The most widely used fungal antagonist for plant disease management in India, *Trichoderma viride* is *Trichoderma asperellum* as confirmed by oligonucleotide barcode and morphological characters

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Trichoderma asperellum **was described as a new species in 1999 for the isolates that produce finely warted conidia, with temperature optima of 30°C and survive by producing chlamydospores, differentiating them from** ***Trichoderma viride*** **that produces conspicuously warted conidia, with low temperature optima (20°C) and with no record of chlamydospores production. However, use of the taxonomic name** ***T. viride*** **is still being continued for all the isolates with warted conidia in many countries, including India. Thirty isolates identified earlier as** ***T. viride*** **were selected and characterized using morphological characters and their identification was confirmed by oligonucleotide barcode that employed amplification of ITS and** ***tef1*** **regions. All the isolates were confirmed to be** ***T. asperellum*****. Analysis using sequence polymorphism-derived markers not only confirmed their identity as** ***T. asperellum*****, but also showed new patterns among Indian isolates and presence of cryptic species** ***T. asperelloides*****. Analysis of sequences submitted from India to GenBank as** ***T. viride*** **or** ***T. asperellum*** **using barcode also showed that they belong to** ***T. asperellum*** **and not to true** ***T. viride*****. We confirm that the most widely used fungal antagonist in India,** ***T. viride*** **is actually** ***T. asperellum*** **or its cryptic species** ***T. asperelloides*****.**

Keywords: Finely warted conidia, Indian isolates, *Trichoderma asperellum*, *Trichoderma viride*, warmer regions.

AMONG the fungal biocontrol agents, the genus *Trichoderma* has been the most explored and commercially utilized one. *Trichoderma* is used in different crops like rice, wheat, pulses (pigeonpea, chickpea, cowpea, black gram, green gram), vegetables (tomato, brinjal, beans, chillies, cabbage, carrot), coconut, black pepper, cardamom, ginger, banana, sugarcane, sunflower, groundnut, soybean, cotton, castor and tobacco against a wide range of plant pathogens. One of the popular species *T. viride* is effective against all soil-borne fungal pathogens like *Pythium*, *Phytophthora*, *Fusarium*, *Rhizoctonia*, *Sclerotium*, *Macrophomina*, etc.[1–3]. A review of the status of biopesticides showed that India has the capacity to produce 1850 MT of *Trichoderma* formulations per annum while the requirement is 22038 MT with a market value of Rs 260 crores[4]. There are around 150 registered manufacturers of *Trichoderma* in India, out of which *T. viride* has a major share. There are 86 firms who have received *T. viride* culture from Tamil Nadu Agricultural University (TNAU), Coimbatore and registered with Central Insecticide Board, India for commercial production[5].

In late nineties of the last century, Lieckfeldt *et al.*[6] indicated the presence of two groups within *T. viride* and named them as type I (named as true *viride*, with natural distribution in the very cool conditions in the northern and southern hemispheres) and type II (later described as *T. asperellum*, present in warmer regions). Samuels *et al.*[7] described the new species *T. asperellum* for the isolates with finely warted conidia that grow at higher temperature (30°C and above) and produce abundant chlamydospores. Further, recent classification by Samuels *et al.*[8] indicated the presence of two cryptic species of *T. asperellum*, namely *T. asperelloides* and *T. yunnanense*.

Use of the nomenclature *T. viride* for the isolates with warted conidia has been continuing for a long time in India[9–11]. However, after the description of *T. asperellum*, Indian isolates with warted conidia should have been studied systematically for their correct identification. Even the recent publications from India use the nomenclature *T. viride* in place of *T. asperellum*. Devi *et al.*[12] report the use of *T. viride* isolates for which the ITS accession numbers have been deposited in GenBank as *T. asperellum* (JN104481 to JN104484 for the isolates 4177 from Tamil Nadu; 5544 from Andhra Pradesh and 5593, 6703 collected from Assam respectively). Further, there are 253 applications received for the commercial registration of talc formulations of *T. viride* in India and 20 of

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them have been issued permission for completion registration (www.cibrc.nic.in); many of them have temporary registration. Since the correct systematic identity is important for issues related to diversity, benefit sharing, etc. the isolates should be checked for their taxonomic identity before registration.

In the present study 30 isolates of *Trichoderma* species with finely warted conidia that grow at high temperature with production of chlamydospores, which have been earlier identified as *T. viride* were selected and the morphological characterization was done as described by Samuels[13] and molecular identification was carried out using oligonucleotide barcode[14] which employs the amplification of ITS sequences followed by *in silico* analysis using the software TrichOKEY 2.0 developed by International Sub-commission on *Trichoderma* and *Hypocrea* taxonomy (ISTH). The molecular identity was confirmed by *tef1* sequence amplification. Phylogenetic analysis of accessions of ITS and *tef1* sequences obtained in the present study and oligonucleotide barcode analysis of accessions submitted to GenBank by other workers as *T. viride* or *T. asperellum* from India showed that the Indian isolates were *T. asperellum*. Further, sequence polymorphism marker analysis was carried to confirm the identity of *T. asperellum* which revealed that besides *T. asperellum* the cryptic species *T. asperelloides* is also present in India.

Materials and methods

Cultures and their maintenance

Trichoderma isolates maintained at the National Bureau of Agriculturally Important Insects, Bangalore, having warted conidia and tentatively identified earlier as *T. viride* based on morphological characters were used. Two isolates TNAU MNT7 and TNAU Tv6 were received from the Department of Plant Pathology, TNAU, Coimbatore. *Trichoderma* cultures were earlier isolated from the soil by serial dilution method using *Trichoderma*-specific medium[15] at ambient temperature (25–30°C). The cultures were maintained on slants of potato dextrose agar at 5° under refrigerated condition.

Morphological characterization

For each character 30 samples of each isolate were studied and average of 30 measurements was used for comparison. Morphological characterization was done as described earlier[13]. All measurements were taken on fungi grown on Spezieiller Nährstoffarmer Agar (SNA) which is a low nutrient medium or cornmeal dextrose (CMD) agar medium. The slides were mounted first in 3% (aq.) KOH, which was replaced by distilled water as the mount dried. Cultures were incubated in 9 cm petri plates at 25°C under 12 h darkness/12 h cool white light. Dimensions are reported as minimum and maximum values of all measurements. Growth was determined on the basis of cultures grown on potato dextrose agar (PDA) and SNA at 30°C with intermittent light. The inoculum was a 10 mm diameter plug taken from the edge of a 4–5-day-old actively growing culture on CMD. Colony radius was measured at intervals of approximately 24 h over 96 h. Growth rate trials were repeated thrice and the results of the trials were averaged.

For morphological characterization of these isolates, characters describing conidia, conidiophore, conidia shape (globose to sub-globose, sub-globose to ovoidal, ellipsoidal, oblong or clavate), length and width of conidia (in µm), ornamentation (smooth, finely warted, conspicuously warted and tuberculate), and pigmentation on plates (green to grey-green, colourless or white in mass, yellow or white to yellow) were recorded[13]. For conidiophores, presence or absence of sterile and fertile hairs arising from conidiophore or pustle, if present, their orientation (spiralled, straight or sinuous) and presence or absence of pustules on CMD or SNA medium were recorded. Hydrophobicity or hydrophillic nature of the conidia was also recorded. For phialides, length (in µm), size of midpoint (in µm), base (in µm), supporting cell (in µm), ratios of length to widest point, length to supporting cell and widest point to width of supporting cell, and presence of intercalary phialides were recorded. Presence or absence of chlamydospores, as well as width and nature of chlamydospores (unicellular or multicellular; intercalary or solitary) were recorded. Colony growth on PDA at 30°C and 35°C after 72 h in darkness was recorded. Since only anamorph stages were observed in the present study, teleomorph characters were not recorded. The characters recorded were fed to the web-based interactive system developed by Samuels and his team (http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm) and correct identity of the species was recorded. Twenty-four isolates were used for recording the morphological characters. The procedure given by Samuels[13] was followed for slide-making. For recording the observations in scanning electron microscope, cell suspensions were dropped on poly-L-lysine-coated glass slides. Immediately after drying, samples were fixed with 4% formaldehyde – 1% glutaraldehyde (v/v) in 0.2 M phosphate buffer (pH 7.4) for 1 h. Next the samples were rinsed three times in 0.2 M phosphate buffer (pH 7.4), dehydrated in ethanol (50/75/100/100%), transferred to formaldehyde dimethyl acetyl (FDA), critical point-dried, sputter-coated with gold-palladium for 200 s and analysed using a DSM 950 scanning electron microscope (SEM; Zeiss) at the Indian Agricultural Research Institute, New Delhi.

DNA extraction and amplification of ITS and tef genes

DNA was extracted from the selected isolates of *Trichoderma* and isolation was carried out using Eukaryote DNA

isolation kit with cetyl trimethyl ammonium bromide (CTAB; Himedia, India). The nuclear rDNA containing ITS 1, 2 and 5.8S rRNA gene was amplified using the primer ITI-1 5′-TCTGTAGGTGAACCTGCGG-3′ITS-4 and 5′-TCCTCCGCTTA TTGATATGC-3′, and the PCR conditions were as described by Kullnig-Gradinger *et al.*[16]. Large intron *tef1* was also amplified using primer pair EF1 728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1986R (5′-TACTTGAAGGAACCCTTACC-3′), and the conditions were as described by Druzhinina *et al.*[14]. Amplification of the respective genes was confirmed by agarose gel electrophoresis.

Nucleotide sequencing and in silico analysis

Sequencing of the PCR product was carried out[16], and the sequences obtained were submitted to NCBI GenBank and their accession numbers were obtained. The *Trichoderma* isolates were identified using the molecular data obtained from sequencing ITS1 and ITS2 regions and by feeding the data in the TrichOKEY 2.0 program (www.isth.info), where species identification of *Trichoderma* is done using thoroughly revised and validated sequence data. The sequence data with respect to *tef1* were analysed using TrichoBLAST for confirmation of identification. Previously published sequences available online (at www.isth.info) were retrieved and used for multiple sequence analyses and phylogenetic analyses. The multiple sequence analyses and construction of phylogenetic trees were performed using CLUSTAL and MEGA version 5.05 respectively.

Differentiation of T. asperellum isolates based on sequence polymorphism-derived typing patterns

The isolates identified as *T. asperellum* in the present study were differentiated based on the amplification of sequence polymorphism-derived (SPD) typing markers developed earlier[17,18], which were employed to describe the new cryptic species of *T. asperellum* by Samuels *et al.*[8]. The SPD typing patterns of 30 isolates of *T. asperellum* were determined. The primers used were: (i) UP-PCR marker F: CACCTTGCTTATCGAATGGGG R: ATCTTCAACACTGGATGGCAATA that amplifies 167 bp product; (ii) ISSR marker F: CTGCAGACGAAGTTATTCAAAG, R: CTTCCTTGCTCGAAGCTGAGG that amplifies 104 bp product; (iii) ISSR marker F: ATATTGCAGAGGTTCCAGCCG, R: GACAGACAATCCTCGAGACATTG that amplifies 273 bp product; (iv) ISSR marker CTGCAGACGAAGTTATTCAAAG that amplifies 456 bp product and (v) UP-PCR marker F: CAGCGTCGATGGAAGATGCC, R: CTTCAACACCGGATGGCAGTG that amplifies 322 bp product. A multiplex PCR was carried out as described earlier by Samuels[8]. After amplification, 8 µl PCR1 products were mixed with 8 µl PCR2 products plus 3 µl 6X loading dye and loaded on 2% agarose gel in 40 mM Tris-acetate buffer, pH 8.3, containing 1 mM EDTA and 3.3×10^{-6} %ethidium bromide. Electrophoresis was conducted at 50 V. The gels were documented using Biorad Gel Documentation Unit.

Results and discussion

Identification based on morphological features, SEM studies and molecular characterization

Morphological characters of 30 selected isolates of *Trichoderma* which were previously identified as *T. viride* were recorded (Supplementary data 1). The conidia length ranged between 3.1 and 4.5 µm, whereas the width was between 2.1 and 3.9 µm. The L/W ratio was 1.1–1.4. The standard descriptions for the conidia of *T. asperellum* size are (2.8–) 3.5–4.5 (–7.0) µm × (2.5–) 3.0–4.0 (–6.0) µm, L/W 1.0–1.4 (mean = 1.2). The phialides length ranged from 7.1 to 11.1. The phialides midpoint ranged between 1.4 and 3.5 µm, whereas the phialides base was between 1.4 and 326 µm. The supporting cell width was 1.4–3.6 µm. The phialides L/W ratio was between 2.5 and 5.0. The ratio of widest point in phialides to width of supporting cell was 0.4 to 1.3. The standard descriptions for the phialides of *T. asperellum* are (4.6–) 6.5–11.5 (27.5) µm long, (2.0–) 2.7–4.2 (–6.8) µm in the middle, (1.3–) 1.8–2.8 (–4.7) µm wide at the base, *l*/*w* 1.6–3.6. The typical hook-like structure expected in the conidiophores of *T. viride* was not conspicuously observed in these isolates. In all the isolates the conidia shape was sub-globose or sub-globose to ovoid (Figures 1 and 2) and conidia ornamentation was finely warted as also evident from SEM observations (Figure 2). The lateral branches of conidiophores were branched. The conidial colour was either olive green or dark green and the dry conidia were produced on CMD agar. Radial growth on PDA at 30°C after 72 h in darkness ranged between 24 and 80 mm. Pustle formulation was noticed in most of the isolates and they were found to bear conidiophores. In three isolates (TaCu1, TaTN1 and TaTN6C) coconut odour was recognized, whereas in others distinct odour could not be recognized. In all the isolates abundant chlamydospores were produced (Figure 3). The growth rate of standard isolates of *T. asperellum* described was colony radius of (7–)54(–64) mm at 30°C and (0–)27 (–42) mm at 35°C after 72 h on PDA. Samuels[13] does not describe coconut odour in *T. asperellum*. The observations made in this study match with the standard descriptions or are close to them.

Molecular characterization of the 30 isolates used in the morphological characterization was done based on the ITS and *tef1* sequences, and the sequences were deposited in GenBank (Supplementary data 2). Six more isolates (Ta Cu15, Ta11, Ta17, Ta23, Ta29 and Ta36) were used

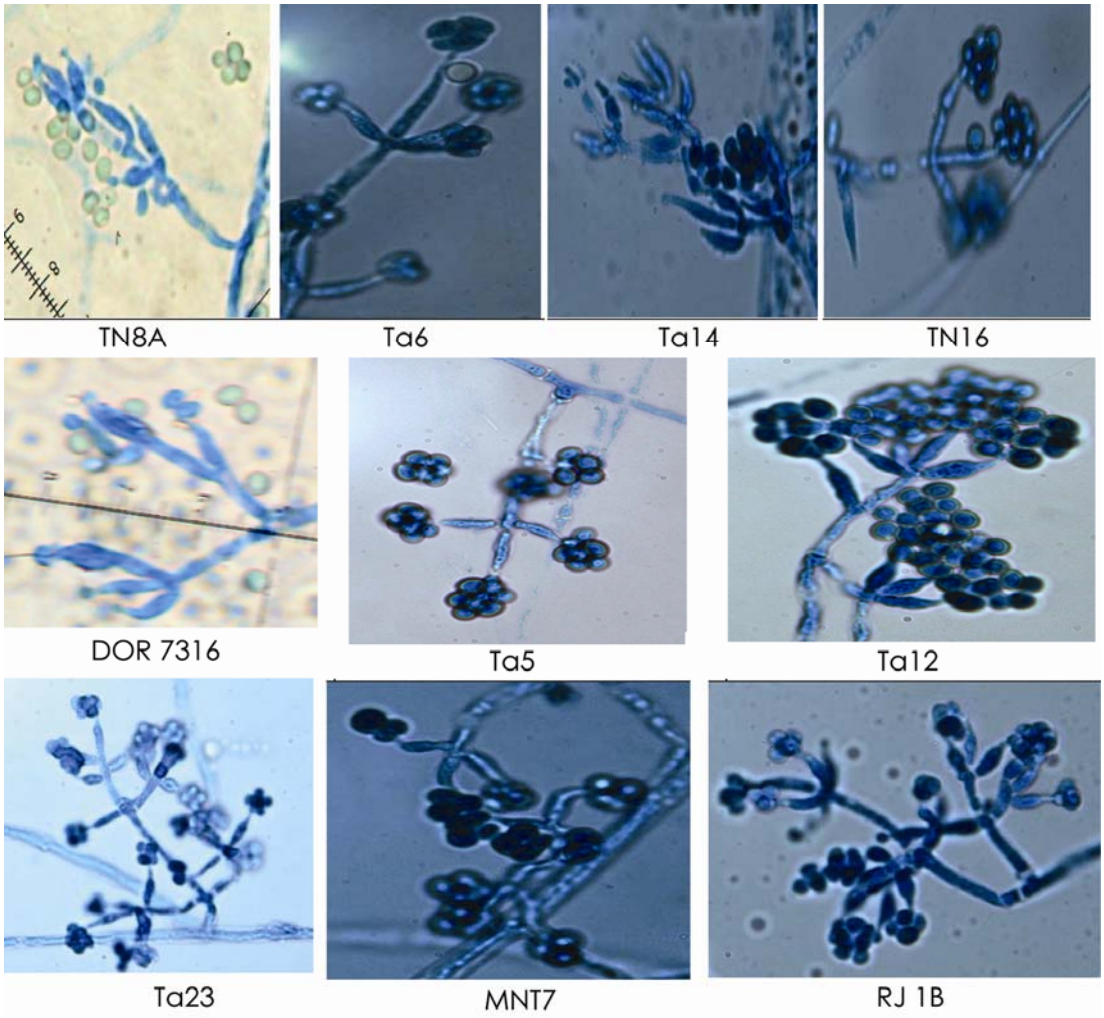

Figure 1. Conidiophores of selected *Trichoderma asperellum* isolates used in the present study.

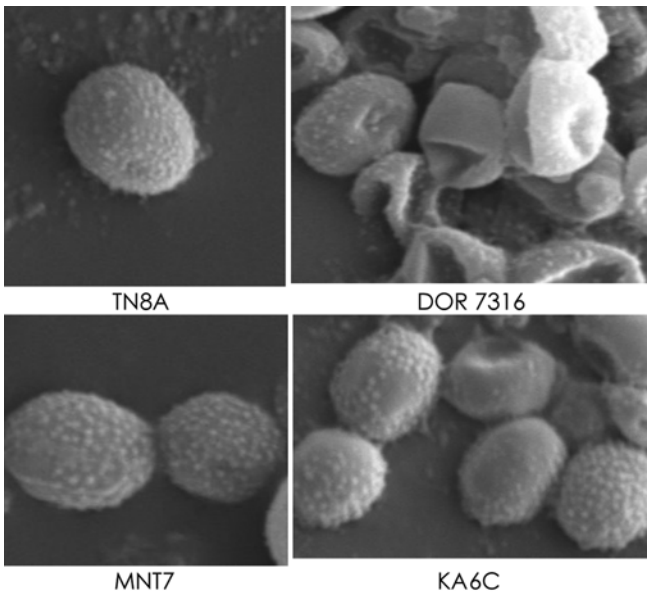

Figure 2. Conidia of selected *T. asperellum* isolates used in the present study observed under SEM.

for molecular characterization. The TrichOKEY 2.0 identification confirmed that all of them belonged to *T. asperellum* and comparison of *tef1* sequences using BLAST also confirmed their identity as *T. asperellum*.

Phylogenetic analysis

Phylogenetic analysis with ITS sequences of *T. asperellum* from this study and isolates submitted as *T. viride* along with standard *T. viride* isolates from ISTH was carried out. Phylogenetic analysis methods like maximum likelihood (ML), maximum parsimony (MP), neighbour-joining (NJ), minimum evolution (ME); UPGMA and Bayesian approach are used by different workers. ME gives more importance to the recent process of evolutionary variations. MP involves analysis of sequence data without employing a model for evolution and therefore has difficulties in analysing data of very closely or very distant taxa, besides in some cases being sensitive to the order in which sequences are added to the tree. Besides,

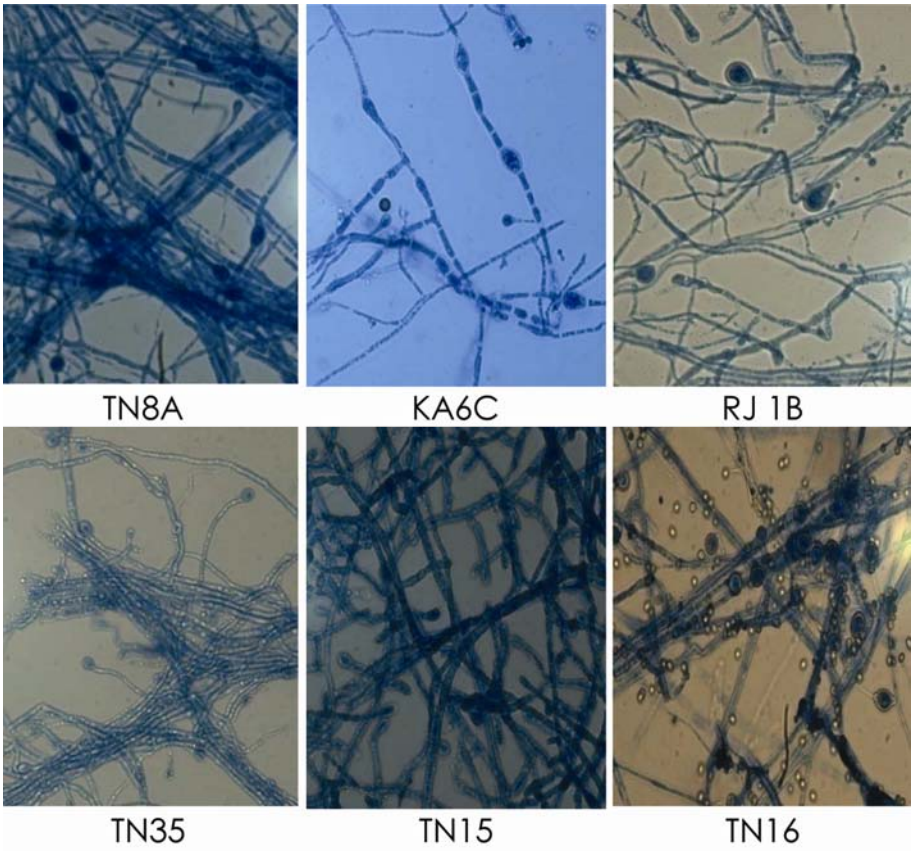

Figure 3. Chlamydospore production in selected *T. asperellum* isolates used in the present study.

MP provides numerous most parsimonious trees[19,20]. NJ, though faster, does not consider intermediate ancestors, assuming that there is no requirement for an internally consistent evolutionary model. The main disadvantage of the NJ model is that it misses homoplasies, especially if the evolutionary distances are longer. The Fitch/Margoliash method also misses homoplasies. Druzhinina *et al.*[19] recommended the Bayesian approach to phylogenetic inferences which has been recently employed in many genera, including *Trichoderma*/*Hypocrea*[21–25], where there were analyses of very closely and very distantly related genera and the studies resulted in the description of new genera and grouping taxa into clades. The advantages of ML method are that it reconstructs ancestral nodes thereby using all the evolutionary data, generates branch lengths and statistical estimate of significance of each branch. Though ML method suffers from the log computation time, it is adequate for phylogenetic analysis. In the present study we compared only *T. asperellum*, *T. viride* and *T. asperelloides* sequences, while other species were used as out groups. We used the ML and MP methods for phylogenetic analysis.

The phylogenetic analysis of the ITS sequences was carried out with sequences of *T. viride* (GJS 91-62), *T. reesei* (ATCC 13631), *T. virens* (CBS249.59), *T. velutinum* (TUB F-801), *T. harzianum* (CBS 226.95) and *T. aggresivum* (CJS 99.29) isolates downloaded from the database (www.isth.info) as outgroups. Besides, standard *T. asperellum* isolate CBS 433.97 was used for comparison.

The phylogenetic analysis of ITS sequences of all *T. asperellum* isolates was performed after editing the sequences using multiple alignment software TrichoMark (available at www.isth.info). The sequences of other *Trichoderma* species downloaded from ISTH as mentioned were used as outgroups. The phylogenetic tree constructed using ML method (Supplementary data 3) showed clustering of *T. viride*, *T. reesei*, *T. virens*, *T. velutinum*, *T. harzianum* and *T. aggresivum* in one clade of the fourth node, whereas *T. asperellum* isolates were arranged on both sides of this clade differentiated with nodes. The standard isolate CBS433.97 from ISTH was close to the isolate TaTN1. TaKa6B and TN6C were distinct from others and separated into different clades. Ta23, Ta36, TaCu15, Ta30, RJ1B, TN8A, Ta35, TaCu1, Ta6, TaTN1 and standard isolate from ISTH were grouped into subgroups of one main clade. Isolate TaCu6 was distinct and grouped separately. Another group of *T. asperellum* consisted of the isolates Ta13, TNAU MNT7, Ta15, Ta17, Ta11, Ta ASS12, Ta14, Ta5, Ta29, Ta12 and Ta16. The separation of *T. asperellum* isolates into two main groups shows that evolutionarily there are two main groups.

Alternatively, there may be cryptic species like *T. asperellum*, *T. asperelloides* and *T. yunnanense*, which are not separated by ITS sequences as reported by Samuels *et al.*[8]. Phylogenetic tree by ML method does not group isolates only based on sequence similarity, but also based on the evolutionary aspects. There are only 17 nucleotide changes between the ITS sequences of *T. viride* and *T. asperellum*. The TrichOKEY 2.0 identification is based on the species-specific hallmarks identified for each species in the oligonucleotide barcode[14]. The phylogenetic tree based on maximum parsimony method (Supplementary data 4) clustered all other species of *Trichoderma* into one clade. The standard isolate of *T. asperellum* (CBS433.97) was close to *T. viride* isolates (GJS-91-62). The next clade close to standard isolate from ISTH consisted of TaRJ1B, Ta6, Ta17 and Ta30. The second clade consisted of TaTN1, Ta23, TaASS12A and TaTN6B. The third clade consisted of TaKa6B, Ta Cu1, Ta14, Ta13, Ta15 and Ta3, and the next clade included TaTN8A, Ta5, Ta16 and Ta35. Isolates Ta12, Ta36, TaCu6, TaTNAU MNT7, Ta11, TaCu13 and Ta Cu15 were in one clade with different sub-clades.

The alignment of ITS sequences of *T. viride* and *T. asperellum* revealed that there were only 17 nucleotide changes. The changes noticed were presence of G in place of A at sites 69 and 327; A in place T at 109; T in place of C at 111 and 342; G in place of T at 341 and 374, A in place of G at 348; A in place of C at 355 and 406; G in place of C at 357; C in place of G at 365; G in place of T at 374; additional A at 363 and additional triplet TCG at 343–345. Thus in the phylogenetic tree using ML that analyses the phylogenetic components also, *T. asperellum* was grouped along with *T. viride*, whereas the tree based on maximum parsimony method grouped all other species together and *T. asperellum* isolates separately.

Phylogenetic analysis of *tef1* sequences of *T. asperellum* isolates was performed along with *tef1* sequences of standard isolate of *T. asperellum* (ISTH03CBS) and isolates of *T. viride* (27CBS, CPK998, CPK999, CBS1110994 and CBS111096). According to the description of cryptic species of *T. asperellum*[8], we included the *tef1* sequences of seven *T. asperelloides* isolates also in the phylogenetic analysis. The analysis by ML method (Supplementary data 5) revealed that five *T. asperelloides* isolates (GJS-06-158, GJD2009, GJS-08-87, TR31 and GJS-99-9) were grouped together. *T. viride* isolates CBS111094 and CBS 111096 from ISTH were grouped into a clade. A clade of *T. asperellum* isolates Ta23, TaCu4, Ta11 was close to these *T. viride* isolates. *T. viride* isolates CPK998 and CPK999 were grouped separately. *T. asperellum* isolate Ta30 was phylogenetically close to these two *T. viride* isolates. Isolates CBS111094 and CBS111096 were grouped separately, and a clade of three *T. asperellum* isolates Ta23, TaCu4 and Ta11 was close to this clade and isolate Ta15 was the next closest to this clade. The sequences of accessions mentioned in the study are available at GenBank and phylogenetic trees have been submitted to TreeBASE.

The grouping of *T. viride* or *T. asperelloides* isolates in ML method indicated the occurrence of homoplasies. The variation in the characters, viz. finely warted conidia, growth at 30°C or above, and production of abundant chlamydospores was phylogenetically independent and probably responsible for the homoplasies.

The clustering of *T. asperellum* isolates along with *T. asperelloides* isolates in the phylogenetic tree constructed with *tef1* sequences confirms the observation of Samuels *et al.*[8] that *T. asperellum sensu lato* consisted of two cryptic species, viz. *T. asperelloides* and *T. yunnanense*. The identification of these two cryptic species has been done with SPD markers using the loci of *tef1*, *rpb2*, *act*, *ITS1* and ITS2 and 5.8 S rRNA.

Differentiation of T. asperellum isolates using SPD markers

The variation in ITS sequences alone is too small to differentiate these cryptic species. This is evident from the fact that oligonucleotide barcode for *Trichoderma* does not have any hallmarks specific to *T. asperelloides* or *T. yunnanense*. Hence it is not possible to differentiate the isolates of these cryptic species using TrichOKEY 2.0. In our study also we expected that probably there would be some *T. asperelloides* isolates identified as *T. asperellum.* Hence we attempted to differentiate the *T. asperellum* isolates by SPD typing patterns. There were four typing patterns proposed by Samuels *et al.*[8]. Patterns I–III represented *T. asperellum*, while pattern IV represented *T. asperelloides* and *T. yunnanense*. Pattern IV is made of one reproducible amplicon of 273 bp that was present in patterns I and III also. Pattern I was made of two amplicons of 167 and 273 bp, while pattern II was made of two amplicons of 104 and 456 bp, and pattern II was made of two amplicons of 273 and 322 bp. However in our study, new patterns were also observed (Figure 4 and Table 1). The isolate Ta15 clearly showed pattern IV and was identified as *T. asperelloides*. The remaining isolates showed different combinations of two or three amplicons, indicating that Indian isolates of *T. asperellum* and its cryptic species differ in their SPD typing patterns.

Isolate TaASS12A did not have amplification of any of these products. Only isolates belonging to pattern II were supposed to show the amplification of 104 bp product. But in the present study out of 30 isolates tested, 28 showed the amplification of the same. Isolates Ta30, Ta101, Ta102, DOR7316, Ta12 and TaRJ1B were found to be variants of pattern II that does not have the 456 bp product. Isolates Ta13, Ta35, Ta36, TNAU 06, TNAU MNT7, KA6C and TN1 were found to be variants of pattern II that had 167 bp product additionally, but they did not have the 456 bp product that was present in

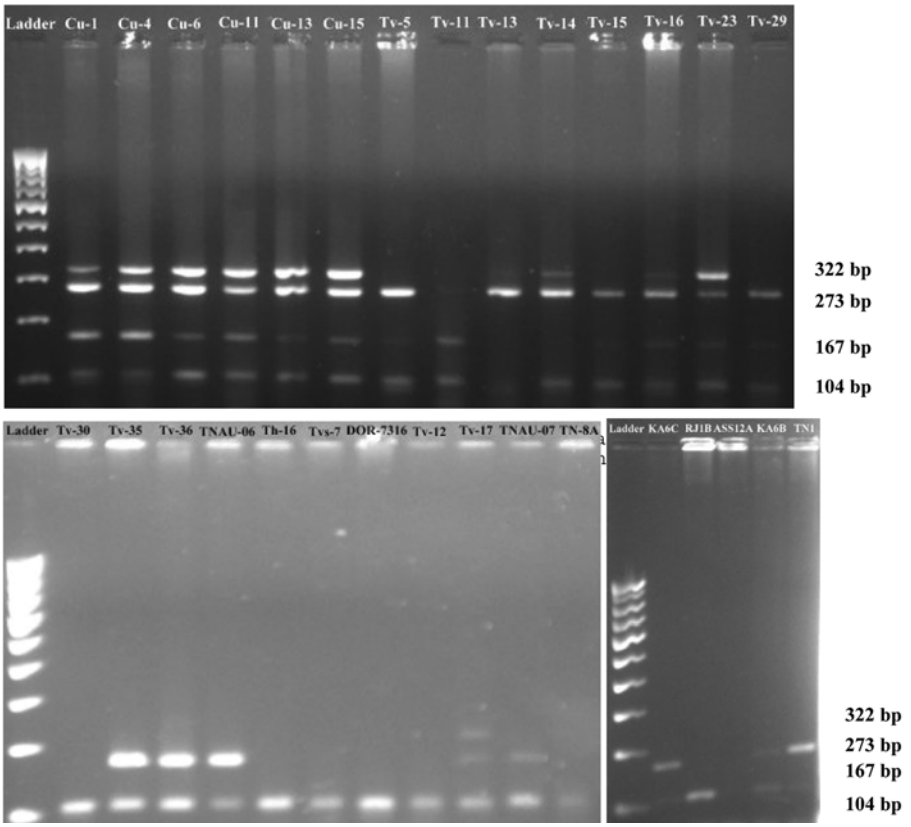

Figure 4. Sequence polymorphism-derived pattern in isolates identified as *T. asperellum* by oligonucleotide barcode.

pattern II. Isolate Ta14 was found to be the variant of pattern III with additional product of 104 bp. Similarly, isolates Ta11, Ta29 and Ta17 were variants of pattern I with the additional product of 104 bp. Isolates Cu1, Cu4, Cu6, Cu11, Cu13, Cu15, Ta16 and Ta23 formed a new pattern with four products of 322, 273, 167 and 104 bp respectively. The differentiation of these isolates into cryptic species of *T. asperellum* is difficult considering the variation in the patterns after multiplex PCR with SPD markers. Some of the isolates that do not have 273 bp products may be *T. asperelloides*, but we need confirmation in this regard with other evidences. The new patterns obtained with Indian isolates show the phylogenetic influences and variability in *T. asperellum* and their cryptic species.

Thus, we propose that the pattern with only one amplicon of 273 bp is *T. asperelloides* and others with additional amplicons is *T. asperellum*. The presence of new cryptic species may be proposed later based on these patterns and proteomics work using MALDI-TOF. Morphological characters and proteomics methods will be needed to differentiate *T. asperelloides* and *T. yunnanense*.

The classical approach to identify *Trichoderma* species based on differences in morphology and growth characters is difficult because of homoplasy of morphological characters. Homoplasy is a character shown by a set of species but not present in their common ancestor due to convergent evolution. As mentioned by Druzhinina *et al.*[14], the mistaken identity of *Trichoderma* species is due to the lack of quality control while deposition of sequences within NCBI/GenBank and some sequences are submitted with original name and not under the names they have identified subsequently.

There are 56 GenBank accessions for the ITS sequences submitted as *T. viride* isolates from India (Supplementary data 6). These sequences were downloaded from GenBank and subjected to TrichOKEY 2.0 for molecular identification using oligonucleotide barcode. Out of 56 submissions, six isolates 01PP-8315/11, APT01, T4, APT09, MML3116 and TNAUTV1 were found to be *T. asperellum* according to TrichOKEY 2.0. Other isolates belonged to *T. harzianum* (2), *T. koninigiopsis* (10), *H. Sulphurea* (3), *T. longibrachiatum* (10), *T. koninigi* (2), *T. atroviride* (2), *T. ovalisporum* (8), *T. virens* (3) and *T. paucisporum* (1). Seven isolates could not be identified as the submissions were only 18S or 28S rRNA sequences. The marine isolate SKS-2 collected from Gulf of Mannar in the Bay of Bengal was identified as *H. rufa*, the perfect stage of *T. viride*. Jaklitsch *et al.*[26] reassessed this species and described cryptic species *Hypocrea vinosa* with anamorph *T. vinosum*, *T. gamsii*, *T. neokoningii* and *T. scalesiae*. Hence the identity of the anamorph of this isolate has to be confirmed. The marine isolates do not have the same phylogeny and habitat as those of biocontrol strains. The accessions of ITS sequences submitted as *T. asperellum*

Table 1. Sequence polymorphism-derived patterns obtained for the isolates identified as *Trichoderma asperellum* by oligonucleotide barcode

Isolate	Pattern	Amplification of PCR products (bp)				
		456	322	273	167	104
ASS12A	Variant of pattern II without 456 bp PCR product	–	–	–	–	–
Ta30	–do–	–	–	–	–	+
Ta101	–do–	–	–	–	–	+
Ta102	–do–	–	–	–	–	+
DOR7316	–do–	–	–	–	–	+
Ta12	–do–	–	–	–	–	+
TN8A	–do–	–	–	–	–	+
RJ1B	–do–	–	–	–	–	+
Ta35	Variant of pattern II without 456 bp, but with 167 bp product	–	–	–	+	+
Ta36	–do–	–	–	–	+	+
TNAU 06	–do–	–	–	–	+	+
TNAU 07	–do–	–	–	–	+	+
KA6C	–do–	–	–	–	+	+
KA6B	–do–	–	–	–	+	+
TN1	–do–	–	–	–	+	+
Ta13	Pattern IV	–	–	+	–	–
Ta15	Pattern IV with additional 104 bp product	–	–	+	–	+
Ta14	Variant of pattern III with additional 104 bp product	–	+	+	–	+
Ta11	Variant of pattern I with additional 104 bp product	–	–	+	+	+
Ta29	–do–	–	–	+	+	+
Ta17	–do–	–	–	+	+	+
Cu1	New pattern	–	+	+	+	+
Cu4	–do–	–	+	+	+	+
Cu6	–do–	–	+	+	+	+
Cu11	–do–	–	+	+	+	+
Cu13	–do–	–	+	+	+	+
Cu15	–do–	–	+	+	+	+
Ta16	–do–	–	+	+	+	+
Ta23	–do–	–	+	+	+	+

isolates from India were checked. Besides the accessions submitted from this study, there are 99 accessions from India for *T. asperellum*. Seventy-nine of them were for the ITS sequences, while 19 were for *tef1* and one for the endochitinase gene. These submissions were made by different organizations. Details of accession numbers, sequences and organizations (Supplementary data 7) indicate the occurrence of *T. asperellum* across the country in different states from Jammu & Kashmir to Tamil Nadu and Assam to Gujarat.

We conclude based on our observations on morphology and supported by oligonucleotide barcode-based molecular identification using TrichOKEY 2.0 that Indian isolates of *Trichoderma* with finely warted conidia, abundant chlamydospore production, good growth at high temperature belong to *T. asperellum*. This confirms the report of Lieckfeldt *et al.*[6] on the occurrence of type I (= *T. viride*) in the northern or southern temperate locations and type II (= *T. asperellum*) isolates in warm regions. The differentiation of these isolates into cryptic species using SPD markers of Samuel *et al.*[8] as *T. asperellum* and *T. asperlloides* proves that Indian isolates of *Trichoderma* with finely warted conidia belong to *T. asperellum* and its cryptic species *T. asperelloides*, and not to *T. viride*.

1. Harman, G. E., Howell, C. R., Viterbo, A., Chet, I. and Lorito, M. *Trichoderma* species – opportunistic, avirulent plant symbionts. *Nature Rev. Microbiol.*, 2004, **2**, 43–56.
2. Samuels, G. J., *Trichoderma*: systematics, the sexual state, and ecology, *Phytopathology*, 2006, **96**, 195–206.
3. Sriram, S. and Ray, R. C., *Trichoderma:* systematic and molecular taxonomy and application in agriculture and industry. In *Microbial Biotechnology in Agriculture and Aquaculture, Volume 1* (ed. Ray, R. C.), Science Publishers, New Hampshire, USA, 2005, pp. 333–370.
4. Rabindra, R. J., Ramanujam, B. and Hussaini, S. S., *Microbial Biopestices: Formulations and Applications*, Project Directorate of Biological Control, Bangalore, 2005, p. 269.
5. Nakeeran, S., *Trichoderma* – production and use in India. In 7th International IPM Symposium (Memphis) organized by Tamil Nadu Agricultural University (TNAU) and Integrated Pest Management–Collaborative Research Support Programme (IPM–CRSP) held at TNAU, Coimbatore, March 2012, http://www.oired.vt.edu/ipmcrsp/documents/presentations/TrichodermaInIndia-Nakeeran-March 2012.pdf
6. Lieckfeldt, E., Samuels, G. J., Nirenberg, H. I. and Petrini, O., A molecular and morphological perspective of *Trichoderma viride*:

is it one or two species? *Appl. Environ. Microbiol.*, 1999, **65**, 2418–2428.
7. Samuels, G. J., Lieckfeldt, E. and Nirenberg, H. I., *Trichoderma asperellum*, a new species with warted conidia, and redescription of *T. viride*. *Sydowia*, 1999, **51**, 71–88.
8. Samuels, G. J., Ismaiel, A., Bon, M., de Respinis, S. and Petrini, O., *Trichoderma asperellum sensu lato* consists of two cryptic species. *Mycologia*, 2010, **102**, 944–966.
9. Bilgrami, K. S., Jamaluddin and Rizvi, M. A., *Fungi of India*, Today and Tomorrow Publications, New Delhi, 1971.
10. Bilgrami, K. S., Jamaluddin and Rizvi, M. A., *Fungi of India*. *Part II. Host Index Addenda*. Today and Tomorrow Publications, New Delhi, 1981.
11. Sarabhoy, A. K., Agarwal, D. K. and Varshney, J. C., *Fungi of India 1977–81*. Associated Publishing Company, New Delhi, 1992.
12. Devi, T. P., Kamil, D., Prabhakaran, N. and Pandey, P., Development of genus specific rDNA based marker for detection of *Trichoderma* species. *J. Mycol. Plant Pathol*., 2011, **41**, 600–605.
13. Samuels, G. J., *Trichoderma – A Guide to Identification and Biology*, Beltsville, Maryland: United States Department of Agriculture, USA, 2004, p. 40.
14. Druzhinina, I., Koptchinski, A., Komon, M., Bissett, J., Szakacs, G. and Kubicek, C. P., An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet. Biol*., 2005, **42**, 813–828.
15. Elad, Y. and Chet, I., Improved selective media for the isolation of *Trichoderma* spp. or *Fusarium* sp. *Phytoparasitica*, 1983, **11**, 55–58.
16. Kullnig-Gradinger, C. M., Szakacs, G. and Kubicek, C. P., Phylogeny and evolution of the fungal genus *Trichoderma*: a multi gene approach. *Mycol. Res.*, 2002, **106**, 757–767.
17. Bon, M. C., Hurard, C., Begoude, D., Tondje, P. and Samuels, G., Molecular typing of mycoparasitic isolates of *Trichoderma asperellum* and implications for effective biological control of black pod disease. In Proceedings of 15th International Cocoa Research Conference, San Jose, Costa Rica, 9–14 October 2006. Cocoa Producers' Alliance, Abidjan, Nigeria, 2007, vol. II, pp. 1315–1320, ISBN 978-065-959-5, 2007.
18. Deberdt, P. *et al.*, Impact of environmental factors, chemical fungicide and biological control on cacao pod production dynamics and black pod disease (*Phytophthora megakarya*) in Cameroon, *Biol. Control*., 2008, **44**, 149–159.
19. Druzhinina, I. S., Kopchinskiy, A. G. and Kubicek, C. P., The first 100 *Trichoderma* species characterized by molecular data. *Mycoscience*, 2006, **47**, 55–64.
20. Salemi, M. and Vandamme, A., *The Phylogenetic Handbook. A Practical Approach to DNA and Protein Phylogeny*, Cambridge University Press, Cambridge, 2003.
21. Chaverri, P., Castlebury, L. A., Overton, B. E. and Samuels, G. J., *Hypocrea/Trichoderma*: species with conidiophore elongations and green conidia. *Mycologia*, 2003, **95**, 1100–1140.
22. Rannala, B. and Yang, Z., Probability distribution of molecular evolutionary trees: a new method of phylogenetic interference. *J. Mol. Evol.*, 1996, **43**, 304–331.
23. Huelsenbeck, J. P. and Ronquist, F., MYBAYES: Bayesian inference of phylogeny. *Bioinformatics*, 2001, **17**, 754–755.
24. Lutzoni, F., Pagel, M. and Reeb, V., Major fungal lineages are derived from lichen symbiotic ancestors. *Nature*, 2001, **411**, 937–940.
25. Druzhinina, I., Chaverri, P., Fallah, P., Kubicek, C. P. and Samuels, G. J., *Hypocrea flaviconidia*, a new species from Costa Rica with yellow conidia. *Stud. Mycol.*, 2004, **50**, 401–407.
26. Jaklitsch, W. M., Samuels, G. J., Dodd, S. L., Lu, B. and Druzhinina, I. S., *Hypocrea rufa/Trichoderma viride*; a reassessment, and description of closely related species with and without warted conidia. *Stud. Mycol.*, 2006, **55**, 135–177.

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