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Genetic Diversity of *Trichoderma harzianum* Isolates in Sunflower Rhizosphere: The Application of the URP Molecular Marker

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Abstract: The genetic diversity of 77 *Trichoderma harzianum* isolates collected from sunflower rhizosphere soils in Urmia, Khoy, and Salmas in West Azerbaijan province, Iran, was evaluated by using the Universal Rice Primer (URP) molecular marker. The DNA band pattern of the isolates was developed using seven primers of this marker. These primers produced 186 gene loci, out of which 182 loci were polymorphic. Accordingly, the genetic diversity of the isolates was calculated, and their kinship relations were determined by cluster analysis using the NTSYS software package. URP-6R had the highest marker index among the studied primers, followed by URP-1F, URP-4R, and URP-25F, implying their higher efficiency in discriminating between the isolates. The results showed that the URP marker could discriminate between isolates using macroscopic morphological characteristics, such as color and colony type, potential of pigment production in the culture medium, and colony growth rate. Furthermore, there was no significant relationship between the geographical distribution of the isolates and the band patterns generated by the primers except for a few cases. The results generally revealed that the URP marker was an efficient tool for determining the genetic diversity of *T. harzianum*.

Keywords: *Helianthus* L.; universal rice primer marker; DNA band pattern; morphological characteristics

Citation: Rezaee Danesh, Y.; Pellegrini, M.; Kariman, K.; Boyno, G.; Djebaili, R.; Farda, B.; Najafi, S. Genetic Diversity of *Trichoderma harzianum* Isolates in Sunflower Rhizosphere: The Application of the URP Molecular Marker. *Sustainability* **2022**, *14*, 15111. https://doi.org/10.3390/su142215111

Academic Editors: Mukesh Meena, Prashant Swapnil and Andleeb Zehra

Received: 8 October 2022
Accepted: 14 November 2022
Published: 15 November 2022

1. Introduction

Oil imports account for a significant part of the sunflower oil supply in Iran and Italy. Improving sunflower production is a promising approach for offsetting vegetable oil shortages [1]. Sunflower cultivation is hampered by several phytopathogens. Among these, fungi are considered the dominant ones worldwide [2]. One of the most critical sunflower fungal pathogens which causes high losses in crop yields in most regions is *Sclerotinia sclerotiorum*, which can destroy 100% of sunflower crops [3,4]. However, its control is challenging, and even chemical measures have failed to control sunflower crown and root rot disease caused by this fungus [5,6]. Therefore, scientists have focused on developing the capability of beneficial microorganisms with plant pest and disease control abilities [7,8]. Soil-borne fungi belonging to the *Trichoderma* genus are the most effective mycoparasites against *S. sclerotiorum* [9].

Trichoderma harzianum is one of the most used bio-control agents due to its high aggressiveness against pathogens. Several antagonistic mechanisms are described for this species (e.g., competition, parasitism, and antibiosis). *T. harzianum* stimulates growth and plant resistance [10]. High reproduction capacity and adaptability to different substrates

and rhizosphere modulation have also been described [10]. *T. harzianum* is one of the most abundant species of the genus *Trichoderma*, which can be found in diverse ecological niches [11]. This species is widely applied in the commercial formulations of herbicides [12] and has extensive applications in the biological control of plant pathogens. This species also induces plant resistance against phytopathogens and improves plant growth [13–16].

Given the significance and influence of this species in food production, it is necessary to understand its genetic structure. However, despite the high significance of this species, it does not have taxonomically known bounds due to its complexity [17]. Research on the genetic and phylogenic diversity of *T. harzianum* worldwide has revealed that the species is complex and highly capable of living in diverse habitats [18].

Advanced molecular methods have been used to precisely distinguish different fungi, especially those highly diverse in shape and color at the species level [19,20]. Molecular information has shown heterogeneity within the *Trichoderma* isolates [17,21]. It is difficult to identify the intraspecific diversity of *T. harzianum* using morphological attributes [22]. For this reason, researchers have employed different molecular methods to study its intraspecific genetic diversity, such as Restriction Fragment Length Polymorphism (RFLP) [23], Random Amplification of Polymorphic DNA (RAPD) [24,25], and Inter-Simple Sequence Repeat (ISSR) [26]. DNA molecular markers provide a fast and precise method for research on genetic diversity to gain an insight into the complex structure of *Trichoderma* populations [27].

Trichoderma isolates have a high level of genetic diversity and can be used to produce commercial products with ecological benefits [28,29]. Since *Trichoderma* has been considered a bio-control agent and biotechnological application [29–31], it is imperative to define its isolates further and more precisely [32]. Additionally, if the dominant *Trichoderma* species genetic diversity is high, this index can be used to select the isolates with high antagonistic potential.

The genetic diversity of this species has not been studied in the sunflower rhizosphere soils in West Azerbaijan. This research was designed to study the intraspecific diversity of *Trichoderma* isolates using the Universal Rice Primer (URP) marker. Based on repetitive sequences of wild Korean rice and given the suitability of the URP marker to be used in the polymerase chain reaction (PCR)-based fingerprinting of different organisms, we hypothesized that it would be suitable for the description of the genetic diversity of *Trichoderma* isolates. Although URP markers are based on random primers, the high annealing temperature and length of the primer in the polymerase chain reaction make the primer specific and the technique reproducible compared to other methods such as RAPD [33].

2. Materials and Methods

2.1. Fungal Isolates

The *T. harzianum* isolates were provided by the fungal culture collection at the Department of Plant Protection, Faculty of Agriculture, Urmia University, Iran. These isolates were collected from the sunflower rhizosphere in the West Azarbaijan province, Iran, during the year 2019–2020 (Urmia, Khoy, and Salmas regions). From a total of 105 *T. harzianum* identified isolates, 77 isolates were selected for genetic diversity studies based on several characteristics, i.e., culture medium's growth pattern, high-quality DNA, and geographical distribution.

2.2. Preparation of Mycelia Mass

Isolates of *T. harzianum* were cultured in a potato dextrose agar (PDA) medium (Merck, Darmstadt, Germany) supplemented with chloramphenicol (300 μg L^{-1}) and were transferred to Petri dishes to grow at 20–25 °C. Three days after growth, three 5 mm discs from the margins of young colonies of the fungal isolates were transferred to flasks containing 70 mL of liquid potato dextrose broth (PDB) medium (Merck, Germany). The flasks were placed in a shaker at 120 rpm at room temperature for three days. Next, the flask contents

were filtered through Whatman grade 1 filter paper on a Büchner funnel using a vacuum pump. Then, the mycelial mass was separated from the liquid culture medium. After separating agar discs, they were washed with sterilized distilled water to remove the residual liquid medium from the mycelia surface.

2.3. DNA Extraction

The genomic DNA was extracted from the samples using a DNA extraction kit (#K0512, Fermentas, Waltham, MA, USA) with the protocol proposed by Safaei et al. [34]. After extraction, DNA quality was assessed by 0.8% agarose gel (Bishop Inc., Calgary, AB, Canada) electrophoresis. DNA quantity was determined spectrophotometrically at 260 and 280 nm (OMNIDOC, Cleaver Scientific LTD., Rugby, UK). The final DNA concentration was obtained using the coefficient of dilution.

2.4. PCR and Electrophoresis

Seven primers (Cinnagen, Tehran, Iran) were used in the fungal isolates' PCR. The DNA was amplified in an Eppendorf thermocycler gradient at a volume of 20 µL of the reaction mixture, including 2 µL of the PCR buffer, 0.7 µL $MgCl_2$, 0.4 µL of dNTPs, 1 µL of the primer, and 0.5 µL of the Taq polymerase enzyme. The DNA fragments were amplified by initial denaturation at 94 °C for 4 min. The denaturation was followed by 35 cycles. Each cycle was set with 1 min denaturation at 94 °C, 1 min annealing at the optimal temperature of each primer, and 2 min extension at 72 °C. The final extension was performed for 10 min at 72 °C [32]. A distinctive temperature gradient was set and selected based on the best annealing temperature for each primer to determine the optimum annealing temperature (Table 1). The PCR products were analyzed on 1.4% agarose gel electrophoresis using TBE buffer at 90 V for 135 min, followed by visualizing by ethidium bromide (1 µg/mL) staining under UV radiation.

Table 1. The sequence and annealing temperature of each URP (Universal Rice Primer) used for polymerase chain reaction (PCR).

Primer	Sequence	Annealing Temperature (°C)
URP-1F	5′-ATCCAAGGTCCGAGACAACC-3′	51
URP-2F	5′-GTGTGCGATCAGTTGCTGGG-3′	47
URP-2R	5′-CCCAGCAACTGATCGCACAC-3′	49
URP-4R	5′-AGGACTCGATAACAGGCTCC-3′	54
URP-6R	5′-GGCAAGCTGGTGGGAGGTAC-3	53
URP-13R	5′-TACATCGCAAGTGACACAGG-3′	46
URP-25F	5′-GATGTGTTCTTGGAGCCTGT-3′	51

2.5. Assay of Morphological Traits of the Fungi

New cultures were prepared from all samples to explore the relationship between the morphological traits and the genetic traits and the capability of the marker to discriminate between the isolates based on morphological traits. The criteria to evaluate the growth rate of the colony and the time required for filling 6 cm Petri dishes were pigment production, green–yellow colony production, sporulation, and the growth patterns of different isolates. The daily growth rate was measured every 24 h until the Petri dishes were filled. The growth patterns and production of aerial hyphae and pigments were also tested after 5–6 days at the time of full sporulation of the isolates.

2.6. Data Analysis

The sizes of the visible bands were specified for all isolates. Results were used and processed with the common bands to build the data matrix. Since the URP marker was dominant, the band's presence was scored as 1, and its non-presence was scored as 0. The data were ranked with a 1–0 contingency table, which was recorded in MS Excel. The weight of the bands was determined with a 1-kilobase DNA genomic ladder (Fermentas, Waltham,

MA, USA). The NTSYS-pc software was used for cluster analysis [35]. The polymorphism information content (PIC) shows the extent to which a marker is polymorphic. This parameter was calculated using the sum of the square of allele frequency (P) and the sum of the square of allele non-presence (q) and the elimination of monomorphic loci [36] as follows:

$$PIC = 1 - (\frac{\sum_{i=1}^{n} pi2}{n} + \frac{\sum_{i=1}^{n} qi2}{n}).$$

The marker index (MI) was also calculated as:

$$MI = PIC \times EMR$$

The EMR (effective multiplex ratio) is an effective multiplex index and represents the number of polymorphic gene loci in a genotype, calculated by the following formula:

$$EMR = n_p \times \beta$$

in which n_p is the total number of polymorphic gene loci, and β is calculated by:

$$\beta = \frac{n_p}{n_p - n_{np}}$$

The n_p represents the number of polymorphic bands, and n_{np} represents the number of monomorphic bands [37]. The number of alleles, effective alleles, Nei's genetic diversity, and the Shannon diversity indexes were examined for the studied population using the POPgen 32 software package [38]. Molecular variance analysis, a method for studying intraspecific molecular diversity, was performed for three different populations by the GenALEX version 6.1 software package. SPSS version 19 was used to statistically analyze the morphological data.

3. Results

Seven random primers, including URP-1F, URP-2F, URP-2R, URP-4R, URP-6R, URP-13R, and URP-25F, were used based on reproducibility and polymorphism to examine the genetic diversity of the *T. harzianum* isolates. The polymerase chain reaction was repeated for each isolate twice to ensure the reproducibility of the band pattern. All the studied primers produced distinctive band patterns (Figure 1). None of the seven primers could fully discriminate the isolates in their geographical regions. However, isolates with similar geographical regions were put in the same groups. The analysis of band patterns resulting from the seven studied primers classified the isolates into three groups at the 36% similarity level (Figure 2). The first group included 14% of the isolates, including those from Urmia and Khoy. The second group included 84% of the isolates, including those from all three regions of Urmia, Khoy, and Salmas. The third group only included one isolate from Urmia. At the 55% similarity level, the number of the groups increased to 22, showing high genetic diversity among them. The URP marker put the isolates at a close distance from one another based on characteristics such as the type of similar colony, high growth rate, low growth rate, the generation of the green–yellow colonies, and the production of pigments in the PDA culture medium.

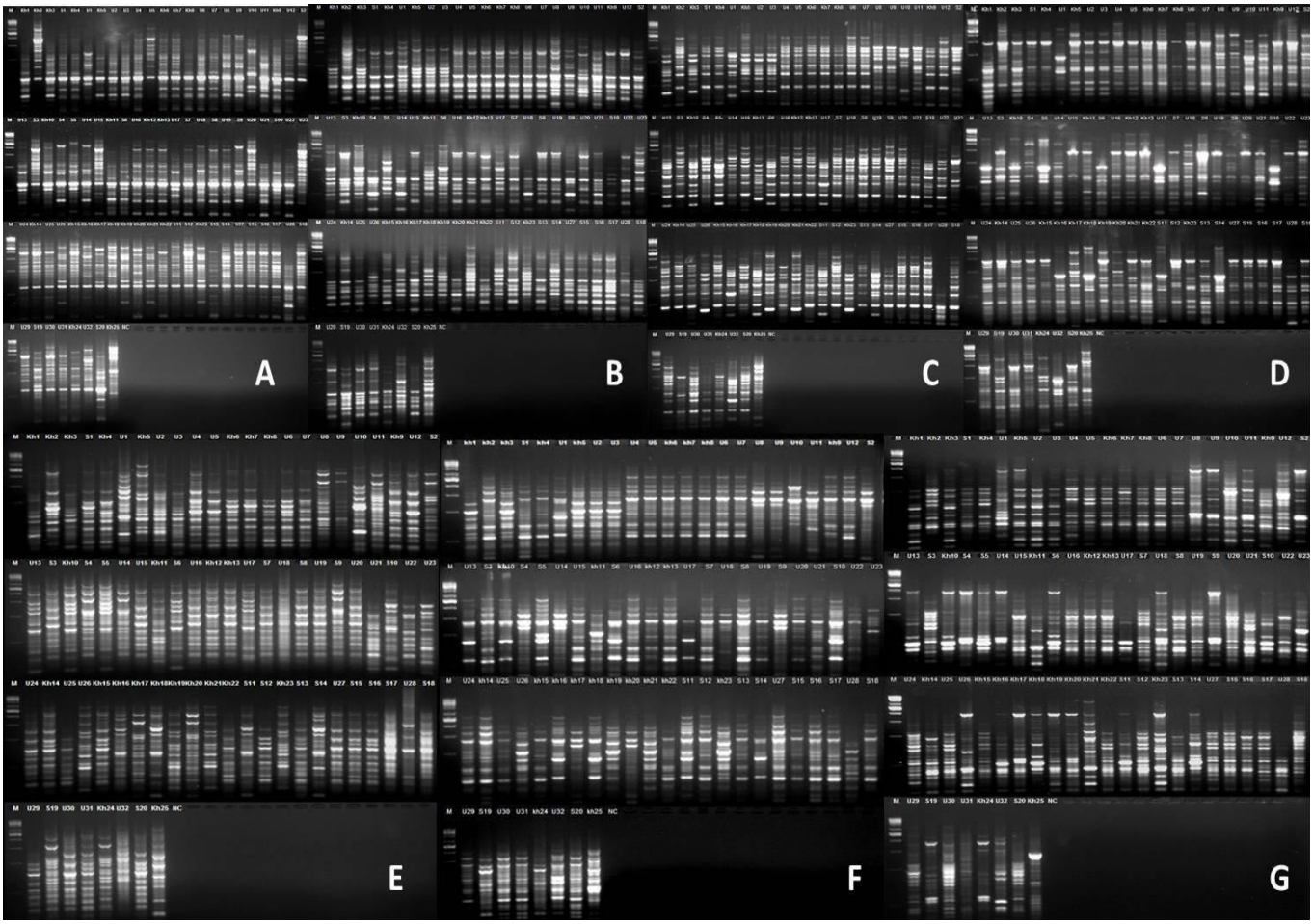

Figure 1. Using seven URP primers for studying the genetic diversity of 77 isolates of *T. harzianum*. (**A**): URP-1F primer; (**B**): URP-2F primer; (**C**): URP-2R primer; (**D**): URP-4R primer; (**E**): URP-6R primer; (**F**): URP-13R primer; (**G**): URP-25F primer. M: DNA size marker (1 kB).

The two isolates, S19 and Kh24, which had similar colony types, were placed in the same group at both similarity levels (Figure 3A). The S20, U29, U30, and U31 isolates, which had lower growth rates in the culture medium and produced green–yellow colonies, were classified in the same group (Figure 3B). The Kh1, Kh3, Kh5, and Kh11 isolates, which had similar colony types in the culture medium and belonged to the Khoy region, were put in the same group (Figure 3C). The S17, U25, Kh22, S13, S15, S7, S12, U19, U24, and Kh12 isolates, which had similar colony types in the PDA medium, were placed in the same group at close distance to one another (Figure 3D). The S2, S4, U11, U14, U18, Kh17, and Kh20 isolates, which filled the 6 cm Petri dishes in 48 h and were among the fast-growing isolates, were placed in the same group at close distance to one another (Figure 3E). The S2, U11, U13, U18, and U23 isolates, which had similar colony types in the culture medium, were classified in the same group (Figure 3F). The S12, U16, U22, Kh6, and Kh13 isolates, the most slowly growing isolates in the solid and liquid media among the 77 studied isolates, were all classified in the same group at close distances from one another (Figure 3G).

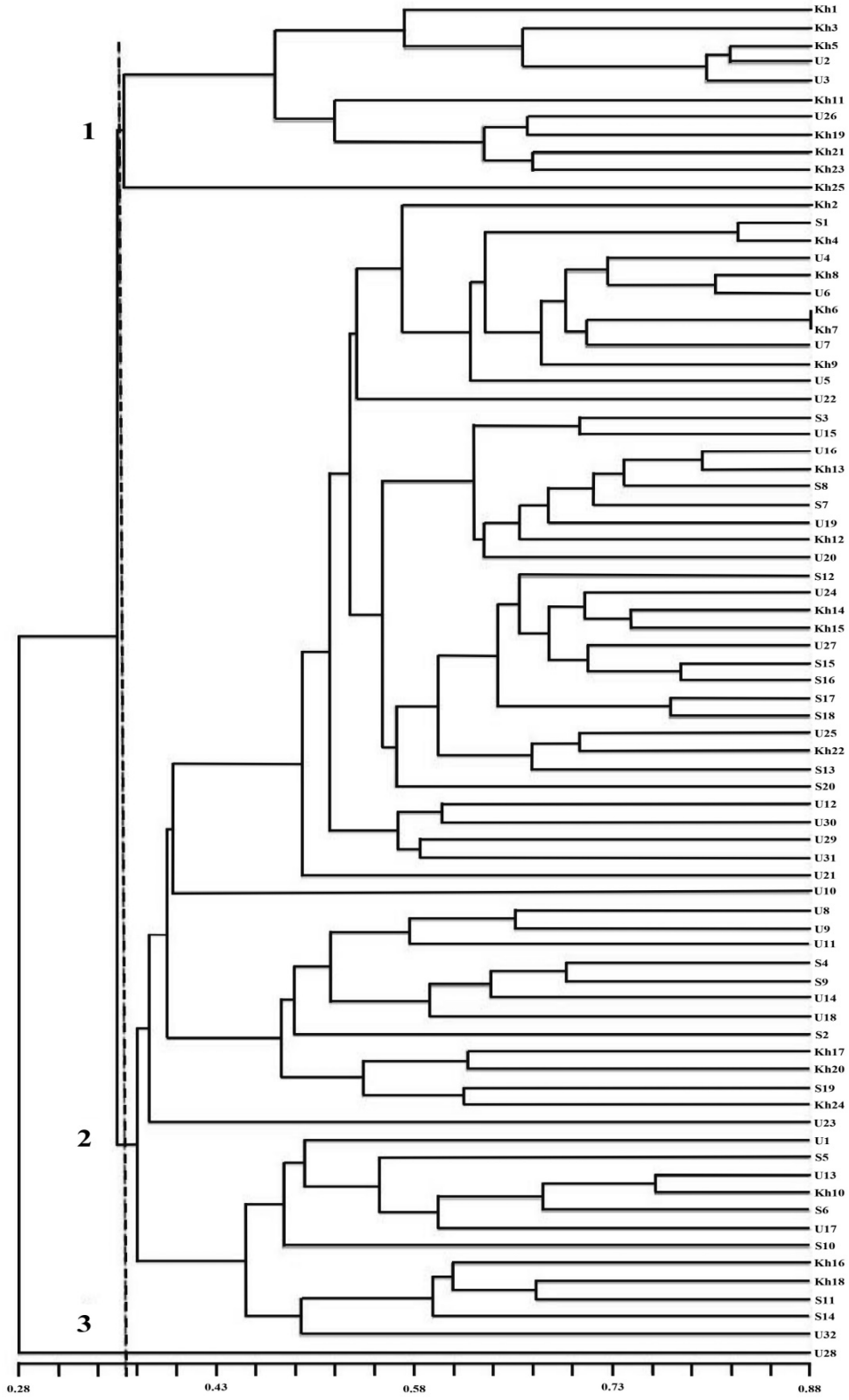

Figure 2. Dendrogram drawn based on the UPGMA method and Jaccard similarity index for 77 selected isolates of *T. harzianum* resulting from the integration of the band pattern data of the seven URP primers. The seven studied primers classified the isolates into three groups (1, 2 and 3) at the 36% similarity level.

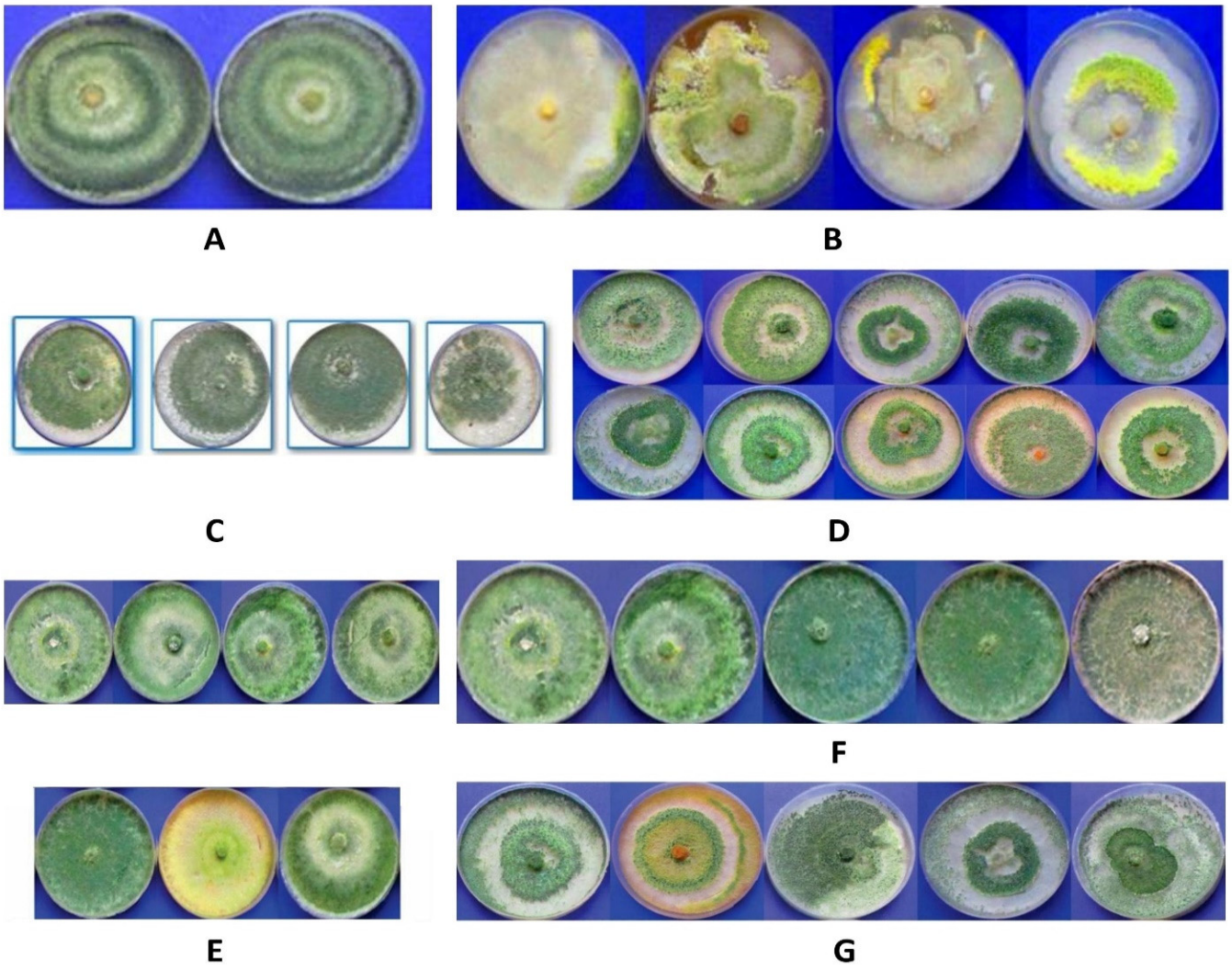

Figure 3. (**A**) The isolates with a similar growth pattern in the PDA culture medium including Kh24 (**left**) and S19 (**right**), (**B**) the isolates producing green–yellow color in the culture medium including S20, U29, U30, and U31 from left to right, (**C**) the isolates with a similar growth pattern including Kh1, Kh3, Kh5, and Kh11 from left to right, (**D**) the isolates with a similar growth pattern in the PDA culture medium including S17, U25, Kh22, S13, S15, S7, S12, U19, U24, and Kh12 from left to right (**E**) the isolates with a fast growth rate in the culture medium including S2, S4, U11, U14, U18, Kh17, and Kh20 from left to right, (**F**) the isolates with a similar growth pattern in the PDA culture medium including S2, U11, U13, U18, and U23 from left to right, and (**G**) the isolates with a slow growth rate in the solid and liquid culture media including S12, U16, U22, Kh6, and Kh13 from left to right.

The highest number of gene loci was obtained from primers 1F and 25F (32 gene loci) and the lowest number from primer 13R (20 gene loci). Table 2 presents the percentage of polymorphic bands, EMR, PIC, and marker index (MI), which represent the primers' capability of discriminating isolates. In total, the band patterns of the URP marker produced 186 gene loci, among which 182 loci were polymorphic, showing a polymorphic rate of 98%. The highest similarity was 88% between the two isolates, Kh6 and Kh7.

Table 2. The number of gene loci, polymorphism bands (β), polymorphism information content (PIC), effective multiplex ratio (EMR), and marker index (MI) resulting from the URP-PCR marker in the study of the genetic diversity of *T. harzianum* isolates.

Primer	Number of Gene Loci	β	PIC	EMR	MI
URP-1F	32	91%	0.36	29.33	10.60
URP-2F	24	72%	0.36	17.40	6.34
URP-2R	22	100%	0.29	22	6.41
URP-4R	27	100%	0.35	27	9.68
URP-6R	29	100%	0.37	29	10.98
URP-13R	20	100%	0.34	20	6.84
URP-25F	32	100%	0.28	32	9.12

The Nei's genetic diversity and the Shannon diversity index were used to investigate the genetic diversity of *T. harzianum* in the three populations of Urmia, Khoy, and Salmas (Table 3). The Urmia population had the highest (percentage of polymorphic loci) PPL (95.70), representing the polymorphism of all gene loci for each population, and the highest H (0.33), which represents Nei's genetic diversity. These findings suggest that the Urmia region has very high genetic diversity. The Salmas region, with a PPL value of 89.78, an H value of 0.31, and I value of 0.47, had lower genetic diversity than the other two populations. The Shannon diversity index (I) was the same for the Urmia and Khoy populations, estimated at 0.49 for these two populations. The gene flow between the studied populations was estimated by calculating the indices of inter-population genetic discrimination (Fst) and inter-population gene flow (Nm). Fst was found to be 0.03 and Nm was estimated at 13.64. A gene flow value of >1 implies low discrimination between populations and vice versa. The *T. harzianum* isolates had very high genetic diversity (0.96–0.98) and a meager genetic distance (0.02–0.03) in different regions of Khoy, Urmia, and Salmas (Table 4). The Khoy and Urmia isolates were highly similar (0.98). The results revealed that the genetic diversity was almost similar between the Urmia isolates and the Khoy isolates and between the Urmia isolates and the Salmas isolates, and that the genetic diversity was higher between the Salmas and Khoy isolates. These aspects show that isolates collected from the Salmas region differ from those collected from Khoy and Urmia.

Table 3. The indices used to evaluate the inter-population diversity of *T. harzianum* in different regions of West Azerbaijan.

Population	Number of Samples	Ne	Na	H	I	PPL
Urmia	32	1.56	1.95	0.33	0.49	95.70
Khoy	25	1.56	1.94	0.32	0.49	94.62
Salmas	20	1.49	1.89	0.29	0.44	89.78
Mean	-	1.53	1.92	0.31	0.47	94.36

In the table: Ne = Mean number of effective alleles; Na = Mean number of observed alleles; H = Mean Nei's gene diversity; I = Mean Shannon index; PPL = Percentage of polymorphic loci without considering allele frequency.

Table 4. Genetic similarity and distance of the studied populations based on Nei's factor (Popgene-Nei32)—genetic similarity between the populations (above the table's diagonal) and genetic distance between the populations (below the table's diagonal).

Population	Khoy	Salmas	Urmia
Khoy	-	0.96	0.98
Salmas	0.035	-	0.97
Urmia	0.020	0.021	-

The analysis of molecular variance (AMOVA) by GenALEX version 6.1 showed that the intra-population diversity accounted for 99% of the total genetic diversity. In contrast, the genetic diversity among the populations was estimated at only 1%. The goodness-of-fit

for clustering with the similarity matrix (the cophenetic coefficient) was at an excellent level for the URP data. The correlation coefficient between the cophenetic matrix and the initial similarity matrix (r) was obtained at 0.91. If the cluster analysis can fit data well, there will be a close correlation between this matrix and the initial similarity matrix. A correlation coefficient of >0.91 shows excellent fitness, values between 0.8 and 0.9 show good fitness, and the lowest values show weak fitness.

4. Discussion

This study investigated for the first time the genetic diversity of *T. harzianum* isolated from the sunflower rhizosphere in different regions in the north of West Azerbaijan province, Iran, using the URP molecular marker. When there are no reliable morphological characteristics, molecular markers are the best and most important instrument for studying the genetic diversity of isolates. Since the differences among the isolates of this fungus are difficult to identify even if morphological methods are revised [39], molecular markers are suitable instruments to help precisely analyze the genetic diversity of *Trichoderma* isolates [40].

The PPL index, which shows polymorphism in all gene loci for any population, was found by the studied marker to be the highest for the Urmia population, which shows that the Urmia region has very high diversity. The high similarity (0.98) between the isolates of the Khoy and Urmia regions reveals that they have similar ancestors. It can also be claimed that there has been a gene flow between the isolates of these two regions. The Nm is an evolutionary force that plays a vital role in the genetic diversity of a population. Gene flow and genetic fall are sources of the differences in the frequency of alleles in neutral gene loci within populations [41].

The results failed to discriminate between the isolates based on their geographical regions. The lack of relationship between genetic diversity and the geographical origin of the isolates may be related to the fact that the isolates were initially created from one genetic line. Then it was expanded closely with another genetic line by the selection phenomenon under specific environmental and ecological conditions. A lack of relationship among isolates and geographical regions may be associated with the dominance of the gene flow over the effects of genetic drift and the inhibition of the discrimination of isolates in a region. In other words, the discrimination of isolates in a region decreases by the migration of the isolates, the mixture of genotypes between populations, and abundant gene flow and exchange [24].

Based on the estimation of genetic information within and between the populations, e.g., the number of observed alleles per gene locus, the number of effective alleles, gene diversity, and gene diversity index, high genetic diversity was observed within the *T. harzianum* populations. In this research, the mean number of effective alleles per gene locus was 1.53 for all gene loci studied by the marker. Since this figure is close to the number of actual alleles, i.e., 1.92, it can be a reason for the good effect of alleles on polymorphism and the estimation of genetic diversity. The isolates were classified into 22 groups at the 55% similarity level, showing a high diversity. The genetic diversity of the *T. harzianum* isolates corroborates with the results of Sharma et al. [24], Siameto et al. [25], and Hernandez [42], who used the RAPD markers, and the results of Kumar and Sharma [43] and Hassan et al. [26], who used the ISSR markers.

The origin of this diversity is unknown because the *T. harzianum* isolates are diploid and heterothallic, and their diversity might have increased during sexual reproduction and recombination processes. Indeed, migration and sexual reproduction may significantly escalate the intra-population genetic diversity and population dynamic of different regions [17,24]. The genetic diversity was found to be high within the individuals of each population or region but very slight between the populations. This aspect can be attributed to the factors underpinning genetic variations, e.g., mutation and gene or genotype migration due to different factors.

The polymorphic percentage calculated for the URP marker was 97.85%. Our results confirm the efficiency of genomic fingerprinting by the URP method in studying the genetic diversity of the *T. harzianum* isolates. The URP marker has been used for other fungi with a high polymorphism index [44,45]. URP is characterized by simplicity, the generality of the primers used, and high reproducibility. Among the primers used in the test, the primer URP-6R had the highest MI, followed by URP-1F, URP-4R, and URP-25F primers. The primers whose MIs were higher in this research are recommended to be used in the large-scale investigation of *T. harzianum* isolates using the URP-PCR marker.

5. Conclusions

Our findings underlined that, among the studied primers, URP-6R had the highest marker index, followed by URP-1F, URP-4R, and URP-25F, implying their higher efficiency in discriminating between the isolates. The results showed that the URP marker could discriminate isolates by macroscopic morphological characteristics, such as color and colony type, potential of pigment production in the culture medium, and colony growth rate. No significant relationship between the geographical distribution of the isolates and the band patterns generated by the primers was found, except for a few cases. The results generally revealed that the URP marker was an efficient tool for determining the genetic diversity of *T. harzianum*. The literature did not present comprehensive information on the genetic diversity of *T. harzianum* isolates in the sunflower rhizosphere. No previous reports investigated the different regions of West Azarbaijan province. Thus, our findings enrich the knowledge of *T. harzianum* genetic diversity, describing isolates obtained from West Azerbaijan province sunflower rhizospheres for the first time. Genetic structure results might help when using isolates for biotechnological purposes (e.g., bio-stimulants and bio-control agents). Future studies should address the biotechnological application of these isolates in different fields (e.g., food industry and sustainable agriculture). Future research should also investigate the suitability of the URP marker and the primers used in this study for *T. harzianum* genetic diversity studies with large-scale investigations and considering different additional variables (e.g., different vegetal species and pedoclimatic conditions).

Author Contributions: Conceptualization, Y.R.D. and M.P.; methodology, K.K. and G.B.; software, R.D. and S.N.; formal analysis, B.F.; investigation, G.B. and S.N.; resources, Y.R.D.; data curation, M.P.; writing—original draft preparation, G.B. and K.K.; writing—review and editing, Y.R.D. and M.P.; visualization, S.N.; supervision, Y.R.D. and M.P.; project administration, Y.R.D.; funding acquisition, Y.R.D. and M.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from corresponding authors on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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