European Journal of Biology
Eur J Biol 2023; 82 (1): 59–69
DOI: 10.26650/EurJBiol.2023.1279151

RESEARCH ARTICLE

Morphological and Molecular Identification of *Trichoderma* Isolates Used as Biocontrol Agents by DNA Barcoding

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ABSTRACT

Objective: *Trichoderma* genus are environmentally friendly, targeted biocontrol agents used in organic agriculture. Currently, due to the increasing number of organic farming practices, *Trichoderma* species form a good market as commercial biocontrol agents. This study aims to make morphological and molecular identification of *Trichoderma* isolates, which were found to be potential biocontrol agents against plant pathogenic fungi, and to perform phylogenetic diversity analyses of these species using different bioinformatics tools.

Materials and Methods: Two different gene regions (the nuclear ribosomal internal transcribed spacer (ITS) and translation elongation factor 1 (EF) were used for molecular identification of *Trichoderma* isolates in this study. Polymerase Chain Reaction (PCR) related regions were amplified and sequenced using primers specific to these gene regions. Following molecular identifications based on these two different gene regions, phylogenetic trees were drawn and polymorphic regions in the nucleotide sequences of these genes were determined.

Results: As a result of the study, *Trichoderma* isolates were determined as *T. citrinoviride* Bissett and *T. atroviride* P. Karst. at the species level. This study not only provides essential information about the biodiversity of *Trichoderma* species, which is a biocontrol agent, but also allows the design of new species-specific primers based on the polymorphic regions of both species.

Conclusion: It will be possible to make fast and low-cost molecular identification independent of sequence analysis by using primers unique to these species in the future.

Keywords: *T. citrinoviride*, *T. atroviride*, BLAST, TrichOKEY, TrichoBLAST, targeted biocontrol agents, DNA barcoding

INTRODUCTION

Trichoderma genus are recognized as environmentally friendly, targeted biocontrol agents. For this reason, there are extensive studies on the biology of *Trichoderma* species, their enzyme production and their use in different biotechnological fields.[1–10] Due to the importance of the *Trichoderma* genus in the biological control of plant diseases, it is crucial to identify the species in this genus correctly.

Morphological identification of *Trichoderma* species is based on a number of parameters, such as colony colors, characteristics and growth rates, sporulation degree, sclerotic formation, color of mycelium and spores.[11] Colors ranging from white to green are observed in different *Trichoderma* species. Growth culture used, inoculation technique and incubation conditions may affect morphological characteristics.[12–15] Commonly used parameters in microscopic observations are the conidiophore character, conidiophore branching and the shape of the conidium (spore). However, the fact that some species have similar morphology and similar cultural characteristics is not sufficient for a clear and precise definition of the species belonging to the *Trichoderma* genus.[15,16] For this reason, morphological methods should be used together with molecular techniques. Today, molecular techniques have proven to be extremely important in the taxonomy of fungi, and the application of these techniques has led to the re-evaluation of many types.[15–18] For this reason, precise, accurate, rapid identification and classification of the species in the *Trichoderma* genus are still the subjects of intense

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Submitted: 07.04.2023 • **Revision Requested:** 02.05.2023 • **Last Revision Received:** 02.06.2023 • **Accepted:** 05.06.2023

study.[19–22] New *Trichoderma* species isolated from different geographical regions are on the increase and new nucleotide sequences of these new species have been obtained.[23–25] The Nuclear Ribosomal Internal Transcribed Spacer (ITS) is the most reliable gene region used for species-level identification of *Trichoderma*. Differences between closely related species can be detected using this gene region. In addition to this gene, The Translation Elongation Factor 1 (EF) gene is also used in the identification of species and it reflects the differences between closely related species. Studies show that most of the species-level definitions are made clearly and accurately by using the ITS and EF1 gene regions together.[26,27] In this study, mycelial production was carried out in liquid cultures of 11 *Trichoderma* isolates, which were determined to be biological control agents isolated from local sources. Then, genomic DNA isolation from these mycelia, followed by Polymerase Chain Reaction (PCR) and sequencing studies were performed. Nucleotide sequences were compared using different bioinformatics tools (BLAST, TrichOKEY, TrichoBLAST). Differences were determined by phylogenetic analyses and molecular identifications were carried out at the species level.

MATERIALS AND METHODS

Fungal Isolates

All *Trichoderma* spp. were isolated from the lumbering industry in Turkey. The soil dilution plating method was used for the isolation of *Trichoderma* spp. The diluted samples were directly plated onto Rose Bengal Chloramphenicol agar as a selective medium. After 6 days of incubation at 28°C, fungal colonies were transferred to "Malt Extract Agar (MEA)" and "Potato Dextrose Agar (PDA)" slants and they were stored +4°C for further investigations.[28–30]

Activation and Cultivation of *Trichoderma* Isolates

In order to activate a total of 11 *Trichoderma* isolates, three-point sowing was performed on MEA and PDA media stored in MEA slanted agar at +4 °C in the host mold collection. The cultivated petri dishes were incubated at 28 °C for 3-5 days and observed at 24-hour intervals during this time.

Morphological and Microscopic Characterization

In order to make the morphological and microscopic characterization of *Trichoderma* spp. in the study, they were cultivated in ”MEA” and ”PDA” media and incubated at 28°C for one week. For observation, the microscopic slides were stained with tryphan blue. *Trichoderma* hyphae, clamydospores, spores and phialides were observed with the OLYMPUS BX53 light microscope.[30–32]

Molecular Analysis

Liquid Culture Production

In order to produce *Trichoderma* spp. in liquid culture and to obtain mycelia, spore solution of each isolate was prepared initially. 0.1% Tween 80 was used for the homogeneous spore solution. Tween 80 was added to cover the surface of the media containing the spores of the activated *Trichoderma* isolates, and the spores were gently scraped from the surface with the help of an L baguette and taken into previously numbered sterile glass tubes with caps. For liquid culture production, 1% of the prepared spore solutions were inoculated into 250 ml flasks containing 50 ml MEB. They were then incubated for 4 days in a shaker at 28 °C and 120 rpm. During this time, pellet/mycelia formation was observed at 24-hour intervals.

Genomic DNA Extraction

Mycelia obtained as a result of liquid culture production were separated from the production medium by using filtration under aseptic conditions and then they were used for genomic DNA isolation. In this study, the manual genomic DNA isolation method was modified and used.[33] Polymerase Chain Reaction Amplification: PCR studies were performed for two different gene regions (ITS and EF gene). GeneMark 5X PCR Dye Master Mix II (GeneMark, Taiwan) was used for PCR. The primer pairs of the ITS and EF gene regions used in the study and the base sequences of these primers are as follows, respectively: ITS-V9G-F (TTACGTCCCTGCCCTTTGTA) and ITS-LS266-R (GCATTCCCAAACAACTCGACTC), EF1-728F-F-Tric (CATCGAGAAGTTCGAGAAGG) and TEF- 1-LLerev-R-Tric (AACTTGCAGGCAATGTGG).[34–36] The PCR condition used is 94 °C 3 min for ITS gene region, [94 °C 30sec, 58 °C 30sec, 72°C 1.5 min] (35 cycles), 72 °C 5min, 4 °C and 94 °C 3min for EF gene region, [94 °C 30 sec, 59 °C 1 min, 74 °C 50 sec] (35 cycles), 74 °C 7 min, 4 °C. PCR products obtained in the studies were purified using the Invitrogen Pure Link Quick Gel Extraction Kit (Invitrogen, USA). Genomic DNA and PCR products were run on 1% and 2% agarose gel, respectively. The bands were then visualized on the G-BOX gel imaging device (G-Box Syngene, UK).

Sequence Analysis

PCR products of the ITS and EF gene regions of each isolate were sent to REFGEN Biotechnology Limited Company (Ankara) for sequence analysis.

Bioinformatics Studies

The similarity percentages of the base sequences belonging to both different gene regions were determined using the NCBI

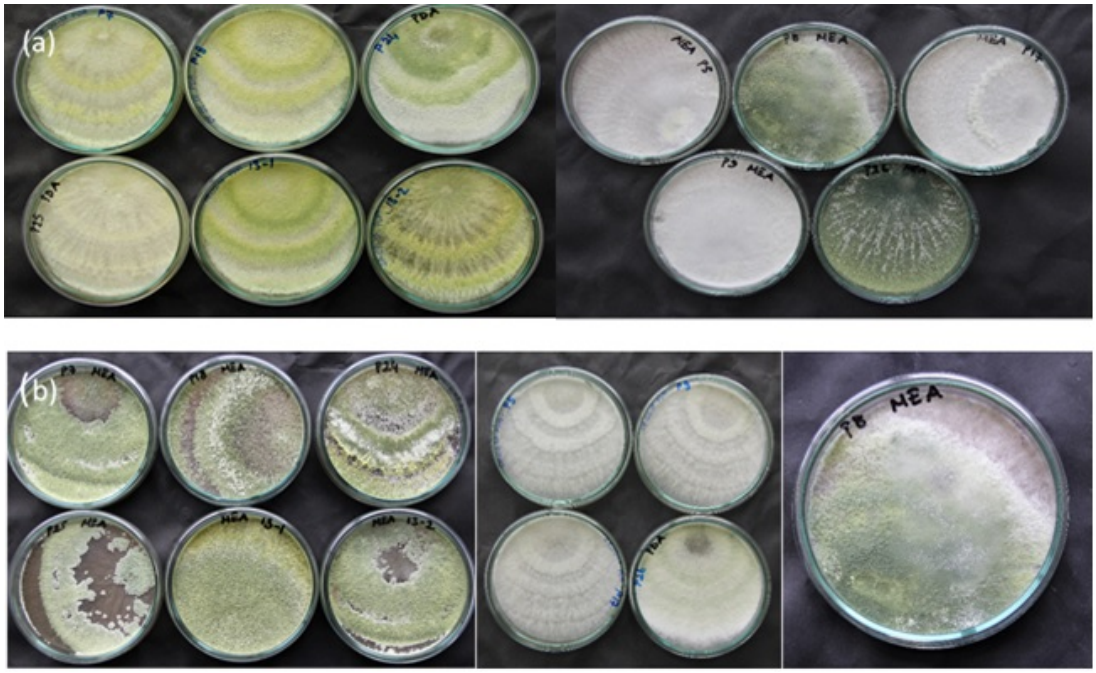

Figure 1. (a) The growth and sporulation on PDA medium and (b) MEA medium of *Trichoderma* species (*T. citrinoviride* P7, P13-1, P13-2, P18, P24, P25 and *T. atroviride* P5, P8, P9, P17, P26).

(National Center of Biotechnology Information) database programs BLAST (http://www.blast.ncbi.nlm.nih.gov), TrichOKEY (an oligonucleotide barcode for *Hypocrea* and *Trichoderma*) and TrichoBLAST (a sequence similarity search tool for *Hypocrea* and *Trichoderma*) and an accession number was received.[37–39] DNA sequences were aligned using Molecular Evolutionary Genetics Analysis Version X (MEGA X).[40,41] ClusterW program and conserved regions between species were determined by comparing with each other.[42] In addition, polymorphic areas belonging to two different gene regions were identified and gene motifs were determined. Phylogenetic trees were constructed with 1000 replication Bootstrap analysis using the Maximum Likelihood (GTR + G) method in MEGA X: Molecular Evolutionary Genetics Analysis Version X.

RESULTS

As a result of the study, Trichoderma isolates, which were isolated from the lumbering industry were determined as *T. citrinoviride* and *T. atroviride* at the species level.

Morphological and Microscopic Characterization of *Trichoderma* Isolates

T. citrinoviride species showed faster growth when compared to *T. atroviride* species (Figures 1a and 1b).

When *Trichoderma* species were compared in terms of growth and sporulation, MEA medium was found to be better than the PDA medium (Figures 2A and 2B). Morphological and microscopic views of all isolates are given in Figure 2. The color of the colonies varied from light green to dark green. However, colony characteristics were not sufficient for the identification of the species level. Colonies of *T. citrinoviride* became light greenish and all characteristics are shown in Figure 1. Green conidia were dispersed on the whole plate. Mature cultures of *T. atroviride* were developed from white to green pigmentation (Figure 1). Morphology of the spores and sporulating structures of the *Trichoderma* isolates were more or less similar.

Molecular Identification of *Trichoderma* Isolates

Two different gene regions (ITS and EF) were used for molecular identification of *Trichoderma* isolates in this study. The binding temperatures of both gene regions were determined as the first step. As a result of PCR studies, the binding temperature was determined as 58 °C for the ITS gene region and 59 °C for the TEF gene region. PCR products with a length of approximately 1000 bp for the ITS gene region (Figure 3a) were obtained. If it belonged to the EF gene region, PCR with a length of approximately 1400-1500 bp products were obtained (Figures 3b and 3c). Figures 3a, 3b and 3c show the single band image of the PCR products obtained as a result of PCR.

Bioinformatics Studies

The nucleotide sequences of both gene regions were compared using the BLAST, TrichOKEY and TrichoBLAST bioinfor-

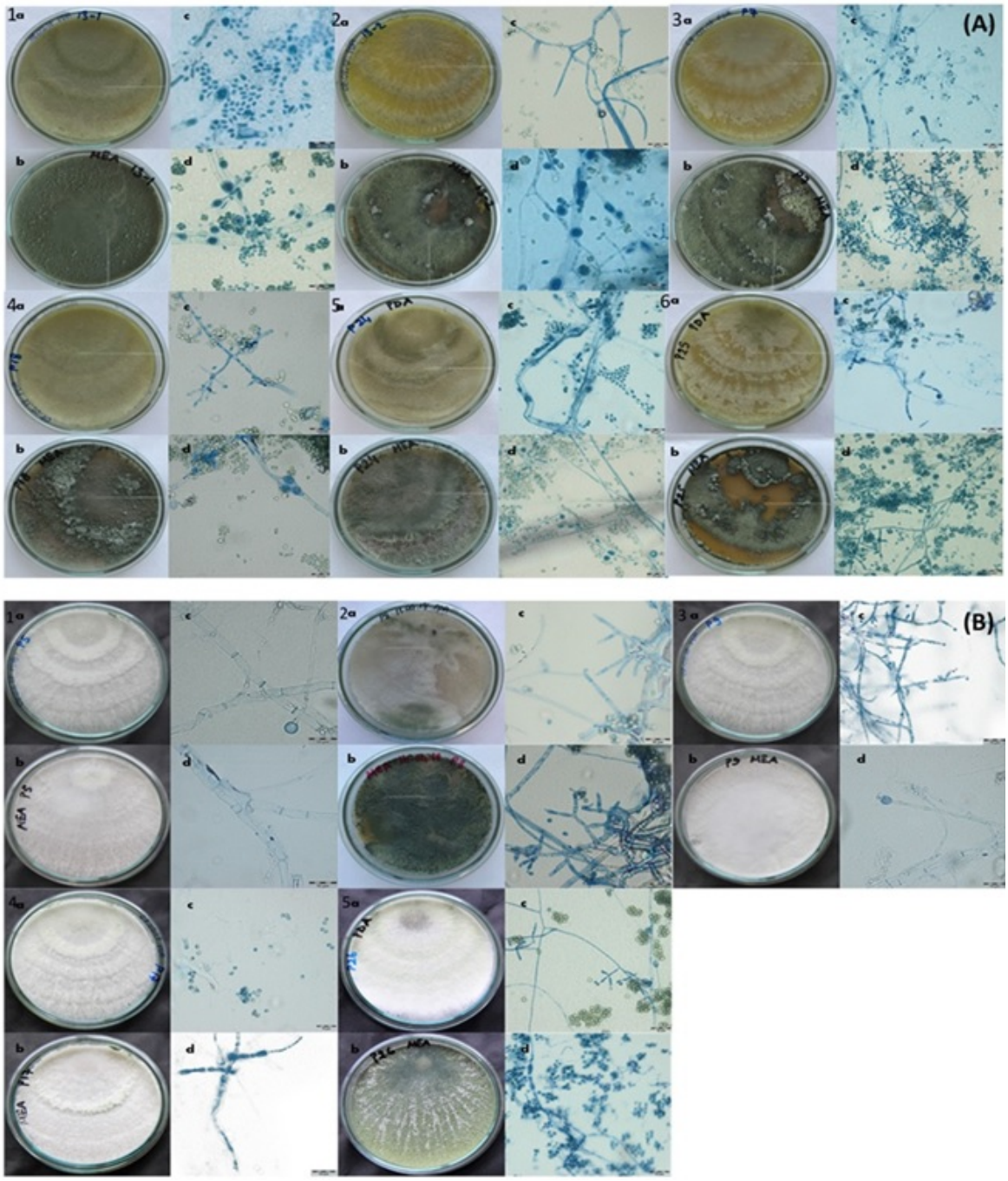

Figure 2. (A) Morphological and microscopic characterization of *T. citrinoviride* (P7, P13-1, P13-2, P18, P24 and P25). (B) Morphological and microscopic characterization of *T. atroviride* isolates P5, P8, P9, P17 ve P26.

matics tools and the percentages of similarity were determined (Table 1).[37–39]

As a result of the analyses made accordingly, the isolates were determined as *T. atroviride* and *T. citrinoviride*. Sequences of both gene regions for all species were registered in NCBI-GenBank and their accession numbers were obtained (Table 2).

By comparing base sequences in *T. atroviride* and *T. citrinoviride* species, polymorphic areas were identified at the gene level and gene motifs belonging to two different gene regions were determined for both species. Table 3 reflects the bases that differ between the two species in bold.

In order to compare phylogenetic similarities, *H. schweinitzii* CBS 818.91 (Accession no: Z31013, 647 bp) and *Hypocrea atroviridis* strain NBRC 8436 (Accession no: JN943354, 1144

Table 1. Molecular identification of *Trichoderma* isolates by using BLAST, TrichOKEY, TrichoBLAST and the calculating the similarity percentages of *ITS* and *EF* gene regions of species.

Isolate names	Gene	NCBI/BLAST		TrichOKEY	TrichoBLAST
		Similarity (%)	Species	Species	Species
P5	***ITS***	99	*T. atroviride*	*T. atroviride*	-
P8		99	*T. atroviride*	*T. atroviride*	-
P9		99	*T. atroviride*	*T. atroviride*	-
P17		99	*T. atroviride*	*T. atroviride*	-
P26		99	*T. atroviride*	*T. atroviride*	-
P7		99	*T. citrinoviride*	*T. citrinoviride*	-
P13-1		100	*T. citrinoviride*	*T. citrinoviride*	-
P13-2		99	*T. citrinoviride*	*T. citrinoviride*	-
P18		99	*T. citrinoviride*	*T. citrinoviride*	-
P24		99	*T. citrinoviride*	*T. citrinoviride*	-
P25		99	*T. citrinoviride*	*T. citrinoviride*	-
P5	***EF***	99	*T. atroviride*	-	*T. atroviride*
P8		99	*T. atroviride*	-	*T. atroviride*
P9		99	*T. atroviride*	-	*T. atroviride*
P17		99	*T. atroviride*	-	*T. atroviride*
P26		99	*T. atroviride*	-	*T. atroviride*
P7		99	*T. citrinoviride*	-	*T. citrinoviride*
P13-1		99	*T. citrinoviride*	-	*T. citrinoviride*
P13-2		99	*T. citrinoviride*	-	*T. citrinoviride*
P18		99	*T. citrinoviride*	-	*T. citrinoviride*
P24		99	*T. citrinoviride*	-	*T. citrinoviride*
P25		100	*T. citrinoviride*	-	*T. citrinoviride*

bp) registered in GenBank were selected for the ITS gene region.[43,44] In addition, *T. harzianum* strain CBS 226.95 (Accession no: AF057606, 548 bp) was used in the analyses since it is an outgroup located outside the species in this study.[45] In order to compare phylogenetic similarities, the *T. atroviride* strain CBS 693.94 (Accession no: KJ786838.1, 1249 bp), and *T. citrinoviride* strain DAOM 139758 (Accession no: EU338334.1, 619 bp) registered in GenBank, were selected for the EF gene region.[2,46] In addition, the *T. harzianum* strain CBS 226.95 (Accession no: AY605833.1, 534 bp) was used in the analyses since it is also an outgroup located outside the species in the present study.[47] Numbers on the phylogenetic tree indicate Bootstrap values (Figure 4). Looking at the trees in Figure 4, it is seen that there is a clear distinction between *T. atroviride* and *T. citrinoviride* species for both gene regions. As a result, the amplicon lengths and accession numbers obtained using primers specific to the ITS and EF gene region of *Trichoderma* species are also presented in Table 4.

DISCUSSION

Trichoderma spp. have been known for quite a long time and the species within this genus are wide. However, there has been considerable confusion about the identification of different species. In the past, identification of various strains was mainly based on morphological methods. However, today, molecular identi-

Table 2. Accession numbers of ITS and EF gene regions of *Trichoderma* spp species in this study.

Species	Isolates names	Genbank Accession Number	
		ITS Gene	EF Gene
T. atroviride	P5	MG972794	MH393753
T. atroviride	P8	MG972795	MH393751
T. atroviride	P9	MG972796	MH393750
T. atroviride	P17	MG972797	MH393749
T. atroviride	P26	MG972798	MH393752
T. citrinoviride	P7	MG972799	MH393744
T. citrinoviride	P13-1	MG972800	MH393747
T. citrinoviride	P13-2	MG972801	MH393748
T. citrinoviride	P18	MG972802	MH393745
T. citrinoviride	P24	MG972803	MH393743
T. citrinoviride	P25	MG972804	MH393746

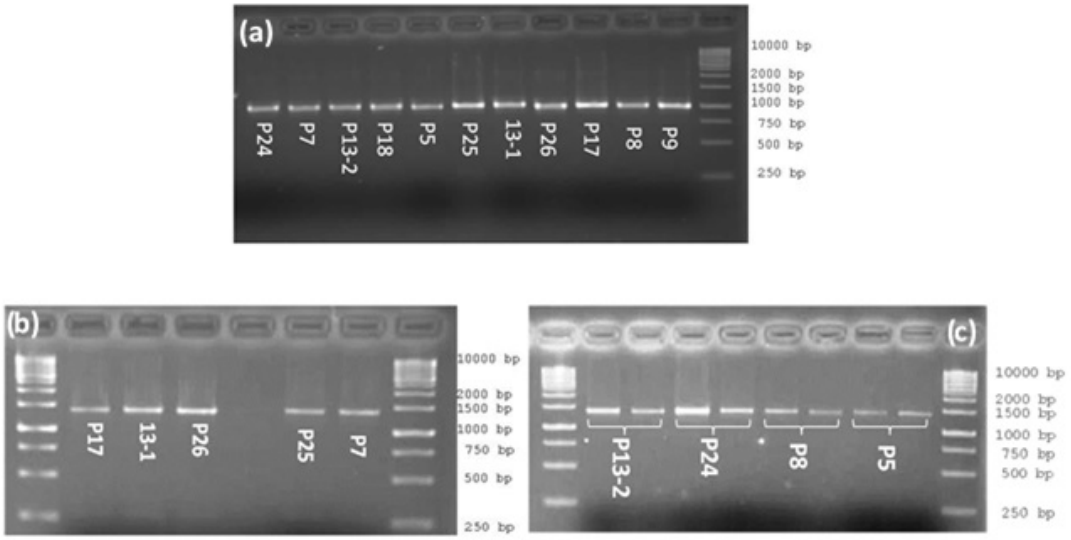

Figure 3. (a) PCR was performed by using (ITS-fw and ITS-rev) primers to amplify ITS region. PCR products that is averagely 1000 bp in length are showed. (b and c) PCR was carried out by using (EF1-728F-F-Tric and TEF -1-LLErev-R-Tric) primers to amplify EF region PCR products which are 1400-1500 bp in length were obtained from *Trichoderma* isolates. 1 kb DNA Ladder (Fermentas SM03131) was used.

fication techniques are preferred, enabling the analysis of the multilocus gene sequences of ITS, TEF-1α and Calmodulin (Cal), to differentiate between different strains.[20–22] The differences between the sequences of the conserved gene regions of different species serve as DNA barcodes for the species. These barcodes emerge as a method based on the use of protected DNA regions for the determination of biological diversity and species, and provide a clear distinction at the species level.

Table 3. The determined gene motifs in ITS and EF gene of *Trichoderma* species and the comparison of base differences in this gene. The different base was displayed in bold to show the base difference in the conserved region in both species.

Gene Region	Base Interval	*Trichoderma* species	
		T. atroviride motifs	***T. citrinoviride motifs***
ITS	61-80	AGGTGGGCAA C**T**ACCACTCA	AGGTGGGCAA C**C**ACCACTCA
	191-210	CAATGTGAAC **CA**TACCAAA**C**	CAATGTGAAC **GT**TACCAA**T**C
	221-240	GCGGG**GTCAC** - - GCCCCGGG	GCGGGA**TTCT CT**GCCCCGGG
	241-260	**T**GCGTCGCAG CCCCGGA**A**CC	**C**GCGTCGCAG CCCCGGA**T**CC
	261-280	**A** - - GGCGCCC GCCGGAGG**GA**	**CAT**GGCGCCC GCCGGAGG**AC**
	281-300	C**CAAC**CAAAC TCTTTT**CTG**T	C**AACT**CAAAC TCTTTT**TTC**T
	301-320	**AGTCCC**C - - - - - - - - - - TCG	**CTCCGTCGCG GCCTACG**TCG
	321-340	CGG**ACGTTA**T TT**CTTACA**GC	CGG**CTCTGT**T TTA**TTTTT**GC
	341-360	TCTGAGC - - - - - - - - - - - - -	TCTGAGC**CTT TCTCGGCGAC**
	361-380	- - - - - - - - **AA AAATTC**AAAA	**CCTAGCGGGC GTCTCG**AAAA
EF	252-270	G**T**ACTC**AAT T**GC**A**TC**GTCT**	G**C**ACTC**GTC C**GC**G**TC**A**TC**A**
	271-290	TC**TCCATCT**C TGT**GTGG**TTC	TC**ACTGCAG**C TGT**A**T - - TTC
	291-310	**ATTG**TGCTAA **T**CAT**GCTTCA**	**GCGA**TGCTAA **C**CAT**CTTCCC**
	311-330	**ATC**AA**T**AGGA AGCCGCCGA**G**	**CTTAAC**AGGA AGCCGCCGA**A**
	341-360	GTTC**T**TTCAA GTATGCGTGG	GTTC**C**TTCAA GTATGCGTGG
	391- 410	CGTGG**T**ATCA CCATCGACAT	CGTGG**C**ATCA CCATCGACAT
	461-480	TGT**TTTCGCT** TT**T**CCTCA**TT**	TGT**GGATCCA** TT**G**CCTCA**CC**
	481-500	G**ATA**CT**TGGA** GA**C**CAA**GATT**	G**CGT**CT**CTTC** G**GA**CA**CGGCA**
	501-520	CTAACG**TGC**C **GCTCTGT**AGA	CTAACG**ATTC CCG**C - **AC**AGA
	521-540	CGCTCCCGG**T** CACCGTGA**TT**	CGCTCCCGG**C** CACCGTGA**CT**
	571-590	AGGC**T**GACTG CGCTAT**C**CT**G**	AGGC**C**GACTG CGCTAT**T**CT**C**
	591-610	AT**T**AT**C**GC**T**G CCGGTACTGG	AT**C**AT**T**GC**C**G CCGGTACTGG

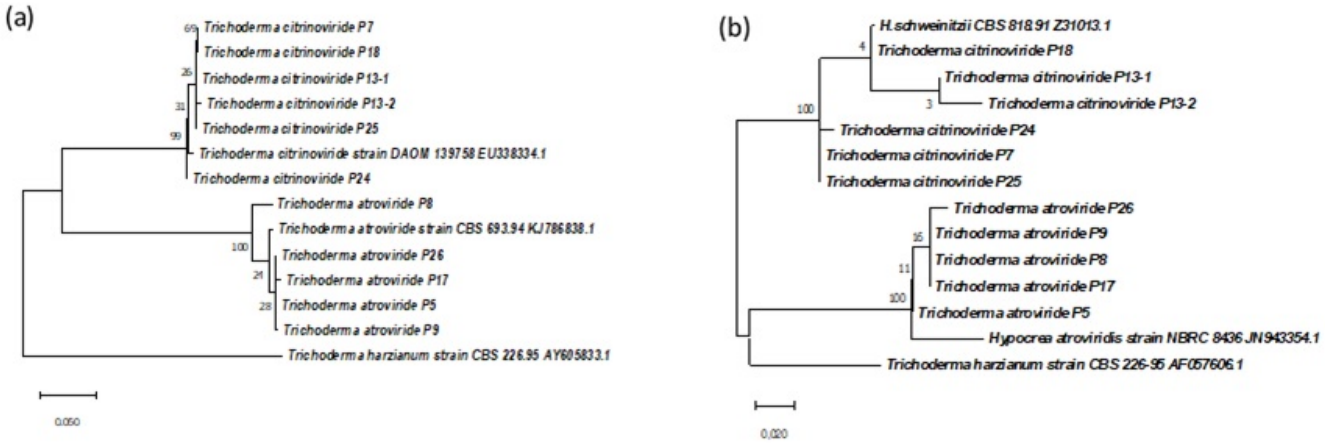

Figure 4. (a) Phylogenetic trees based on partial EF sequence data (b) ITS sequence data of *Trichoderma* species.

Both morphological and multi-gene-based molecular identification of *Trichoderma* isolates were performed in this study. As a result of the comparative analyses of the sequences of two different gene regions (ITS, EF) amplified by PCR on genomic

Table 4. The comparison of ITS and EF gene regions of different *Trichoderma* spp.

Gene	Primer	Amplicon leght (bp)	Accession number	Species
ITS	*ITS*-V9G-F ve *ITS*-LS266-R	848 880 899 933 912	MG972794, MG972795, MG972796, MG972797, MG972798	*T. atroviride*
	ITS-V9G-F ve *ITS*-LS266-R	536 902 773 725 798 688	MG972799, MG972800, MG972801, MG972802, MG972803, MG972804	*T. citrinoviride*
	ITS-1- ALR0	548	AF057606	*T. harzianum strain* CBS 226.95
	SR6R & 5.8S -5.8SR & LR1- *ITS*s (*ITS*-1 & *ITS*-2)	621	Z48811	*T. harzianum /atroviride*
	*ITS*6 ve *ITS*4 primerleri	605	KJ786740	*T. atroviride* strain TRS31
EF, TEF1 α	*EF*1-728F-F-Tric-*TEF* -1-LLErev-R-Tric	754 785 598 696 704	MH393749, MH393750, MH393751, MH393752, MH393753	*T. atroviride*
	*EF*1-728F-F-Tric-*TEF* -1-LLErev-R-Tric	600 696 680 527 780 716	MH393743, MH393744, MH393745, MH393746, MH393747, MH393748	*T. citrinoviride*
	*EF*1-728F TEF1-LLErev	1019	KP008873	*T. harzianum*
		1026	KP008948	*Trichoderma* sp. TRS4
		1026	KP008949	*Trichoderma* sp. TRS29
		1026	KP008950	*Trichoderma* sp. TRS33
	*EF*728M EF2	488	KT275197	*T .harzianum*
		488	KT275198	*T. harzianum*
		488	KT275199	*T. harzianum*

DNA, the isolates were successfully identified on a species basis as *T. citrinoviride* (n=6) and *T. atroviride* (n=5).

The ITS gene region is one of the most reliable genes used for species-level identification. By comparing the sequences of its gene region with the sequences registered in the NCBI GenBank, *Trichoderma* isolates can be identified at the species level with a high percentage of homology. However, some sequences in the NCBI GenBank are stored under the name from which the strain was originally obtained, not the name by which the strain was later identified. In addition, the high similarity of the base sequences as well as the intraspecific variability of the sequence should be known. Even though it is known that a particular strain may exhibit, such as 1% nucleotide (nt) variation, this may not be the case for the entire sequence and the nt's at some positions do not change. For the accurate identification of *Hypocrea/Trichoderma* members on the basis of species and eliminating the problems mentioned, the publicly available TrichoBLAST database was developed, powered by sequence identification and similarity tools.[39] This database distinguishes most species using sequences from the TEF-1α gene region used in taxonomic and phylogenetic studies.[48] In the same year, TrichOKey version 1.0 was published, the first

fungal oligonucleotide barcode database for the identification of *Hypocrea* and *Trichoderma* species.[38] They have mentioned that the GenBank database contains sequences of many misnamed and misidentified *Trichoderma* isolates.[38] Therefore, they formed the publicly available TrichOKEY database, which specifically compares only the ICH1 and ITS1 gene sequences for the reliability of the results and the correct identification of *Trichoderma* species.[49] The program provides an online opportunity for rapid molecular identification of an isolate at genus, division, and species levels based on the diagnostic combination of several oligonucleotides (pronounced markers) specifically nested within ITS1 and -2. However, identification based on sequences from the gene ITS region sometimes fails to distinguish closely related taxa as more than one species can share the same ITS genotype. For this reason, the base sequences of the ITS and EF gene regions in the present study were compared using different bioinformatics tools (BLAST, TrichOKEY, TrichoBLAST) and differences between species were determined by phylogenetic analyses (Table 3).

In line with the findings obtained in the study, these two gene regions can be used together for molecular identification of *Trichoderma* species. The amplicon lengths and accession numbers obtained using primers specific to the ITS and EF gene region of different *Trichoderma* species are presented in Table 4.

Today, PCR-based molecular techniques/new strategies are required for rapid, accurate and low-cost sequence-independent identification of *Trichoderma* species. There are limited studies on this subject. One of these studies was carried out by and PCR-based molecular identifications were performed by designing new primers specific to the TEF1 gene region for two *Trichoderma* species (*T. citrinoviride* and *T. reesei*).[50]

Conserved and polymorphic regions of ITS and EF gene regions of *T. atroviride* and *T. citrinoviride* species in the present study are shown in Table 3.

CONCLUSION

In the light of the data obtained, new species-specific primers can be designed based on different base regions between the two species. With the guidance of these species-specific primers/DNA Barcode, PCR-based molecular identification will be possible and fast and inexpensive methods will be developed independent of sequence analysis.

Acknowledgment:: This study is supported by Ege University Scientific Research Projects Coordination Unit. Project Number: 15-MÜH-059

Peer Review:: Externally peer-reviewed.

Author Contributions:: Conception/Design of Study- Y.G., R.E.; Data Acquisition- Y.G., G.G., A.B.B.; Data Analysis/Interpretation- Y.G., G.G., A.B.B.; Drafting Manuscript- Y.G.; Critical Revision of Manuscript- ; Final Approval and Accountability- Y.G., G.G., A.B.B., R.E.

Conflict of Interest:: Authors declared no conflict of interest.

Financial Disclosure:: Authors declared no financial support.

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How cite this article

Gezgin Y, Guralp G, Bercin Barlas A, Eltem R. Morphological and Molecular Identification of *Trichoderma* Isolates Used as Biocontrol Agents by DNA Barcoding. Eur J Biol 2023;82(1): 59-69. DOI: 10.26650/EurJBiol.2023.1279151