Genetic diversity of *Trichoderma atroviride* strains collected in Poland and identification of loci useful in detection of within-species diversity

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**Abstract** Molecular markers that enable monitoring of fungi in their natural environment or assist in the identification of specific strains would facilitate *Trichoderma* utilization, particularly as an agricultural biocontrol agent (BCA). In this study, sequence analysis of internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) of the ribosomal RNA (rRNA) gene cluster, a fragment of the translation elongation factor 1-alpha (*tefl*) gene, and random amplified polymorphic DNA (RAPD) markers were applied to determine the genetic diversity of *Trichoderma atroviride* strains collected in Poland, and also in order to identify loci and PCR-based molecular markers useful in genetic variation assessment of that fungus. Although *tefl* and RAPD analysis showed limited genetic diversity among *T. atroviride* strains collected in Poland, it was possible to distinguish major groups that clustered most of the analyzed strains. Polymorphic RAPD amplicons were cloned and sequenced, yielding sequences representing 13 *T. atroviride* loci. Based on these sequences, a set of PCR-based markers specific to *T. atroviride* was developed and examined. Three cleaved amplified polymorphic sequence (CAPS) markers could assist in distinguishing *T. atroviride* strains. The genomic regions identified may be useful for further exploration and development of more precise markers suitable for *T. atroviride* identification and monitoring, especially in environmental samples.

**Introduction**

*Trichoderma* is a fungal genus that includes more than 200 species that occur all over the world in different geographical regions and climatic zones (Atanasova et al. 2013; Hoyos-Carvajal et al. 2009; Kredics et al. 2014). They are remarkable for their rapid growth under various environmental conditions, as well as their ability to use different substrates as carbon sources (Kubicek et al. 2003; Atanasova and Druzhinina 2010); thus, they are recognized as efficient decomposers of woody and herbal materials and other organic matter (Atanasova et al. 2013; Schuster and Schmoll 2010).

*Trichoderma* interacts with other microorganisms and plants in diverse ways (Harman et al. 2004; Lorito et al. 2010; Druzhinina et al. 2011; Woo et al. 2006). *Trichoderma* strains, including *Trichoderma atroviride* strains, that are promoting efficient plant growth and stress resistance are used as biological control agents (BCAs) in sustainable farming systems (Chet and Inbar 1994; Benítez et al. 2004; Stewart and Hill 2014).

Various molecular techniques have been used in *Trichoderma* research, in order to investigate genetic diversity within the genus. These include restriction fragment length polymorphism (RFLP) analysis (Dodd et al. 2004a), random amplified polymorphic DNA (RAPD) analysis (Hermosa et al. 2001), amplified fragment length polymorphism (AFLP) analysis (Buhariwalla et al. 2005), sequence-characterized amplified region (SCAR) analysis (Hermosa...
et al. 2001; Dodd et al. 2004b; Cordier et al. 2007), microsatellite markers (Naef et al. 2006), and sequence analysis (Kindermann et al. 1998; Dodd et al. 2003; Druzhinina et al. 2005; Blaszczzyk et al. 2011; Atanasova et al. 2013). One of the simplest and cheapest techniques is RAPD, which is characterized by low repeatability and transferability between different laboratories. Nevertheless, it delivers solid data at minimal expense when used strictly in a single laboratory. Moreover, RAPD markers can be easily transformed into PCR-based SCAR markers, as demonstrated in several studies for *T. atroviride* (Hermosa et al. 2001; Cordier et al. 2007; Feng et al. 2011).

Application of molecular techniques in *Trichoderma* taxonomy solved inaccuracies related to phenotypical *T. atroviride* species identification, as well as demonstrated interspecies genetic variability. In order to reevaluate biocontrol strains reported as *Trichoderma harzianum* and *Trichoderma viride*, Hermosa et al. (2000) utilized sequences of internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) of the ribosomal RNA (rRNA) gene cluster and analyzed the hybridization patterns of mitochondrial DNA. Authors identified four groups of *Trichoderma* including *T. atroviride*. Identification of mycoparasitic strains performed with assistance of ITS1, ITS2, mtSSUrDNA, and partial chi18-5 gene sequences revealed that the strains belonged to *T. harzianum*, *T. atroviride*, and *Trichoderma asperellum* (Kullnig et al. 2001). Based on the sequences of ITS1, ITS2, and translation elongation factor 1-alpha (tef1) gene fragments, four major clades (A, B, C, and D) of *T. atroviride* were distinguished, and the fifth clade E was suggested (Dodd et al. 2003; Jaklitsch et al. 2006; Samuels et al. 2006; Mulaw et al. 2010; Gal-Hemed et al. 2011).

Application of *Trichoderma* BCA strains raised questions about fungal survival and spread in the environment (Dodd et al. 2004a, b). The molecular strategies were applied to develop markers useful for biocontrol monitoring at different levels: genus-specific (Hagn et al. 2007; Devi et al. 2011; Chakraborty et al. 2011; Friedl and Druzhinina 2012), species-specific (Chen et al. 1999; Kredics et al. 2009; Miyazaki et al. 2009; Friedl and Druzhinina 2012) and strain-specific (Hermosa et al. 2001; Rubio et al. 2005; Cordier et al. 2007; Savazzini et al. 2008). Strain-specific molecular markers proposed for *Trichoderma* BCA strain monitoring included RAPD, RFLP (Zimand et al. 1994; Bowen et al. 1996; Abbasi et al. 1999), and SCAR markers (Hermosa et al. 2001; Dodd et al. 2004b; Rubio et al. 2005; Cordier et al. 2007; Savazzini et al. 2008; Longa et al. 2009; Feng et al. 2011; Nacini et al. 2011). Due to the complex taxonomy of *Trichoderma*, development of strain-specific markers is a long and laborious process (Druzhinina and Kubicek 2005). The most beneficial is development of species-specific markers, which can also be used to simplify and assist in taxonomic identification, based on sequence differences at the interspecies level, useful for elaboration of such markers (Chen et al. 1999; Friedl and Druzhinina 2012; Kredics et al. 2009; Miyazaki et al. 2009).

The aim of this study was to utilize sequences of ITS1 and ITS2 region, a fragment of the tef1 gene, and RAPD markers to analyze the genetic diversity of *T. atroviride* strains collected in Poland, develop PCR-based markers, and identify genomic regions to enable genetic diversity studies and further development of reliable methods of *T. atroviride* identification. In this study, RAPD markers were converted into PCR-based markers suitable for detection of *T. atroviride*. Additionally, application of CAPS markers developed in this study is expected to improve the accuracy of *T. atroviride* classification.

### Materials and methods

**Fungal material and strain identification**

All *Trichoderma* strains used in this study were obtained from a stock culture collection maintained by the Microbiology Laboratory at the Research Institute of Horticulture in Skierniewice, Poland. Stock cultures were deep-frozen in glycerol and stored at −80 °C. Forty strains of *T. atroviride* were used in this study; these included 38 strains of *T. atroviride* collected at different locations in Poland, and the two reference strains CBS 693.94 (Dodd et al. 2003) and MIM206040 (Kubicek et al. 2011) (Table 1). Strain identification was based on the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) of the rRNA gene cluster sequences and *TrichO*Key v. 2.0 (Druzhinina et al. 2005) and sequences of the fragment of the gene encoding translation elongation factor 1-alpha (tef1) and *TrichoBLAST* v. 1.0 analysis (Kopchinskiy et al. 2005). All sequences were obtained by PCR and direct amplicon sequencing from both directions. ITS1 and ITS2 regions were PCR-amplified with ITS6 and ITS4 primers (Cooke and Duncan 1997; White et al. 1990). For the tef1 sequencing, primers EFl-728 F and TEFLLerrev were used (Carbone and Kohn 1999; Jaklitsch et al. 2005). ITS and tef1 sequences of the strains are available in the NCBI GenBank (Accession numbers are provided in Table 1). The following sequences were used for the PCR-based marker species specificity evaluation: *Trichoderma aggressivum* f. aggressivum CBS 100528, *T. aggressivum* f. europaeum CBS 100526, *T. asperellum* TRS705, *Trichoderma citrinoviride* TRS119, *Trichoderma cerinum* 38.24.06.2, *Trichoderma crassum* TRS113, *Trichoderma gamsii* TRS123, *Trichoderma hamatum* TRS127, *T. harzianum* sensu lato II subclade CBS 466.94, *T. harzianum* s. l. III subclade TRS60, *T. harzianum* s. l. X subclade CBS 115901, *T. harzianum* s. l. Lixii subclade TRS66, *T. harzianum* sensu stricto TRS71, *Trichoderma longibrachiatum* TRS708,
Trichoderma pleuroticola TRS120, Trichoderma spirale TRS111, Trichoderma tomentosum TRS82, Trichoderma virens Gv29-8, Trichoderma viride TRS575, Trichoderma viridescens TRS35, and Trichoderma velutinum 29.24.06.1. For all above mentioned strains, species/subclade affiliation was confirmed by ITS and tef1 sequencing as described above.

Table 1  Source and origin of *T. atroviride* strains used in this study

| Strain | GenBank accession number of sequences | Source | Location | Origin |
|--------|--------------------------------------|--------|----------|--------|
|        | ITS                                  |        |          |        |
| TRS31  | KJ786740 KJ786821                    | Soil   | Balcerów | Central Poland |
| TRS15  | KJ786724 KJ786805                    | Lumberyard | Głuchów |        |
| TRS36  | KJ786729 KJ786810                    | Soil   | Kątne    |        |
| TRS28  | KJ786721 KJ786802                    | Production hall | Kolonia Bolimowska |        |
| TRS5   | KJ786741 KJ786822                    | Compost | Łódź     |        |
| TRS2   | KJ786749 KJ786830                    | Compost | Łódź     |        |
| TRS1   | KJ786739 KJ786820                    | Mushroom growing hall | Maków |        |
| TRS3   | KJ786743 KJ786824                    | Mushroom growing hall | Maków |        |
| TRS9   | KJ786738 KJ786819                    | Mushroom growing hall | Maków |        |
| TRS18  | KJ786757 KJ786839                    | Compost | Maków    |        |
| TRS32  | KJ786744 KJ786825                    | Production hall | Maków |        |
| TRS45  | KJ786718 KJ786799                    | Compost | Maków    |        |
| TRS20a | KJ786731 KJ786811                    | Production hall | Sierakowice Lewe |        |
| TRS30  | KJ786720 KJ786801                    | Soil    | Skiermiewice |        |
| TRS14a | KJ786725 KJ786806                    | Compost | Skiermiewice |        |
| 35.24.06.4a | KJ786742 KJ786823 | Field soil | Skiermiewice |        |
| TRS68  | KJ786745 KJ786826                    | Mushroom growing hall | Skiermiewice |        |
| TRS17  | KJ786734 KJ786815                    | Mushroom growing hall | Skiermiewice |        |
| TRS21  | KJ786727 KJ786808                    | Soil    | Topoloowo |        |
| TRS22  | KJ786726 KJ786807                    | Soil    | Wiskitki  |        |
| TRS24  | KJ786722 KJ786803                    | Production hall | Iganie Nowe | Eastern Poland |
| TRS25a | KJ786731 KJ786812                    | Production hall | Iganie Nowe |        |
| TRS38  | KJ786733 KJ786814                    | Soil    | Myslibórz | Northern Poland |
| TRS39  | KJ786728 KJ786809                    | Soil    | Myslibórz | Northern Poland |
| TRS42  | KJ786752 KJ786833                    | Soil    | Radzanów  |        |
| TRS7a  | KJ786747 KJ786828                    | Production hall | Bieruń | Southern Poland |
| TRS40  | KJ786755 KJ786836                    | Compost | Rzeczyce  |        |
| TRS26  | KJ786751 KJ786832                    | Soil    | Lipniak   | Western Poland |
| TRS6   | KJ786750 KJ786831                    | Compost | Sława    |        |
| TRS10  | KJ786754 KJ786835                    | Not known | Turek |        |
| TRS43a | KJ786748 KJ786829                    | Forest soil | Wielkopolski Park Narodowy |        |
| TRS11  | KJ786735 KJ786816                    | Not known | Wrocław |        |
| TRS12a | KJ786737 KJ786818                    | Not known | Wrocław |        |
| TRS13  | KJ786736 KJ786817                    | Not known | Wrocław |        |
| TRS16  | KJ786753 KJ786834                    | Not known | Not known | Poland |
| TRS19  | KJ786732 KJ786813                    | Soil    | Not known | Poland |
| TRS34  | KJ786719 KJ786800                    | Not known | Not known |        |
| TRS44  | KJ786756 KJ786837                    | Not known | Not known |        |
| CBS 693.94 (IMI 359825) | AF359263 KJ786838 | Mushroom compost | *T. atroviride* reference straina | Northern Ireland |
| IMI206040 | AF278795.1 KJ786838 | Biocontrol strain | *T. atroviride* reference strainb | USA |

a Strains used in RAPD primer screening part of study
b Not included in main genetic diversity study
c Dodd et al. (2003)
d Kubicek et al. (2011)
for *T. atroviride*. Mycelia taken from stock cultures were placed on potato dextrose agar (PDA) plates (Sigma-Aldrich, St. Louis, MO, USA) and cultured in the dark at 25 °C for 4–7 days. Mycelia grown on PDA were then used to inoculate 250-mL flasks containing 50 mL potato dextrose broth (Sigma-Aldrich), and cultures were incubated in the dark on a rotary shaker at 200 rpm for 7 days at room temperature (21±2 °C).

**DNA extraction**

Total fungal DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions with slight modifications. Fungal cells of tested *Trichoderma* strains were harvested by centrifugation of liquid cultures and dried on filter paper at room temperature (21±2 °C), prior to grinding in liquid nitrogen with quartz sand (Sigma-Aldrich). Proteinase K and RNase A were added to the lysis buffer. The purity and quantity of isolated DNA were evaluated by agarose gel electrophoresis followed by ethidium bromide staining, and by determination of A<sub>260</sub>/A<sub>280</sub> using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Finally, the DNA concentration was adjusted to 1 ng/μL by diluting the samples with 10 mmol/L Tris-HCl, pH 8.0.

**RAPD analysis**

A set of 520 decamer primers (primer kits OPA to OPZ from Operon Technology, Alameda, CA, USA) was used for initial RAPD screening of eight strains of *T. atroviride*: 35.24.06.4, TRS7, TRS12, TRS14, TRS20, TRS25, TRS40, and TRS43. Then, 68 primers that had given the best results were further tested on 39 strains to assess the genetic diversity of *T. atroviride* strains collected in Poland. RAPD-PCR was performed as described by Olczak-Woltman et al. (2009). Reactions contained 1× Green PCR Buffer containing 2 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L each dNTP, 1 μg bovine serum albumin, 0.5 U Taq polymerase (Fermentas, Vilnius, Lithuania), 1 μmol/L arbitrary primer, and 2 ng DNA template. PCR was carried out under conditions of initial incubation at 95 °C for 1 min, followed by 10 cycles of 95 °C for 5 s, 37 °C for 30 s, and 72 °C for 30 s; 35 cycles of 95 °C for 3 s, 37 °C for 30 s, and 72 °C for 60 s; and a final extension at 72 °C for 7 min. RAPD-PCR was carried out in a PTC-200 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Amplified DNA fragments were separated by electrophoresis with 2 % agarose–0.5× Tris-borate-EDTA (TBE) gels containing ethidium bromide and visualized under UV light. Gel images were edited in CorelDRAW version 12 (Corel Corporation, Ottawa, Ontario, Canada). The binary matrices were constructed based on evaluation of clear RAPD amplicons. Amplicons were scored for presence (1) or absence (0) for each strain. Cluster analysis of the binary data was performed using the NTSYS-pc v.2.1 program (Exeter Software, Setauket, NY, USA). Similarity matrices were generated using Jaccard’s coefficients, and an unweighted pair-group method using arithmetic averages (UPGMA) was chosen to generate the dendrogram from RAPD similarity matrices. To determine how faithfully the dendrogram preserves pairwise distances between original unmolded data, the cophenetic correlation coefficient parameter (*r*) was calculated.

**Cloning and sequencing**

Polymorphic RAPD amplicons were excised from the gel, and DNA was purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer’s protocol. Next, purified amplicons were reamplified under the conditions described for RAPD-PCR, resolved using a 1 % agarose–0.5× TBE gel, excised from the gel, and purified. Efficiently reamplified RAPD amplicons were cloned using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Recombinant plasmids were isolated, and two independent plasmids were used for sequencing of each amplicon (with the exception of Q01-420, for which only one clone was available) using universal M13 forward and reverse primers. The Sequencher 5.1 program (Gene Codes Corporation, Ann Arbor, MI, USA) was used to assemble single reads to obtain consensus sequences in FASTA format. The consensus sequences were deposited at the National Center for Biotechnology Information (NCBI), and GenBank accession numbers are summarized in Table 2 (RAPD-PCR amplicons) and Table 3 (CAPS amplicons).

**Bioinformatics analysis**

For phylogenetic reconstruction, sequences of the fragment of *tef1* gene were assembled with ClustalX (Thompson et al. 1997) and manually edited in CLC Genomic Workbench 7.5 (CLCBio, Aarhus, Denmark), and final block alignment was prepared with a less stringent selection option using GBlocksServer (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). The unconstrained GTR+I+G substitution model, estimated nucleotide frequencies, and substitution values were selected according to Samuels et al. (2006). Metropolis-coupled Markov chain Monte Carlo (MCMC) analysis was performed with two runs for one million generations with four chains, with the heating coefficient λ=0.1 with MrBayes 3.2.2×64 (Ronquist et al. 2012).

RAPD amplicon sequences were compared using basic local alignment search tool (BLAST) (Altschul et al. 1997) with publicly available sequences deposited at both NCBI and the Genome Portal of the Joint Genome Institute (JGI, Grigoriev et al. 2012). At NCBI, blastn and blastx were used
to explore the non-redundant protein sequence, nucleotide collection, and high-throughput genomic sequence databases. At JGI, sequences were aligned using blastn to the T. atroviride IMI206040 genome version 2 unmasked assemblies. Proteins containing amino acid sequences similar to those encoded by RAPD amplicons were collected and further analyzed using CLC Genomics Workbench 7.5 (CLCBio), and Pfam database search (Punta et al. 2012). RAPD sequences were scanned for sequence repeats using RepeatMasker (http://www.repeatmasker.org/) and mfold (Zuker 2003).

Cleaved amplified polymorphic sequence marker development

The consensus RAPD sequences were mapped on the T. atroviride IMI 206040 v. 2 genome sequence deposited at JGI (http://genome.jgi-psf.org/Triat2/Triat2.home.html).

### Table 2

| RAPD-PCR amplicon | NCBI GenBank Acc. No. | Scaffold | e-value | Similarity | e-value | Protein NCBI BLAST hits (non-redundant database) | Pfam domains (database v. 27) |
|-------------------|-----------------------|---------|---------|------------|---------|-----------------------------------------------|-------------------------------|
| A10-933           | KF364331 contig_23    | 0.0     | 9.13E-8 | NS         | NA      | Not found                                     |                              |
| B07-500           | KF364332 contig_29    | 0.0     | 1.17E-132 | Hypothetical protein | 7e-42   | Not found                                     |                              |
| E03-391           | KF364333 contig_28    | 0.0     | NA      | NS         | NA      | Not found                                     |                              |
| E04-458           | KF364334 contig_27    | 0.0     | NA      | NA         | NA      | Not found                                     |                              |
| H08-917           | KF364335 contig_16    | 0.0     | 2.54E-12 | Tetratricopeptide (TRP) protein | 8e-116 | Tetratricopeptide repeat, PF13414 and PF07719 |
| M06-768           | KF364336 contig_22    | 0.0     | 9.13E-176 | NS         | NA      | Not found                                     |                              |
| Q01-420           | KF364337 contig_26    | 0.0     | NA      | NS         | NA      | Not found                                     |                              |
| Q01-885           | KF364338 contig_24    | 0.0     | NA      | NA         | NA      | Not found                                     |                              |
| R08-1283          | KF364339 contig_29    | 5.06E-53 | 2.31E-18 | Putative ankyrin repeat-containing protein | 1e-175 | Ankyrin repeats (3 copies) PF12796 |
| T07-429           | KF364340 contig_27    | 0.0     | 6.01E-157 | Putative ankyrin repeat-containing protein | 7e-429 | Not found                                     |                              |
| X16-1030          | KF364341 contig_14    | 0.0     | 2.72E-173 | Hypothetical protein | 3e-142 | Magnesium transporter NIPA PF05653 |
| X18-634           | KF364342 contig_23    | 0.0     | 2.99E-173 | Hypothetical protein | 3e-35  | Not found                                     |                              |
| Z04-873           | KF364343 contig_20    | 0.0     | 2.99E-123 | Hypothetical protein | 6e-54  | Not found                                     |                              |

NS no significant similarity, NA not applicable

### Table 3

| CAPS amplicon | Primer names and sequencesa (5′-3′) | Amplicon length (bp)b | Optimized PCR conditions (annealing temperature/number of cycles) | Restriction enzyme | GenBank Accession number/strain |
|---------------|-------------------------------------|-----------------------|---------------------------------------------------------------|-------------------|--------------------------------|
| Q01-4         | Q01_4F-GCACACCAACTGCTGGAGCTTTQ01_4R-CACGCTGACAATGACCACGAC | 1017                  | 66 °C/27 cycles                                              | BstI              | KF576213/TRS15/KF576210/TRS22/KF576211/TRS40 |
| X18-35        | X18_3F-AGGCACAGTCCCCCCTTTTAGTX18_5R-TGACGATCTGGAAGGTTTTG | 358                   | 65 °C/35 cycles                                              | TaqI              | KF576212/TRS36/KF576213/TRS42 |
| Z04-2         | Z04_2F-TTACCCAGTGGCAGATCCTCCACATTGZ04_2R-TATACGGGCCTCCACATTG | 1450                  | 66 °C/27 cycles                                              | DraI              | KF576214/CBS 693.94/KF576215/TRS45 |

CAPS cleaved amplified polymorphic sequence

a Sequences of PCR amplicons of representative strains were deposited at NCBI

b Amplicon length is based on genome sequence of T. atroviride reference strain IMI20604
Several sets of primers were designed to amplify RAPD regions or extended RAPD regions based on genome sequence. A standard PCR was performed using Taq polymerase (Fermentas) according to the manufacturer’s instructions. PCR was carried out under conditions of initial incubation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, optimized annealing temperature or gradient temperature for 30 s, and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The amplified products for selected T. atroviride strains were analyzed on agarose gels, purified, and sequenced. Derived sequences were compared and searched for polymorphic restriction sites using Sequencher 5.1 (Gene Codes). PCR amplification was performed using selected primer pairs. PCR products were digested with corresponding restriction enzyme (BsuI, DraI, and TaqI; all from Fermentas) according to the manufacturer’s instructions, and digestion products were separated on 1.5 % agarose–0.5× TBE gel. The respective primer pairs with optimized PCR conditions and corresponding restriction enzymes are shown in Table 3. To confirm specificity of the CAPS PCR to T. atroviride species, multiplex PCR was performed with DNA of 19 Trichoderma species mentioned above, CAPS primers, and additional primers 5.8S-R and LR6 (Vilgalys and Hester 1990). The pair of primers 5.8S-R and LR6 amplified a 1.45-kb fragment of the ITS region and served as a positive control for the PCR.

Results and discussion

Molecular identification of strains based on sequences of ITS1, ITS2, and the fragment of tef1 gene confirmed that all of the strains used in this study belong to T. atroviride. Based on tef1 sequences, all strains were classified to clade A described by Dodd et al. (2003), except for TRS18 that did not fit any clade. TrichOKEY 2 analysis revealed one new ITS1 sequence variant that possesses a single nucleotide change in comparison to the IMI206040 sequence. Six strains, TRS15, TRS24, TRS28, TRS30, TRS34, and TRS45, were characterized by the new ITS1 variant. Seven variants of tef1 sequence which differed at least by a single nucleotide were found within the studied T. atroviride strains. Bayesian phylogeny analysis of tef1 sequences allowed strain clustering (Fig. 1). TRS18 strain was found to be the most distant one. Four groups of strains were distinguished, with the two biggest groups differing within by a single nucleotide change only. Strains TRS43 and CBS 693.94 did not fall into any groups (Fig. 1).

In addition, RAPD markers were used for further evaluation of genetic diversity of T. atroviride strains. A total of 520 primers were tested on eight T. atroviride strains, including 35.24.06.4, TRS7, TRS12, TRS14, TRS20, TRS25, TRS40, and TRS43, in order to select RAPD primers useful for analysis. Amplicons were obtained for 482 primers, with an average of eight amplicons of different sizes identified for each primer. Based on electrophoretic evaluation of amplification pattern quality, 68 primers that produced polymorphic amplicons were chosen for the assessment of genetic diversity of the T. atroviride strains. RAPD PCR was performed with selected primers on 38 strains of T. atroviride collected at different locations in Poland (Table 1). As a control, T. atroviride reference strains CBS 693.94 (Dodd et al. 2003) and IMI 206040 (Kubicek et al. 2011) were used. High-quality amplification products were obtained for 55 of the 68 RAPD primers. Only two primers (OPK11 and OPX09) did not show any polymorphisms among tested strains. The greatest number of polymorphic amplicons distinguishing groups of strains was obtained with primer OPA10. Amplification profiles of three primers, OP18, OPQ01, and OPZ04, showed that the T. atroviride strains, with the exception of strain TRS18, can be classified into two main groups. The amplification profile generated using OPZ04 is shown in Fig. 2.

RAPD amplicons were scored for presence and absence, and 391 amplicons differentiating tested strains were used to construct a binary matrix, and then a dendrogram illustrating genetic diversity (Fig. 3). The cophenetic correlation coefficient (r) for the dendrogram was 0.979, indicating a good fit between the unmodeled data and the dendrogram. Tested T. atroviride strains, of which all except TRS18 belonged to clade A, were classified into two main groups. The first group was represented by the reference strain IMI206040, as well as 28 of the tested strains, and corresponds to the two biggest groups of strains including TRS43, distinguished based on tef1 sequence. The second group contained reference strain CBS 693.94 and the following eight tested strains: TRS2, TRS6, TRS10, TRS16, TRS26, TRS40, TRS42, and TRS44. This group corresponds to CBS 693.94 and remaining two small groups of strains distinguished based on tef1. The observed variation among strains within the two major groups was rather limited; however, some subgroups could be distinguished (Fig. 3). T. atroviride strain TRS18 was clearly distinct from both groups of strains, based on both tef1 and RAPD analysis, and also did not fit any previously described clades. TRS18 tef1 intron 4 sequence BLAST similarity search with non-redundant nucleotide sequence database at NCBI revealed 95 % identity with any sequence deposited in GenBank. This suggests that TRS18 strain is characterized by a unique and distinct genotype that could represent new clade of T. atroviride.

Twenty polymorphic RAPD markers were chosen for cloning, and 13 RAPD-PCR products were successfully cloned and sequenced (Table 2). Resulting sequences were aligned with the T. atroviride IMI206040 genome at the JGI portal. Twelve RAPD sequences were identical to corresponding T. atroviride IMI206040 sequences (e-value=0). Sequence
R08-1283 showed some variation, but the e-value was very low, indicating strong similarity to the *T. atroviride* genome (Table 2). Seven sequences showed strong similarity to hypothetical proteins of *T. atroviride* deposited in the NCBI database, and Pfam protein domains were clearly identified for three of these (Table 2). The H08-917 sequence showed a high degree of similarity to tetratricopeptide repeat (TPR)-containing proteins. TPR motifs are short amino acid repeats that occur in many proteins and not only are responsible for interactions between proteins but also facilitate protein complex formation and protein transport within cells. TPR-containing proteins play a role in protein assembly and therefore have a significant impact on the cell cycle and a plethora of cellular processes (D’Andrea and Regan 2003). Interestingly, the H08-917 sequence also possessed short and degenerate DNA repeats that were identified by RepeatMasker and mfold. The R08-1283 and T07-429 sequences were recognized as proteins with ankyrin (ANK) repeats, which play a role in protein-protein interactions (Rubtsov and Lopina 2000). Moreover, the X16-1030 sequence was similar to the NIPA membrane protein, which functions as a magnesium transporter (Goytain et al. 2007, 2008).

Using the identified RAPD marker sequences and the *T. atroviride* genome sequence, pairs of specific primers were designed, followed by optimization of PCR conditions and testing of the primers on a set of *Trichoderma* strains belonging to different species, including *T. atroviride* (Table 3). Unique PCR amplification products for three pairs of primers, Q01-4, X18-35, and Z04-2, were obtained for *T. atroviride* strains and not for 16 other *Trichoderma* species/clades (Fig. 4). In the case of X18-35, a very faint PCR amplicon was obtained for *T. gamsii*. These suggest that the amplicons Q01-4, X18-35, and Z04-2 are specific to *T. atroviride*. We
propose that pairs of primers Q01-4, Z18-35, and Z04-2 could be used for T. atroviride detection.

For selected T. atroviride strains representing two main groups of strains, amplicons Q01-4, X18-35, and Z04-2 were sequenced, and sequences were deposited in GenBank (Table 3). These sequences were compared, and polymorphic restriction sites were identified. Based on identified sequence differences (single nucleotide polymorphisms and insertions/}

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**Fig. 3** Dendrogram generated using Jaccard’s coefficient and the UPGMA clustering method. Binary matrices for T. atroviride strains were constructed based on evaluation of RAPD amplicons generated using 55 primers. Cluster analysis of the binary data was performed using NTSYS-pc 2.1 software. Similarity matrices were generated using Jaccard’s coefficient and an unweighted pair-group method using arithmetic averages (UPGMA) was used to generate the dendrogram.

**Fig. 4** Amplification of Q01-4, X18-35, and Z04-2 fragments in different Trichoderma species. M, DNA size markers; amplicon 5.8S-R-LR6, PCR positive control.
deletions), restriction enzymes were selected to digest PCR amplicons and develop CAPS markers useful for *T. atroviride* identification (Table 3). In the case of CAPS marker Q01-4 (Q01-4FR amplification product digested with *Bsl*I), a two-band pattern was obtained for 29 strains of *T. atroviride* belonging to group 1, and a single band corresponding to the non-restricted Q01-4 F-4R amplicon was obtained for group 2 strains and TRS18 (Fig. 5). This CAPS marker allows the distinction of strains belonging to *T. atroviride* group 1. For Z04-2 amplification products, *Dra*I digestion resulted in banding patterns reflecting the RAPD-based grouping. However, the products for strain TRS6 and TRS18 were digested and these strains were not classified to the group 1 as in the RAPD study (Fig. 5). We propose that analysis of both CAPS markers can be used to extend ITS/tef1-based *T. atroviride* identification and can assist in classification of strains within clade A into one of the two main groups of *T. atroviride*: group 1 (IMI206040-like), characterized by both Q01-4 and Z04-2 amplicons digested, and group 2 (CBS 693.60-like), characterized by both amplicons non-digested. In the case where Q01-4 amplicon is not digested and Z04-2 is digested, additional markers have to be developed to distinguish TRS6-like from TRS18-like strains. Developed CAPS markers have to be further tested on a larger number of strains that will confirm their usefulness in *T. atroviride* genetic studies. The variable RAPD loci found in this study and characterized at the sequence level may be useful in the development of more complex assays, such as multiplex PCR, real-time PCR, or next-generation sequencing (NGS)-based assays, that will be valuable in identification and monitoring of *T. atroviride* strains, as well as in genetic diversity studies including metagenomics approaches and further *T. atroviride* pan-genome understanding.

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