Mycologia Iranica 8(2): 41– 50, 2021 Original Article DOI: 10.22092/MI.2022.358333.1213

A cost-effective molecular approach for classification of large *Trichoderma* collections

S. Dehghan

A. Mirshamsi Kakhki ✉

M. Farsi

A. Seifi

Department of Biotechnology and Plant Breeding, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran

Abstract: Molecular techniques are necessary for reliable identification of *Trichoderma* species. For this, sequencing of several genomic loci, such as ITS, *TEF1* and *RPB2*, are advised, but this is costly when a lot of isolates are to be analyzed. RFLP of the ITS region, on the other hand, is a simple and cost-effective method for differentiation between large numbers of *Trichoderma* species. In the current work, 77 *Trichoderma* isolates were classified using the ITS-RFLP technique by combination of three restriction enzymes (*Msp*I, *Nla*III and *Nla*IV). Regarding the results, the isolates were divided into 8 groups. Out of these, 14 representative isolates were selected for sequencing of ITS, *TEF1* and *RPB2*. In order to evaluate the enzymes used in this study, 227 ITS sequences of *Trichoderma* species from the RefSeq database of NCBI were analyzed. Based on these, about 65% of the tested *Trichoderma* sequences can be categorized into small groups including less than 10 species and thus allowing a low-cost pre-classification. Our results showed that the ITS-RFLP method and the enzymes used in this study can make easy the differentiation of a large numerous of *Trichoderma* species. We present the ITS-RFLP method using the combination of three restriction enzymes as an efficient and reliable method for the identification of *Trichoderma* isolates.

KEYWORDS: ITS-RFLP, *Trichoderma*, Classification, Cost-effective

INTRODUCTION

Trichoderma reesei is one of the most important microorganisms with industrial applications, due to its ability to degrade plant biomass and production of industrial enzymes such as cellulase enzymes (Sivasithamparam and Ghisalberti (1998); Martinez et al. 2008). More than 250 *Trichoderma* species have been reported from different climate zone using several methods such as morphological and molecular methods (Atanasova et al. 2013; Bissett et al. 2015). Because of the incorrect identification, morphological characters is not sufficiently accurate to distinguish between individual *Trichoderma* species (Kubicek and Harman 2002; Hagn et al. 2002). Therefore, molecular methods must be done to validate the initial observations (Druzhinina et al. 2005; Druzhinina et al. 2006; Cai and Druzhinina 2021). One of the most widely used gene loci for differentiation of species is the ITS region including ITS1 and ITS2 (White et al. 1990). One way to screen for differences in this region is by ITS-RFLP. As a low-cost alternative to sequencing particularly for the screening of larger numbers of isolates, ITS-RFLP can differentiate species by detecting sequence polymorphisms in the ITS region (Dean et al. 2005b; Diguta et al. 2011; Koffi et al. 2019). While sequencing of the ITS region has become a standard in many labs worldwide, identification solely based on the ITS region is also not sufficient for distinguishing closely related *Trichoderma* species. In addition, the available sequences for comparison in GenBank (https://https.ncbi.nlm.nih.gov/genbank/) can be incorrect due to some misidentified or incomplete sequences (Kopchinskiy et al. 2005). Recent studies emphasize to use some other informative loci, such as the translation elongation factor 1-α (*TEF1*) and RNA polymerase II subunit B2 (*RPB2*) for identification of *Trichoderma* strains at the species level (Hoyos-Carvajal and Bissett 2011). Nevertheless, this is costly when a high number of *Trichoderma* isolates have to be identified.

The main objective of the current work was to developing a simple and cost effective method for grouping of large scale *Trichoderma* isolates. To this end, and considering the arguments above as well as high cost of sequencing in low-budget projects, the ITS-RFLP technique was used as a complementary method besides sequencing of selected isolates. For evaluating the efficiency of the restriction enzymes (*Msp*I, *Nla*III and *Nla*IV) used for ITS-RFLP, an analysis was done by grouping 227 ITS sequences of *Trichoderma* species from the RefSeq database of

Submitted 16Aug 2021 accepted for publication 24 Oct 2021
✉ Corresponding Author: E-mail: mirshamsi@um.ac.ir

https://mij.areeo.ac.ir

NCBI, based on the polymorphisms between these sequences. The ITS-RFLP method and the enzymes used in this study facilitated the initial grouping of our larger-scale *Trichoderma* isolation (77 isolates) into relatively small groups of related species.

MATERIALS AND METHODS

Isolates

77 *Trichoderma* isolates, which were used in this study, were isolated from forests soils in Iran in another study (Suppl. Table.1) (Dehghan et al. In press).

Identification of polymorphic restriction enzymes

Three restriction enzymes were chosen based on an initial analysis, in which 20 ITS sequences of *Trichoderma* (randomly selected from the RefSeq of NCBI database) were analyzed with all potentially commercially available restriction enzymes. The enzymes with the highest number of cutting sites in the ITS regions and thus showing the best polymorphism to group the sequences were selected.

Grouping of 77 *Trichoderma* isolates using ITS-RFLP method

DNA extraction was carried out from mycelia of 5 days old cultures (Doyle 1991) of all 77 identified *Trichoderma* isolates by morphological and microscopic methods in another study (not published). Then ITS regions including ITS-1, 5.8S rRNA, and ITS-2 were amplified by PCR using the universal primers ITS1 (5′-TCCGTAGGTGAA CCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTG ATATGC-3′). PCR reactions were done using 50-100 ng of genomic DNA as a template, 0.5 µM of each primer, 0.2 mM of dNTPs, 1X Phusion HF Buffer, and 1 unit Phusion DNA polymerase in a total volume of 50 µl. PCR program was consisted of an initial denaturation step of 98 ˚C for 30 sec, then 35 cycles of denaturation at 94 ˚C for 10 sec, primer annealing at 54 ˚C for 30 sec and elongation at 72 ˚C for 30 sec and final elongation step at 72 ˚C for 10 min. For ITS-RFLP, all ITS-PCR fragments of *Trichoderma* isolates were digested separately with three restriction enzymes - *Msp*I, *Nla*III and *Nla*IV -according to the manufacturer's instructions (New England BioLabs, Germany). All of the restriction fragments were separated on 2.5% agarose gel (TAE.1X). For better detection of smallest bands, we used the Midori Green Direct (NIPPON Genetics EUROPE) which was added into each sample before loading on the gel. Then all isolates were compared based on the length polymorphism of fragments on the gel and were divided into some groups resulted from ITS-RFLP results of all three enzymes. Some isolates from each group were selected for sequencing.

Sequencing of ITS, *TEF1* and *RPB2* gene loci

ITS-PCR, *TEF1*-PCR and *RPB2*-PCR were done in the selected isolates, purified and sent to the Eurofins Genomics Company for sequencing. The representative isolates from each group were selected based on the number of isolates in each group (three or four isolates from the groups including 30 isolates and one or two isolates from the smaller groups). A fragment of the large intron of the *TEF1* gene encoding translation elongation factor 1-alpha and a fragments of RNA polymerase II subunit B gene (*RPB2*), were amplified by PCR using the primer pair EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′) (Druzhinina et al. 2004) for *TEF1* fragment and the primer pair fRPB2-5f (5′-GAYGAYMGWGATCAYTTYGG-3′) and fRPB2-7cr (5′-CCCATRGCTTGTYYRCCCAT-3′) (Cai and Druzhinina 2021) for the *RPB2* fragment. PCR reactions were done as described for ITS-PCR reactions except that the annealing temperature was 56 ˚C for *TEF1*-PCR and 53 ˚C for *RPB2*-PCR. The sequences were identified in this study were deposited to NCBI databank. The phylogenetic trees were drawn using MEGA 5.2 (Tamura et al. 2011) and the maximum likelihood method based on Kimura-2 parameters with 1000 bootstrap replications. For multigene phylogenetic tree a Single gene alignment was done by MEGA version 5.2 for each loci. Then, a multigene dataset was created using SequenceMatrix 1.8.1 and the output was used for data partitioning using MrBayes 3.2.7 (1000000 generation). The phylogram was imported to Fig Tree version 1.4.4 and multigene phylogenetic tree was drawn. *Nectria eustromatica* was used as outgroup.

Validation of the polymorphic restriction enzymes: *Nla*III, *Msp*I and *Nla*IV

To evaluate the efficiency of the ITS-RFLP method and also of the restriction enzymes used in this study for distinguishing our *Trichoderma* species, 227 *Trichoderma* ITS sequences (Supple. Table.2) were collected from the RefSeq database from NCBI (https://www.ncbi.nlm.nih.gov/refseq/) and digested by the three enzymes *Msp*I, *Nla*III and *Nla*IV using Benchling software. All strains were grouped based on the resulting restriction length polymorphism between the species.

RESULTS

ITS-RFLP method on *Trichoderma* species isolated from soil

The ITS amplicon size using primers ITS1 and ITS4 was around 600 bp for all 77 *Trichoderma* isolates. Digestion of the ITS-PCRs by the three restriction enzymes: *Nla*III, *Msp*I, and *Nla*IV, allowed to divide the *Trichoderma* isolates into groups according to the resulting band pattern (Supple. Table. 3). The ITS-RFLP experiment was repeated for each enzyme two times to test the reproducibility of this technique. All results for each enzyme were the same (Supple. Fig. 3). With four different restriction patterns, the digestion with *Nla*III detected higher polymorphism between the isolates compared to *Msp*I and *Nla*IV. A total of 41 isolates fell into group 1 (250 bp / 380 bp), 20 isolates in sub-group 1-1 (260 bp /370 bp), 6 isolates in sub-group 1-2 (260 bp / 400 bp), 2 isolates in group 2 (115 bp / 130 bp / 265 bp), 2 isolates in group 3 (120 bp / 130 bp /350 bp) and 5 isolates in group 4 (100 bp / 245 bp / 275 bp).

Digestion with *Msp*I resulted in three groups and one sub-group. 35 isolates were placed in group 1, which showed three bands (100 bp / 215 bp / 280 bp). Two isolates (H1N27 and H1N29) were similar to group 1 in their banding patterns with slight differences (100 bp / 200 bp / 300 bp) and thus were classified as a subgroup of group 1. The second group included 35 isolates with two bands (60 bp / 100 bp / 260 bp), and four isolates were grouped as group 3 with two bands (70 bp / 100 bp / 290 bp).

Three distinct band patterns were obtained based on the *Nla*IV digestion that classified 30 isolates as group 1, 12 isolates as group 2, and 34 isolates as group 3. It should be noted that one of the strains (K1N29) did not show any band on agarose gel with any of the three enzymes which reflect that it was not digested with these enzymes.

Subsequent to the individual digestions, a final analysis was performed based on the PCR-RFLP results for each enzyme so that all isolates with the same restriction patterns for all three enzymes were grouped together. According to this, all 77 *Trichoderma* isolates could be divided into eight major groups (Table. 1).

Table 1. Final grouping based on the ITS-RFLP results of all three enzymes (*Msp*I, *Nla*III and *Nla*IV). The isolates selected for sequencing are highlighted in bold.

Number of group	Name of Isolates
1	A2N2, A2N17, D1N1, D1N2, D1N3, D1N5, F1N5, F2N4, F2N7, G2N1, G2N3, H1N1, H1N2, **H1N3**, H1N24, H1N36, I2N18, I2N26, **I2N27**, I2N30, K1N3, K1N2, K1N5, **K1N6**, K1N9, K1N20, K1N21, K2TN1
1-1	G2N2, H1N39
1-2	H1N27, H1N29
2	C2N9, **D2N3**
3	**F2N1**, F2N8
4	E1N17, E1N19, **F1N3**, H1N31, H1N47, I1N13, M1N2, M1N7, M1N8, **M1N12**, M2N9
4-1	G1N5, **H1N16**, H1N17, H11N8, H1N19, H1N21, H1N23, H1N26, H1N28, H1N43, **H1N44**, H1N46, H2N5, H2N6, I2N28, K1N10, K1N26, K1N27, K2N13
5	E1N16, F1N11
6	**F1N1, F1N2**
7	H1N12, **H1N20**, H1N22, **H1N40**, H1N45
8	**H2N2**

ITS, *TEF1* and *RPB2* sequencing results of 14 representative strains

Sequencing analysis of 14 isolates selected as representatives from ITS-RFLP grouping using BLASTN and *TrichOKEY* programs identified these as being derived from eight species (Table 2): *T. koningii*, *T. koningiopsis*, *T. atrobrunneum*, *T. harzianum*, *T. orientale*, *T. chlamydosporium*, *T. zayuense and T. tomentosum* (Fig. 1 and suppl. Fig. 1, supple. Fig. 2 and suppl. Tables 7 to 9). Multigene phylogenetic analysis was coordinated to the single gene analysis (Fig.2).

Table 2. The sequenced isolates in this study with their accession number.

Name of isolate	Recognized strain	Accession number		
		ITS	*TEF1*	*RPB2*
H1N3	*T. atrobrunneum*	MZ707740	OK087569	OK087585
I2N27	*T. harzianum*	MZ707742	OK087570	OK087586
K1N6	*T. harzianum*	MZ707741	OK087571	OK087587
F2N1	*T. orientale*	MZ707746	OK087572	OK087584
F1N3	*T. koningiopsis*	MZ707736	OK087565	OK087580
H1N16	*T. koningiopsis*	MZ707737	OK087566	OK087581
H1N44	*T. koningiopsis*	MZ707738	OK087567	OK087582
M1N12	*T. koningiopsis*	MZ707739	OK087568	OK087583
F1N1	*T. koningii*	MZ707734	OK087563	OK087578
F1N2	*T. koningii*	MZ707735	OK087564	OK087579
D2N3	*T. zayuense*	MZ707743	OK087573	OK087577
H2N2	*T. chlamydosporium*	MZ707747	OK087574	OK087590
H1N20	*T.tomentosum*	MZ707744	OK087575	OK087588
H1N40	*T.tomentosum*	MZ707745	OK087576	OK087589

The isolate F2N1 was represented as *T. orientale* based on the single gene analysis and separated from *T.lognibrachiatum* and *T. reesei*. The isolate was also clustered close to the *T. orientale* according to the multigene phylogenetic analysis. The results were similar for H1N3 which identified as *T. atrobrunneum* in both single and multigene analysis. For other isolates the multigene analysis showed similar results with the single gene analysis.

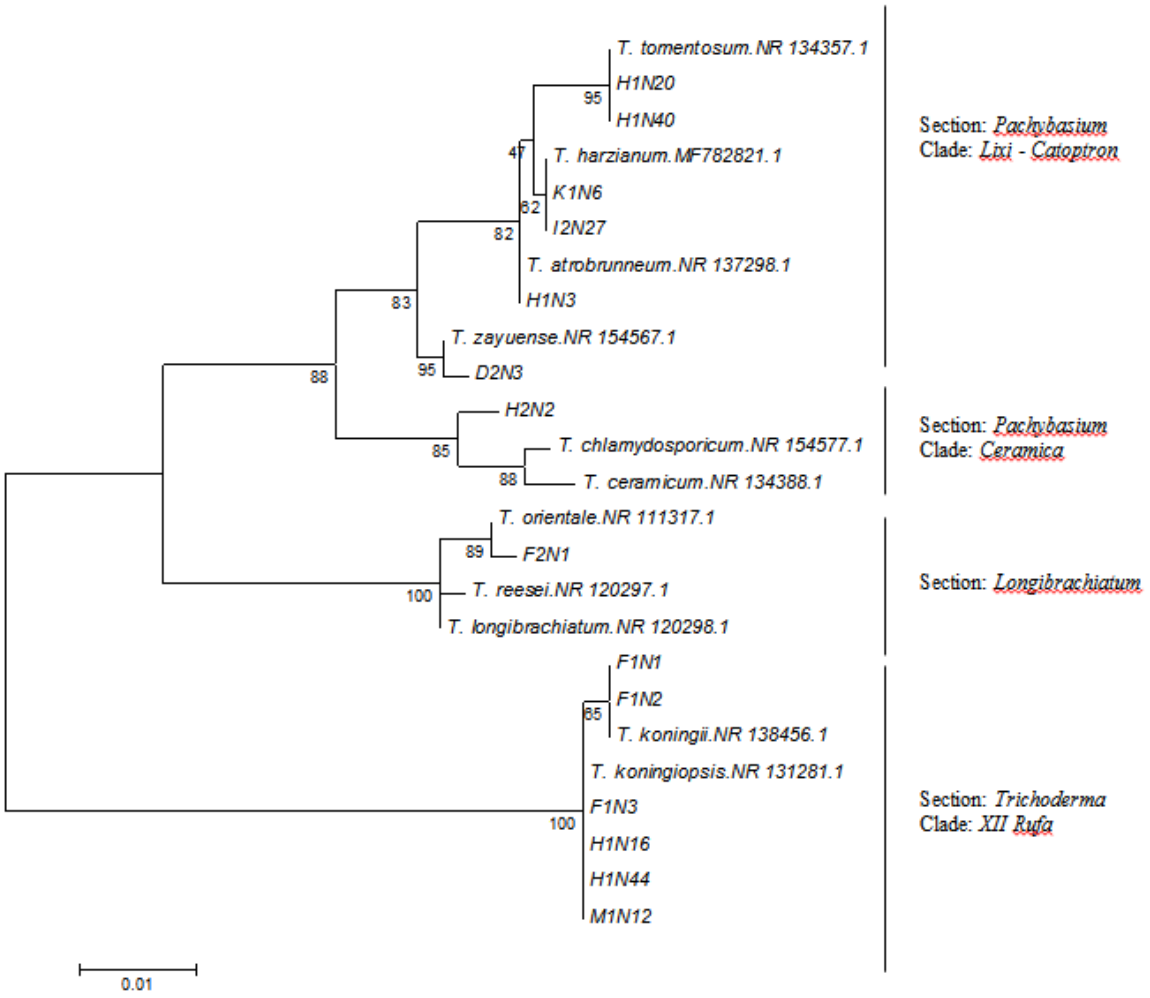

Fig. 1. Phylogenetic tree based on the ITS sequencing results for the 14 isolated species from this study and including 11 reference sequences obtained from GenBank that are marked with accession number next to the species name. *Trichoderma* species are classified as five sections (Bisset 1991a). *T. harzianum* complex and *T. tomentosum* belong to the section *Pachybasium*, clade *Lixi - Catoptron*. *T. orientale*, *T. reesei* and *T. longibrachiatum* are classified as section *Longibrachiatum*, *T. koningii* aggregate is placed in section *Trichoderma*, clade *XII Rufa*. *T. chlamydosporicum* and *T. ceramicum* belong to the section *Pachybasium*, clade *Ceramica* (Druzhinina and Kubicek 2005; Druzhinina et al. 2005).

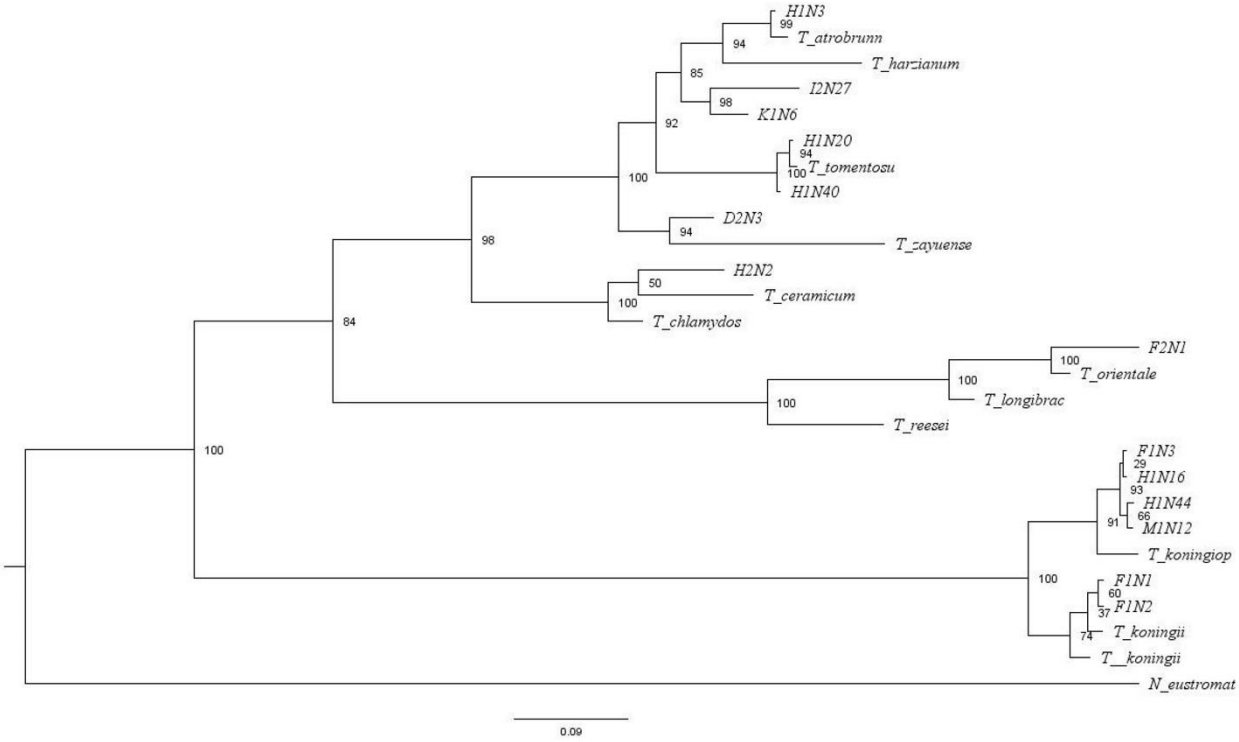

Fig. 2. Phylogenetic construction of a combined sequences including ITS, *TEF1* and *RPB2* gene fragments. The tree is rooted to *Nectria eustromatica.* Because of the limitation for SequenceMatrix which is important the names are no longer than 11 charactes, the names of some species are written incompletely. You can see their full names in the following: *T. atrobrunneum, T. tomentosum, T. zayuense, T. ceramicum, T. chlamydosporium, T. orientale, T. lognibrachatum, T. reesei, T. koningiopsis, T. koningii and N. eustromatica.*

Validation of the *Nla*III, *Msp*I and *Nla*IV restriction enzymes

Evaluating of the combination of the restriction enzymes for ITS-RFLP (Fig. 3) showed that the *Nla*III digestion divided the strains into 15 groups named A to O (Supple. Table.4). Groups A, C and D included many species, but group F contained only three and groups G, H and I only two species. Groups J to O included only one species. The groups obtained from *Nla*III analysis were divided into smaller groups based on the two other enzymes. First, the larger groups were broken down by the *Msp*I enzyme digestion pattern named A1 to H1 (Supple. Table. 5), and then the *Msp*I-derived groups were again divided into smaller groups by the *Nla*IV enzyme (Supple. Table. 6). Groups A1, C1, D1, D2, D3 and D4 of *Msp*I included many species, but groups B1, B2, B3, D5, D6, D7, E2, F1 and H1 are small groups with 4, 3 or 2 species, and nine groups (A2, C2, C3, D8, D9, E3, F2, G1 and G2) have only one species. Finally, the results of using the *Nla*IV enzyme divided the species into the smallest groups. According to the *Nla*IV results, only one group included more than 20 species, which is D1-1 and four groups have 11-20 species (C1-1, D1-2, D3-1 and D4-1). Other groups (17 groups) are small groups with less than 10 species and 32 groups have only one species. Therefore, *Nla*IV was able to separate 32 groups from other species by distinctive band patterns in combination with the other two enzymes (Table 3).

.

DISCUSSION

We used the ITS-RFLP method using a combination of only three restriction enzymes: *Msp*I, *Nla*III, and *Nla*IV, which grouped the 77 *Trichoderma* isolates into eight distinct groups. Using ITS-RFLP, Dean et al. (2005b) were able to distinguish the genera *Stachybotrys*, *Penicillium*, *Aspergillus* and *Cladosporium* at the species level by four restriction enzymes (*EcoRI*, *HaeIII*, *Msp*I, and *HinfI*). Koffi et al. (2019) used six endonucleases to identify isolates of filamentous fungi on pineapple fruits, and Diguta et al. (2011) used nine enzymes for complete discrimination of filamentous fungi isolated from grapes. Five enzymes were also used by Dupont et al. (2006) to distinguish 12 species of *Penicillium* from 65 isolates. Five enzymes were also used by Dupont et al. (2006) to distinguish 12 species of *Penicillium* from 65 isolates.

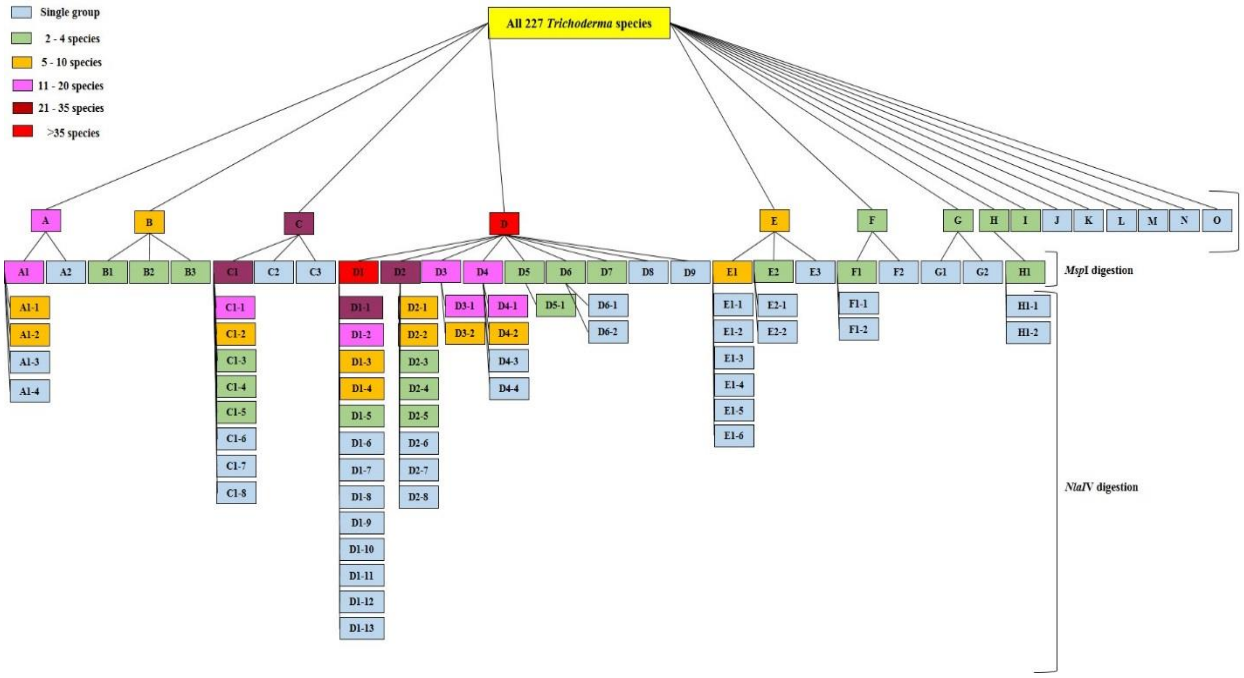

Fig.3. Dendrogram grouping 227 species of *Trichoderma* based on the ITS-RFLP results using restriction enzymes *Nla*III, *Msp*I and *Nla*IV. The first row of boxes (A to O) represents the groups obtained from cleavage with the *Nla*III enzyme. The second row of boxes represents the smaller groups by digestion patterns based on the *Msp*I enzyme (A1 to H1). Finally, the *Nla*IV enzyme cleavage patterns led to the final groups shown as A1-1 to H1-2. For example, the species in group A (yielded from grouping based on the cutting sites of the *Nla*III enzyme) were broken down to two sub-groups, A1 and A2, based on the cutting sites of the *Msp*I enzyme. Then A1 was broken further down into four sub-groups, A1-1 to A1-4, based on the cleavage patterns of *Nla*IV. The species in each group are listed in Suppl. Table. 4 to 6.

In another study, ITS-RFLP showed interspecies variation among 65 isolates of *Trichoderma* using the three restriction enzymes *Mob1*, *Taq1* and *Hinf1* (Kamala et al. 2015). Out of all 77 isolates, 14 strains were selected for sequencing (highlighted in bold script) representing species from all groups except group 5, which fell by ITS-RFLP into a single-species group (group D4-3 including only *T. neorufoides*). Comparison of the RFLP results showed that the isolates in groups 1 and 7 could not be differentiated by *Msp*I digestion but they were separated by *Nla*III and *Nla*IV digestion. Moreover, also the species in groups 2 and 3 and groups 4, 5, 6 and 8 were placed in a same group and not separated by *Msp*I while they were discriminated by *Nla*III. *Nla*IV was not able to separate the groups 2, 3, 7 and 8 as well as groups 4, 5 and 6 from each other, however it separated group 1 from all others. The *Nla*III-mediated RFLP turned out to be the best for discriminating the isolates and enabled to separate the groups 3 (*T. orientale*), 6 (*T. koningii*) and 7 (*T. tomentosum*) from the others as distinctive groups.

For evaluating the efficiency of the enzymes used in this study, an *in silico* analysis was performed on a high number of species. According to this, *Nla*III successfully could separate six species as individual groups from the others without needing any other enzyme. *Msp*I showed a higher degree of polymorphism compared to the *Nla*III in all 227 sequences and was also able to distinguish the species in small groups with only three, two or one species. Nine species can be discriminated as individual groups with *Nla*III and *Msp*I digestion without needing *Nla*IV. *Nla*IV was the best enzyme for the differentiation of the 227 species compared with *Nla*III and *Msp*I. *Nla*IV was more polymorphic than two others and able to distinguish 32 individual species. The results demonstrate that 65% (149 species) of the 227 *Trichoderma* ITS sequences obtained from the NCBI RefSeq database can be divided into small groups including one to ten species.

Table 3. The single groups and their banding patterns were obtained from grouping of 227 species using digestion by three restriction enzymes (*Nla*III, *Msp*I and *Nla*IV).

Species name	**Expected pattern of bands on agarose gel**		
	***Nla*III**	***Msp*I**	***Nla*IV**
T. gliocladium	115/155/225		
T. luteoeffusum	110/130/160/212		
T. placentula	180/ 240		
T. kunmingense	180/350		
T. gelatinosum	105/215		
T. stipitatum	60/245/310		
T. tsugarense	125/250/270	100/180/290	
T. tawa	110/370	100/280	
T. aurantioeffusum	110/370	60/100/300	
T. brunneoviride	250-260/350-380	65/100/210	
T. tremelloides	250-260/350-380	75/100/200	
T. sambuci	160/210/240-250	70/115/220	
T. alutaceum	70-100/210/240	100/170/270	
T. britannicum	85/165/215	100/280	
T. aerugineum	85/165/215	65/100/280	
T. tawa	110/370	100/280	
T. ghanense	125/250/270	60-70/100/ 260-280, 290	120/170/335
T. ampulliformis	125/250/270	60-70/100/ 260-280, 290	120/170/250
T. fertile	110/370	60-70/100/260-280, 290	130/170-180/280
T. albolutescens	110/370	60-70/100/260-280, 290	120/150/320
T. sulawesense	110/370	60-70/100/260-280, 290	70/100/140/250
T. minutisporum	250-260/350-380	60-70/ 100/ 260-280, 290	70/125/170/210
T. leucopus	250-260/350-380	60-70/ 100/ 260-280, 290	90/120/210
T. hongkongense	250-260/350-380	60-70/ 100/ 260-280, 290	135/170/300
T. megalocitrinum	250-260/350-380	60-70/ 100/ 260-280, 290	100/290
T. parmastoi	250-260/350-380	60-70/ 100/ 260-280, 290	80/120/290
T. pseudonigrovirens	250-260/350-380	60-70/ 100/ 260-280, 290	75/125/175/245
T. voglmayrii	250-260/350-380	60-70/ 100/ 260-280, 290	90/120/225
T. peltatum	250-260/350-380	60-70/ 100/ 260-280, 290	100/170/300
T. hirsutum	250-260/350-380	100/170-180/270-280	75/125/175/245
T. phyllostachydis	250-260/350-380	100/170-180/270-280	80/100/165/230
T. phellinicola	250-260/350-380	100/170-180/270-280	130/170-180/280
T. neorufoides	250-260/350-380	100/260-280	115/175/275
T. cinnamomeum	250-260/350-380	100/260-280	60/125/175/210
T. peltatum	250-260/350-380	60-70/ 100/ 260-280, 290	100/170/300
T. patellotropicum	250-260/350-380	80/100/320	120/150/320
T. patella	250-260/350-380	80/100/320	120/175/340
T. tropicosinense	160/210/240-250	65/100/280	125/170/240
T. rosulatum	160/210/240-250	65/100/280	125/170/260
T. danicum	160/210/240-250	65/100/280	90/200/250
T. spinulosum	160/210/240-250	65/100/280	90/125/245
T. luteocrystallinum	160/210/240-250	65/100/280	125/170/245
T. foliicola	160/210/240-250	65/100/280	75/130/170/210
T. compactum	160/210/240-250	100/280	80/125/420
T. catoptron	160/210/240-250	100/280	75/125/150/245
T. bavaricum	70-100/210/240	65/ 100/ 270	75/125/150/210
T. ceciliae	70-100/210/240	65/ 100/ 270	75/125//150/245
T. sinoluteum	70/210/ 240	65/ 100/ 275	75/130/170/210
T. pachypallidum	70/210/ 240	65/ 100/ 275	125/150/280

As mentioned, some single species can be discriminated by digestion with only one enzyme and some by combination of two or three enzymes. Six species can be seperated by only *Nla*III digestion, nine species by combination of *Nla*III and *Msp*I digestion, 10 species by only *Nla*IV digestion and 22 species by combination of all three enzymes (*Nla*III as the first enzyme, then *Msp*I as the second and *Nla*IV as the third). Out of 227 species, 60 species (26%) were placed in groups including 5-10 species by combination of the three enzymes and 42 species (18.5%) were placed in groups including 2-4 species using digestion by only one enzyme or a combination of two or three enzymes. Interestingly, 47 species (20% of 227 species) were differentiated as single groups. They also were comprised 50% of all groups. Despite its drawbacks, the ITS-RFLP method could therefore help in the identification process without the need for DNA sequencing for large number of *Trichoderma* isolates. Nevertheless, for absolute verification and particularly for those species falling into groups including many candidate species (even after digestion by the three enzymes), a molecular analysis by sequencing should be performed (Cai and Druzhinina 2021).

According to the restriction digestions of the 77 *Trichoderma* isolates, the isolates in groups 1, 2, 4, and 8 were placed in a group obtained from the *Nla*III digestion of all 227 species with a lot of candidate species (more than 100). But in the next steps, they separated into several smaller groups including less species and they were realised after sequencing.

According to the ITS-PCR sequencing results, the isolate H1N3 identified as *T. harzianum*. But, *TEF1*-PCR and *RPB2*-PCR sequencing results indicated that it is likely to be *T. atrobrunneum.* These results was coordinated with grouping suggested by the phylogenetic analysis which was separated *T. atrobrunneum* from *T. harzianum* in the trees based on the *TEF1*-PCR and *RPB2*-PCR also multigene phylogenetic analysis. The isolate in group 8 (H2N2) obtained from ITS-RFLP grouping of our 77 *Trichodrma* isolates was placed in group D1-4 (from in *silico* digestion of all 227 species) with seven species. This isolate could have corresponded to either *T. chlamydosporicum* or *T. ceramicum* based on the sequencing of the ITS region as well as multigene analysis and could also not clearly be identified based on the *TEF1* sequencing result due to lack of *TEF1* reference sequences of *T. chlamydosporicum* in the RefSeq database. However, the combination of ITS-RFLP results and *in silico* analysis favored the classification of this isolate as *T. chlamydosporicum*, since the banding pattern of H2N2 using *Nla*IV digestion was more similar to the banding pattern of the corresponding reference sequence (NR_154577.1). This suggestion was confirmed by the additional *RPB2* sequencing, and H2N2 was thus tentatively identified as *T. chlamydosporicum*. The two isolates in group 3 (F2N1 and F2N8) were placed in group I (obtained from digestion of 227 species) including only two species (*T. orientale, T. floccosum*). Sequencing also single and multigene phylogenetic analysis showed that the isolate F2N1 clustered close to the *T. orientale* reference. As already mentioned above, due to the RFLP digestion results of the two isolates in group 5 being indicative of RFLP group D4-3 with only one species, these isolates are likely *T. neorufoides* and were not further investigated by sequencing.

CONCLUSION

In conclusion, we used an ITS-RFLP method using the combination of three restriction enzymes for a simplified identification of *Trichoderma* isolates from a large isolation effort. By grouping the species and thereby reducing the number of candidates when a high number of isolates should be identified, it was able to facilitate downstream identification of new *Trichoderma* isolates in contrast to the time-consuming morphological and high cost of sequencing methods. For final clarification and in some ambiguous cases, a combination of several methods including the sequencing of at least three genetic loci is nevertheless necessary.

ACKNOWLEDGEMENTS

The authors would like to express special thanks to Prof. Dr. Philipp Benz (Technische Universität München) for his help with sequencing and critical reading of the manuscript. Furthermore, we would like to thank Sabrina Klasen (Technische Universität München) for her help in sequencing. This work was funded by the Ferdowsi University of Mashhad, Iran (Grant number 3/43048) and Iran National Science Foundation (INSF) (Grant number 95849817).

REFERENCES

Atanasova L, Le Crom S, Gruber S, Coulpier F, Seidl-Seiboth V, Kubicek CP, Druzhinina IS. 2013. Comparative transcriptomics reveals different strategies of *Trichoderma mycoparasitism*. BMC genomics. 14(1):1-5. https://doi.org/10.1186/1471-2164-14-121.

Bissett, J.; Gams, W.; Jaklitsch, W.; Samuels, G.J. Accepted *Trichoderma* names in the year 2015. IMA Fungus 2015, 6, 263–295.

Cai F, Druzhinina IS. 2021. In honor of John Bissett: authoritative guidelines on molecular identification of *Trichoderma*. Fungal Diversity. 107(1):1-69. https://doi.org/10.1007/s13225-020-00464-4.

Dean TR, Roop B, Betancourt D, Menetrez MY. 2005. A simple multiplex polymerase chain reaction assay for the identification of four environmentally relevant fungal contaminants. Journal of Microbiological Methods. 61(1):9-16. https://doi.org/10.1016/j.mimet.2004.10.015.

Diguta CF, Vincent B, Guilloux-Benatier M, Alexandre H, Rousseaux S. 2011. PCR ITS-RFLP: A useful method for identifying filamentous fungi isolates on grapes. Food Microbiology. 28(6)

:1145-54. https://doi.org/10.1016/j.fm.2011.03.006.

Doyle J. DNA protocols for plants. 1991. In Molecular techniques in taxonomy (pp. 283-293). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-83962-7_18.

Druzhinina IS, Chaverri P, Fallah P, Kubicek CP, and Samuels GJ. 2004. *Hypocrea flaviconidia*, a new species from Costa Rica with yellow conidia. Studies in Mycology. 50: 401-407.

Druzhinina IS, Kopchinskiy AG, Komoń M, Bissett J, Szakacs G, Kubicek CP. 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genetics and Biology. 42(10):813-28. https://doi.org/10.1016/j.fgb.2005.06.007.

Druzhinina I, Kubicek CP. 2005. Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters?. Journal of Zhejiang University. Science. B. 6(2):100. https://doi.org/10.1631/jzus.2005.B0100.

Druzhinina IS, Kopchinskiy AG, Kubicek CP. 2006. The first 100 Trichoderma species characterized by molecular data. Mycoscience. 47(2):55. https://doi.org/10.1007/S10267-006-0279-7.

Dupont J, Dennetière B, Jacquet C, Roquebert MF. 2006. PCR-RFLP of ITS rDNA for the rapid identification of *Penicillium* subgenus *Biverticillium* species. Revista iberoamericana de micología. 23(3):145-50. https://doi.org/10.1016/S1130-1406(06)70034-1.

Hagn A, Geue H, Pritsch K, Schloter M. 2002. Assessment of fungal diversity and community structure in agricultural used soils. Kerala, India: Research Signpost. 21:3-5.

Hoyos-Carvajal L, Bissett J. 2011. Biodiversity of *Trichoderma* in neotropics. The dynamical processes of biodiversity-case studies of evolution and spatial distribution. InTech. 303-20.

Kamala T, Devi SI, Sharma KC, Kennedy K. 2015. Phylogeny and taxonomical investigation of *Trichoderma* spp. from Indian region of Indo-Burma biodiversity hot spot region with special reference to Manipur. BioMed research international. 2015. https://doi.org/10.1155/2015/285261.

Koffi YF, Diguta CA, Alloue-Boraud MI, Koffi LB, Dje M, Gherghina EV, Matei F. 2019. PCR-ITS-RFLP identification of pineapple spoilage fungi [J]. Rom Biotechnol Lett. 24:418-24.

Kopchinskiy A, Komoń M, Kubicek CP, Druzhinina IS. 2005. TrichoBLAST: a multilocus database for *Trichoderma* and *Hypocrea* identifications. Mycological research. 109(6):658-60. https://doi.org/10.1017/S0953756205233397

Kubicek CP, and Harman GE, editors. 2002. *Trichoderma* and *gliocladium*: basic biology, taxonomy and genetics. Vol. 1. CRC Press.

Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, Danchin EG. 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nature biotechnology. 26(5):553-60. https://doi.org/10.1038/nbt1403.

Samuels GJ, Petrini O, Kuhls K, Lieckfeldt E, Kubicek CP. 1998. The Hypocrea schweinitzii complex and *Trichoderma* sect. *Longibrachiatum*. Centraalbureau voor Schimmelcultures.

Sivasithamparam K, Ghisalberti EL. 1998. Secondary metabolism in *Trichoderma* and *Gliocladium*. Basic biology 1: 139-191.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution. 28(10):2731-9. https://doi.org/10.1093/molbev/msr121.

White TJ, Bruns T, Lee SJ, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications. 18(1):315-22.

یک روش مولکولی کم هزینه جهت طبقه‌بندی مجموعه‌های بزرگ تریکودرما

سارا دهقان، امین میرشمسی کاخکی✉، محمد فارسی، علیرضا سیفی
گروه بیوتکنولوژی و به‌نژادی گیاهی، دانشکده کشاورزی، دانشگاه فردوسی مشهد، ایران

چکیده :برای شناسایی دقیق و قابل اعتماد گونه‌های قارچ تریکودرما استفاده از تکنیک‌های مولکولی کاملا ضروری است. با این که تعیین توالی همزمان مکانهای ژنومی ITS، *TEF1* و *RPB2* برای این منظور توصیه می‌شود، با این حال زمانی که قرار است جدایه‌های زیادی مورد تجزیه و تحلیل قرار گیرند، این کار بسیار پر هزینه خواهد بود. از طرف دیگر، ITS-RFLP یک روش ساده و مقرون به صرفه برای تمایز بین تعداد زیادی از گونه های تریکودرما است. در مطالعه حاضر با استفاده از تکنیک ITS-RFLP و ترکیب سه آنزیم محدودکننده (*Msp*I, *Nla*III و *Nla*IV) تعداد ۷۷ سویه تریکودرما مورد مطالعه به ۸ گروه طبقه‌بندی و از این میان، ۱۴ سویه برای تایید کارایی روش پیشنهادی، برای مکان های ژنومی ITS، *TEF1* و *RPB2* موردتوالی یابی قرار گرفتند. به‌منظور ارزیابی ترکیب سه آنزیم محدود کننده استفاده شده در این مطالعه، ۲۲۷ توالی ITS گونه‌های تریکودرما از پایگاه داده RefSeq پایگاه NCBI مورد آنالیز قرار گرفتند. بر این اساس، حدود ۸۰ درصد از توالی های تریکودرمای مورد آزمایش به گروه های کوچک‌تر از ۱۰ گونه طبقه بندی و امکان یک پیش طبقه بندی کم هزینه فراهم گردید. نتایج این مطالعه نشان داد که این روش و آنزیم های مورد استفاده می تواند تمایز دقیق تعداد زیادی از گونه های قارچ تریکودرما را با هزینه کم تسهیل نماید. بطور کلی، روش ITS-RFLP با استفاده از ترکیب سه آنزیم محدود کننده می تواند به عنوان یک روش کم هزینه، کارآمد و قابل اعتماد برای شناسایی تعداد بالای جدایه‌های تریکودرما معرفی می‌شود.

کلمات کلیدی: ITS-RFLP، تریکودرما، طبقه‌بندی، کم‌هزینه

مکاتبه کننده: امین میرشمسی کاخکی Email: mirshamsi@um.ac.ir
تاریخ دریافت: ۱۴۰۰/۵/۲۵ تاریخ پذیرش: ۱۴۰۰/۸/۲