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# Genetic diversity analysis of *Gaeumannomyces graminis* var. *tritici* in Kermanshah Province of Iran using RAPD markers

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**Abstract**

Take-all disease caused by *Gaeumannomyces graminis* is the most destructive disease on cereals in the world that has been reported from different areas in Iran. Ninety-seven isolates were obtained from diseased plants collected from Kermanshah Province. To evaluate the genetic diversity 60 isolates were selected and studied using RAPD molecular finger-print. All samples were collected during 2010 and 2011 summer. RAPD-PCR was carried out to investigate genetic diversity. Fifteen RAPD primers which had more polymorphism and higher repeatability were used for DNA replication. The dendrogram obtained from cluster analysis of isolates divided strains into eight groups at the similarity level of 32%. This grouping was somewhat according to the geographical distribution. The polymorphism obtained for RAPD markers was calculated as 100%. To ensure the correct identification of isolates, specific primers were used to identify varieties of *G. g.* var. *tritici*, *G. g.* var. *avenae* and *G. g.* var. *graminis*. These primers did not replicate any DNA fragment for *G. g.* var. *graminis* varieties. The results showed that specific primers are sensitive and useful for identification of different varieties of *G. graminis*. This is the first report of using RAPD markers to assess genetic variation of *G. graminis* in Iranian isolates isolated from wheat and barley.

**Key words** – geographical distributions – Iranian varieties – molecular finger-print – take all

## Introduction

Take-all disease caused by *Gaeumannomyces graminis* var. *tritici* is a fungal disease of wheat that starts as a root rot, causing stunting and nutrient deficiency-like symptoms in the tops, and progressing upward into the base of the stems where it can then disrupt the flow of water to the tops and cause premature death of the plant (Cook 2003, Elliott & Landschoot et al. 1991). Take-all is the second most destructive disease of wheat in the world (Trolldenier et al. 1981). It was first reported as a disease of wheat in Sweden in 1823 (Nilsson et al. 1969). In Iran, it was first reported from Dasht naz farms in Sari and also in other regions of Mazandaran and Gorgan provinces (Forotan et al. 1989). The disease is now present in most regions of Iran (Safaei et al. 2007). In some infected farms up to 80% loss of crop has been estimated (Kazemi et al. 2008). The genus *Gaeumannomyces* contains seven species that have different hosts (Freeman & Ward 2004,

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Rachdowang et al. 1999). *Gaeumanomyces graminis* is the most important species in the genus. This species is divided into four varieties: *G. graminis* var. *tritici* (Ggt), *G. graminis* var. *avenae* (Gga), *G. graminis* var. *maydis* (Ggm) and *G. graminis* var. *graminis* (Ggt). *G. graminis* var. *tritici* is the major agent of take-all that also infects barely but not oats. *G. graminis* var. *avenae* additionally causes disease on oat. *G. graminis* var. *maydis* causes disease on corn and *G. graminis* var. *graminis* attacks some grass weeds, including Bermuda grass and rice (Freeman & Ward 2004, Fouly et al. 1996).

Host range, vast geographical spread, viability and complexity of soil environment have led to inefficient chemical control and difficulty in managing this disease. Many ways to control and contain the disease have been recommended including removing plant debris and weeds, use of symbiotic microorganism, deep tillage, chemical control, use of resistant varieties and crop rotation (Tilson 2005, Mathre 1998, Mathre 1992, Cook et al. 2003).

The aim of this study is to recognize *G. graminis* var. *tritici* strains in wheat and barley in Kermanshah Province and also to investigate the amount of genetic diversity from 14 different areas of Kermanshah Province using RAPD-PCR molecular markers.

## Materials & Methods

### Sampling

During 2010 and 2011, sampling of infected plants from Kermanshah, Dalahoo, Salas, Eslam abad, Giulan gharb, Sarpol-e Zahab, Ravansar, Javanrud and other cities were conducted. After removing excess plant parts infected samples were placed inside a paper bag and transported to the laboratory.

### Isolation of pathogen

Segments of infected plant tissues were washed under tap water for 10 minutes. The washed segments were sliced into smaller pieces and surface sterilized by dipping in 1% sodium hypochlorite for one minute. The surface sterilized pieces were placed on 2% water agar and 2% potato dextrose agar (PDA) and then incubated at 25 ℃ for 10 days. Spores generated on surface sterilized leaves at 25 ˚C, were transferred to 2% water agar and then fungal colonies were produced by hyphal tip method on PDA (Freeman & Ward et al. 2004). Ninety-seven *G. graminis* isolates were selected according to geographical distribution and were used in this study (Table 1).

### Identification of isolates

Preliminary detection of pathogen isolates was done due to the characteristics of colony morphology, growth pattern and form of mycelium branches and also according to the ability to produce perithecia on agar medium containing germinated seeds of wheat, wheat extract and stems and pods extract of bean plants (Crozier 1999, Holden & Hornby 1981, Thomas et al. 2004). The production of hyphopodia was also studied (Fig. 1). However, the specific primer was used to ensure correct identification of isolates.

DNA was extracted from 97 fungal isolates following the protocol outlined by Thomas et al. (2004). Then, reverse PCR primers GGT-RP and GGA-RP, with NS5 (White et al. 1990) as the forward primer was used for identification of *G. graminis* isolates (Fouly & Wilkinson et al. 2000). Amplification by NS5/GGT-RP can distinguish between isolates of Ggt and isolates of Gga on the basis of PCR product size.

PCR primers GG1 and GG2 were designed for diagnosis of take-all patch of turf-grasses (Goodwin et al. 1995). Three isolates of *Rhizoctonia* sp., *Fusarium* sp. and *Pythium* sp. as a negative control were used in this test. They were amplified with primers NS5: 5' AACTTAAAGGAATTGACGGAAG 3' and GT-RP:5' TGCAATGGCTTCGTGAA 3', (GGA-RP: 5' TTTGTGTGTGACCATAC 3), respectively, in a 25 ml reaction containing 1 units Taq DNA polymerase, 2/5 µl PCR buffer provided by the manufacturer, 1/6 µl $MgCl_2$, 10 mM each dNTP, 1/5 µl each primer and 10 ng template DNA, using a thermocycler (Corbett Research, Australia)

programmed for 3 min at 93 ˚C, 30 cycles of 35 s at 93 ˚C, 60 s at 52 ˚C, and 1 min at 72 ˚C, followed by 5 min at 72 ˚C (Fouly & Wilkinson et al. 2000a). Electrophoresis of PCR products with voltage 120 and for an hour in agarose gel 4.1% was done. After completion of electrophoresis, the gels were placed in ethidium bromide solution (0/5 µg/ml) for 20 minutes. Shooting with UVP gel imaging system was conducted.

**RAPD PCR**

According to host variety and geographical distribution, 60 isolates were selected and the genetic diversity of these analyzed using RAPD technique. Of the 20 RAPD primers that were tested initially, 15 primers which showed greater repeatability and polymorphism were used to test all isolates (Table 2). For the RAPD primer pairs PCR assays were done in a total volume of 25 µl with a final concentration of 2.5 µl of XPCR buffer, 0.5 µl of each dNTP (10 mM), 1.6 µl MgCl2, 0.2 µl of Taq (1U) DNA polymerase, 2.5 µl of each primer (10 µM) and approximately 2.5 µl of fungal template DNA (10 ng/ µl) were used. Reactions were performed in a CORBETT Research model thermal cycler using the following PCR conditions: denaturation at 95 ºC for 5 min, 35 cycles of denaturation at 94 ºC for 40 sec, annealing at 40 ºC for 50 sec, extension at 72 °C for 1 min, final extension at 72 °C for 5 min, followed by cooling at 4 °C until recovery of the samples. Amplification products were visualized in 1.2% agarose gels stained with ethidium bromide (Mule et al. 2004).

**Statistical analysis**

Due to use of the specific markers to identify varieties it can be separated from the other variety by produced band during RAPD PCR, so the electrophorese gels can be scored by existence (1) or not existence (0) of the bands on the gel in this method. Cluster analysis was done by WPGMA method, Jaccard index and MVSP ver. 3.131, NTSYSpc ver. 2.02, GenAlex ver. 6.1 software programs (Peakall & Smouse 2006). For the two-dimensional observation of distance between the isolates, principal component analysis was used to supplement the clustering method.

**Table 1** Information of infected samples collected from different parts of Kermanshah.

| Code | Location | Host | Culture mode | Code | Location | Host | Culture mode |
|---|---|---|---|---|---|---|---|
| G1=1* | Sare pol Zahab | Wheat | Irrigated | G50=26* | Ravansar-Pave | Barely | Rain-fed |
| G2 | Salas Babajani | Wheat | Rain-fed | G51 | Javanrood | Barely | Rain-fed |
| G3=2* | Sare pol Zahab | Wheat | Irrigated | G52 | Javanrood | Barely | Rain-fed |
| G4 | Gilan Gharb | Barely | Rain-fed | G53=27* | Koozaran | Barely | Rain-fed |
| G5=3* | Gilan Gharb | Barely | Rain-fed | G54=28* | Koozaran | Barely | Rain-fed |
| G6 | Gilan Gharb | Barely | Rain-fed | G55=29* | Koozaran | Barely | Rain-fed |
| G7 | Gilan Gharb | Barely | Rain-fed | G56=30* | Koozaran | Barely | Rain-fed |
| G8=4* | Gilan Gharb | Wheat | Rain-fed | G57 | Koozaran | Barely | Rain-fed |
| G9=5* | Gilan Gharb | Wheat | Rain-fed | G58 | Koozaran | Barely | Rain-fed |
| G10 | Faraman village | Barely | Rain-fed | G59 | Koozaran | Barely | Rain-fed |
| G11=6* | Negivaran | Barely | Rain-fed | G60=31* | Javanrood | Barely | Rain-fed |
| G12 | Ghazanchi | Barely | Rain-fed | G61 | Koozaran | Barely | Rain-fed |
| G13=7* | Ghazanchi | Barely | Rain-fed | G62 | Koozaran | Barely | Rain-fed |
| G14 | Ghazanchi | Barely | Rain-fed | G63=32* | Mahidasht | Barely | Rain-fed |
| G15=8* | Ghazanchi | Barely | Rain-fed | G64=33* | Bistoon | Wheat | Irrigated |
| G16=9* | Ghazanchi | Barely | Rain-fed | G65=34* | Bistoon | Wheat | Irrigated |
| G17 | Ghazanchi | Barely | Rain-fed | G66=35* | Bistoon | Wheat | Irrigated |
| G18 | Ghazanchi | Barely | Rain-fed | G67=36* | Bistoon | Wheat | Irrigated |

**Table1 (continued)**

| Code | Location | Host | Culture mode | Code | Location | Host | Culture mode |
|---|---|---|---|---|---|---|---|
| G19 | Ghazanchi | Barely | Rain-fed | G68=37* | Bistoon | Wheat | Irrigated |
| G20=10* | Ghazanchi | Barely | Rain-fed | G69 | Bistoon | Wheat | Irrigated |
| G21=11* | Ghazanchi | Wheat | Irrigated | G61 | Bistoon | Wheat | Irrigated |
| G22 | Ghazanchi | Wheat | Irrigated | G70 | Harsin | Wheat | Irrigated |
| G23=12* | Sarab Ghanbar | Barely | Rain-fed | G71=28* | Harsin | Wheat | Irrigated |
| G24=13* | Sarab Ghanbar | Barely | Rain-fed | G72=39* | Harsin | Wheat | Irrigated |
| G25=14* | Sarab Ghanbar | Barely | Rain-fed | G73=40* | Harsin | Wheat | Irrigated |
| G26 | Sarab Ghanbar | Barely | Rain-fed | G74=41* | Harsin | Wheat | Rain-fed |
| G27=15* | Sarab Ghanbar | Barely | Rain-fed | G75=42* | Mahidasht | Wheat | Irrigated |
| G28 | Sarab Ghanbar | Barely | Rain-fed | G76 | Mahidasht | Wheat | Irrigated |
| G29=16* | Sarab Ghanbar | Barely | Rain-fed | G77=43* | Mahidasht | Barely | Rain-fed |
| G30 | Sarab Niloofar | Wheat | Irrigated | G78=44* | Eslam Abad | Barely | Rain-fed |
| G31=17* | Sarab Niloofar | Barely | Rain-fed | G79=45* | Sahne | Wheat | Irrigated |
| G32=18* | Sarab Niloofar | Wheat | Irrigated | G80=46* | Faraman | Barely | Rain-fed |
| G33=19* | Koozaran | Barely | Rain-fed | G81=47* | Faraman | Wheat | Rain-fed |
| G34 | Koozaran | Barely | Rain-fed | G82=48* | Faraman | Wheat | Rain-fed |
| G35 | Koozaran | Barely | Rain-fed | G83=49* | Faraman | Barely | Rain-fed |
| G36=20* | Koozaran | Barely | Rain-fed | G84=50* | Faraman | Barely | Rain-fed |
| G37=21* | Koozaran | Barely | Rain-fed | G85=51* | Mian Rahan | Barely | Rain-fed |
| G38=22* | Koozaran | Barely | Rain-fed | G86 | Mian Rahan | Barely | Rain-fed |
| G39 | Koozaran | Barely | Rain-fed | G87=52* | Songhor | Barely | Rain-fed |
| G40 | Koozaran | Barely | Rain-fed | G88=53* | Songhor | Barely | Rain-fed |
| G41 | Koozaran | Barely | Rain-fed | G89=54* | Songhor | Barely | Rain-fed |
| G42=23* | Koozaran | Barely | Rain-fed | G90=55* | Kerend | Wheat | Irrigated |
| G43=24* | Koozaran | Barely | Rain-fed | G91=56* | Kerend | Wheat | Irrigated |
| G44 | Koozaran | Barely | Rain-fed | G92=57* | Gilan Gharb | Barely | Rain-fed |
| G45 | Koozaran | Barely | Rain-fed | G93=58* | Gilan Gharb | Wheat | Rain-fed |
| G46 | Koozaran | Barely | Rain-fed | G94 | Gilan Gharb | Wheat | Rain-fed |
| G47=25* | Koozaran | Barely | Rain-fed | G95 | Kamiaran | Wheat | Rain-fed |
| G48 | Koozaran | Barely | Rain-fed | G96=59* | Pave | Wheat | Irrigated |
| G49 | Ravansar-Pave | Barely | Rain-fed | G97=60* | Eslam Abad | Wheat | Irrigated |

* Selected isolates used in the study of genetic diversity.

**Table 2** Details of the primers used to assess genetic diversity of *Gaeumannomyces graminis.*

| Primer | Sequence 5'-3' | Annealing temperature ˚C |
|---|---|---|
| OPC07 | GTCCCGACGA | 40 |
| E10 | CACCAGGTGA | 40 |
| U11 | AGACCCAGAG | 40 |
| A7 | GAAACGGGTG | 40 |
| OPC13 | AAGCCTCGTC | 40 |
| E17 | CTACTGCCGT | 40 |
| OPC10 | TGTCTGGGTG | 37 |
| E16 | GGTGACTGTG | 40 |
| ABI | CCGTCGGTAG | 40 |
| OPC04 | CCGCATCTAC | 40 |
| E7 | AGATGCAGCC | 40 |
| E19 | ACGGCGTATG | 40 |
| C16 | CACACTCCAG | 40 |
| OPC15 | GACGGATCAG | 40 |
| OPC08 | TGGACCGGTG | 40 |

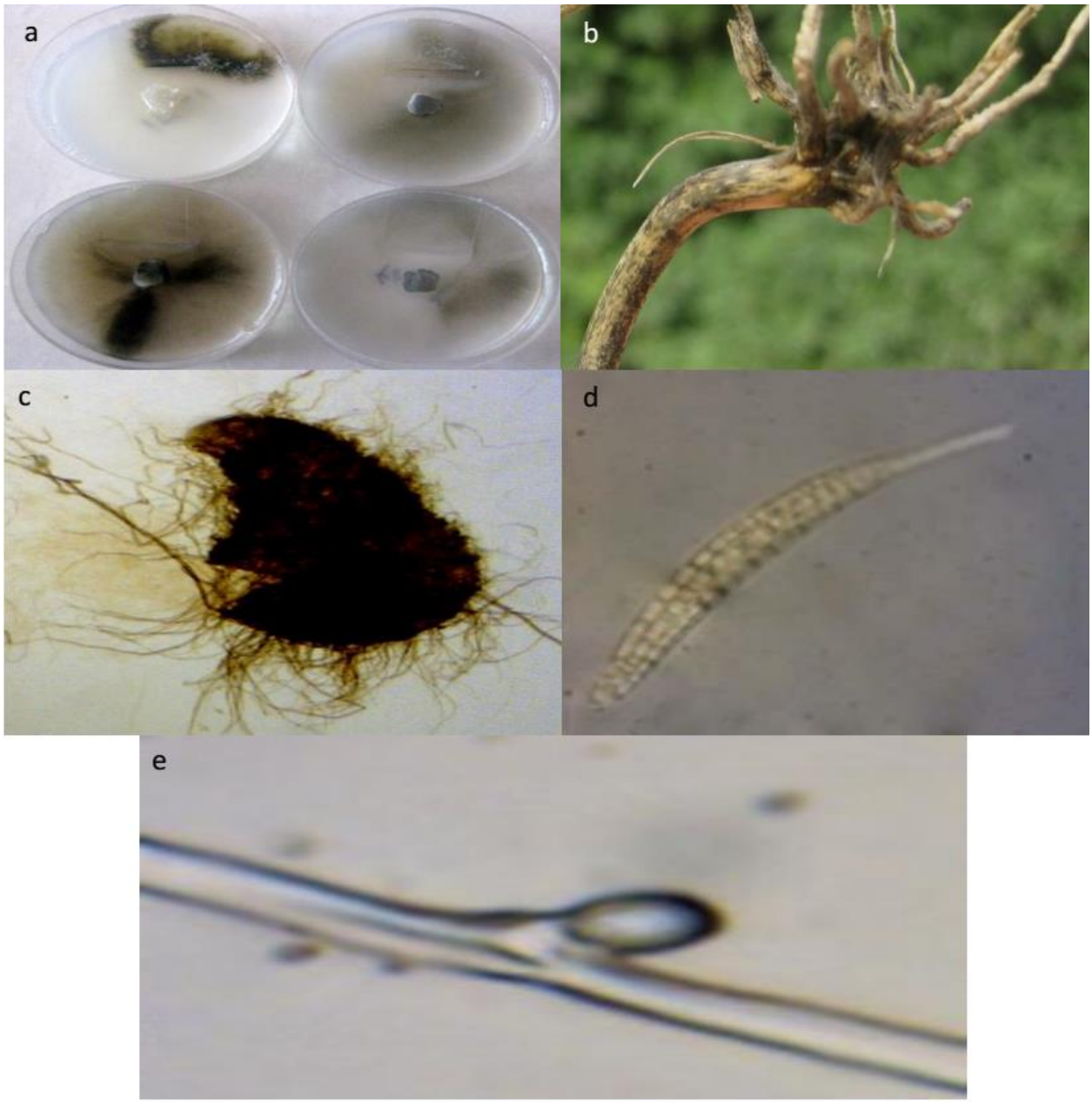


**Fig 1** – Morphological characters of *Gaeumannomyces graminis* var. *tritici*. a. Morphological variety of colony appearance in different isolates of *Gaeumannomyces graminis* var. *tritici* at $25^0$ on PDA. b. Black stolon of *Gaeumannomyce graminis* var. *tritici* on rotted wheat root. c, d. Asci and ascospores exiting from perithecia. e. Moving hypha with simple hyphopodium.

**Results**

The presence of *G. graminis* was found in 139 out of 307 farms visited. For all isolates, application of upstream NS5 primer with downstream GGT-RP primer lead to a band in the range of 410 bp and the application of upstream NS5 primer with downstream GGA-RP primer lead to a band in the range of 400 bp. The results in both cases showed that all isolates were *G. g*. var. *tritici*. The use of both downstream primers was done in the presence of three witnesses, including *Pythium* sp., *Rhizoctonia* sp. and *Fusarium* sp. The results showed that *G. g*. var. *avenae* was not found among collected isolates and all isolates belong to varieties *G. g*. var. *tritici* (Fig. 2).

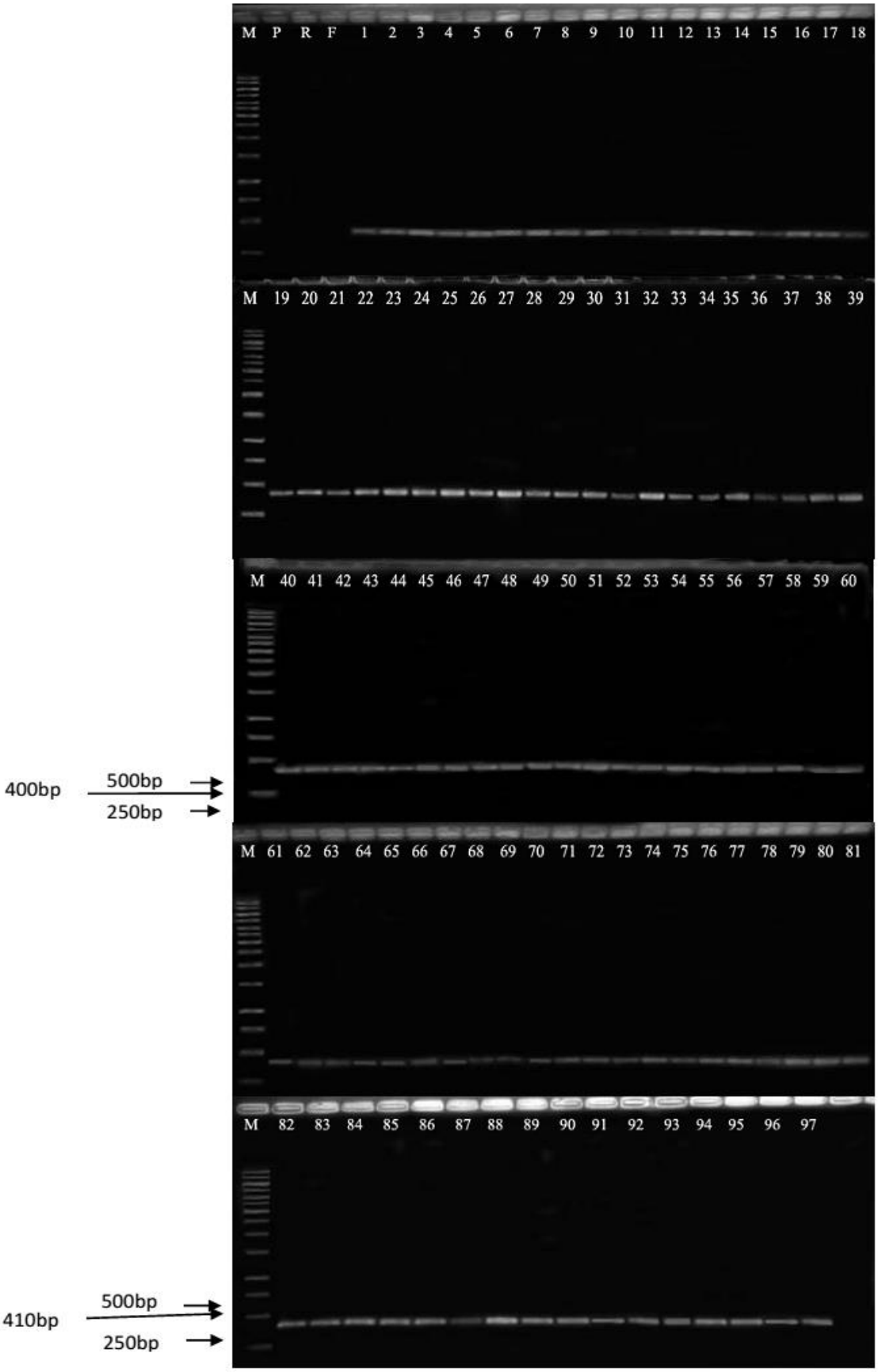


**Fig 2** – Banding patterns attained from 97 selected isolates of *Gaeumannomyces graminis* using specific primers, with control. M: 1kb molecular marker.

**Analysis of DNA fingerprinting RAPD PCR**

Out of 20 tested RAPD primers, 15 primers showed a greater repeatability and polymorphism, thus they were used for all strains (Fig. 3).

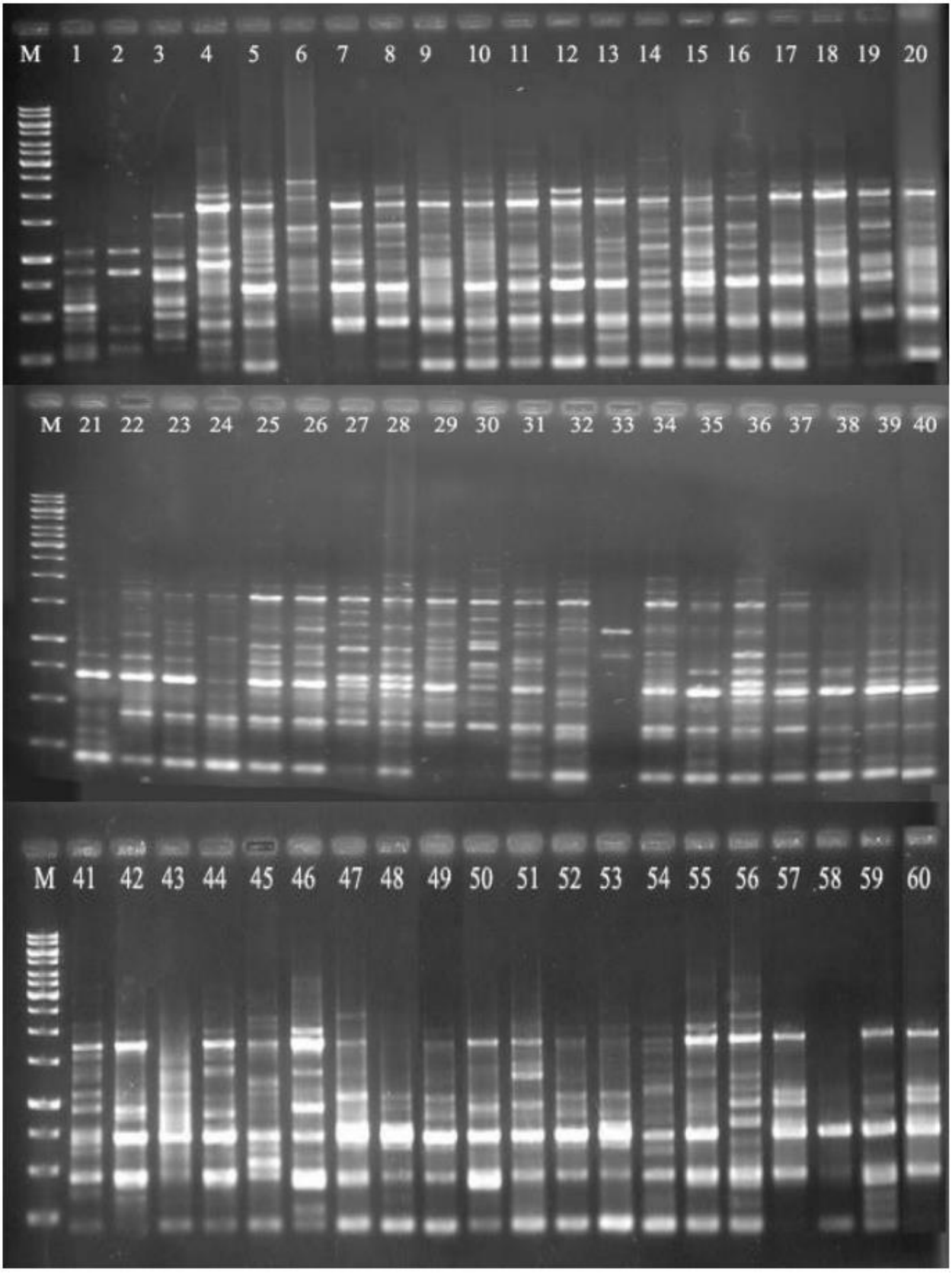


**Fig 3** – Banding patterns attained from 60 selected isolates of *Gaeumannomyces graminis* isolated from wheat and barley using primer E7. M: 1kb molecular marker.

The number and range of bands produced by each primer is shown in Table 3. The results of the analysis of the banding pattern of all RAPD primers divided the isolates into eight groups, in similarity level of 32%, also confirmed by two-dimensional plot obtained from the main coordinate analysis (Figs 4, 5).

**Table 3** Percentage of the polymorphism and information content of 15 primers used for 60 isolates of *Gaeumannomyces graminis*.

| Primer | PIC[1] | MI[2] | Total bands | Number of polymorphic bands | Polymorphism percentage |
|---|---|---|---|---|---|
| OPC07 | 33.0 | 95.6 | 21 | 21 | 100 |
| E10 | 38.0 | 09.8 | 21 | 21 | 100 |
| U11 | 42.0 | 72.6 | 16 | 16 | 100 |
| A7 | 34.0 | 6.6 | 19 | 19 | 100 |
| OPC13 | 30.0 | 15.6 | 20 | 20 | 100 |
| E17 | 27.0 | 15.5 | 19 | 19 | 100 |
| OPC10 | 33.0 | 66.4 | 14 | 14 | 100 |
| E16 | 34.0 | 49.7 | 22 | 22 | 100 |
| ABI | 38.0 | 14.6 | 16 | 16 | 100 |
| OPC04 | 38.0 | 53.8 | 22 | 22 | 100 |
| E7 | 38.0 | 57.9 | 25 | 25 | 100 |
| E19 | 33.0 | 07.6 | 18 | 18 | 100 |
| C16 | 34.0 | 04.8 | 23 | 23 | 100 |
| OPC15 | 36.0 | 74.9 | 27 | 27 | 100 |
| OPC08 | 36.0 | 57.9 | 21 | 21 | 100 |
| Average | 34.0 | 23.7 | 304 | 304 | 100 |

1- Polymorphic Information Content
2- Marker Index

Three hundred and four loci were obtained from all RAPD band patterns, all of which had polymorphism. Maximum and minimum polymorphic loci were attained using OPC 15 primer (27 loci) and OPC 10 primer (14 loci), respectively. Among these primers, OPC 15 primer was allocated the highest ability of separation. Maximum and minimum amount PIC was 0.42 belonging U11 primer and 0.27 belonging E11 primer. The average of calculated PIC was 0.34. High levels of PIC in this study show the high level of distinction. Marker index were calculated on the basis of polymorphic bands per primer and was variable from 4.66 to 9.74 which belonging OPC 10 primer and OPC 15 primer, respectively. The results showed that the highest and lowest genetic similarity based on Jaccard coefficient was between isolate 1 from sarpol-e Zahab (wheat) and isolate 2 sarpol-e Zahab (wheat) in amount of 53/0 and between isolate 22 from Kuzaran (barley) and isolate 56 from Islamabad (barley) in amount of 18/0. Attained results of RAPD cluster analysis did not match with the geographical location. Cophenetic coefficient was calculated at a rate of 527/0, for obtained results of RAPD analysis.

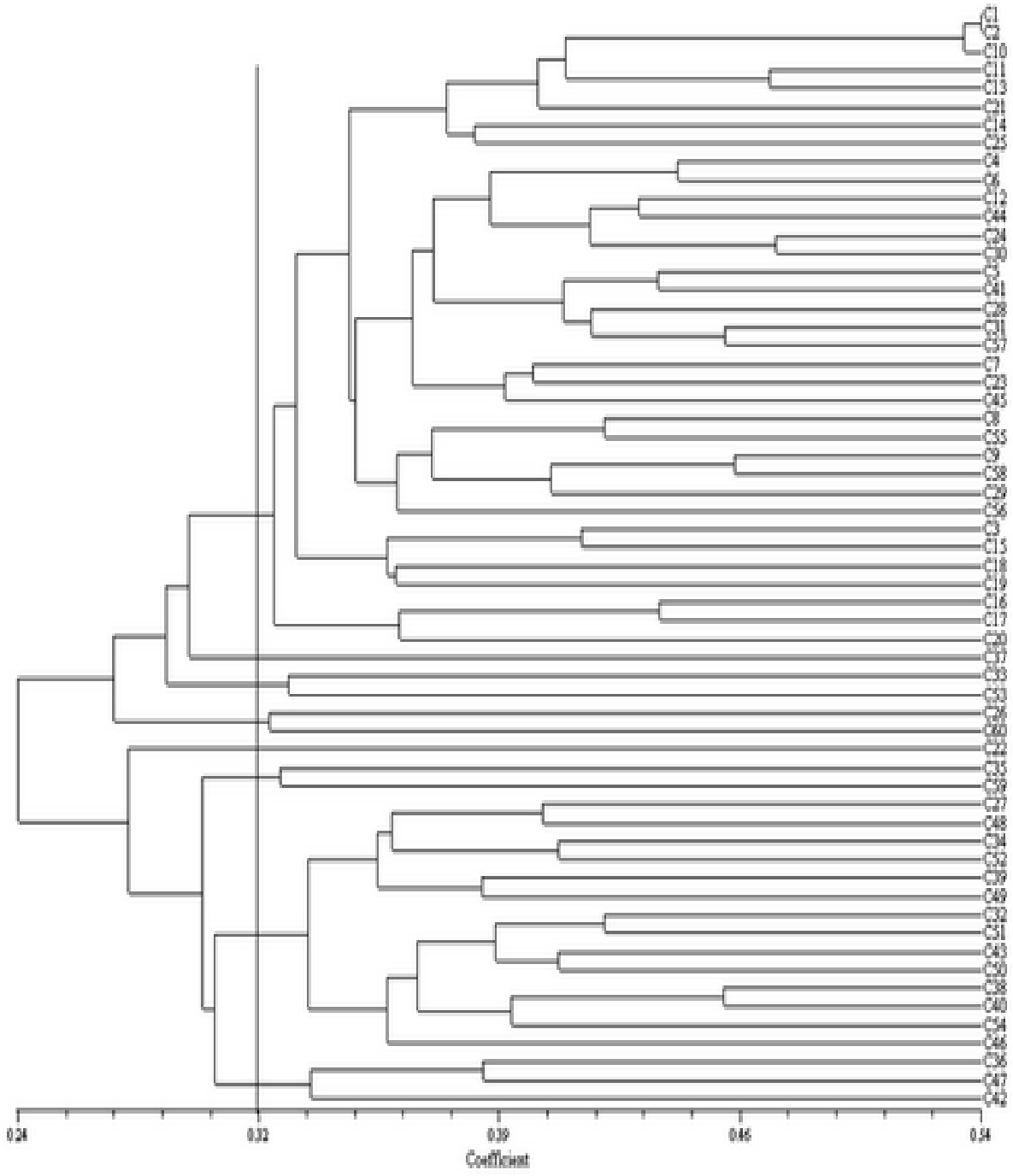


**Fig 4** – Dendrogram based on UPGMA using combined data from RAPD banding patterns.

**Discussion**

The results of this research confirm effectiveness of DNA fingerprinting using RAPD markers and use of specific primers to identify the fungus *G. graminis*. Conventional identification of *G. graminis* is time consuming and unreliable, and differences in the medium materials can lead to uncertain identification of fungus and its varieties (Asher & Shipton 1981). Because of the similarity of morphological and reproductive characteristics, it is difficult to distinguish varieties *G*. g. var. *tritici* and *G*. g. var. *avenae*, despite the use of morphological and reproductive characteristics such as stolon and size of ascospores in identification process. Although the pathogenicity test is not easy because of its long and time-consuming process. In general, use of proprietary medium can distinguish varieties of *G. graminis*, but these methods are costly. Since this fungus is destructive and difficult to control. An accurate, rapid and sensitive method which

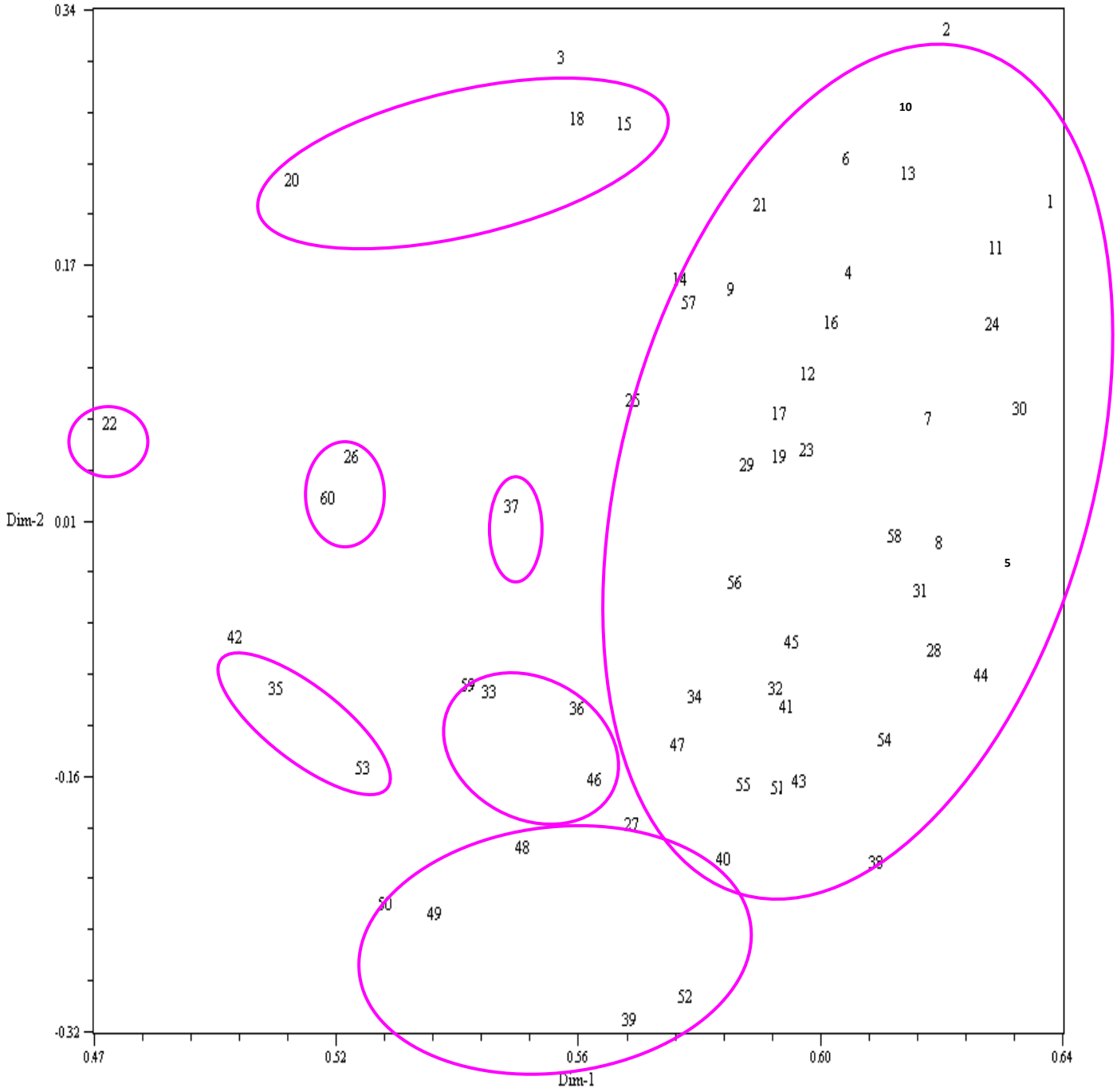


**Fig 5** – Two-dimensional plot of the original coordinate analysis using Jaccard matrix for 60 selected isolates of *Gaeumannomyces graminis*.

cans diagnosis the fungus even in soil and plant tissue is necessary. In addition to the differentiation of *G. graminis* from other species, species-specific primers can also separate varieties *G. g.* var. *tritici* from *G.* g. var. *graminis* and *G. g.* var. *avenae*. So, in order to ensure the correct identification of obtained isolates, specific primers were used to separate *G. g.* var. *tritici* varieties from *G. g.* var. *graminis* and *G. g.* var. *avenae* varieties. In PCR progress NS5: GGT-Rp and NS5: GGA-RP were used as specific primers. The results showed that 97 studied isolates were *G. g.* var. *tritici*, according to production of 410 and 400 bp bands by this isolate. As well as the results it can be deduced that, no observation of band 300 bp between the produced bands indicate the lack of variety G. g. var. avenae among the isolates. All tests were conducted in the presence of three controls (*Fusarium* sp., *Rhizoctonia* sp., *Pythium* sp.) for which no band was attained (Fig. 1).

These results confirm the results of other researchers (Irzykowska 2007, Thomas 2004, Fouly & Wilkinson 1999, White et al. 1990). Specific primers used in this study, do not produce any bands for *G*. g. var. *graminis*., according to Fouly & Wilkinson (2000). To study the genetic diversity of *G. graminis*, 60 isolates were selected from the 97 isolates and were analyzed by RAPD markers. The polymorphism amount attained for each primer was equal to 100 percent. The results of this study demonstrate that RAPD marker is a good marker for studying genetic diversity of *G. graminis* isolates. For example, Augustin et al. (1999) used 26 different RAPD primers to study 48 isolates of *G. graminis* by analyzing of lac or existence of bands and they can classify the isolates into four groups. According to host and geographical location, Weber et al. (2005) classified 70 isolate of *G. graminis*, isolated from wheat, into two main groups by using 10 different RAPD markers. The results showed that, high diversity level was observed among the isolates and also cluster analysis of RAPD markers was partly consistent with the geographical distribution of the isolates. This study suggests that locating the isolates from different regions in a same group indicates their genetic similarity. This study identified considerable variation among isolates of *G. graminis*, and also isolates grouped by geographical origin can be due to pathogen transmission and since the fungus is soil-born it can spread by agricultural implements or irrigation water through the soil to other areas, thus creating strains with the same genetic pattern from different areas of the province. The results showed that the vast genetic diversity in this species have seen, not only among isolates with distinct geographical areas but also in the population of isolates collected from a plant host and even in isolate from same region. This study also produces considerable information in relation to identification and genetic diversity of Iranian isolates of *G. graminis* through specific primers and RAPD molecular markers. In the case of an opportunistic disease, high genetic diversity helps it to survive in nature. So, the assessment of genetic diversity of Iranian strains of *G. graminis* made understanding the pathogen population structure easier and as a result can impact disease management.

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