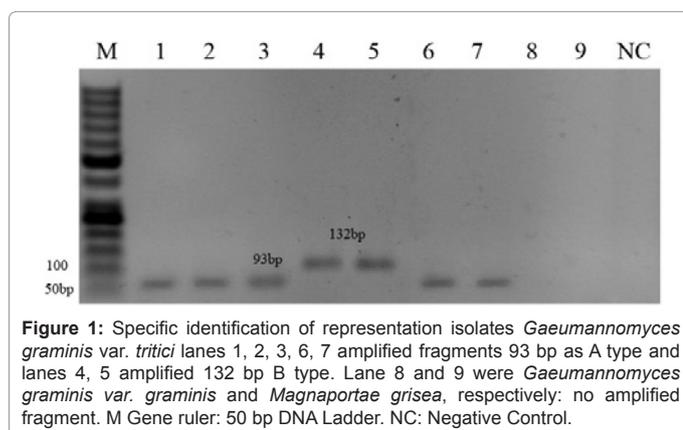
The comparison of each profile for each primer was performed on the basis of the presence (1) and absence (0) of the same length bands. Each band was assumed to represent a single genetic locus. The resulting data matrices were used for cluster analysis of isolates by the UPGMA algorithm and Jaccard's similarity coefficient by NTSYSpc software version 2.02 [17]. The resulting data matrices were used and the discrimination power of each marker was evaluated by the Polymorphism Information Content (PIC) [3] as $PIC = 1 - \sum_{i=1}^n p_i^2$, where p is the frequency of the each band. Moreover, the distinguishing power of each marker was described by the Resolving Power (Rp) index as $R_p = \sum I_b$, in which I_b is the "band in formativeness" of each marker. In this formula, I_b is calculated by $I_b = 1 - (2 \times |0.5 - p|)$, where p is the proportion of the 24 isolates containing the band [18]. The structure of the genetic diversity of the isolates was tested by analysis of molecular variance (AMOVA) [19]. This method provides an estimate of the fraction of between-population diversity (i.e. Φ_{st}), analogous to the F_{st} [20], which can be tested by a permutational procedure. In this study, AMOVA was performed to test the structure (i) among weak and strong isolates in terms of their pathogenicity, and (ii) among locations (provinces) of isolates were their samples are taken. The structure of the genetic diversity was further analyzed by a principal component analysis (PCA) from the Nei's genetic distance matrix. AMOVA and PCA analyses were performed with Gen Alex software version 6.3 [21]. Sequence of primers tested with RAPD and ERIC markers are presented in table 2.

Results, Discussion and Conclusion

Take-all of wheat caused by the fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*) is the most significant root disease of wheat throughout the world. The same pathogen also affects triticale, barley and rye, but to lesser extents [1,22-24]. Twenty four isolates of *Ggt* were recovered from infection samples of pathogen collected from different provinces of Iran including Fars, Markazi and Mazandaran. This disease has been recognized for more than 30 years ago in Iran

Primer	Sequence primer (5'-3')	No. of bands	No. of polymorphic bands	Percentage polymorphism	PIC	Rp	Size range of bands (bp)
OPC-08	CCC GTC AGC A	17	17	100	0.28	9	4000-250
OPC-19	GTA GAC CCG T	38	32	84.21	0.23	10.58	5000-230
Cs1503	TGG ACC GGT G	29	28	96.55	0.29	15.58	3086-290
P5	GAG TGG TGA C	28	28	100	0.3	13	4643-334
P6	CGG CCC ACG T	19	19	100	0.3	7.75	3640-188
ERIC	RI- CACTTAGGGGTCCTCAATGT I2-AAGTAAGTGACTGTGAGCG	31	26	83.87	0.25	8.5	2370-250

Table 3: Names and sequences of tested primers, for RAPD and ERIC markers.



[22] and since then reported from different regions including Golestan, Hamedan, Ghazvin, Markazi, Fars, Khuzestan, Tehran and Kurdistan provinces [13]. According earlier study, the results of pathogenicity assays showed that, all *Ggt* isolates were pathogenic on wheat and nine of them were pathogenic on oat as well [14]. Statistical analyses of the pathogenicity assay indicated virulence among isolates were significantly different. Previous studies had displayed considerable

variation in virulence among *Ggt* isolates [11,25,26]. Some *Ggt* isolates found in Australia reported to be capable of causing disease on oats [7]. All isolates identified with specific primers of *Ggt* for rapid and confident identification. One isolate of *Ggg* and *Magnaporthe grisea* did not amplify any fragment. Two isolates T-1, T-9 of *Ggt*, amplified fragment 132 bp as B type, and remainder of *Ggt* isolates, amplified fragment 93 bp as A type, where only five representative isolates of this type (Figure 1) are taken for specific identification of representation isolates of *Gaeumannomyces graminis* var. *tritici*. For investigation of genetic diversity, five primers of RAPD and one pair of ERIC primers were used to determine the DNA polymorphisms for 24 isolates of *Ggt* and one isolate of *Ggg* (Figure 2, Table 3). The results of cluster analysis showed that with the exception of OPC-08 primer, the remaining all RAPD primers and ERIC separated T-28 (having lobed hyphopodia) from other *Ggt* isolates with simple hyphopodia. Additionally, these primers identified and readily separated the two varieties of pathogen, *Ggg* and *Ggt*. At 39.5% similarity, the tested isolates were divided into three groups including a single member group of T-28 isolate and rest of *Ggt* isolates with simple hyphopodia divided into two groups, the first group was belonged to Markazi province with a 12.5% frequency consisting of three isolates including T-1, T-7, T-9 while the second group contained 84% of the remaining isolates (Figure 3). Cluster analysis for combined RAPD-PCR and ERIC data separated the isolates with simple hyphopodia into two main groups. Association among the isolates revealed by PCA was represented in figure 4. The first three components explained about 65% of the total variation, with 32.83, 16.45, and 15.25% for the first, the second, and the third components, respectively. Different pathogenicity levels of isolates did account for 6% of the total variation revealed by AMOVA analysis (Table 2). The population pair-wise F-statistics matrix between weak and strong pathogenic isolates was significant at 0.05 (999 permutations). In graphical representation showed by PCA, the weak isolates were more spread along the first and two components (axis 1 vs. axis 2), i.e. had a higher genetic diversity, compared with isolates with strong pathogenicity. On second structure, 5% of the total variation was observed between three locations (provinces) of taken samples. T-1 and T-9 and lobed hyphopodium isolate (T-28) separated from other isolates by principle component analysis. The F-statistic was significant at 0.05 between Fars and Markazi provinces (Table 2). These two groups were relatively separated by the second and third components (axis 2 vs. axis 3) as is shown in figure 4b.

A total of 140 polymorphic fragments were detected at the 6 RAPD and ERIC markers with an average number of 17 to 28 per RAPD primers and 26 polymorphic for ERIC primer and polymorphism information content (PIC) value varied between 0.23-0.3. (Table 2). Weber et al. [12] used five RAPD primers to evaluate genetic

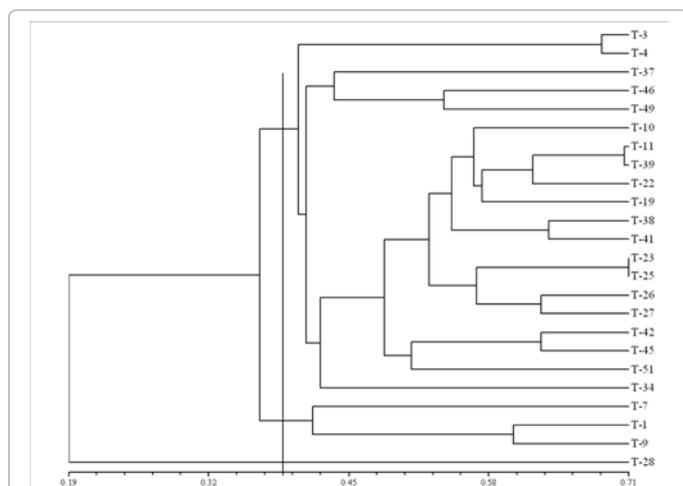


Figure 3: Dendrogram showing genetic relationships among 24 *Gaeumannomyces graminis* var. *tritici* based on combined data for the 5 RAPD and one pair ERIC primers. Cluster analysis was performed by the UPGMA method on a Jaccard's similarity matrix.

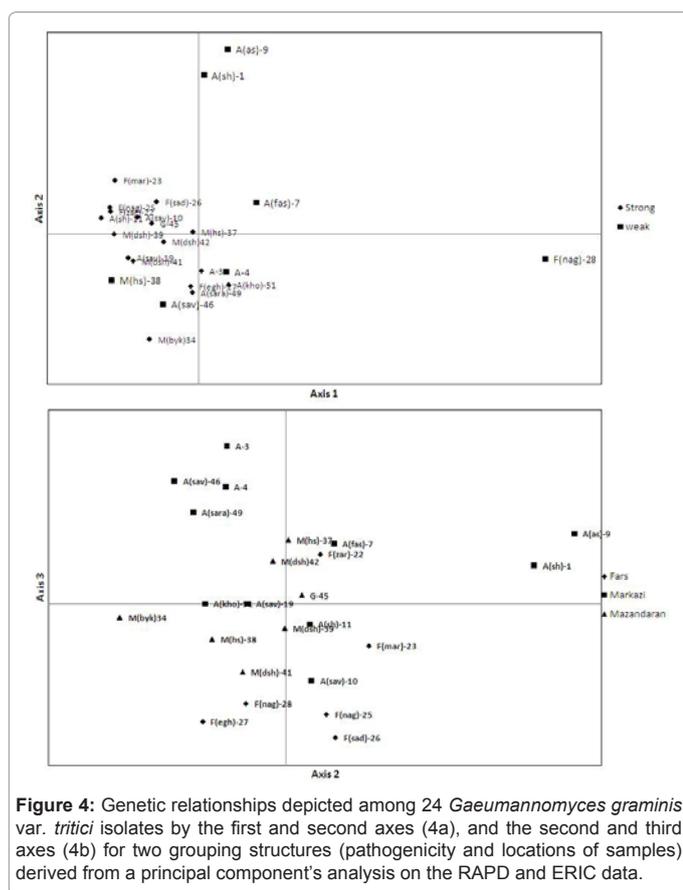


Figure 4: Genetic relationships depicted among 24 *Gaeumannomyces graminis* var. *tritici* isolates by the first and second axes (4a), and the second and third axes (4b) for two grouping structures (pathogenicity and locations of samples) derived from a principal component's analysis on the RAPD and ERIC data.

variability among 70 isolates and related them to sensitivity to silthiofam fungicide. RAPD analysis suggested a high level of genetic variation and identified two groups at the 54% similarity level. Studies of Freeman et al. [8] based on the polymorphisms in ITS2, identified 144 isolates of *Ggt* as A- or B-type and were all tested for sensitivity to Silthiofam. There was a highly significant correlation between B type isolates. Also sensitivity to Silthiofam was demonstrated in a collection of 358 *Ggt* populations in winter wheat. This may be important in order

to pathogen populations with high genetic variation that are potentially proficient of rapidly evolving responses to changing environmental conditions [27]. Consequently, it could be difficult to find long-lasting effective fungicide against this kind of pathogen. In our results, majority of isolates was related to one group that belong to A type. Also according to previous study by Sadeghi et al. [14], comparison pathogenicity resulting with the molecular showed the weaker virulence by the first group while the second group comprising 84% of isolates, explained over 65% disease severity. Also all nine isolates pathogenic on oat belonged to the second group. These results indicated that the dominant population of *Ggt* isolates provides the pathogen with the ability to fight or overcome any commonly used control measures such as chemical or resistant cultivars. Similar divisions of take-all fungus populations into two main groups based on our data were observed that have been reported by other researchers. Augustin et al. [5] used RAPD markers to differentiate *G. graminis* at the intravarietal and intervarietal levels. After amplifying with OPB-07 primer, *Ggt* isolates were separated into two main groups A1 and A2 with different banding pattern. The same researchers disclosed that colonies of subgroup A1 produced dense, darkly pigmented aerial mycelium and those of subgroup A2 had only little aerial mycelium ranging from light grey to dark grey [12,16]. In these groups, the average mycelial linear growth is different from one another. Furthermore researches of Bateman et al. [2] used a mitochondrial rDNA probe distinguished two groups of the pathogen associated with different host-crop species. Also Lebreton et al. [4] examined 120 *Ggt* isolates using RAPD and AFLP markers and separated them into two groups, G1 and G2. The aggressiveness of isolates from group G2 was significantly greater than those from G1 group. In this study, oat attaching isolates which belonged to second group also were more aggressive.

Some fungal genomes have been examined with REP primers [11,28]. Edel et al. [29] showed that using REP primers polymorphism among isolates of *Fusarium oxysporum* can be well revealed. Crozier et al. [11] for the first time used REP primers for the fingerprinting of this pathogen which exhibited valuable polymorphisms among isolates tested. RAPD markers are useful tools especially for fungi that are obligate parasites or that have a population structure composed of clonal lineages and as advanced PCR-based markers turn in to available, it may become possible to amplify specific DNA sequences from soil or root samples and make a direct assessment of the genetic structure of populations of soil borne fungi without first making pure cultures [27]. Using this marker as fast and easy method can be a powerful tool for investigating the occurrence and distribution of this pathogen as well as studying changes in the populations of subgroups of *G. graminis* in cereal cropping systems. Moreover, molecular and pathogenicity test results confirmed studies of the other researchers [14,23] and demonstrated that population of *Ggt* in the wheat growing regions of Iran are highly virulent and also showed that over 80% of isolates examined belong to second group (A-type) which were more aggressive than the first group. In this study with RAPD and ERIC markers, moreover, two isolates of B- type were discernible at other *Ggt* isolate. T-28 isolate with lobed hyphopodium and *Ggg* isolate were detectable at other *Ggt* isolates.

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