Mycologia Iranica 10(2):67–78 , 2023 Original Article DOI: 10.22043/MI.2023.362910.1264

Isolation and identification of Harzianum clade species of *Trichoderma* from Khorramabad County

Z. Mirzaeipour
E. Bazgir✉
Department of Plant Pathology, Faculty of Agriculture, Lorestan University, Khorramabad, Iran
D. Zafari
Department of Plant Protection, Faculty of Agriculture, Bu-Ali Sina University, Hamedan
M. Darvishnia
Department of Plant Pathology, Faculty of Agriculture, Lorestan University, Khorramabad, Iran

Abstract: The genus *Trichoderma* consists of globally distributed fungi. Among them, *T. harzianum*, is one of the most commonly collected *Trichoderma* species, which had been known as an aggregate species. In the present study, using a *Trichoderma* selective medium, 20 isolates belonging to 5 *Trichoderma* species of the Harzianum clad were isolated from soil samples collected from diverse geographical regions of Khorramabad County, Iran. Comparing the cultural and phenotypic criteria combined with molecular tests of the *tef1* gene sequences of isolated fungi, five species namely, *T. afroharzianum*, *T. atrobrunneum*, *T. guizhouense*, *T. harzianum* and *T. pholiotae* were identified and their descriptions and figures are presented in this paper. *T. harzianum* had the highest frequency among the 5 species. *T. pholiotae* is reported for the first time from Iran.

Keywords: Iran, Morphology, Phylogeny, Taxonomy, *tef1*-α, *Trichoderma*

INTRODUCTION

Trichoderma species are generally common parts of different ecosystems and are present in diverse climatic conditions. Some species are restricted to certain geographic locations, while some other species have worldwide distribution. *Trichoderma* species are commonly antagonistic of other fungi. They are living on wood, rotting organic materials, in soil and rhizosphere, on sponges and as endophytes of herbaceous and woody plants (López-Bucio 2015).

Some *Trichoderma* species are frequently used as biocontrol agents for plant diseases, plant growth-promoting agents, biofertilizers, enzymes and antibiotic producers and as bioremediatory of polluted soil and water (Zheng et al. 2021). Some species are used for nanoparticle production of gold or silver in nanotechnology (Maliszewska et al. 2009; Vahabi et al. 2011). Unfortunately some *Trichoderma* species are pathogens and devastating agents of commercially cultivating mushrooms (Park et al. 2006; Sandoval-Denis et al. 2014).

The latest classification of fungi had placed the *Trichoderma* genus under the phylum Ascomycota, subphylum Pezizomycotina, class Sordariomyctes, order Hypocreales and family Hypocreaceae (Lumbsch and Huhndorf 2007; Jaklitsch and Voglmayr 2015; Waghunde et al. 2016). *Hypocrea* has been described as the teleomorph for *Trichoderma* and presently *Trichoderma* genus encompasses 441 species (Barerra et al 2021; Cai and Druzhinina 2021).

Eight subgenric clades have been identified under *Trichoderma* genus, among them the Harzianum is the largest clade with over 95 described species. These species have heterogenic and complicated morphology and phylogeny.

Various studies have suggested that *T. harzianum* is a species complex and only a few cryptic species are named. Regarding its antifungal properties, *T. harzianum* has a long history in agricultural applications. It is effective in the control of soil-borne plant diseases and is the active ingredient for several commercially available biological control and plant growth-enhancing products (Chaverri et al. 2015).

Submitted 13 April 2023, accepted for publication 3 June 2023
✉ Corresponding Author: E-mail: Bazgir.ei@lu.ac.ir

http://mij.areeo.ac.ir

Rifai (1969) identified *Trichoderma harzianum* as one of the 9 species aggregate of *Trichoderma* genus and claimed that in each species aggregate there may be 2 or more cryptic phenotypes which are distinctive biological species. The Harzianum clade was previously known as Catoptron/LixiiClade'.this clade encompass several phenotypically a like species which are related to *T. harzianum* in their phylogeny and together they form the Harzianum Clade (Chaverri et al. 2015). Previous studies showed that the translation elongation factor 1-α (*tef1-α*) is needed to identify species in this complex (Chaverri et al. 2015). The present study was carried out to identify the species of the Harzianum clade of the *Trichoderma* genus in Khorramabad County, Iran, based on the morphological characterizations, as well as the translation elongation factor1-α (*tef1-α*) gene sequence alignment.

MATERIALS AND METHODS

Isolation of *Trichoderma*

For *Trichoderma* isolation, soil samples were randomly collected from 5-30 cm depth of different geographical regions of Khorramabad County and is carried in plastic bags. *Trichoderma* isolation carried out using the dilution plate method (Ávila-Miranda et al. 2006; Samuels et al. 2011). In this method, one gram of each soil sample was added to 9 ml of sterile distilled water as stock and different were dilutions prepared from it and placed on a shaker for one hour, 1 ml of each dilution was scattered on *Trichoderma* selective medium (TSM) plates and the plates incubated at 25 ºC (Davet 1979; Elad and Chet 1983).

Observations and morphological studies of *Trichoderma* isolates

The observation and comparison of the morphological and morphometric characteristics of *Trichoderma* isolates were carried out using an optical microscope. The macroscopic characteristics *viz* colony color, growth rate, smell and growth pattern of on potato dextrose agar (PDA; extract of 200g potato, 18 g dextrose, 18 g agar and l L distilled water), synthetic low nutrient agar or SNA (KH_2PO_4 1 gr lit^{-1}; KNO_3 1gr, KCL 0.5 g, , $MgSo_4$-$7H_2O$ 0.5 gr, sucrose 0.2 g, glucose 0.2 g, agar 18 g and distilled water 1000 ml), and corn meal dextrose agar or CMD medium (cornmeal 40 g, glucose 20 g, agar 18 g and distilled water 1000ml).

Media at 25, 30 and 35ºC and 12h darkness/12h photoperiod of fluorescent light were recorded. Then microscopic characters of conidia, phialides, conidiophores, and chlamydospores were also observed and recorded according to scientific descriptions and microphotographs were taken by a Kecam camera system connected to an Olympus BX53 microscope (Bissett 1984; Gams and Bissett 2002; Samuels 2011; Sharma and Singh 2014).

DNA extraction of *Trichoderma* isolates

For molecular identification, the genomic DNA of *Trichoderma* isolates was extracted using the CTAB (Cetyl trimethyl ammonium bromide) method with minor modifications (Doyle 1990).

The EF1 (ATGGGTAAGGARGACAAGAC) and EF2 (GGARGTACCAGTSATCATGTT) (primers were used for amplification of the translation elongation factor 1-alpha (*tef1*-α) fragments O'Donnell et al. 1998).PCR was performed in a thermocycler using a 25µL reaction system consisting of template DNA (100ng) 1µL, Master Mix2x 12.5 µL, Forward primer (100 µM) 1 µL, Reverse primer (100 µM) 1µL and double sterilized H_2O 9.5µL. For *tef1* amplifications, the PCR programmed as 3 min at 95°C for initial step and 32 cycles of 15 s at 95°C, 15 s at 53°C, and 1 min at 72°C, followed by 5 min at 72°C (Cai and Druzhinina 2021). The PCR products were evaluated using 1% (W/V) agarose gel electrophoresis in a TAE buffer and were observed under UV using the gel-documentation system (Bio-Rad, Gel Doc XR system).

Phylogenetic analyses

Tef1- α sequences were subjected to TrichoBLAST (WWW. ISTH.INFO) or NCBI nucleotide BLAST (http://blast.ncbi.nlm.gov/Blast.cgi) to identify similar sequences of related species. The data sets used for phylogenetic analyses included 20 isolates of *Trichoderma harzianum* complex, which were isolated from different sampling sites of Khorramabad County and 16 reference sequences were assembled from GenBank (NCBI), namely: 2 *T. afroharzianum*, 5 *T. atrobrunneum*, 2 *T. harzianum*, 3 *T. guizhouense*, 3 *T. pholiotae* and 1 *T. atroviride*, which was selected as the out-group taxon. The Phylogenetic tree was constructed using the MEGA version 7.0 (Gouy et al. 2010). The bootstrap value was estimated at 1,000 replications of bootstrap resembling the original nucleotide sequence alignments.

RESULTS AND DISCUSSION

In this study, 100 isolates related to the *Trichoderma* section were isolated, of which 60 isolates belonged to the Harzianum clade.

Alignment, comparison and analysis of the *tef1-α* gene for the 20 isolates has resulted in the NJ tree in Fig. 1. A phylogram was constructed to understand the relationship among the isolates and various species of *Trichoderma*. The phylogenetic tree showed that all of the isolates were separated into one clade.

The analyses of *tef1*-α phylogeny revealed that there was high bootstrap support for the Harzianum clade. The reference isolates are all related together and clearly distinct at species level. There was no vagueness in species identification (Table 1 and Fig 1).

Taxonomy

Trichoderma afroharzianum P. Chaverri, F.B. Rocha, Degenkolb & I. Druzhinina, sp. nov. Mycologia 107930: 580. (2014) (Figure 2).

Table 1. The species identified in this study

Species	Isolate	Accession numbers in GenBank
T. afroharzianum	LT133	OQ702635
T. atrobrunneum	LT6	OQ702628
	LT33	OQ525980
	LT64	OQ702633
	LT133	OQ702636
	LT184	OQ702644
T. guizhouense	LT12	OQ702630
	LT104	OQ702639
	LT148	OQ469500
	LT156	OQ702640
	LT168	OQ702641
	LT172	OQ702642
T. harzianum	LT8	OQ504831
	LT27	OQ702631
	LT36	OQ702632
	LT140	OQ525912
	LT141	OQ702638
	LT180	OQ702643
	LT196	OQ702645
T. pholiotae	LT199	OR102907

On PDA after 72 h mycelium covered the plate after 3 d at 25 °C (Table 2). After 96 h at 25 ºC on PDA medium aerial mycelium abundant, cottony, radiating; conidiation appeared within 48–72 h. typically abundant and disposed of two or three concentric rings around the point of inoculation; dark green to pale olive green colored; diffused olive pigment, in old cultures; Conidiophores pyramidal, branches opposing, the main axis and each branch terminating in a cruciate whorl or verticil of 2–5 phialides. Phialides lageniform to ampulliform, 6.5–12×2.5–3.7 µm. Conidia subglobose to ovoid 2.7–3.7× 2.4–3.2 µm, smooth, green to dark green with age, infrequently yellow. Chlamydospores globose to subglobose, 4.7–7.0 × 5.0–7.5 µm and produced intercalary or terminal (Fig. 2). As shown in Fig 2,

The widely spaced, usually verticillate conidiophores are the distinguishing feature of *Trichoderma afroharzianum*. It has the longest phialides the narrowest supporting cell in the complex. *Trichoderma afroharzianum* is a widespread species isolated from soil, roots and other fungi; possibly fungicolous (Chaverri and Samuels 2013; Chaverri et al. 2015).

Trichoderma afroharzianum, OQ702635 of the present study was very similar to the description of other workers (Chaverri et al. 2015; Jang et al. 2018). It has high parsimony with the type isolate and they are monophyletic. It is difficult to distinguish this species from other species the complex species of T. *harzianum* based on morphological characters. This species has been isolated and identified in some parts of Iran, including Kerman province (Barahui, 2015), and this is the first report of this species from Lorestan province.

Trichoderma atrobrunneum F.B. Rocha, P. Chaverri & W. Jaklitsch, Mycologia 107(3): 580. (2014) (Figure 3).

This species has the slowest growth at 35 ºC compared to other species of this collection (Table 2). Aerial mycelium are abundant after 96 h incubation on PDA at 25 ºC, mycelium is wooly or cottony, conidia formed in wide concentric rings and they are abundant during 48-72 incubation on PDA ; conidiation sometimes sparse or absent, colony color white to green and reverse color light yellow, a sweet odor often detected at 30 ºC. Conidiophores pyramidal, with often opposing, often somewhat widely spaced branches, the main axis and each branch terminating in a cruciate, sometimes verticillate, terminating in a whorl of 2–5 phialides. Phialides ampulliform to lageniform, 2.8–3.7×4.5–7.5 µm. Conidia subglobose to ovoid, 2.3–2.6×2.8–3.8 µm smooth, hyaline when young becoming green to dark green with age. Chlamydospores common, they were globose, 6.5–7.2×6.6–7.5 µm and produced intercalary or terminal.

Trichoderma atrobrunneum LT6 (OQ702628), LT33 (OQ525908), LT64 (OQ702633), LT133 (OQ702636), LT184 (OQ702644) isolates were similar to the descriptions of other study (Haouhach et al. 2020), but the presence of chlamydospores which were observed both intercalary and terminally in present study.

This species is isolated from soil, decaying wood and fungi; possibly is fungicolous (Chaverri and Samuels 2013; Chaverri et al. 2015). *T. atrobrunneum* has been isolated and identified in different regions of the world, including Europe, North America, and Korea, as well as some parts of Iran, including East Azarbaijan province (Azizi 2015), and this is the first report of this species from Lorestan province.

Trichoderma guizhouense Q.R. Li, E.H.C McKenzie & Yong Wang, Mycol. Prog. 12:170 (2012) (Figure 4).

Maximum mycelia growth obtained at 25–30 ºC on PDA (Table 2). Cottony aerial mycelium whether radiating or not are abundant. Conidia are formed in wide concentric rings on aerial hyphae, occasionally on thick mats during 24 h. they turn yellowish afterwards green and usually produce a yellow dispersing pigment; no distinctive odor noted. Conidiophores branching is pyramidal with opposite branches, the interbranch distance is short or occasionally more open, the terminal branches have a cruciate whorl of 2–4 phialides, occasionally arising singly; conidiophores are rarely nodose and phialides are in more or less botryose sets. Phialides are ampulliform, usually very constricted below their tips, rarely lageniform and then usually terminal and unsymmetrical to heavily curved, 2.3–3×4.9–9 µm, Conidia one-celled globose to subglobose, 2.2–2.5×2.5–2.7 µm, smooth-walled, appearing pale yellow-green in the microscope.

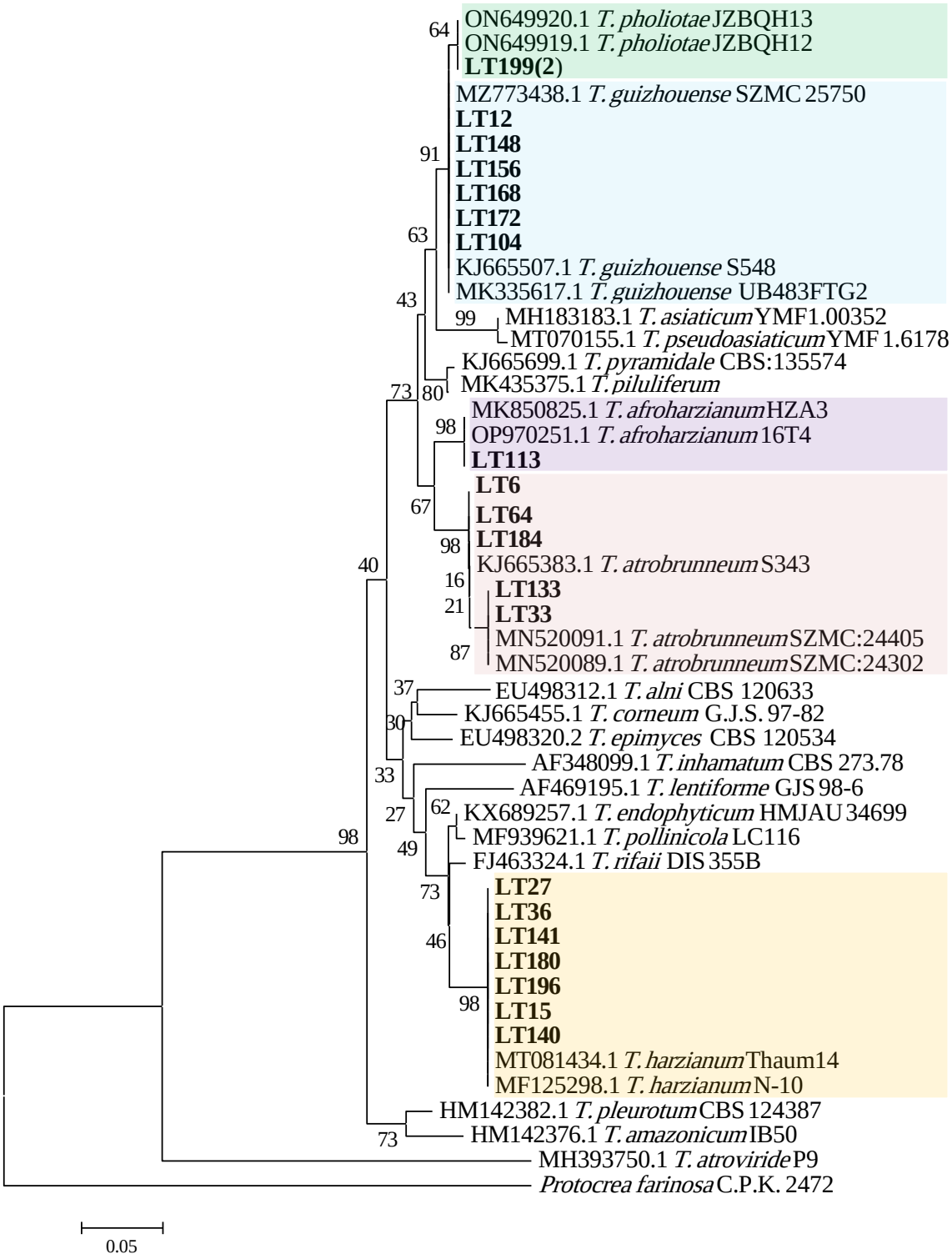

Fig. 1. Phylogenetic tree based on *EF1-α* gene sequences of forty-seven species of *Trichoderma* using the Neighbor-joining method. The numbers above each branch indicate the amount of bootstrap support from a 100-time phylogenetic tree drawing using the Neighbor-joining method *Protocrea farinosa* (C.P.K 2472) is an outgroup sample. The tested species are marked in bold.

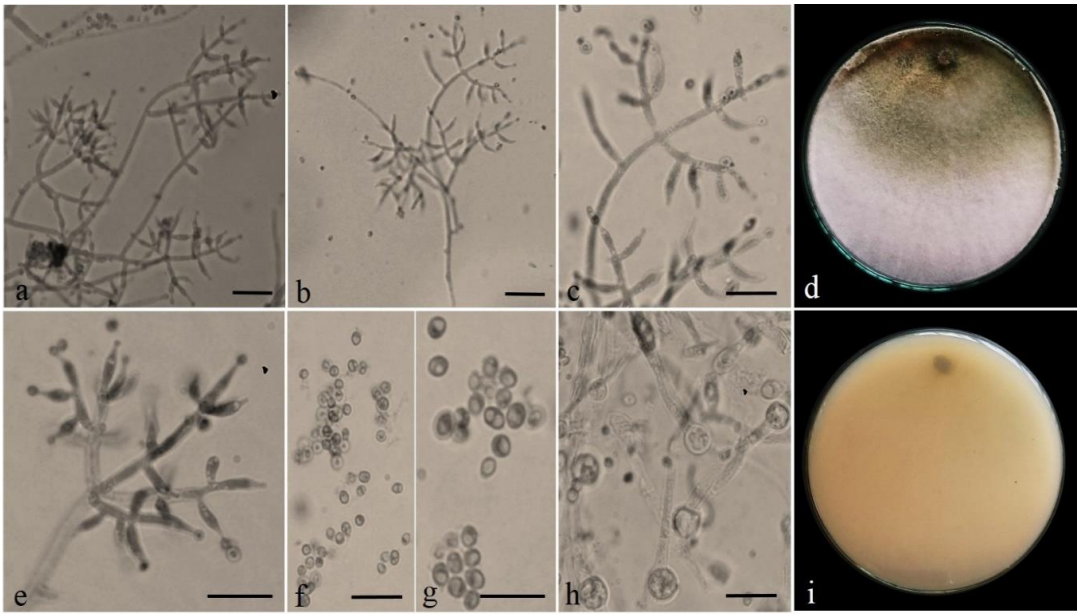

Fig. 2. *Trichoderma afroharzianum*. Asexual morph and cultures at 25 °C. a–c. Conidiophores. d. Culture after 7 days on PDA; e. Conidiophores; f and g. Conidia; h. Chlamydospores. i. Revers of the colony. Bars= l0 µm

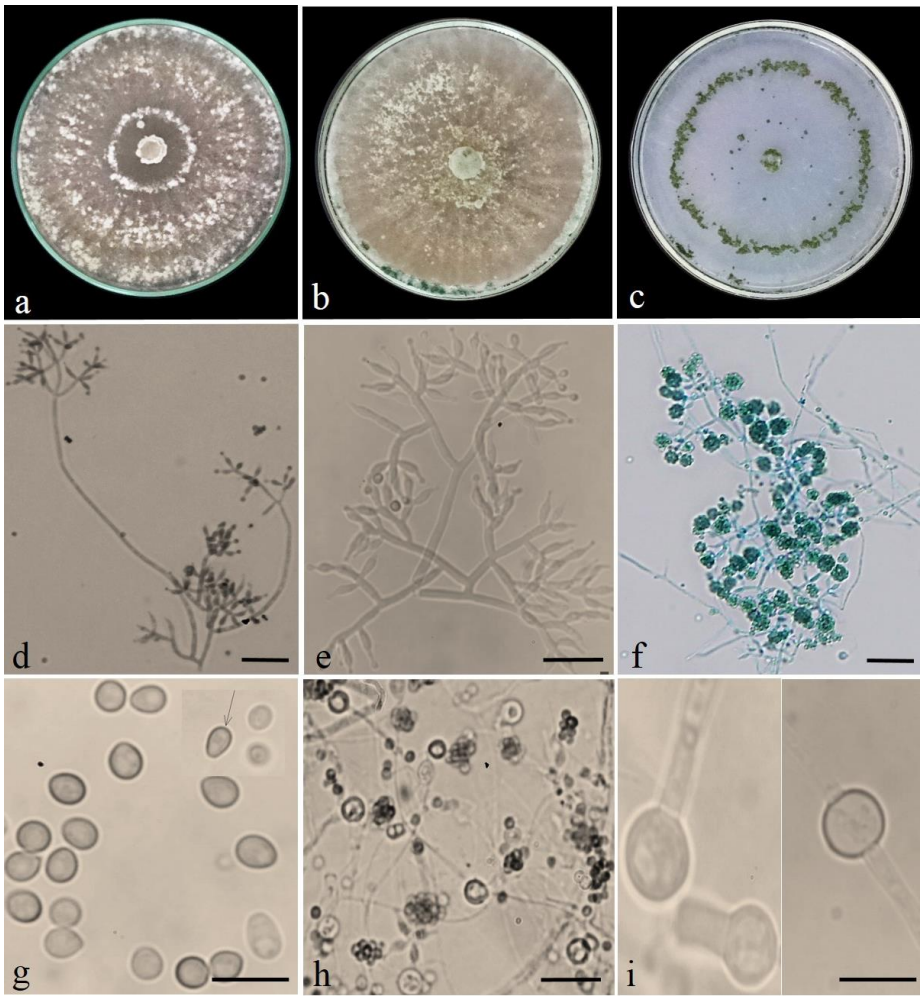

Fig. 3. *Trichoderma atrobrunneum*. Asexual morph and cultures at 25 °C a–c. Cultures after 7 d (a. on PDA; b. on CMD; and c. on SNA); d-f. Conidiophores; g. conidia; h and i. Chlamydospores. Bars= 10µm.

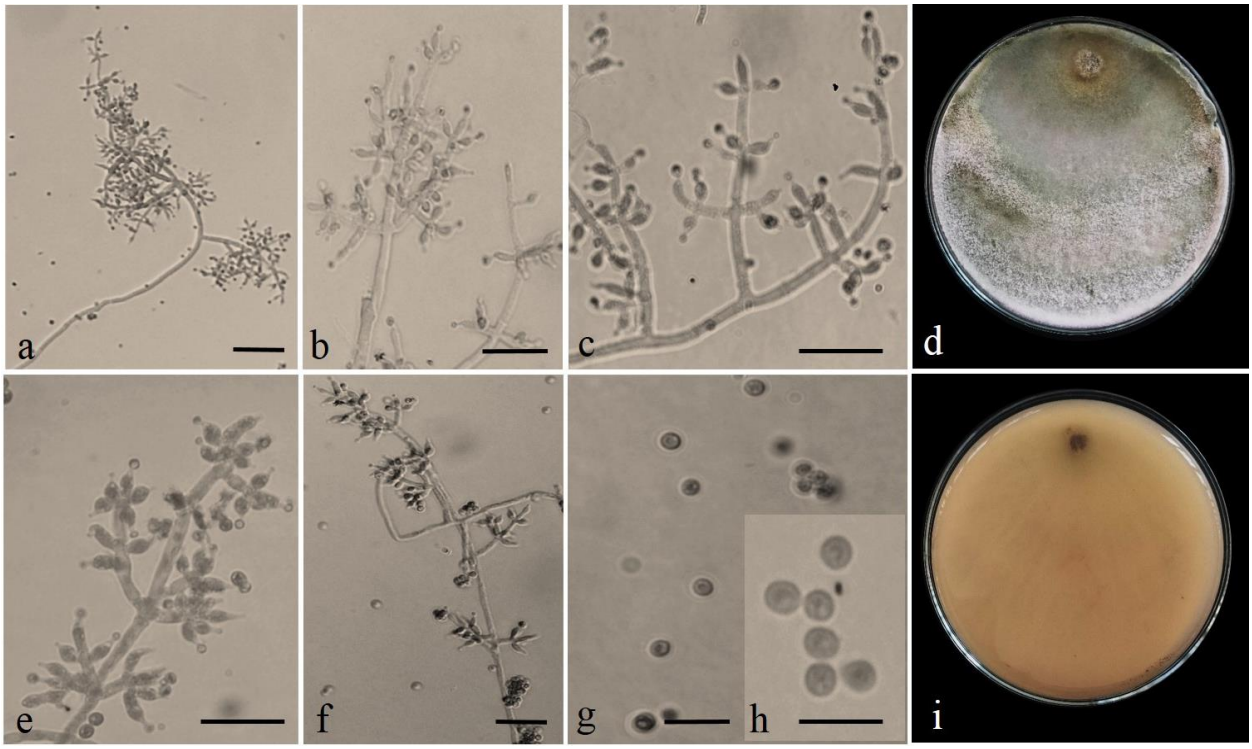

Fig. 4. *Trichoderma guizhouense*. Asexual morph and cultures incubated at 25 °C. a- c. Conidiophores, d. 7 days cultures on PDA, e and f. Conidiophores and phialides, g and h. Conidia, i. revers colony. Bars= 10µm.

Chlamydospores common, they were globose, 6.2–6.3×6.6–6.8 µm and produced intercalary or terminally. Terminal phialides are in a whorl or single, often cylindrical or at least not obviously swollen in the middle and taller than the subterminal phialides.

Trichoderma guizhouense LT12 (OQ702630), LT104 (OQ702639), LT148 (OQ702639), LT156 (OQ702640), LT168 (OQ702641), LT172 (OQ702642) isolates were similar to the descriptions of other researches, but the presence of chlamydospores which were observed both in the intercalary or terminal in present study. *T. guizhouense* is one of the slowest growing species of the species complex, particularly on PDA at 15 ºC. It has been isolated from decaying wood, tree bark and fungi, also it has been isolated from soil samples and from *Ancistrocladus korupensis* and *Cola* spp. stems as an endophyte. It is likely a fungicolous fungus (Chaverri and Samuels 2013).

This species has been isolated and identified in most parts of the world, including Africa, Europe and Asia, *T. guizhouense* was reported for the first time in Iran from chickpea fields in Kermanshah province (Younesi et al. 2021), and this is the first report of this species from Lorestan province.

Trichoderma harzianum Rifai, Mycol. Pap. 116:38 (1969) emend P. Chaverri, G.J. Samuels & F.B. Rocha (Figure 5).

The best temperature for mycelium growth on PDA was 25–30 ºC (Table 2), colony on PDA, abundant aerial mycelium which are cottony; conidia produced compactly over the center and in undulating concentric bands toward the edge; no pustules; conidia are often firstly yellow and turn yellow-green afterward. No distinctive odor on all media, but a sweet odor is sometimes detected. Conidiophores are often uniformly branched and the length of terminal branch is usually more than 150 µm, branching system is pyramidal, the branching angles of the top branches are is 90º, but decreases in the lower branches, the longest branches are at the base and the shortest at the end of main axis. Pyramidal conidiophores have opposing widely spaced branches, terminating in a set of 2–5 phialides, whorls are cruciate or somewhat verticillate. The production of phialides starts from the base of the conidiophore, and for this reason, the tip of the conidiophore appears sterile in the early stages of growth. Phialides ampulliform to lageniform, straight to curved, 2.3–2.8×5–11 µm.

Terminal phialides are in a whorl or lonely, often cylindrical or at least not remarkably swollen in the middle and taller than the intermediate phialides. Phialides in this species are usually very diverse both in terms of shape and size. conidia gray-green when young, becoming dark green; sometimes a pale yellow diffusing pigment after 48 h at 35 ºC; Conidia forming abundantly, round at the upper end and narrow and slightly pointed at the base, subglobose to ovoid, 2.3–3×2.4–3.8 µm, smooth, green. Chlamydospores are small in some isolates and abundant in others and they were globose to subglobose, 8.5–10.5 × 12.5–13.2 µm and produced intercalary or terminal in hyphae.

The characteristics of the colony as well as the macroscopic and microscopic characteristics of this species are very diverse. Distinguishing features: this species may be dignified from other species of this clade by having small conidia and low-density conidiophores. *T. harzianum* is one of the fastest growing species and at 35 ºC on SNA and PDA its average colony radius after 72 h was more than 35 and 40 mm, respectively. It has been isolated mainly from soil; mushroom compost and rarely as an endophyte of stems; It is conceivably a fungicolous species (Chaverri and Samuels 2013). This species has been isolated and identified by different researchers in different parts of the world including in Iran, which was reported first time by Zafari (2012). In the present study, the highest frequency belongs to *T. harzianum* species.

Trichoderma pholiotae Z.J. Cao & W.T. Qin, sp. nov. 31; 8 (11):1154. (2022) (Figure 6).

The optimum temperature for mycelial growth of this isolate was 25–30 C° (Table 2). On CMD, colony radius 71-72 mm after 72h at 25˚C and 73–74 mm at 30˚C and 12 h photoperiod.

The diameters of its colony at 25˚C and 30˚C on PDA were 68–69 and 71–73 mm, respectively, and mycelium covered the 9cm plate after 3 days incubation at 30 ˚C. At 35 C° its growth slows down on PDA, CMD, MEA and SNA media. The isolate completely covered the 9 cm PDA plates at 25 C° after 4 days of incubation.

The center of colonies are white but have circular green zone around their central part. Aerial hyphae are evidently radial, crowded, and hirsute to cottony, there is light yellowish green spreading dye, slightly fruity aroma, colonies are hyaline, fan shaped and tending to aggregate at the periphery. Sporulation started after 2–4 days of incubation at 25 ºC, the reverse of the colony was colorless، odor slightly fruity, and chlamydospores were common, they were ellipsoid, globose, 4.7–7.0×5.0–7.5 µm and produced intercalary or terminally. Conidiophores are straight or curved, often pyramidal with opposite branches, formed crowd intricate reticulum, having a terminal whorl of generally 3–4 phialides and usually paired side branches, less frequently solitary. Branches are mostly at right angles with the main axis with conspicuous septa. Phialides produced in regular levels around the axis, they are ampulliform or lageniform and others apex and not symmetrical to curved with dimensions of 5.0–11.0 × 2.5–4.5 µm, in the terminal part of the conidiophore phialides are usually formed singly. Conidia are often ovate to spheroid, less globose, green, smooth, 2.5–4×2.4–3.3 µm.

Analysis of DNA sequences of the *tef1-α* gene located this isolate close to the *T. harzianum* species complex, and as a result, by combining the morphological characteristics and the sequence related to the *tef1-α* gene, this isolate was registered as a *T. pholiotae* species in the Genbank with the accession code OQ847848. The results of the phylogenetic analysis showed that the studied isolate is placed next to other isolates of *T. pholiotae* and other species of this genus (Fig 1), and to the best of our knowledge, it is the first report of this species from Iran**.**

Phylogenetically, *T. pholiotae* (OR102907) had a linage with *T. asiaticum* and *T. guizhouense* with strong support value (95%–99%), and it was located as a highly supported separate terminal branch among *T. guizhouense* in the Harzianum clade. But, comparing this isolate with *T. guizhouense*, *T. pholiotae* (OR102907) possesses narrower phialides (2.0–3.0 µm) and spherical conidia (Li et al. 2013). *T. asiaticum* has shorter phialides (3.0–) 4.0–6.0 (–7.0) µm (Zheng et al. 2021). This species was first isolated from China. This is the first report of *T. pholiotae* from Iran with comprehensive description and illustration.

Table 2. Colony growth (mm) the species identified in this study in three temperature ranges after 72 hours.

Species	Temperatures ºC	Cultures media (mm)		
		PDA	CMD	SNA
Trichoderma afroharzianum	25	55–66	53–66	45–48
	30	52–61	52–61	43–47
	35	35–44	33–42	30–38
Trichoderma atrobrunneum	25	57–75	53–60	35–45
	30	55–75	55–73	33–42
	35	9–10	7–9	6–10
Trichoderma guizhouense	25	48–77	50–74	58–58
	30	48–75	50–70	58–58
	35	13–21	10–20	8–18
Trichoderma harzianum	25	50–65	55–65	53–60
	30	50–67	54–64	51–61
	35	37–50	37–52	43–52
Trichoderma pholiotae	25	68–69	71–72	48–50
	30	71–73	73–74	55–57
	35	9–14	14–18	7–12

DISCUSSION

In the present work, a total of 70 isolates belonging to the Harzianum clade of *Trichoderma* were isolated from different sampling sites in Khorramabad County. It is concluded that all the studied *Trichoderma* species formed a strongly supported group, which was generally in agreement with the previous researches (Chaverri and Samuels 2003; Gu et al. 2020)

The morphological analysis of isolates was compatible with the *Trichoderma* genus description according to the common taxonomic phenotypical criteria (Chaverri and Samuels 2003; Chaverri et al. 2015; Gu et al. 2020)

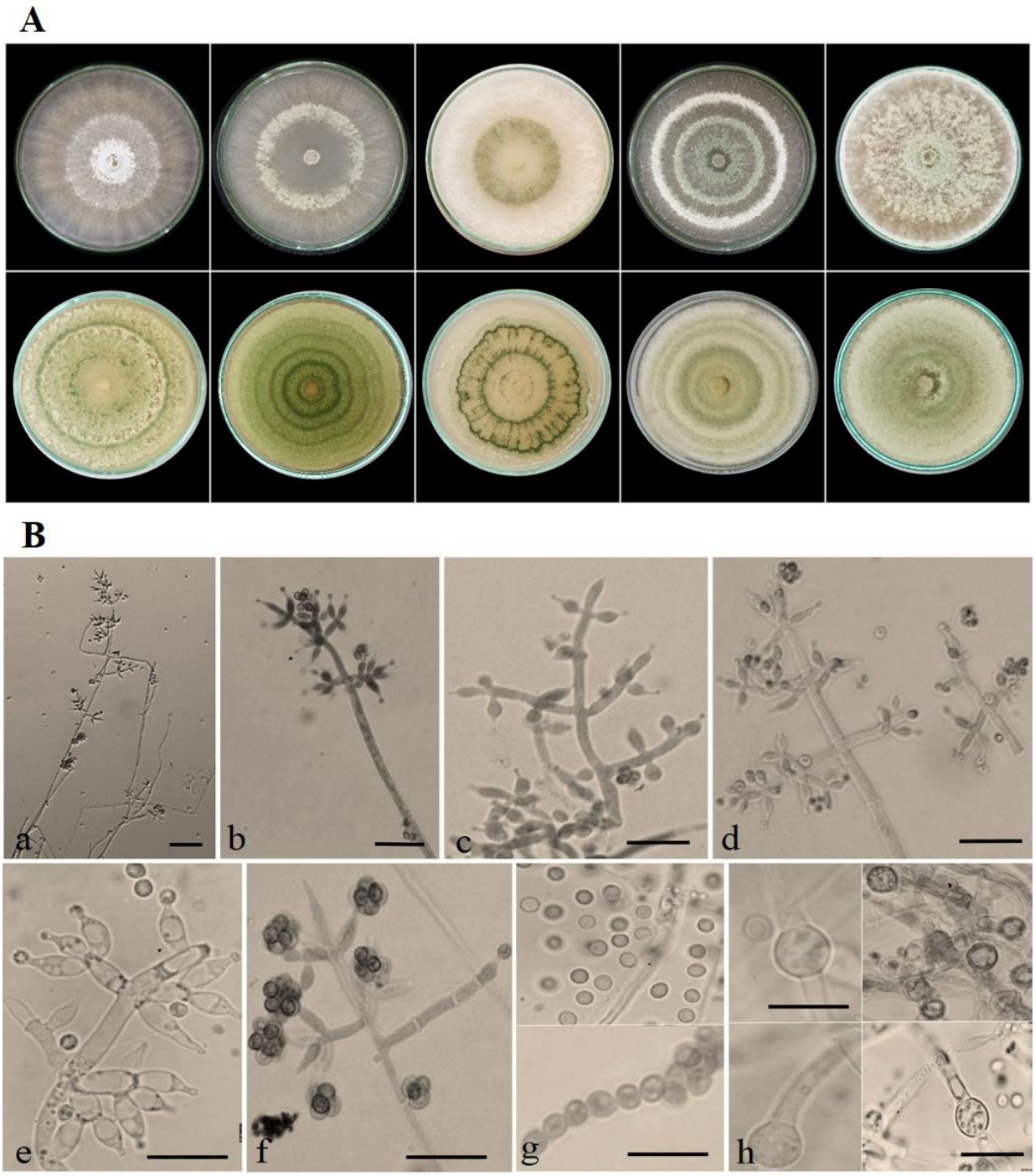

Fig. 6. *Trichoderma harzianum*. **A,** Asexual morph and cultures at 25 °C. Cultures after 4-7 days on PDA; **B**. a-f. Conidiophores and phialides; g. conidia; h. Chlamydospores. Bars= 10µm

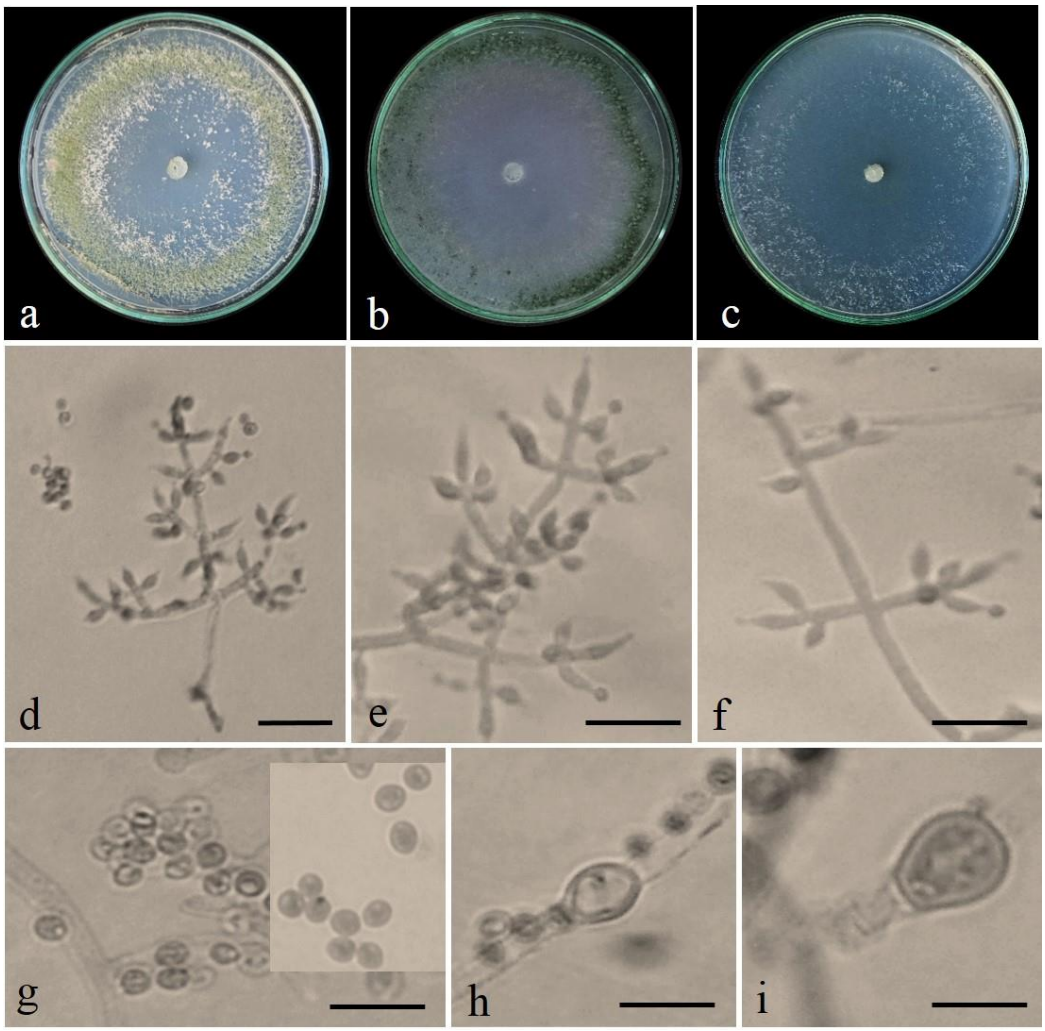

Fig. 6. *Trichoderma pholiotae*. Asexual morph and cultures at 25 °C a–c. Cultures after 7 d (a. on PDA; b. on CMD; and c. on SNA); d-f. Conidiophores; g. conidia; h and i. Chlamydospores. Bars= 10µm.

Since there is high uncertainty and stability in the morphological characters of *Trichoderma* spp. scientists have reached an accord that accurate identification of *Trichoderma* species cannot be based only on the morphological characteristics. (Chaverri and Samuels 2003; Jaklitsch et al. 2006). Therefore, it is hypothesized that *T. harzianum* isolates which were originally distinguished by ITS sequence and morphological characters in previous works probably belonged to the *T. harzianum* complex. However, the present research showed that the complex still encompass many taxa, indicating that further works are needed for more accurate identification of this clad of *Trichoderma*.

As the new of species appearing in the Harzianum clade are increasing, our understanding of *Trichoderma* spp. is getting more trailblazing and comprehensible and complicated, so logical species concepts will be unwaveringly settled only by more researches.

Species identification was performed based on DNA gene sequencing analysis, including *tef1*-α based NCBI BLAST analysis. The molecular approach was adopted because the morphological and cultural studies could not efficiently distinguish *Trichoderma* isolates at species level. Phenotypic attribute are not stable and are affected by culturing conditions (Mazrou et al. 2020) and secondary metabolite production (Hermosa et al. 2014) and, especially in *Trichoderma*, they can often be confusing because of the amalgamation of many types of genes through horizontal gene transfer and mycoparasitism from plant-associated filamentous fungi belonging to different phylogenetically close taxa of Ascomycota hosts.

Sequences available for analysis, suggest that for most cases the only reliable way to identify a species of this complex is with *tef*-1α sequences. The *tef*-1α contribute much more resolution of the representatives of the *T. harzianum* complex than the ITS sequences, as what has been claimed by

O'Donnell (2000) found in *Fusarium*. Several researches indicate that even the restricted morphological concept of *T. harzianum* (Bissett 1991, Gams and Meyer 1998) is genetically and phenotypically diverse. In the present work *T. harzianum* was shown to be highly variable in most of its phenotype features, the same have been concluded by Grondona et al (1997).

These phylogenetic analyses made it possible to identify LT113 as *T. afroharzianum*, LT6, LT33, LT64, LT133 and LT184 as *T. atrobrunneum*, LT12, LT104, LT148, LT156, LT168 and LT172 as *T. guizhouense*, LT15, LT27, LT36, LT140, LT141, LT180 and LT196 isolates as *T. harzianum* and LT199 as *T. pholiotae*. These species have been dissociated from the *T. harzianum* complex (Chaverri et al. 2015; Cao et al. 2022). In fact, these species were regarded (and a few isolates were registered for commercial application) as *T. harzianum*. It should be said that the name *T. harzianum* is an old single species that encompass isolates of today's known *T. afroharzianum* and *T. atrobrunneum*, *T. guizhouense*, *T. pholiotae* and many other species which are very closely related new species. It may be bewildering that an important part of the members of the old *T. harzianum* species complex is still called *T. harzianum*. Accurate identification is especially important for registration of active ingredients for commercial applications. In this paper, it has been resulted that it is difficult to define *Trichoderma* species only based on phenotypic characters, especially for the presence of species complexes and cryptic species,. Phylogenetic analyses have to be done to ascertain the placement of ambiguous species.

However, the present study was limited to a few *Trichoderma* cultures isolated from narrow habitats. So, it is essential to re-examine "*T. harzianum*" cultures from more diverse habitats: soil, marine environments, plants and other fungi. It is believed that more cryptic species will be revealed by future studies with greater numbers of isolates from diverse sources.

ACKNOWLEDGMENTS

We are grateful to the Department of Plant Protection, Lorestan University, Khorramabad, Iran for supporting this project.

REFERENCES

Azizi H. 2015. Identification of *Trichoderma* species isolated from walnut orchards in East Azarbaijan province and investigation of the biocontrol effect of effective isolates on walnut anthracnose. Master's thesis. Vali Asr University (AJ) Rafsanjan, Faculty of Agriculture.

Ávila-Miranda, M. E., Herrera-Estrella, A. and Peña-Cabriales, J. J. 2006. Colonization of the rhizosphere, rhizoplane and endorhiza of garlic (*Allium sativum* L.) by strains of *Trichiderma harzianum* and their capacity to control allium white-rot under field conditions. Soil Biology & Biochemistry. 38(7): 1823-1830. Barahui N. 2015. Identification of *Trichoderma* isolates from the soil of citrus orchards in Kerman province and investigation of the enzyme activity of isolates effective in controlling citrus gomoz disease. Master's thesis. Vali Asr University (AJ) Rafsanjan, Faculty of Agriculture.

Barrera VA, Iannone L, Romero AI, Chaverri P. 2021. Expanding the *Trichoderma harzianum* species complex: Three new species from Argentine natural and cultivated ecosystems. Mycologia. 113, 1136–1155.

Bisset J.1984. A revision of the genus *Trichoderma*. I. Section Longibrachiatum sect. nov. Can. J. Bot. 62:924-31..

Bissett, J. 1991. A revision of the genus *Trichoderma*. II. Intrageneric classification. Canadiation. Journal of Botany. 69, 2357–2372. https://doi.org/10.1139/b91-297.

Cai F, Druzhinina IS. 2021. In honor of John Bissett: authoritative guidelines on molecular identification of *Trichoderma*. Fungal Diversity, 107, 1-69.

Cao ZJ, Qin WT, Zhao J, Liu Y, Wang SX, Zheng SY. 2022. Three New *Trichoderma* Species in Harzianum Clade Associated with the Contaminated Substrates of Edible Fungi. Journal of Fungi. 31; 8 (11):1154.

Chaverri P, Branco-Rocha F, Jaklitsch W, Gazis R, Degenkolb T, Samuels GJ. 2015. Systematics of the *Trichoderma harzianum* species complex and the re-identification of commercial biocontrol strains. Mycologia. 1; 107 (3):558-90.

Chaverri, P.; Samuels, G.J. 2003. *Hypocrea*/*Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae): Species with green ascospores. Stud. Mycol. 48, 1–116.

Chaverri P, Samuels GJ. 2013. Evolution of habitat preference and nutrition mode in a cosmopolitan fungal genus with evidence of interkingdom host jumps and major shifts in ecology. Evolution. 1; 67(10):2823-37.

Davet PA .1979. technique for analyzing soil populations of *Trichoderma* spp. and *Gliocladium virens*. In Annales de Phytopathologie.

Doyle JJ (1990) Isolation of plant DNA from fresh tissue. Focus. 12: 13-5.

Elad Y, Chet I .1983a. Improved selective media for isolation of *Trichoderma* spp. or *Fusarium* spp. Phytoparasitica. 11**:**55–58.

Gams W, Meyer W. 1998. What exactly is *Trichoderma harzianum*? Mycologia. 1; 90(5):904-15.

Gams W, Bissett J (2002) Morphology and identification of. *Trichoderma* and *Gliocladium*. 3-31.

Gouy M, Guindon Ph, Gascuel O. 2010. SeaView Version 4: A Multiplatform Graphical User Interface for Sequence Alignment and Phylogenetic Tree Building. Molecular Biology and Evolution 27: 221-224.

Grondona I, Hermosa R, Tejada M, Gomis MD, Mateos PF, Bridge PD, Monte E, Garcia-Acha I. 1997. Physiological and biochemical characterization of *Trichoderma harzianum*, a biological control agent against soilborne fungal plant pathogens. Applied and Environmental Microbiology. 63(8):3189-98.

Gu, X.; Wang, R.; Sun, Q.; Wu, B.; Sun, J.-Z. 2020. Four new species of *Trichoderma* in the Harzianum clade from northern China. Mycokeys, 73, 109–132. https://doi.org/10.3897%2Fmycokeys.73.51424.

Haouhach S, Karkachi N, Oguiba B, Sidaoui A, Chamorro I, Kihal M, Monte E. 2020. Three new reports of *Trichoderma* in Algeria: *T. atrobrunneum*, (South) *T. longibrachiatum* (South), and *T. afroharzianum* (Northwest). Microorganisms. 23; 8(10):1455. https://doi.org/10.3390/microorganisms8101455.

Hermosa, R.; Cardoza, R.E.; Rubio, M.B.; Gutiérrez, S.; Monte, E. 2014. Secondary metabolism and antimicrobial metabolites of *Trichoderma*. In Biotechnology and Biology of *Trichoderma*; Gupta, V.S., Schmoll, M., Herrera-Estrella, A., Upadhyay, R.S., Druzhinina, I., Tuohy, M., Eds.; Elsevier: Amsterdam, the Netherlands. pp. 125–137.

Jaklitsch WM, Voglmayr H. 2015. Biodiversity of *Trichoderma* (Hypocreaceae) in Southern Europe and Macaronesia, Mycology. 80:1-87.

Jaklitsch, W.M.; Samuels, G.J.; Dodd, S.L.; Lu, B.S.; Druzhinina, I.S. 2006. *Hypocrea rufa*/*Trichoderma viride:* A reassessment, and description of five closely related species with and without warted conidia. Stud. Mycol. 56, 135–177. Jang S, Kwon SL, Lee H, Jang Y, Park MS, Lim YW, Kim C, Kim JJ. 2018. New report of three unrecorded species in Trichoderma harzianum species complex in Korea. Mycobiology. 3; 46(3):177-84. https://doi.org/10.1080/12298093.2018.1497792.

Li, Q.R.; Tan, P.; Jiang, Y.L.; Hyde, K.D.; Mckenzie, E.H.C.; Bahkali, A.H.; Kang, J.C.; Wang, Y. 2013. A novel Trichoderma species isolated from soil in Guizhou, *T. guizhouense*. Mycol. Prog. 12, 167–172. López-Bucio J, Pelagio-Flores R, Herrera-Estrella A. 2015. *Trichoderma* as biostimulant: exploiting the multilevel properties of a plant beneficial fungus, Scientia Horticulturae 196, 109–123. https://doi.org/10.1016/j.scienta.2015.08.043.

Lumbsch HT, Huhndorf S. 2007. Whatever happened to the pyrenomycetes and Loculoascomycetes. Mycological Research. 111:1064– 1074.

Maliszewska I, Aniszkiewicz Ł, Sadowski Z. 2009. Biological Synthesis of Gold Nanostructures Using the Extract of *Trichoderma koningii*. Acta Phys. Pol. 116, 14. Mazrou, Y.S.A.; Makhlouf, A.H.; Elseehy, M.M.; Awad, M.F.A.; Hassan, M.M. 2020. Antagonistic activity and molecular characterization of biological control agent *Trichoderma harzianum* from Saudi Arabia. Egyptian Journal of Pest Control**.** 30, 1.

O'Donnell K, Cigelnik E, Nirenberg HI (1998). Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* **90**: 465–493

O'Donnell K, Kistler HC, Tacke BK, Casper HH. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proceedings of the National Academy of Sciences. 5; 97(14):7905-10. Park, MS, Bae KS, Yu SH. 2006. Two New Species of *Trichoderma* Associated with Green Mold of Oyster Mushroom Cultivation in Korea. Mycobiology. 34, 111–113. https://doi.org/10.4489%2FMYCO.2006.34.3.111

Rifai MA. 1969. A revision of the genus *Trichoderma*. Mycological Papers. 116:1–56.

Samuels G, Chaverri P, Farr D, McCray E (2011) *Trichoderma* Online, Systematic Mycology and Microbiology Lab. ARS, USDA.

Sandoval-Denis M, Sutton DA, Cano-Lira JF, Gené J, Fothergill AW, Wiederhold NP, Guarro J. 2014. Phylogeny of the Clinically Relevant Species of the Emerging Fungus *Trichoderma* and Their Antifungal Susceptibilities. Journal of. Clinical Microbiology. 52, 2112–2125.

Sharma KK, Singh US (2014) Cultural and morphological characterization of rhizospheric isolates of fungal antagonist *Trichoderma*. Int. j. appl& nat. sci. 6(2): 451-6.

Vahabi K, Mansoori G, Karimi S. 2011. Biosynthesis of Silver Nanoparticles by Fungus *Trichoderma reesei* (A Route for Large-Scale Production of AgNPs). *Insciences J*ournal 1, 65–79. https://dx.doi.org/10.5640/insc.010165.

Waghunde RR, Shelake RM, Sabalpara AN. 2016. Review *Trichoderma*: A significant fungus for agriculture and environment. African Journal of Agricultural Research. 11(22): 1952-1965. https://doi.org/10.5897/AJAR2015.10584.

Zafari, D. 2012. "Taxonomic study of the genus *Trichoderma* in Iran". PhD Thesis, Faculty of Agriculture, Tarbiat Modares University, Tehran.

Zheng H, Qiao M, Lv Y, Du X, Zhang KQ, Yu Z. 2021. New species of *Trichoderma* isolated as endophytes and saprobes from southwest china. Journal of fungi, 7(6), p.467. https://doi.org/10.3390%2Fjof7060467.

جداسازی و شناسایی گونه‌های کلاد *Harzianum* تریکودرما از شهرستان خرم‌آباد

زهرا میرزایی‌پور[1]، عیدی بازگیر✉[1]، دوستمراد ظفری[2] و مصطفی درویش‌نیا[1]

۱- گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه لرستان، خرم آباد

۲-استاد، گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه بوعلی سینا همدان

چکیده : جنس تریکودرما شامل قارچ‌های هست که دارای پراکنش جهانی می‌باشند. در میان آنها، *T. harzianum*، یکی از رایج‌ترین گونه‌های تریکودرما جمع‌آوری شده، به عنوان یک مجموعه گونه شناخته شده است. در مطالعه حاضر با استفاده از محیط انتخابی تریکودرما، ۲۰ جدایه متعلق به ۵ گونه تریکودرما از کلاد Harzianum از نمونه‌های خاک جمع‌آوری‌شده از مناطق جغرافیایی مختلف شهرستان خرم‌آباد جداسازی شد. آنها بر اساس مطالعات تمام خصوصیات فنوتیپی، ویژگی‌های کشت و آنالیزهای مولکولی توالی‌های ژن *tef1α* شناسایی شدند. در نتیجه، پنج گونه مختلف *T. afroharzianum*، *T. atrobrunneum*، *T. guizhouense*، *T. harzianum* و *T. pholioate* شناسایی شدند. در این کار، این گونه‌ها را شرح و تصاویر ارائه دادیم. *T. harzianum* بیشترین فراوانی را در بین ۵ گونه داشت. *T. pholiotae* برای اولین بار از ایران گزارش می‌شود.

کلمات کلیدی: ایران، مورفولوژی، فیلوژنی، تاکسونومی، *tef1-α*، تریکودرما

مکاتبه کننده: عیدی بازگیر Email: Bazgir.ei@lu.ac.ir

تاریخ دریافت: ۱۴۰۲/۱/۲۴ تاریخ پذیرش: ۱۴۰۲/۳/۱۳