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TRICHODERMA AFROHARZIANUM SPECIES ASSOCIATED WITH THE ANTHROPOGENICALLY POLLUTED SOILS IN UZBEKISTAN

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SUMMARY

Genus *Trichoderma* classifies as a broad-based, saprotrophic fungi and a prime component of the fungal community of various soil ecosystems. The diversity of this genus has non-extensive studies in Uzbekistan, and the prevalence of investigations relied on phenotypic traits. Here, the isolation of four Trichoderma strains (BZS-T1, BZS-T2, BZS-T3, and BZS-T4) from the anthropogenic pollution zone in Uzbekistan succeeded. Using a molecular genetic approach based on the *tef1* gene region enabled us to report for the first time *Trichoderma afroharzianum* species in the polluted soils of Uzbekistan. Twelve reference strains obtained from the NCBI database underwent the phylogenetic analysis of the BZS-T1 strain. Based on the phylogenetic tree, the isolate BZS-T1 displayed clustering with *T. afroharzianum* strain T-22 (ATCC 20847) from the USA, isolate AG500 from Thailand and isolate Tri-1 procured from China. The promising results confirmed the value of the DNA-based identification process, which was worthwhile to promote and apply, especially for identifying *Trichoderma* species in Uzbekistan and Central Asia. The *T. afroharzianum* isolate BZS-T1 could have considerable potential regarding its antagonistic properties, inducing systemic resistance in plants and as a bioremediation agent for polluted soils and wastewater.

Keywords: *Trichoderma afroharzianum*, identification, *Tef-1a*, molecular genetic approach, phylogenetic analysis, microbial biodiversity, Central Asia

Key findings: Several morphologically based studies of *Trichoderma* in Uzbekistan enunciated that several *T. harzianum* isolates succeeded in segregation. However, the *T. afroharzianum* has no previous reports in Uzbekistan.

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INTRODUCTION

Trichoderma species are of great practical interest in connection with their study and use in human activities. The spacious use of these fungi requires detailed investigations of their ecological characteristics, species diversity, competitive relationships, and geographical distribution. The heavy anthropogenic impact and the dominant urbanization level lead to a decreased ability of urban soil to recover from the adverse effects. In microbial populations, the morphological and structural variations and the varied biochemical activities reflect the anthropogenic impact on the ecosystem and the individual species (Zaynitdinova *et al*., 2022a).

Soil degradation is a deterioration of physical, chemical, and biological soil quality caused by anthropogenic activities. With its effects on global food security and environmental quality, soil degradation has become a chief global issue. The uppermost layer of soil, or the "topsoil," accounts for around 95% of all food produced worldwide. Therefore, it has become the most crucial part of the food chain. However, due to unsustainable farming practices, nearly one-third of the topsoil has disappeared globally in the last 150 years (FAO and ITPS, 2015). Several factors have caused soil degradation. The primary causes are erosion, deforestation, overgrazing, overexploitation for fuel wood, unsustainable land use, and agricultural activities. According to past estimates, the global rates of annual topsoil loss range from 28 to 36 Pg yr−1 (Quinton *et al*., 2010; Borrelli *et al*., 2017).

Moreover, erosion rates in croplands outpace soil's natural replenishment rate by a ratio of 10 to 40 (Pimentel and Burgess, 2013). This phenomenon is also known as accelerated erosion. One-third of top soils have life spans (the amount of time it takes for topsoil to erode) of less than 200 years (Evans *et al*., 2020).

Anthropogenic soil pollution is a global problem that seriously jeopardizes ecosystems and human health (Singh and Singh, 2020). Soil is the key to supplying several ecosystem processes required for life support on Earth. Yet, the worrying truth is that soils' quality and the priceless advantages they provide rapidly disappear (Jie *et al*., 2002). For instance, an estimated 16.1% polluted soils are prominent in China. Anthropogenic soil pollution is also a growing problem in Uzbekistan. An analysis of recent years has shown that more than 100 million tons of industrial waste generated in Uzbekistan occur annually, and household waste accounts for about 35 million tons yearly.

Soil contamination primarily originates from anthropogenic activities. Numerous pathways, such as the application of fertilizers and pesticides, inappropriate wastewater disposal, the use of plastics and medicines, and the burning of fossil fuels, can allow both organic and inorganic contaminants to reach the soil matrix (Peña, 2022; Gautam *et al*., 2023). These pollutants entering the soil matrix make cleanup highly difficult and expensive. Numerous researches have indicated that heavy metals like cadmium and arsenic lower crop yields and drastically impair crop quality and food security (Djahed *et al*., 2018). Furthermore, evidence exists connecting these inorganic and organic contaminants to severe human disorders (Rahman *et al*., 2015).

These alarming negative processes emphasize the pervasiveness of soil degradation and contamination and the pressing need for remedial efforts. Regarding this, one must note that fungi of the genus *Trichoderma* have a high potential to restore soil and water pollution (Harman *et al*., 2004; Ezzi and Lynch, 2005). Its strains contribute to the breakdown of pesticides, herbicides, insecticides, heavy metals, and polycyclic aromatic hydrocarbons.

Trichoderma fungi are naturally widespread and are prevalent on many substrates, but more often, in the soil and dead plants (Kredics *et al*., 2014; Matniyazova *et al*., 2022). These fungi play a primary role in the microbial community, which serves numerous agriculture and biotechnology sectors (Jangir *et al*., 2019). These species are also beneficial for producing cellulolytic and other enzymes, biological control of plant diseases, and biological remediation of

contaminated soils (Harman *et al.*, 2004; Zaynitdinova *et al*., 2022b).

The ability to inhibit the growth and development of phytopathogenic fungi and parasitize them by affecting hyphae and sclerotia, with the inability to damage living plants, applies to the biological control of plant pathogens. Preparations based on species of the genus *Trichoderma* serve to protect plants from a wide range of diseases caused by fungi and nematodes in greenhouse and field conditions stimulate plant growth and development. Several commercial preparations are available based on different species of the genus *Trichoderma*, predominantly complex, with these plant protection products being dynamically developed (Cumagun, 2012; Fraceto *et al*., 2018).

In fungal species taxonomy, the fundamental step is morphological characterization. Classifying closely related fungal species can proceed based on the differences in asexual spore types, i.e., macroconidia, microconidia, and chlamydospores. However, considerable expertise in morphological analyses is necessary for studying the fungal taxonomy. Some fungal species seemed closely related, with no visible differences, and received the name species complex. The term species complex refers to designated groups of organisms with close taxonomic relations, making it difficult to distinguish them. The best-known use of species complex for fungal taxonomy is in the genus *Fusarium* (Van-der-Lee *et al*., 2015) and Trichoderma (Chaverri *et al.*, 2015). Therefore, species complex classification based on morphological analyses is inadequate for taxonomic characterization. The molecular-genetic approaches with morphological analysis effectively apply and provide accurate species identification (Raja *et al*., 2017; Sherimbetov *et al*., 2020a, b).

Recent taxonomic study of the fungi, defined by the most recent MycoBank classification (MycoBank Database, http://www.MycoBank.org), the genus *Trichoderma* belongs to the domain Eukaryota, kingdom Fungi, subkingdom Dikarya, division Ascomycota, subdivision Pezizomycotina, class Sordariomycetes, subclass Hypocreomycetidae, order Hypocreales, and family Hypocreaceae. According to the International Commission on *Trichoderma* Taxonomy, the genus *Trichoderma* already includes 460 species (https://trichoderma.info/trichoderma-taxonomy-2020/).

Trichoderma taxon systematics have caused many controversies due to revising the genus concept and the description of numerous new species. In analogy with the morphological approach, various molecular methods of taxonomy have cropped up, which provided more reliable identification of *Trichoderma* species and offered a new approach to the systematics of this genus (Druzhinina *et al*., 2006; Cai and Druzhinina, 2021; Shenouda and Cox, 2021).

Several DNA regions like actin (ACT), calmodulin (CAL), ITS, RNA polymerase II (RPB2), and TEF1a have provided for molecular-genetic analysis of *Trichoderma* (Samuels *et al*., 2002; Park *et al*., 2005; Jaklitsch, 2009; Huh *et al*., 2011). Among them, TEF1a is the best DNA region for single-region analysis because it has shown high species resolution for the phylogeny of *Trichoderma* (Chaverri *et al.*, 2015; Jang *et al.*, 2017). Based on the above discussion, the presented study carried out the molecular characterization through phylogenetic analysis of some indigenous *Trichoderma* strains in Uzbekistan.

MATERIALS AND METHODS

Isolation of *Trichoderma spp*. from soil

Collected soil samples came from the territory of the Bozsu Aeration Station, Tashkent, Uzbekistan. For isolating fungal strains, soil sample collection comprised digging 12–15 cm depth, with the soil placed in labeled plastic bags before transporting it to the laboratory in an icebox and then stored at 4 °C for further study. Using the serial dilution technique helped isolate *Trichoderma spp*. from the soil samples. The process added 10 g of soil into 90 mL sterile water, mixed for 30 min (200 r min^{-1}), and then diluted to 10^3-fold.

Soil dilution culturing on TSM (*Trichoderma* selective media) contained 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.9 g of K_2HPO_4, 0.15 g of KCl, 1.0 g of NH_4NO_3, 3.0 g of glucose, 0.15 g of rose bengal, 20 g of agar, 0.25 g of chloramphenicol, 0.3 g of p-dimethylamino benzene diazo sodium sulfonate, and 0.2 g of pentachloronitrobenzene (Elad *et al*., 1981). After 48 h, harvesting Trichoderma colonies underwent culturing on potato dextrose agar (PDA) medium (potato infusion from 200 g potatoes, 20 g of dextrose, 20 g of agar, and 1 liter of distilled water) (Eddleman, 1998).

The single spore isolation resulted from purifying *Trichoderma* colonies. Transferring the single spore on a PDA medium further obtained a pure isolate. After 48−72 h culturing on the PDA medium, using light microscopy (NLCD-307B LCD Digital Microscope, Ningbo Yongxin Optics Co., Ltd. Novel, China) enabled to observe Trichoderma conidiophores and conidia according to the Bissett taxonomy system. Four identified *Trichoderma* spp. (BZS-T1, BZS-T2, BZS-T3, and BZS-T4) Isolates based on mycological keys became the samples for further studies (Bissett, 1991a, b).

Then, *Trichoderma* inoculation continued into a 250-mL conical flask containing 100 mL PDB (potato dextrose broth) medium (potato starch infusion from 200.0 g potatoes, 20 g of dextrose, and 1 l of distilled water) with an inoculating loop. The flask on a shaker had a shaking speed of 120 r/min for 48 h. Mycelia gathered from the culture medium by filtering using a gauze.

DNA extraction and PCR amplification

DNA extraction from Trichoderma mycelium used a 'PureLink Genomic DNA Mini Kit' (Thermo Fisher Scientific). Measurement of quantity and quality of genomic DNA employed a spectrophotometer NanoDrop Eight (Thermo Fisher Scientific, USA), and then the DNA samples storage comprised -20 °C temperature until used for PCR. The primers used appear in Table 1.

A region of nuclear DNA containing translation elongation factor 1-a (*Tef-1a*) gene amplification by PCR had the primer combination EF1-983F (5'-GCY CCY GGH CAY CGT GAY TTY AT -3') and EF1-2218R (5'- AT GAC ACC RAC RGC RAC RGT YTG -3') (Rehner and Buckley, 2005). For the PCR process, a mixture included amplification reagents - 'Platinum™ Taq DNA Polymerase' (Thermo Fisher Scientific, USA). The PCR mixture (25 µl) contained the DNA of the investigated strain (4 µl), 14.9 µl dd H2O, 2.5 µl 10xPCR buffer, 0.75 µl 50 mM MgCl2, 0.5 µl 10 mM dNTP mixture, 0.5 µl 10 mM forward primer, 0.5 µl 10 mM reverse primer, 1.25 µl extender KB, and 0.1 µl Platinum Taq DNA polymerase.

The following thermocycling program for PCR comprised initial denaturation (95 °C, 10 min), denaturation (94 °C, 10 s), annealing (54 °C, 30 s), elongation (72 °C, 1 min)– 40 cycles, and final elongation (72 °C, 5 min). The resulting PCR products examination engaged gel electrophoresis with 1xTBE buffer (pH 8.3) in 2% agarose gel, followed by gel staining with 0.5 µg/ml ethidium bromide (EtBr) solution. Electrophoresis used horizontal electrophoresis system SE-1 (Helicon, Russia) at 100V for 100 min. PCR products' visualization employed UV light and photodocumentation using a gel document imaging system BK-AG100 (Biobase Kings Co., Ltd, China).

Sequencing of PCR product

First, PCR amplicon corresponding to isolate BZS-T1 used a razor blade for excising, then transferred to a 1.5 ml centrifuge tube and purified using the PureLink™ Quick Gel Extraction Kit (Invitrogen, USA), according to manufacturer's instructions. The cycle

Table 1. Primers used in this study.

Target region	Primer name	Primer Sequence (5'-3')	Product size (bp)	Reference
Translation elongation factor 1-alpha (*Tef-1a*)	EF1-983F EF1-2218R	GCYCCYGGHCAYCGTGAYTTYAT ATGACACCRACRGCRACRGTYTG	~1000 bp	Rehner and Buckley (2005)

sequencing reaction utilized the BigDye® Terminator v3.1 kit (Applied Biosystems, USA). Cycle sequencing reaction consists of ddH2O - 3.5 m1, BigDye-1 m1, 5x Sequencing buffer-2 m1, sequencing primer - 0.5 m1, and purified PCR product-2 m1. Sequencing used the Lu1 and Lu4 primers.

The following thermocycling program for cycle sequencing reaction included an initial denaturation stage at 96 °C 1 min; denaturation at 96 °C for 10 s, annealing at 50 °C for 10 s, and elongation at 60 °C for 3 min, repeated for 45 consecutive cycles. Afterward, the product of the sequence reaction reached storage at 4 °C. The sequencing reaction products bore purification from fluorescently labeled terminator nucleotides using the Dynabeads Sequencing Clean-Up Kit (Applied Biosystems, USA). Capillary electrophoresis of DNA sequence reaction products continued on Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific).

Phylogenetic analysis

The raw sequence reads tef1α attained checking for quality, trimmed, manually edited, and assembled using the SnapGene5.3.1 program. BLAST (Basic Local Alignment Search Tool) helped search similar sequences present in the Genbank NCBI database (Altschul *et al*., 1990; https://blast.ncbi.nlm.nih.gov/Blast.cgi), with the closely related sequences taken from the NCBI database for subsequent phylogenetic analysis.

The multiple sequence alignment (ClustalW and T-Coffee algorithm) and construction of phylogenetic trees employed the MEGA 11 (Molecular Evolutionary Genetics Analysis version 11) (Tamura *et al.*, 2021). The maximum likelihood method relied on the Tamura-Nei model, taking 1000 bootstrap replicates to test the interior branches' reliability and the constructed tree validity. Then, the phylogenetic tree visualization and display engaged the MEGA11.

RESULTS AND DISCUSSION

Morphological characterization

Four isolates of micromycetes reached segregation during the examination of the soil samples with anthropogenic pollution in the Bozsu Aeration Station region, Tashkent, Uzbekistan. Based on the morphological traits, the isolates belonged to the genus *Trichoderma* (Figures 1 and 2). The colony morphology and microscopic observations of the investigated isolates are available in Table 2.

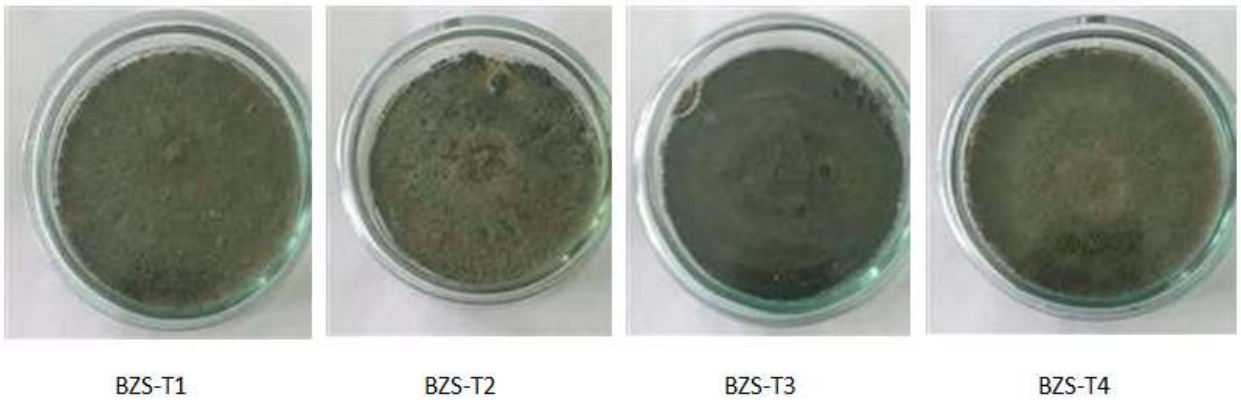

Figure 1. Colonies of the studied *Trichoderma* strains on PDA medium (7 days).

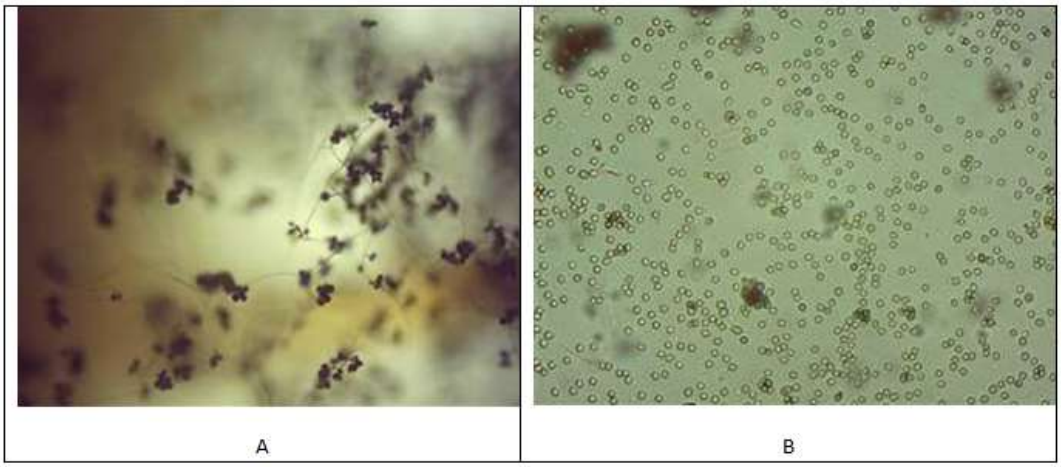

Figure 2. A - Conidiophores of BZS-T1 isolate, B – Conidia of BZS-T1 isolate.

Table 2. Morphological characteristics of the investigated *Trichoderma* isolates.

Isolate	Colony color	Colony reverse color	Colony edge	Conidial shape	Conidial size (µm)		Phialide shape	Phialide size (µm)	
					Length	Width			
BZS-T1	Green	Light yellow	Smooth	Subglobose to ovoid	3.1-3.4	2.7-3.0	Ampulliform to lageniform	3.9-7.2	2.5-3.4
BZS-T2	Yellowish-green	Creamy	Smooth	Subglobose to ovoid	2.4-3.2	2.3-2.9	Lageniform to ampulliform	4.7-7.8	2.3-3.5
BZS-T3	Dark green	Light yellow	Smooth	Subglobose to ovoid	2.7-3.4	2.5-3.2	Lageniform to ampulliform	4.8-8.0	2.3-3.6
BZS-T4	Green	Light yellow	Smooth	Subglobose to ovoid	2.7-3.5	2.6-3.1	Lageniform to ampulliform	4.6-8.0	2.4-3.7

Taxonomy

***Trichoderma afroharzianum*:** Chaverri P, Rocha FB, Druzhinina I. Mycologia 107(3): 568 (2014) [MB#809945].

Description: Colonies on PDA at 28 °C after 72 h, 90 mm diameter, aerial mycelium white, abundant, dense, with yellow pigment often diffused in the medium after five days. Colonies on CMA at 28 °C after 72 h, 75~80 mm diameter, aerial mycelium white, and sparse. Conidiophores are pyramidal, often symmetric, somewhat widely spaced branches, each terminating in a cruciate whorl or verticil of 3~5 phialides. Phialides lageniform to ampulliform, length: 3.9–7.2 µm, width: 2.5–3.4 µm; Conidia smooth, green to dark green, but rarely yellow, subglobose to ovoid, length: 3.1–3.4, width: 2.7–3.0 µm. No chlamydospores observed.

Isolate examined: Republic of Uzbekistan, Tashkent, Bozsu Aeration Station (41°27’65”N, 69°15’39”E), topsoil (BZS-T1, GenBank Acc. No. OR413445).

Molecular genetic analysis

The PCR amplified the region of the *tef-1* gene on four samples. PCR products obtained were with EF1-983F and EF1-2218R primers, as predicted, ~1000 bp long (Figure 3). PCR product of the *tef1* gene of BZS-T1 isolates bore sequencing, with the resulting sequence deposited into the GenBank NCBI under Accession number OR413445.

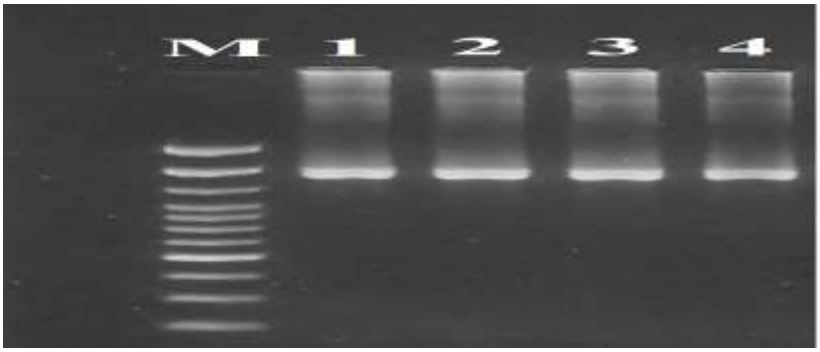

Figure 3. Electropherogram of the results of *Tef-1α* amplification (M - DNA Molecular Weight Marker 100-1000 bp, 1-4 - PCR products of *Tef-1α* obtained from BZS-T1, BZS-T2, BZS-T3, and BZS-T4 *Trichoderma* isolates).

Table 3. Strains and NCBI GenBank accession numbers used for phylogenetic analysis.

Species	Strain	Genbank Accession number	Country
T afroharzianum	BZS-T1	OR413445	Uzbekistan
T afroharzianum	T-22 (ATCC 20847)	KU933430.1	USA
T afroharzianum	AG500	MT929245.1	Thailand
T afroharzianum	Tri-1	OP102131.1	China
T afroharzianum	TRS835	KP008787.1	Poland
T afroharzianum	TB3-12	MW267264.1	China
T afroharzianum	a124	ON934349.1	China
T afroharzianum	TRS861	KP008786.1	Poland
T afroharzianum	TRS821	KP008781.1	Poland
T afroharzianum	HZA3	MK850825.1	China
T afroharzianum	E708	ON934365.1	China
T afroharzianum	TB1-23	MW267268.1	China
T afroharzianum	TB1-26	MW267269.1	China

The BLASTN analysis results of the *tef1* gene nucleotide sequence BZS-T1 against an NCBI nucleotide sequence revealed that this isolate was similar to other isolates of the *T. afroharzianum* at rates ranging from 99.59% to 99.89%. This BZS-T1 isolate showed the highest genetic similarity (99.89%) with the *T. afroharzianum* strain T-22 (ATCC 20847) (GenBank Accession number KU933430) from the USA. The GenBank Accession numbers of sequences used for phylogenetic analysis appear in Table 3.

According to the constructed phylogenetic tree, the BZS-T1 isolate showed clustering with the *T. afroharzianum* T-22 (ATCC 20847) from the USA, isolate AG500 from Thailand, and isolate Tri-1 from China (Figure 4). Alignment of tef1α nucleotide sequences of BZS-T1 isolate with T-22 (ATCC 20847) strain revealed the presence of one SNP in the position 67 (G to T); BZS-T1 isolate has G in this position, while the T-22 strain have T in this position (Figure 5).

Several morphologically alike species proved phylogenetically related to *T. harzianum* and belonged to the *T. harzianum* species complex (Samuels *et al.,* 2002; Chaverri and Samuels, 2003; Park *et al.,* 2005; Jaklitsch, 2009; Samuels and Hebbar, 2015). Thus, species in the *Harzianum* species complex were morphologically very similar and difficult to distinguish. These results were consistent with a prior study conducted in Korea (Jang *et al*., 2018), where identities of the *T. harzianum* strains deposited in the Korea University Culture Collection underwent analysis with EF1a sequences (KUC; Korea Univ., Seoul, Korea). Four species identified as

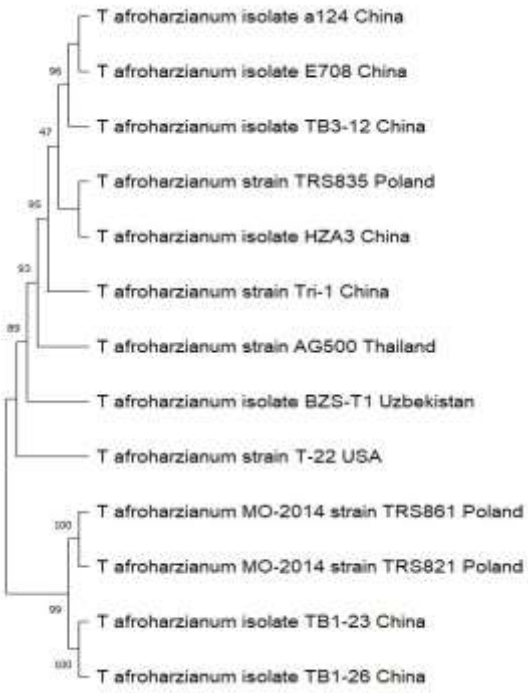

Figure 4. Phylogenetic tree based on the Maximum Likelihood analysis of the *Tef-1a.* Numbers above branches are bootstrap support values based on 1000 replicates.

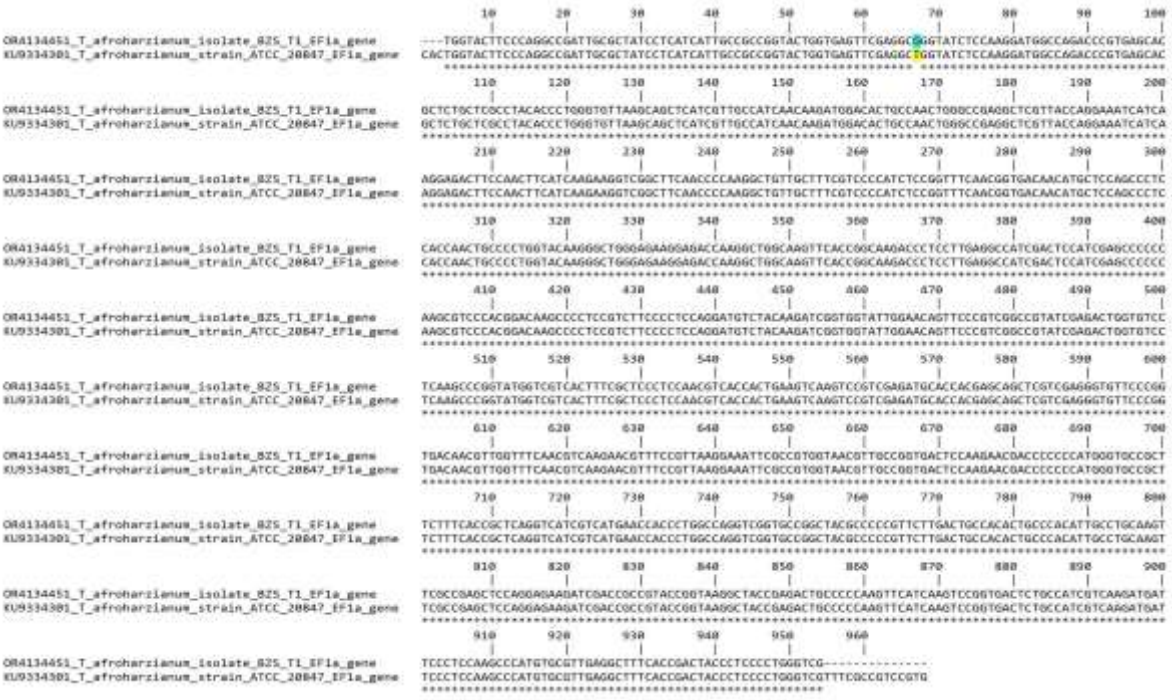

Figure 5. ClustalW Alignment of *Tef-1a* nucleotide sequences of BZS-T1 isolate with T22 strain (ATCC 20847)**.**

T. afroharzianum, T. atrobruneum, T. pyramidale, and *T. harzianum* emerged.

One must also note that *T. afroharzianum* strain T-22 has become a reference (standard) strain (ATCC 20847, LGC Standards, Poland) (Oskiera *et al.*, 2015). Therefore, in the presented study, the reliability of species identification of the BZS-T1 isolate reached significant validity. Notably, *T. afroharzianum* T-22 is one of the most widely used active ingredients in commercial bio-fungicides and bio-fertilizers. It has mycoparasitic activity and can also induce potent systemic resistance to phytopathogens by indirectly altering plant-pathogen interaction (Han *et al.*, 2004). The results also showed that *T. afroharzianum* T-22 has a robust capacity for mycoremediation of soils contaminated with diesel hydrocarbons (Elshafie *et al*., 2020; Ismaiel *et al*., 2022). Therefore, based on the genetic similarity, it was reasonable to suggest that *T. afroharzianum* isolate BZS-T1 could have considerable potential regarding the antagonistic properties, inducing systemic resistance in plants, and as a bioremediation agent for polluted soils.

Systematic names of *Trichoderma* must correspond critically to the genuine biology and evolutionary history of phylogenetically defined lineages in taxonomy. The synthesis of secondary metabolites, growth requirements, antagonistic potential, host ranges, and geographic distributions require consideration for examples of distinctive characteristics phylogenetically defined lineages may possess. Trichoderma-derived secondary metabolites include peptaibiotics, siderophores, diketopiperazines, polyketides, terpenes, pyrones, butenolides, anthraquinones, lactones, and other metabolites (Zeilinger *et al*., 2016; Khan *et al*., 2020). Noteworthily, the production of these substances is species- and even strain-dependent.

Therefore, morphologically identical, phylogenetic sister species of Trichoderma could express diverse biological, antagonistic abilities and host preferences. Incorrectly using a collective name to identify a cryptic taxon could have unfavorable effects on its applications in agriculture, industry, and biotechnology.

Several morphologically based studies of *Trichoderma* materialized in Uzbekistan, and these investigations have isolated several *T. harzianum* isolates. However, one should point out that no reports of *T. afroharzianum* have previously existed in Uzbekistan. Therefore, future studies need to enhance the number of molecular-genetic studies of Trichoderma isolates and strains in Uzbekistan. It will accurately reflect the overall current state of Trichoderma biodiversity in Uzbekistan.

CONCLUSIONS

Using a molecular-genetic approach based on the *tef1* gene region enabled us to report the *T. afroharzianum* species for the first time in Uzbekistan. The diversity of the *Trichoderma* species revealed relatively less studied in Central Asia, particularly in Uzbekistan. The pertinent findings will help future studies broaden the sampling areas to reflect the current state of *Trichoderma* biodiversity in Uzbekistan.

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