RESEARCH ARTICLE

# *Trichoderma* from Brazilian garlic and onion crop soils and description of two new species: *Trichoderma azevedoi* and *Trichoderma peberdyi*

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## Abstract

Fifty four *Trichoderma* strains were isolated from soil samples collected from garlic and onion crops in eight different sites in Brazil and were identified using phylogenetic analysis based on combined ITS region, *tef1-α*, *cal*, *act* and *rpb2* sequences. The genetic variability of the recovered *Trichoderma* species was analysed by AFLP and their phenotypic variability determined using MALDI-TOF. The strain clusters from both typing techniques coincided with the taxonomic determinations made from phylogenetic analysis. The phylogenetic analysis showed the occurrence of *Trichoderma asperellum*, *Trichoderma asperelloides*, *Trichoderma afroharzianum*, *Trichoderma hamatum*, *Trichoderma lentiforme*, *Trichoderma koningiopsis*, *Trichoderma longibrachiatum* and *Trichoderma erinaceum*, in the soil samples. We also identified and describe two new *Trichoderma* species, both in the harzianum clade of section *Pachybasium*, which we have named *Trichoderma azevedoi* sp. nov. and *Trichoderma peberdyi* sp. nov. The examined strains of both *T. azevedoi* (three strains) and *T. peberdyi* (12 strains) display significant genotypic and phenotypic variability, but form monophyletic clades with strong bootstrap and posterior probability support and are morphologically distinct from their respective most closely related species.

## Introduction

One of the most important fungal diseases occurring in garlic (*Allium sativum*) and onion (*Allium cepa*) is white rot, caused by the sclerotium-forming fungus *Sclerotium cepivorum*, often causing severe losses in garlic and onion production worldwide [1]. In Brazil, the states of Paraná, Minas Gerais, São Paulo and Goiás produce 64% of the national *Allium* crop (mostly garlic and onion) [2]. Despite recent advances in *Allium* production in Brazil, production is not sufficient to fulfil internal demand, due to low productivity [3]. Despite the diversity of garlic and onion cultivars available to growers, the favorable humidity and temperature conditions for most of these cultivars are also conducive to white rot disease. In the absence of reliable conventional white rot control methods, biological control is being investigated as a viable option, particularly using species of the fungal antagonist, *Trichoderma* [4].

*Trichoderma* has been widely used in biological control due to its ecological plasticity, easy large-scale production and efficiency against many plant pathogens such as *Fusarium*, *Pythium*, *Rhizoctonia*, *Sclerotinia*, *Botrytis* and *Verticillium* [5–9]. *Trichoderma* species are common in rhizospheric and non-rhizospheric soils and in endophytic relationships with many plants, displaying antifungal properties as well as promoting growth and inducing plant resistance against pathogenic fungi [10–12]. Three *Trichoderma asperellum* strains, one *Trichoderma harzianum* strain and a fifth unidentified *Trichoderma* strain from the rhizosphere of garlic and onion crops in Costa Rica have been tested for their *in vitro* antagonism against *S. cepivorum*, following their identification using ITS sequences [13]. The combination of different biocontrol agents to obtain synergistic or additive effects has also been tested in the field to control *S. cepivorum*, where simultaneous application of four selected species of *Trichoderma* (*Trichoderma hamatum*, *T. harzianum*, *Trichoderma oblongisporum* and *Trichoderma viride*), in association with fungicides, was shown to be effective for the management of white rot disease [14].

The phylogenetic species concept, based on concordance of multiple gene genealogies, has revolutionized fungal taxonomy [15] and exposed weaknesses in traditional morphology-based identification. Taxonomic revisions and the recognition of previously cryptic speciation in *Trichoderma* has also made clear that the universal DNA barcode for fungi, the internal transcribed spacers 1 and 2 of the nuclear ribosomal RNA gene cluster (ITS), is no longer adequate to ensure accurate species determinations in many *Trichoderma* sections [16–18], where a multi-gene approach is now usually adopted. By 2015 [19], there were 256 accepted *Trichoderma* name combinations, a number that is regularly increasing.

Multi-gene phylogenetics provides a gold standard for fungal identification and species delimitation. However, its methodology is time-consuming, technically demanding and expensive. Phenotyping using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) provides an attractive alternative for rapid microbial identification and strain differentiation purposes and has been used in filamentous fungi such as species of *Aspergillus*, *Fusarium*, *Penicillium*, *Trichoderma* and *Metarhizium*, among others [20,21]. The major advantages of MALDI-TOF are its cost effectiveness, rapidity, low error rate and the possibility of distinguishing closely related species [22].

While correct species identification is important in the selection and validation of microbial biocontrol agents [23], assessment of infraspecific variation is also of importance to protect commercial strains and to understand the genetic resources available in natural populations. There are abundant reports of molecular genotyping techniques applied to fungal biocontrol agents available in the literature. One of the most attractive methods, however, due to its ability to efficiently generate large numbers of markers at low cost which are amenable to automated fluorescence-based scoring is AFLP (amplification fragment length polymorphism), which has been used to identify and differentiate closely related species of *Trichoderma* [24].

Due to the potential of *Trichoderma* species to control white rot disease, we aimed to collect strains from crop soils from multiple localities in some of the principal garlic and onion growing areas in Brazil. We also aimed to correctly identify the strains, under the current taxonomic framework, to the species level using multi-gene DNA sequence analysis and assess their genetic and phenotypic variation using AFLP and MALDI-TOF, respectively. Such data will be a valuable resource for ongoing biocontrol research in Brazil.

## Materials and methods

### Collection and isolation of *Trichoderma* strains

*Trichoderma* strains were isolated from soil samples collected from eight distinct garlic or onion crops in the Brazilian states of Santa Catarina (SC), Minas Gerais (MG), Rio Grande do

Sul (RS) and São Paulo (SP) ([Table 1](#); [Fig 1](#) - Map). From each sample, 10 g of soil was placed in a 250 ml Erlenmeyer flask containing 90 ml of sterile distilled water. After stirring at 180 rpm for 40 min, serial dilutions were spread onto plates containing Martin's semi-selective medium (per litre: 18 g agar, 10 g dextrose, 0.5 g $MgSO_4$, 0.5 g peptone, 0.5 g beef extract, 0.05 g bengal pink and 0.3 g chloramphenicol) and incubated at 28˚C for 7 days. Isolated colonies with typical *Trichoderma* morphology were transferred to potato-dextrose agar (PDA; Difco) supplemented with 0.25 ml l$^{-1}$ Triton X100 and 0.3 g l$^{-1}$ chloramphenicol for the subsequent isolation of monosporic cultures. Fungal sample collection was carried out according to Brazilian legislation (IBAMA process 02001.006479/2010-93 and permit n$^o$ 02/2008).

## Morphological characterization

For comparison of growth, colony appearance and morphological features, discs of fresh monosporic *Trichoderma* cultures were transferred to 9 cm Petri dishes containing 20 ml of either PDA, CMD (cornmeal dextrose agar) or SNA (synthetic low nutrient agar), which were cultured at 15, 20, 25, 30 and 35˚C, with 12 hour photoperiod. Morphological characteristics, such as the aspects of phialides, conidia and chlamydospores were observed using a Nikon Eclipse Ci microscope fitted with a Nikon DS Ri2 camera. Microscopical measurements and analysis were carried out using NIS Elements (v. 4.30.01, Nikon) software, where means were based on 30 individual phialides and conidia from each specimen.

## Phylogenetic analysis

Strains were cultivated on PDA for 72 h at 25˚C prior to collection of mycelium, which was scraped from the agar surface, lyophilized and maintained at -80˚C. Genomic DNA was purified from approximately 20 mg of the lyophilized mycelium, using a cetyl trimethyl ammonium bromide (CTAB) extraction method [25]. The nuclear ribosomal ITS1–5.8S rRNA–ITS2 region (ITS), actin (*act*), calmodulin (*cal*), translation elongation factor 1-α (*tef1-α*) and RNA Polymerase II subunit (*rpb2*) markers were amplified by PCR using a mix comprising approximately 2 ng genomic DNA, 1x PCR buffer with 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 1U *Taq* polymerase and 0.3 μM of each primer. Thermal cycling for all markers was standardized as 2 min at 95˚C then 35 cycles of 20 sec at 95˚C, 30 sec at the appropriate annealing temperature for the primers used and 90 sec at 72˚C, followed by 7 min at 72˚C. Primer sequences and annealing temperatures are given in [Table 2](#). The internal *act* sequencing primer, Tact293F, was designed from a conserved region identified in a preliminary alignment of several *Trichoderma act* PCR products sequenced using the amplification primers, along with cognate reference sequences obtained from Genbank. The *tef1-α* reverse PCR primer, tef1080R, was designed from a conserved region identified in an alignment of the 3′ portion of the *tef1-α* gene, amplified from a selection of *Trichoderma* isolates using EF1–1018F and EF1–1620R primers. We obtained fewer artefact bands in PCRs using the tef1080R reverse primer compared with the more widely used tef997R primer. Since the 3′ portion of the *tef1-α* gene is much less variable than the 5′ portion, phylogenetic analysis was restricted to the portion amplified using tef71f and tef1080R primers. Furthermore, there is a richer representation of the 5′ portion of the *tef1-α* gene from related *Trichoderma* species in the databanks, further influencing our decision.

PCR products were verified by agarose gel electrophoresis and were then prepared for sequencing using ExoSAP (Applied Biosystems, Foster City, CA, USA). Both DNA strands were sequenced using the Big Dye v.3.1 kit (Applied Biosystems), using appropriate primers ([Table 2](#)) and an ABI3730 DNA Analyzer (Applied Biosystems). Sequence reads were trimmed for quality, contigs assembled and any base calling mismatches resolved using Chromas Pro

**Table 1. *Trichoderma* species identified from garlic and onion crop soils in Brazil and Genbank accession numbers of their partial actin, calmodulin, *rpb2*, *tef1-α* and ITS sequences used in phylogenetic analysis.**

| Species | Strain | Collection location | Crop | *act* | *cal* | *rpb2* | *tef1-α* | ITS |
|---|---|---|---|---|---|---|---|---|
| *T. koningiopsis* | CEN1386 | Curitibanos, SC | Garlic | MK696725 | MK696671 | MK696779 | MK696617 | MK714859 |
| *T. peberdyi* | CEN1387 | Curitibanos, SC | Garlic | MK696727 | MK696673 | MK696781 | MK696619 | MK714861 |
| *T. peberdyi* | CEN1388 | Curitibanos, SC | Garlic | MK696728 | MK696674 | MK696782 | MK696620 | MK714862 |
| *T. peberdyi* | CEN1389 | Curitibanos, SC | Garlic | MK696729 | MK696675 | MK696783 | MK696621 | MK714863 |
| *T. peberdyi* | CEN1390 | Curitibanos, SC | Garlic | MK696730 | MK696676 | MK696784 | MK696622 | MK714864 |
| *T. peberdyi* | CEN1391 | Rio Paranaíba, MG | Garlic | MK696731 | MK696677 | MK696785 | MK696623 | MK714865 |
| *T. peberdyi* | CEN1392 | Rio Paranaíba, MG | Garlic | MK696732 | MK696678 | MK696786 | MK696624 | MK714866 |
| *T. peberdyi* | CEN1393 | Rio Paranaíba, MG | Garlic | MK696734 | MK696680 | MK696788 | MK696626 | MK714868 |
| *T. hamatum* | CEN1394 | Rio Paranaíba, MG | Garlic | MK696735 | MK696681 | MK696789 | MK696627 | MK714869 |
| *T. hamatum* | CEN1395 | Rio Paranaíba, MG | Garlic | MK696736 | MK696682 | MK696790 | MK696628 | MK714870 |
| *T. asperelloides* | CEN1396 | Rio Paranaíba, MG | Garlic | MK696737 | MK696683 | MK696791 | MK696629 | MK714871 |
| *T. asperelloides* | CEN1397 | Rio Paranaíba, MG | Garlic | MK696738 | MK696684 | MK696792 | MK696630 | MK714872 |
| *T. peberdyi* | CEN1398 | Bueno Brandão, MG | Garlic | MK696740 | MK696686 | MK696794 | MK696632 | MK714874 |
| *T. longibrachiatum* | CEN1399 | São Marcos, RS | Garlic | MK696741 | MK696687 | MK696795 | MK696633 | MK714875 |
| *T. longibrachiatum* | CEN1400 | São Marcos, RS | Garlic | MK696742 | MK696688 | MK696796 | MK696634 | MK714876 |
| *T. longibrachiatum* | CEN1401 | São Marcos, RS | Garlic | MK696744 | MK696690 | MK696798 | MK696636 | MK714878 |
| *T. longibrachiatum* | CEN1402 | São Marcos, RS | Garlic | MK696745 | MK696691 | MK696799 | MK696637 | MK714879 |
| *T. azevedoi* | CEN1403 | São Marcos, RS | Garlic | MK696746 | MK696692 | MK696800 | MK696638 | MK714880 |
| *T. longibrachiatum* | CEN1404 | São Marcos, RS | Garlic | MK696747 | MK696693 | MK696801 | MK696639 | MK714881 |
| *T. koningiopsis* | CEN1405 | São Marcos, RS | Garlic | MK696749 | MK696695 | MK696803 | MK696641 | MK714883 |
| *T. koningiopsis* | CEN1406 | São Marcos, RS | Garlic | MK696750 | MK696696 | MK696804 | MK696642 | MK714884 |
| *T. koningiopsis* | CEN1407 | São Marcos, RS | Garlic | MK696751 | MK696697 | MK696805 | MK696643 | MK714885 |
| *T. asperelloides* | CEN1408 | Monte Alto, SP | Onion | MK696753 | MK696699 | MK696807 | MK696645 | MK714887 |
| *T. asperelloides* | CEN1409 | Monte Alto, SP | Onion | MK696755 | MK696701 | MK696808 | MK696647 | MK714889 |
| *T. afroharzianum* | CEN1410 | Monte Alto, SP | Onion | MK696756 | MK696702 | MK696809 | MK696648 | MK714890 |
| *T. asperelloides* | CEN1411 | Monte Alto, SP | Onion | MK696757 | MK696703 | MK696810 | MK696649 | MK714891 |
| *T. lentiforme* | CEN1412 | Monte Alto, SP | Onion | MK696758 | MK696704 | MK696811 | MK696650 | MK714892 |
| *T. asperelloides* | CEN1413 | Monte Alto, SP | Onion | MK696759 | MK696705 | MK696812 | MK696651 | MK714893 |
| *T. afroharzianum* | CEN1414 | Monte Alto, SP | Onion | MK696760 | MK696706 | MK696813 | MK696652 | MK714894 |
| *T. lentiforme* | CEN1415 | São José do Rio Pardo, SP | Onion | MK696761 | MK696707 | MK696814 | MK696653 | MK714895 |
| *T. lentiforme* | CEN1416 | São José do Rio Pardo, SP | Onion | MK696762 | MK696708 | MK696815 | MK696654 | MK714896 |
| *T. afroharzianum* | CEN1417 | São José do Rio Pardo, SP | Onion | MK696763 | MK696709 | MK696816 | MK696655 | MK714897 |
| *T. asperelloides* | CEN1418 | São José do Rio Pardo, SP | Onion | MK696764 | MK696710 | MK696817 | MK696656 | MK714898 |
| *T. asperelloides* | CEN1419 | São José do Rio Pardo, SP | Onion | MK696765 | MK696711 | MK696818 | MK696657 | MK714899 |
| *T. erinaceum* | CEN1420 | São José do Rio Pardo, SP | Onion | MK696766 | MK696712 | MK696819 | MK696658 | MK714900 |
| *T. erinaceum* | CEN1421 | São José do Rio Pardo, SP | Onion | MK696767 | MK696713 | MK696820 | MK696659 | MK714901 |
| *T. azevedoi* | CEN1422 | Rio Paranaíba, MG | Onion | MK696768 | MK696714 | MK696821 | MK696660 | MK714902 |
| *T. azevedoi* | CEN1423 | Rio Paranaíba, MG | Onion | MK696769 | MK696715 | MK696822 | MK696661 | MK714903 |
| *T. asperelloides* | CEN1424 | Rio Paranaíba, MG | Onion | MK696770 | MK696716 | MK696823 | MK696662 | MK714904 |
| *T. peberdyi* | CEN1425 | Rio Paranaíba, MG | Onion | MK696771 | MK696717 | MK696824 | MK696663 | MK714905 |
| *T. peberdyi* | CEN1426 | Itobi, SP | Onion | MK696772 | MK696718 | MK696825 | MK696664 | MK714906 |
| *T. asperelloides* | CEN1427 | Itobi, SP | Onion | MK696773 | MK696719 | MK696826 | MK696665 | MK714907 |
| *T. lentiforme* | CEN1428 | Itobi, SP | Onion | MK696775 | MK696721 | MK696827 | MK696667 | MK714909 |
| *T. lentiforme* | CEN1429 | São José do Rio Pardo, SP | Onion | MK696776 | MK696722 | MK696828 | MK696668 | MK714910 |
| *T. asperelloides* | CEN1430 | São José do Rio Pardo, SP | Onion | MK696777 | MK696723 | MK696829 | MK696669 | MK714911 |
| *T. asperelloides* | CEN1431 | Sacramento, MG | Onion | MK696778 | MK696724 | MK696830 | MK696670 | MK714912 |

*(Continued)*

**Table 1.** (Continued)

| Species | Strain | Collection location | Crop | *act* | *cal* | *rpb2* | *tef1-α* | ITS |
|---------|--------|---------------------|------|-------|-------|--------|----------|-----|
| *T. peberdyi* | CEN1457 | Curitibanos, SC | Garlic | MK696726 | MK696672 | MK696780 | MK696618 | MK714860 |
| *T. peberdyi* | CEN1458 | Curitibanos, SC | Garlic | MK696733 | MK696679 | MK696787 | MK696625 | MK714867 |
| *T. asperelloides* | CEN1459 | Bueno Brandão, MG | Garlic | MK696739 | MK696685 | MK696793 | MK696631 | MK714873 |
| *T. longibrachiatum* | CEN1460 | São Marcos, RS | Garlic | MK696743 | MK696689 | MK696797 | MK696635 | MK714877 |
| *T. longibrachiatum* | CEN1461 | São Marcos, RS | Garlic | MK696748 | MK696694 | MK696802 | MK696640 | MK714882 |
| *T. longibrachiatum* | CEN1462 | São Marcos, RS | Garlic | MK696752 | MK696698 | MK696806 | MK696644 | MK714886 |
| *T. asperellum* | CEN1463 | Monte Alto, SP | Onion | MK696754 | MK696700 | - | MK696646 | MK714888 |
| *T. asperellum* | CEN1464 | Itobi, SP | Onion | MK696774 | MK696720 | - | MK696666 | MK714908 |

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(v. 1.5, Technelysium Pty Ltd). Sequences, including references obtained from Genbank, were organized into matrices in Bioedit (v. 7.2.6) [26] and aligned using MAFFT v. 7 E-INS-i [27]. A concatenated matrix was assembled using Sequence Matrix (v. 1.8) [28].

An optimal partitioning scheme for each marker was determined in PartitionFinder 2 [35]. Maximum likelihood trees based on data from each marker as well as the concatenated matrix were constructed using IQ-TREE (v. 1.6.5) [36], where optimal nucleotide substitution models
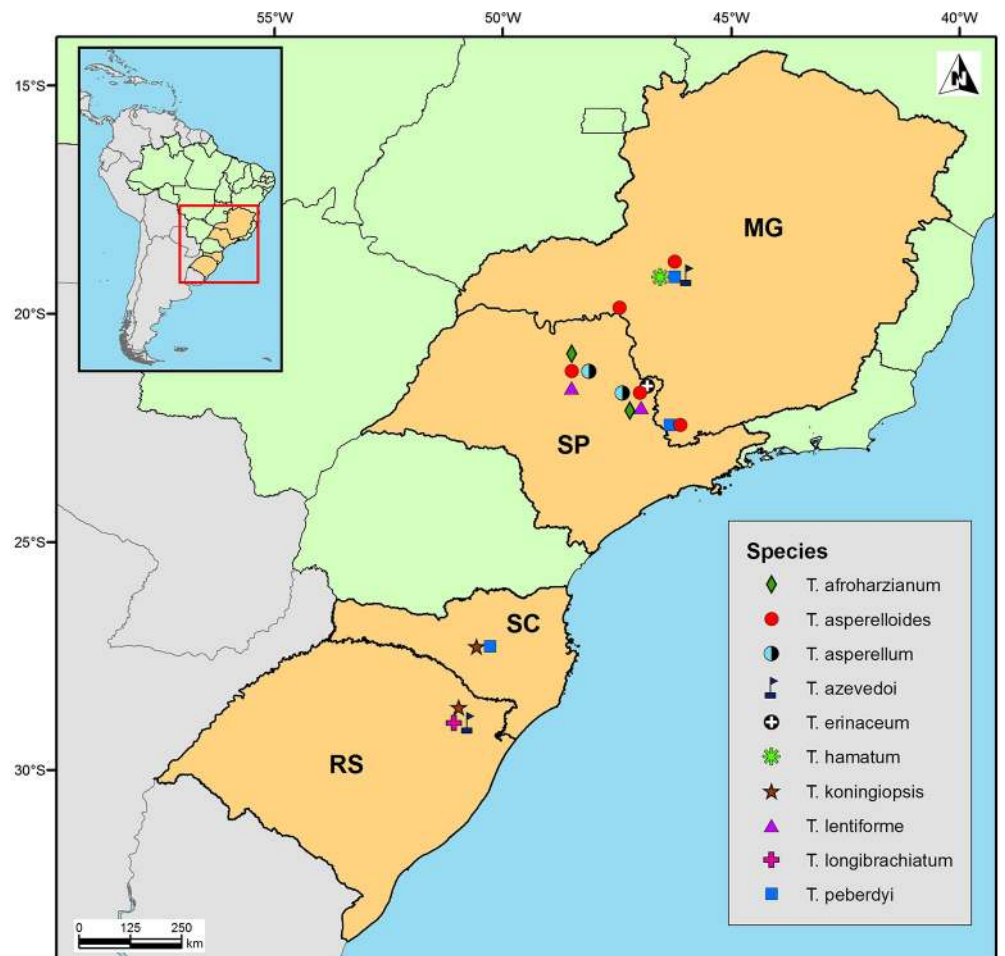


**Fig 1. Map of Southeastern Brazil showing soil collection sites and recovered *Trichoderma* species.**

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**Table 2. Primers used for PCR and sequencing.**

| Locus | Name | Primer sequence 5´-3´ | $T_m$ | Reference |
|---|---|---|---|---|
| ITS | U1 | GGAAGKARAAGTCGTAACAAGG | 55 | [29] |
| | U4 | RGTTTCTTTTCCTCCGCTTA | | " |
| *act* | Tact1 | TGGCACCACACCTTCTACAATGA | 50 | [30] |
| | Tact2 | TCTCCTTCTGCATACGGTCGGA | | [31] |
| * | Tact511R | CTCAGGAGCACGGAAT | | " |
| * | Tact293F | GTGATCTTACCGACTACCTGATG | | This study |
| *cal* | CAL-228F | GAGTTCAAGGAGGCCTTCTCCC | 55 | [32] |
| | CAL-737R | CATCTTTCTGGCCATCATGG | | " |
| * | CAL-235F | TTCAAGGAGGCCTTCTCCCTCTT | | " |
| *tef1-α* | tef71f | CAAAATGGGTAAGGAGGASAAGAC | 50 | [33] |
| * | tef85f | AGGACAAGACTCACATC AACG | | " |
| * | tef954r | AGTACCAGTGATCATGTTCTTG | | " |
| * | tef997R | CAGTACCGGCRGCRATRATSAG | | " |
| | tef1080R | GATACCAGCCTCGAACTCACC | | This study |
| | EF1–1018F | GAYTTCATCAAGAACATGAT | | [34] |
| | EF1–1620R | GACGTTGAADCCRACRTTGTC | | " |
| *rpb2* | RPB2_210up | TGGGGWGAYCARAARAAGG | 48 | Tom Gräfenhan; |
| | RPB2_1450low | CATRATGACSGAATCTTCCTGGT | | http://www.isth.info |
| * | RPB2 1150low | GGTTGTGATCRGGRAARGGAATG | | " |

*Internal primers used for sequencing only.

for each partition were selected using ModelFinder [37]. Branch support was estimated using the Ultrafast bootstrap (UFBoot) [38] with 1000 replicates. Branches with UFboot support of > = 95% were considered credible. Representative sequences of each *Trichoderma* isolate cluster for each marker were used in BLAST [39] searches of the Genbank database in order to make provisional *Trichoderma* species identifications. This information was used to select reference sequences for further analyses, also incorporating strains used in recent taxonomic and molecular phylogenetic treatments of appropriate *Trichoderma* sections [23,40,41], S1 Table.

The partitioned concatenated matrix was also analysed using the Bayesian Metropolis-coupled Markov Chain Monte Carlo method as implemented in MrBayes 3.2.6 [42], running on the CIPRES Science Gateway [43] and utilizing the Beagle library [44]. Model selection for each partition was made in PartitionFinder2 [35]. Two runs of eight MCMCMC chains with a heating temperature of 0.075 were conducted for ten million generations, sampling every 1000 generations. This runtime was sufficient for the convergence diagnostic, the standard deviation of split frequencies, to fall to a minimum of 0.005217. The first 25% of the trees were discarded (burn-in) prior to calculation of the 50% majority rule consensus tree.

## AFLP genotyping

Genetic variability among a representative selection of 46 of the 54 *Trichoderma* isolates was evaluated using the amplified fragment length polymorphism method (AFLP; [45]; adapter and primer sequences given in Table 3) adapted for fluorescent detection. A one-step digestion and adapter-ligation protocol was adopted, which were performed in 20 μl volumes. A single reaction comprised 1X ligase buffer (Promega), 50 mM NaCl, 0.05 μg/μl bovine serum albumin, one unit T4 DNA ligase (Promega), five pmol EcoRI adapter, 50 pmol *Mse*I adapter, five units *Eco*RI (*Eco*RI-HF high fidelity, NEB), five units of *Mse*I and 100 ng genomic DNA. The

**Table 3. AFLP adapter and primer sequences.**

| Primer name | Primer sequence 5′-3′ |
|---|---|
| *Eco*RI-Adapter1 | CTCGTAGACTGCGTACC |
| *Eco*RI-Adapter2 | AATTGGTACGCAGTCTAC |
| *Eco*RI+A | GACTGCGTACCAATTCA |
| *Mse*I_Adapter1 | GACGATGAGTCCTGAG |
| *Mse*I_Adapter2 | TACTCAGGACTCAT |
| *Mse*I+C | GATGAGTCCTGAGTAAC |
| (11) *Eco*RI-AC FAM | FAM+GACTGCGTACCAATTCAC |
| (14) *Eco*RI-AA VIC | VIC+GACTGCGTACCAATTCAA |
| (2) *Mse*I+CTT | GATGAGTCCTGAGTAACTT |
| (3) *Mse*I+CAT | GATGAGTCCTGAGTAACAT |
| (5) *Mse*I+CAG | GATGAGTCCTGAGTAACAG |
| (6) *Mse*I+CAA | GATGAGTCCTGAGTAACAA |

reactions were incubated on a PCR machine at 37°C for two hours, then held at 17°C for one hour and then held at 4°C for two hours. Samples were then diluted five times by the addition of 80 μl $H_2O$ and stored at -80°C. The primers *Eco*RI+A and *Mse*I+C were used for preselective PCR. A 20 μl PCR in 1X PCR Buffer with 2 mM $Mg^{2+}$ contained 1 M Betaine, 0.25 mM dNTPs, 0.5 μM each primer, 1 U *Taq* polymerase and 2 μl of the diluted adapter-ligated DNA. Cycling conditions comprised an initial 72°C for 2 min to allow fill-in of the adapter ends, then 20 cycles of 94°C for 30 sec, 56°C for 1 min and 72°C for 2 min. Ramp rate was limited to 1°C per second. Following cycling, reactions were held at 72°C for 2 min and then 60°C for 30 min. Five μl of PCR products were subsequently analysed on a 1.5% agarose gel, producing a faint smear if the reaction was successful. The preselected DNA was then diluted five times by the addition of 80 μl $H_2O$ and stored at -20°C.

Genomic complexity was further reduced using selective PCR to produce resolvable AFLP profiles, using PCR primer pairs comprising one labelled *Eco*RI+2 and one unlabelled *Mse*I+3 primer (Table 3). Selective primer combinations producing an adequate number of clear fluorescent peaks in preliminary screening were chosen from the range available in the Small Plant Genome Mapping Kit (Applied Biosystems). We found that whilst most *Mse*I+3 primer variations gave satisfactory results, only the *Eco*RI+AC and *Eco*RI+AA primers were efficient in *Trichoderma*. A single 10 μl selective PCR reaction in 1X PCR buffer with 2 mM $Mg^{2+}$ contained 0.15 μM each of *Mse*I+3 primer and fluorochrome-labelled *Eco*RI+2 primer, 0.2 mM dNTPs, 0.5 U *Taq* polymerase and 2 μl of diluted preselected DNA. PCR cycling comprised an initial 94°C for 2 min, then 10 cycles of 94°C for 30 sec, 66°C for 30 sec and 72°C for 1 min. The annealing temperature was reduced by 1°C per cycle (touchdown). Then followed 25 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min. Reactions were then held at 72°C for 3 min and at 60°C for 30 min. Six primer combinations (11x5, 11x6, 14x2, 14x3, 14x5, 14x6; Table 3) were selected for the full analysis. The fluorescent AFLP profiles were detected by mixing 1 μl PCR product with 9 μl HiDi formamide and 0.3 μl of the Genescan 600-LIZ v 2.0 molecular size ladder (Applied Biosystems). Samples were denatured at 95°C for 5 minutes and snap cooled on ice, prior to injection on an ABI 3730 DNA Analyzer (Applied Biosystems).

The raw AFLP data files were processed using PeakScanner (v. 2; Applied Biosystems). The table of peak area data was then imported into the R CRAN library program, RawGeno [46], for peak binning and filtering of low quality or partially overlapping peaks, thereby reducing the risk of size-homoplasy. The filtered AFLP profiles were then converted into a peak

presence or absence binary matrix, totalling 364 characters. Data was analysed under the F81 (restriction; nst = 1 rates = invgamma) model in MrBayes 3.2.6 [42] using two runs of four MCMCMC chains, where two million generations were sampled every 1000 generations. This runtime was sufficient for the average standard deviation of split frequencies to fall to 0.007734. The first 25% of the trees were discarded (burn-in) prior to calculation of the 50% majority rule consensus tree. Intraspecific Nei-Li genetic distances (fragments, length = 4) were calculated in PAUP (v. 4.0a165) [47].

## MALDI-TOF phenotyping and rapid identification

Samples of *Trichoderma* strains were collected from colonies cultivated on PDA plates, which were applied directly to a MSP96 plate (Bruker Daltonics GmbH, Bremen, Germany) and covered with 1 μl of MALDI matrix solution ('Bruker HCCA' or α-cyano-4-hydroxycinnamic acid, at a final concentration of 5 mg HCCA ml$^{-1}$). After sample drying, analyses were performed on a MicroFlex MALDI-TOF mass spectrometer (Bruker Daltonics GmbH), fitted with a nitrogen laser (337 nm) of 20–65% offset intensity and spiral mode of acquisition, where an average of 400 shots (40 laser shots at 10 different regions of the target spot) at 60 Hz were conducted. Signals in the range 2000–20000 m/z were automatically collected with Auto-Converter from the acquisition software (FlexControl 3.3; Bruker Daltonics GmbH). Data were exported to MALDI Biotyper software (3.0; Bruker Daltonics GmbH) and each consensus spectrum incorporated into a profile in the mean spectrum projection (MSP) database.

Based on the results of the phylogenetic analysis, the strains CEN1386, CEN1395, CEN1402, CEN1419, CEN1390, CEN1416 and CEN1420, were selected for the creation of a local *Trichoderma* spectrum database using the Biotyper MBT Explorer Software Module. The library was constructed by sampling colonies grown on individual PDA plates for five days, where material was collected from three distinct regions (colony edge, intermediate and central). The spectra obtained were then used to generate species profiles which were added to the database. Subsequent samples were analyzed in triplicate. Cluster analysis was conducted using the MSP Dendrogram Creation Standard Method (v.1.4) of MALDI Biotyper Software (v. 3.0).

## Nomenclature

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS ONE article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank numbers contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: PubMed Central, LOCKSS.

## Results and discussion

A total of 54 *Trichoderma* strains were isolated from crop soil samples from multiple sites representing the main growing areas of garlic and onion in Brazil. In quantitative terms, 11 strains were isolated from Rio Paranaíba, MG; one from Bueno Brandão, MG; one from Sacramento,

**Table 4. Partition statistics for each sequenced DNA locus generated in IQ-TREE.**

| Partition | Sites | Invariable sites | Parsimony informative sites | Tree length | Best fit model (BIC) |
|-----------|-------|------------------|------------------------------|-------------|----------------------|
| *act* | 754 | 579 | 135 | 1.1454 | TIM3e+I+G4 |
| *cal* | 524 | 216 | 263 | 3.2113 | K2P+I+G4 |
| ITS | 685 | 405 | 144 | 1.8155 | TIM2+F+R3 |
| *rpb2* | 800 | 471 | 295 | 2.7089 | TIMe+I+G4 |
| *tef1-α* | 752 | 272 | 405 | 9.7299 | TN+F+R4 |

https://doi.org/10.1371/journal.pone.0228485.t004

MG; seven from Monte Alto, SP; nine from São José do Rio Pardo, SP; three from Itobi, SP; nine from São Marcos, RS; and five from Curitibanos, SC (Table 1; Fig 1).

All 54 presumptive strains were confirmed as *Trichoderma* species using the ITS oligonucleotide barcode identification program *Trich*OKEY2 [48] http://www.isth.info/. However, confident and unambiguous species identifications were not obtained in the searches, as expected, since previous studies have pointed out the limitations of ITS sequences to delimit *Trichoderma* species [23]. Furthermore, 12 isolates were returned as belonging to unidentified *Trichoderma* species. We therefore performed a full phylogenetic analysis on all 54 isolates with the addition of a further four phylogenetic markers: *act*, *cal*, *tef1-α* and *rpb2*. We first performed a ML phylogenetic analysis on the most highly substituted data set (*tef1-α*; Table 4). Clusters with high sequence similarity were identified and a representative sequence of each cluster used in BLAST [39] searches of the Genbank nucleotide database. Reference sequences for each marker were then selected from the *Trichoderma* (or its sexual morph, *Hypocrea*) species producing top hits, giving preference to type strains and those with most complete representation for our marker selection. We also selected reference sequences based on recent molecular taxonomic treatments of the *Trichoderma* sections and major clades identified in the initial ITS *Trich*OKEY screen (S1 Table).

Among the sequenced markers, the most informative, based on number of parsimony informative characters (PICS), was *tef1-α*, followed by *rpb2*, *cal*, ITS and *act* (Table 4). The *tef1-α* matrix, including reference sequences, was notably rich in indels, presenting a potential risk for alignment ambiguity, which we attempted to minimize by the use of MAFFT E-INS-i, which is among the most accurate of modern consistency-based programs [49]. Among the 54 isolates studied, sequence data were gathered for all five markers, except for two isolates, later identified as *Trichoderma asperellum*, for which the *rpb2* marker could not be amplified, probably due to critical primer mismatch.

The Bayesian phylogram based on the concatenation of ITS, *rpb2*, *act*, *cal* and *tef1-α* sequences (Fig 2) permitted the unambiguous identification of eight species among the 54 isolates, based on their clustering with reference taxa. The isolates identified in the analysis included five *Trichoderma lentiforme*, two *Trichoderma hamatum*, three *Trichoderma afroharzianum*, four *Trichoderma koningiopsis*, two *Trichoderma erinaceum*, 13 *Trichoderma asperelloides*, two *T. asperellum* and eight *Trichoderma longibrachiatum*. The remaining 15 isolates formed two distinct clades: one comprising three isolates, most closely related to *Trichoderma rifaii*, *Trichoderma afarasin*, *Trichoderma endophyticum* and *Trichoderma neotropicale* and a second clade comprising 12 isolates, corresponding to the unidentified group in the TrichOKEY search, most closely related to *Trichoderma ceraceum* and *Trichoderma tomentosum*. Both clades are highly supported in the Bayesian analysis (PP = 1) (Fig 2) and possess 100% ultrafast bootstrap support in a maximum likelihood tree based on the same concatenated matrix (S1 Fig). We therefore suspected that the two unidentified clades represent new *Trichoderma* species, which we confirmed by examination of their distinctive growth and morphological characteristics.
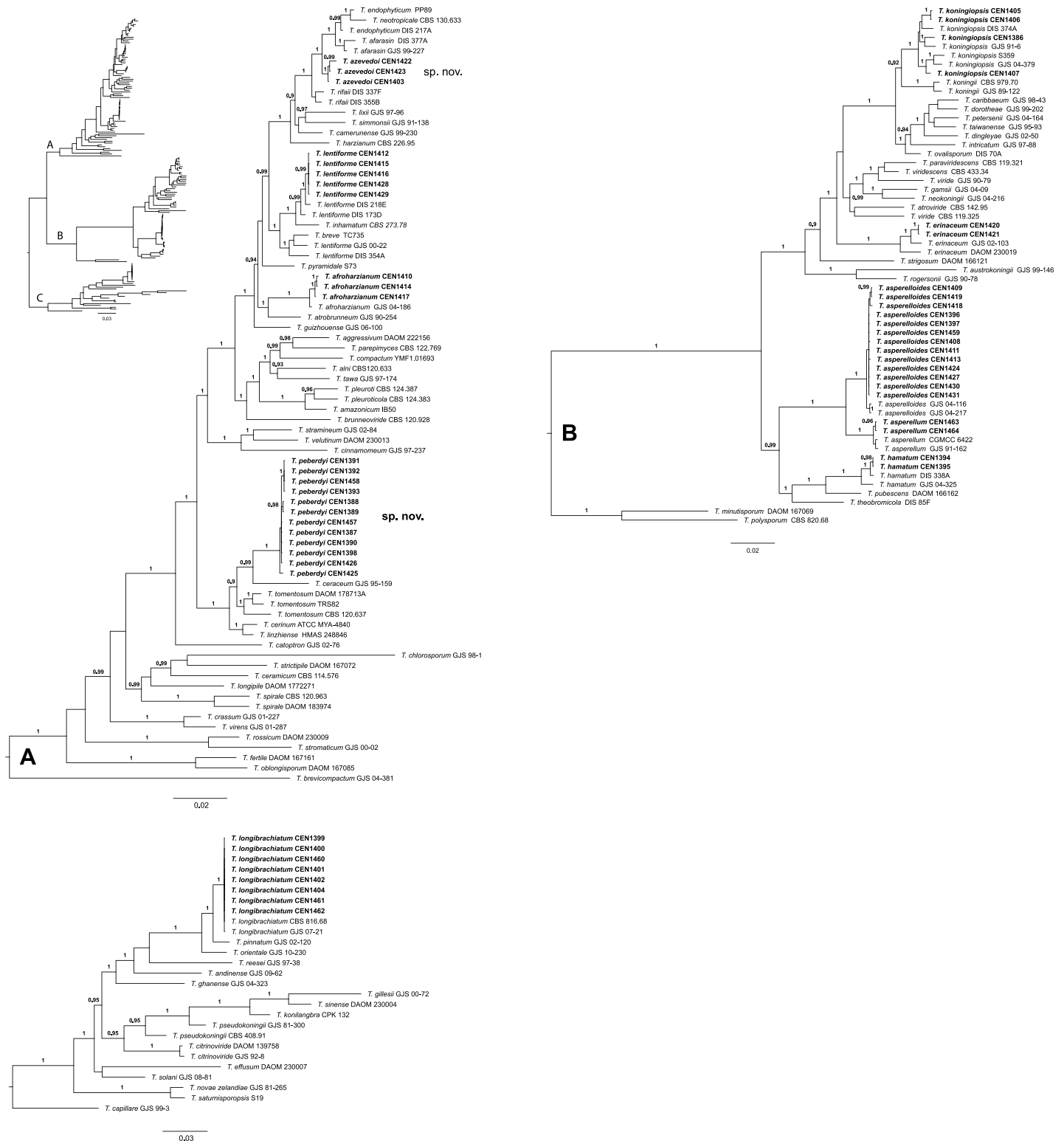
**Fig 2.** A-C. Midpoint rooted Bayesian phylogram (split into three parts as indicated in the overview), based on the concatenation of *act*, *cal*, ITS, *rpb2* and *tef1-α* matrices. Posterior probabilities are given above branches (> 0.9) and the scale bar represents expected changes per site. Strains sequenced in the present study are in bold and are followed by CENxxx numbers. Two new *Trichoderma* species, *T. azevedoi* and *T. peberdyi* are indicated.
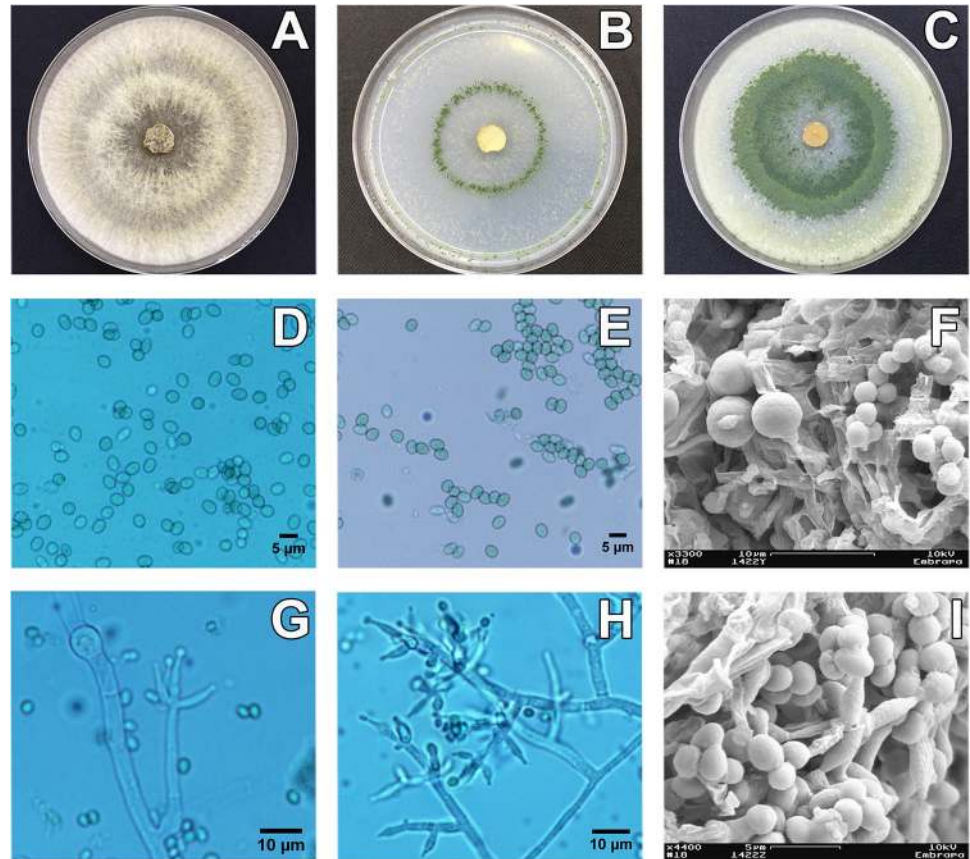
https://doi.org/10.1371/journal.pone.0228485.g002

**Fig 3. Culture characteristics and morphology of *T. azevedoi* sp. nov. strain CEN1422 (holotype).** Panels A-I: Growth on three different media, PDA (A); SNA (B); CMD (C); and morphology of the conidia (D, E), phialides (G, H) and chlamydospores (G) using optical microscopy and conidia and chlamydospores using electron microscopy (F, I).

https://doi.org/10.1371/journal.pone.0228485.g003

## Taxonomy

*Trichoderma azevedoi* Valadares-Inglis, M.C. & Inglis, P.W. sp. nov. [Fig 3](#). Mycobank MB830305. [urn:lsid:mycobank.org: 830305]

**Etmology.** Named in honour of João Lúcio Azevedo (São Paulo University—Brazil) for his contributions to mycology and microbial genetics in Brazil, including the mentoring of numerous professionals in *Trichoderma* studies.

**Holotype.** CEN1422, a freeze dried, metabolically inactive culture deposited in the Herbarium of Embrapa Recursos Genéticos e Biotecnologia (CEN). Collected in Rio Paranaíba—MG state, Brazil, $20^0 \, 05^{'} \, 06^{''}$ S, $51^0 \, 00^{'} \, 02^{''}$ E, from onion crop soil, 02/07/2015, by V. Lourenço Jr. & J.B.T. da Silva. An ex-holotype culture of CEN1422 has been deposited in the Embrapa Coleção de Microrganismos para o Controle de Fitopatógenos e Plantas Daninhas, with the accession number BRM46357.

**Description.** On CMD, colony radius 40 mm after 72h at 25 and 30°C and 12 h photoperiod. Mycelium hyaline with cottony pustules, sporulating heavily after 72 h at 30°C and 96 h at 25°C, turning green after 96 h, more abundantly in a broad ring about half-way to the plate center. At 20°C and 12 h photoperiod, colony radius 25 mm, mycelium hyaline with pustules of spores formed at 96h. No growth observed at 15 and 30°C. On SNA, colony radius 34 mm at 25°C and 40 mm at 30°C after 72 h with 12 h photoperiod. Mycelium hyaline with spores

formed after 72 h in sparse clumps distributed throughout the plate. At 30˚C mid-green spores are formed in a distinct thin concentric ring, approximately one third of the radius from the plate center to the edge. At 20˚C spores are produced after 96 h similarly to 30˚C. On PDA, colony radius 17 mm at 20˚C, 62 mm at 25˚C and 4 mm at 30˚C, at 72 h with 12 h photoperiod. Mycelium cottony, with light green spores formed after 96 h, concentrated in the centre of the plate and in a broad concentric ring approximately half-way to the plate edge.

Conidiophores trichoderma-like, pyramidal with opposing branches or isolated, terminating in groups of three to five phialides. Phialides ampulliform to lageniform, constricted below the tip forming a narrow neck, measuring 7.71 ± 1.42 x 2.52 ± 0.32 μm (overall range 5.45–10.75 x 1.89–3.17 μm), base 1.46–2.55 μm (mean 1.99 μm). Conidia globose, subglobose to ovoid 3.90 ± 0.31 x 2.93 ± 0.22 μm (overall range: 3.54–4.65 x 2.55–3.33 μm). Chlamydospores common, terminal and intercalary, typically globose.

Sexual morph: Unknown. Known distribution: Brazil.

**Other isolates examined.** CEN1403, CEN1423. From garlic or onion crop soils.

**Notes.** *Trichoderma azevedoi* is closely related to *Trichoderma rifaii* (a member of the *Trichoderma harzianum* complex). However, *T. rifaii* is known only as an endophyte of *Theobroma cacao* and *Theobroma gileri*. *T. azevedoi* conidia (mean 3.90 x 2.93 μm) are much larger than *T. rifaii* (mean 2.6 x 2.4 μm) and *T. azevedoi* produces abundant clamydospores, which have not been reported in *T. rifaii*.

*Trichoderma peberdyi* Valadares-Inglis, M.C. & Inglis, P.W. **sp. nov. Fig 4.**

Mycobank MB830304. [urn:lsid:mycobank.org: 830304]

**Etmology.** Named in honour of John F. Peberdy (Nottingham University, UK), for his important contributions to mycology and fungal biotechnology.

**Holotype.** CEN1426, a freeze dried, metabolically inactive culture deposited in the Herbarium of Embrapa Recursos Genéticos e Biotecnologia (CEN). It was isolated in Itobi—SP state, Brazil, 21⁰ 44′ 13" S, 46⁰ 58′ 30" E, from onion crop soil, on 02/09/2015, by V. Lourenço Jr & J.B.T. da Silva. An ex-holotype culture of CEN1426 has been deposited in the Embrapa Coleção de Microrganismos para o Controle de Fitopatógenos e Plantas Daninhas, with the accession number BRM46363.

**Description.** On CMD, colony radius 40 mm after 72h at 25 and 30˚C with 12 h photoperiod. Colony hyaline in sterile zones with cottony aerial hyphae after 72 h at 25 and 30˚C. Spores formed after 120 h at 25˚C in pustules concentrated at the plate edge. Light green spores produced at 30˚C after 120 h. At 20˚C and 12h photoperiod, hyaline mycelium covering entire plate and no spores observed after 120h. No growth at 15 and 35˚C. On SNA, colony radius 40 mm after 96 h at 25˚C and 30˚C, under 12h photoperiod. Colony hyaline with sparse cottony aerial hyphae. Light green spores produced in rays near plate edge after 120 h at 25˚C. Sporulation less dense at 30˚C. No spores formed at 20˚C. No growth observed 15 and 35˚C after 120h. On PDA, colony radius 40 mm at 25 and 30˚C after 72h under 12h photoperiod. Mycelium cottony, with aerial hyphae covering the entire plate with conidia forming under cottony aerial hyphae after 120h at 25 and 30˚C. No diffusible pigments or distinctive odours observed. Conidiophores trichoderma-like, pyramidal with opposing branches or isolated, terminating in groups of two to three phialides. Phialides ampulliform, 7.04 ± 1.01 x 2.67 ± 0.36 (range: 4.91–9.10 x 2.20–3.73 μm), base 1.46–2.55 (mean 1.99 μm). Conidia subglobose to ovoid 3.54–4.65 (3.90) x 2.55–3.33 (2.93) μm, thinning in the proximal region, produced in chains and aggregated in mucilaginous masses. Chlamydospores not observed.

Sexual morph: Unknown. Known distribution: Brazil.

**Other isolates examined.** CEN1387, CEN1388, CEN1389, CEN1390, CEN1391, CEN1392, CEN1393, CEN1398, CEN1457, CEN1458, CEN1425. All from garlic or onion crop soils.
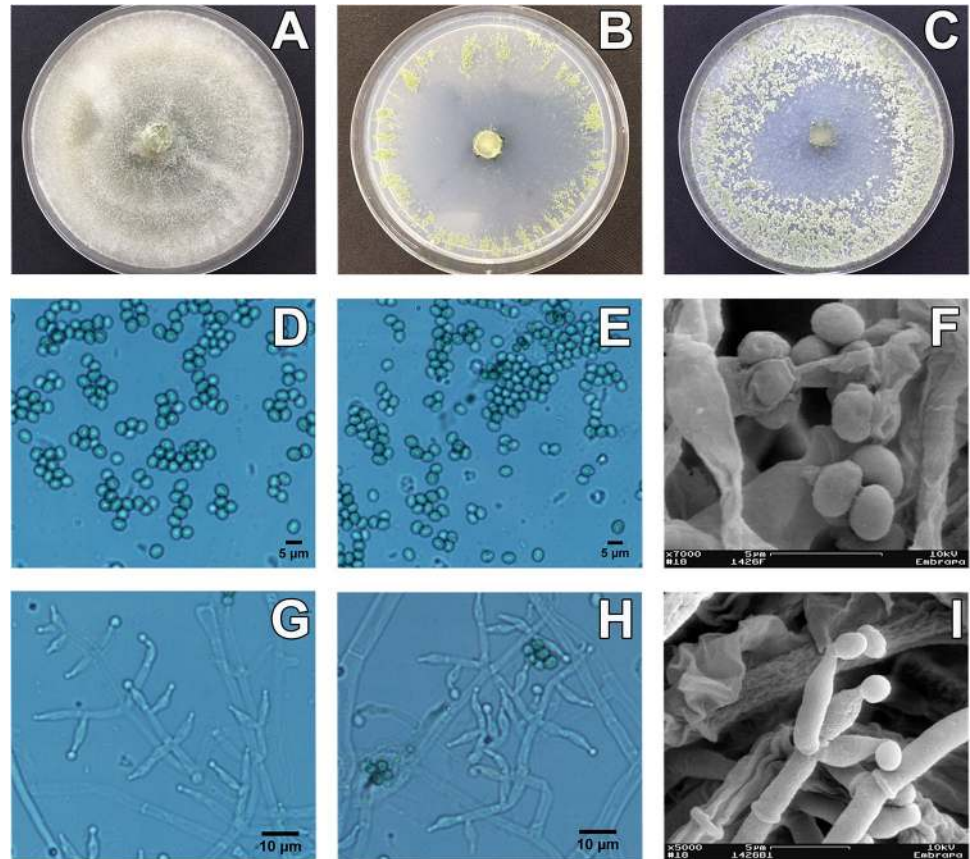
**Fig 4. Culture characteristics and morphology of *T. peberdyi* sp. nov. strain CEN1426 (holotype).** Panels A-I: Growth on three different media, PDA (A); SNA (B); CMD (C); and morphology of the conidia (D, E) and phialides using optical microscopy (G, H) and conidia using electron microscopy (F, I).

https://doi.org/10.1371/journal.pone.0228485.g004

**Notes.** *Trichoderma peberdyi* is closely related to *Trichoderma tomentosum* and *Trichoderma ceraceum*. In comparison with *T. tomentosum*, phialides of *T. peberdyi* are longer and possess a distinct neck, mostly curved towards the tip. *T. peberdyi* conidia are a much lighter green than *T. tomentosum* on SNA media and not produced in distinct concentric rings. *T. peberdyi* conidia are subglobose to ovoid, larger than *T. tomentosum*, a species that produces chlamydospores on CMD, unlike *T. peberdyi*. *T. peberdyi* is distinct from *T. ceraceum* by its lack of diffusible yellow pigment and absence of drops of clear green liquid into which conidia form. *T. ceraceum* is known only from the USA.

In trees based on individual markers (S2–S6 Figs), *T. peberdyi* sp. nov. was distinct from all other *Trichoderma* species and was well-supported in all but the *act* ML tree. *T. azevedoi*, however, appears to be closely related to other neotropical Harzianum clade species, but was clearly distinct and supported in the *act* and *tef1-α* ML trees (S2 and S6 Figs).

## Geographical distribution of *Trichoderma* species in garlic and onion crop soils

The 54 isolates identified to species level, which were collected from eight different sites distributed in four southeastern Brazilian states, fell into three *Trichoderma* sections: *Pachybasium*, *Trichoderma* and *Longibrachiatum* (www.isth.info). The species diversity per collection site varied so that one site yielded a single *Trichoderma* species, two sites yielded two species,

one site yielded three species and four sites yielded four species each (Fig 1; Table 1). In terms of crop, garlic (four sites) yielded six *Trichoderma* species and onion crops (six sites) yielded seven different species. Of the three most frequently isolated species in our analysis, *T. asperelloides* was isolated from six sites, *T. longibrachiatum* was isolated from a single site and one of the new species, *T. peberdyi*, was isolated from four sites (Fig 1).

Inferences on local species diversity are tentative at best, however, and would require a much larger quantitative study, possibly using a meta-barcoding approach (reviewed in Kredics et al., 2018). In a study on the diversity of *Trichoderma* species in the Colombian Amazon region, DNA barcoding of 107 strains using ITS and *tef1* sequences showed that three common cosmopolitan species comprise 68% of the studied isolates, with *T. harzianum sensu lato* representing 38% of strains, followed by *Trichoderma spirale* at 17% and *T. koningiopsis* at 13%, whereas only four putative new taxa were suggested [50]. A larger study of 2078 *Trichoderma* strains collected from agricultural fields in Eastern China, representing four major agricultural provinces, identified 17 known species: *T. harzianum* (429 isolates), *T. asperellum* (425), *T. hamatum* (397), *T. virens* (340), *T. koningiopsis* (248), *T. brevicompactum* (73), *T. atroviride* (73), *T. fertile* (26), *T. longibrachiatum* (22), *T. pleuroticola* (16), *T. erinaceum* (16), *T. oblongisporum* (2), *T. polysporum* (2), *T. spirale* (2), *T. capillare* (2), *T. velutinum* (2), and *T. saturnisporum* (1) [51]. The authors showed that *Trichoderma* biodiversity in agricultural fields varied by region, crop, and season, where, for example, relative frequencies of *T. hamatum* and *T. koningiopsis* from rice crop soil were higher than those from wheat and maize soils, suggesting a crop preference of specific *Trichoderma* species. Although this study principally used ITS sequences to identify species and did not split the *T. harzianum* species complex along the lines of its currently accepted taxonomic framework [23], there is remarkable overlap with the species recovered in the present study of Brazilian garlic and onion crop soils, despite the large geographical separation. There is accumulating evidence that certain *Trichoderma* species have become highly adapted to agroecosystems. Sixty-five percent of *Trichoderma* species associated with the rhizosphere of maize were shared between samples collected from Austria, Tenerife, Madagascar and New Zealand, whereas *Trichoderma* species associated with endemic plants from the same regions were highly specific and diverse. All analysed rhizosphere samples, however, shared a global *Trichoderma* core community dominated by *T. koningii* and *T. koningiopsis* [52].

While a comprehensive worldwide survey of the distribution of *Trichoderma* species under the current rapidly evolving taxonomic framework does not yet exist, recent re-evaluations of existing international culture collections and new collecting efforts in under-sampled geographical locations have greatly expanded our knowledge. Pertinent to the new species recovered herein, those most closely related to *T. azevedoi* include *T. T. rifaii*, *T. endophyticum* and *T. neotropicale*, all of which have been reported to have neotropical distributions [23,33,53]. Species most closely related to *T. peberdyi* include *T. ceraceum*, first reported from the USA [54] and *T. tomentosum*, which is probably cosmopolitan (unpublished Genbank strain data). Among the other *Trichoderma* species recovered in the current study (Table 1), *T. lentiforme* has been reported to be neotropical [23], while the remaining species are of worldwide distribution [23,51,55,56].

## Genotypic and phenotypic variability of *Trichoderma* strains

**AFLP.** AFLP is a powerful and established molecular tool for the analysis of genetic variation in fungal populations [57]. The combination of six selective AFLP primer pairs (Table 3) yielded 364 binary characters in our selected sample of *Trichoderma* isolates, which were analysed using Bayesian phylogenetic inference (Fig 5). The AFLP clusters agreed closely with the

species designations obtained by amplicon sequencing, where each species cluster possessed high posterior support. Closely related species in the Harzianum clade (*T. lentiforme*, *T. azevedoi*, *T. afroharzianum* and *T. peberdyi*), were clearly distinguished. However, posterior supports for some of the deeper branches of the tree were poor, as was topological congruence with the DNA sequence-based tree, suggesting that phylogenetic signals were probably saturated at this level or overcome by homoplasy. Modifications of the AFLP protocol, such as the use of *Eco*RI+3 primers for selective PCR, exclusion of smaller fragments or scoring of only major high rfu peaks, might improve phylogenetic resolution in deeper nodes of the resultant tree, but are likely to be at the expense of the ability to discriminate closely-related taxa. Fragment homology has been shown to decrease with greater time since divergence, so that AFLP data are probably best suited for examining phylogeographic patterns within species and among very recently diverged species [58].

In *T. asperelloides*, which was one of the most commonly sampled taxa, there was no consistent, well-supported monophyletic grouping of strains according to geographical location (Fig 5). All isolates of this species, however, were collected in the south of MG and the north of SP states, which are contiguous regions (Fig 1) and where populations are possibly not clearly structured. In *T. peberdyi* sp. nov., another frequently sampled species, a well-supported clade of four isolates was apparent, all from the municipality of Rio Paranaíba in the mid-west of MG state (Table 1). The clade was further structured so that strain CEN1425, isolated from onion, was differentiated from the other three isolates, which were all isolated from garlic crop soil. The remaining *T. peberdyi* strains lacked clear and supported phylogeographic groupings, although most were isolated in the discontiguous SC state, where the outlier strain, CEN1387, was also isolated. Strain CEN1398 from Bueno Brandão-MG grouped with strain CEN1390 from SC. It is unclear if this pattern represents strain dispersal from SC or is the result of limited sampling of a contiguous population of the lineage, since the geographical range of *T. peberdyi* is currently unknown. *T. peberdyi*, along with our second newly described species, *T. azevedoi* sp. nov., possessed the widest geographical range observed in the current study. The two *T. azevedoi* strains collected from MG state formed a well-supported clade, distinct from the third strain collected from the distant RS state. The other *Trichoderma* species appeared to be common to only one or a few contiguous locations (Table 1; Fig 1), where mixing and dispersal of haplotypes over shorter distances is probably frequent. Elsewhere, in a study of *Trichoderma* spp. associated with the button mushroom, *Agaricus bisporus*, no clear trend was detected between AFLP clustering and geographic origin of isolated materials [59]. In terms of strain distinction, all of the *Trichoderma* species analysed by AFLP demonstrated significant genetic variability in the Bayesian phylogenetic analysis (Fig 5) and in calculated pairwise genetic distances (Table 5). *T. peberdyi* possessed the largest maximum intraspecific genetic distance, although the largest mean intraspecific distance was in *T. koningiopsis*.

**MALDI-TOF.** Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) has become an attractive tool for the identification of microorganisms due to short processing time, reliable identification and low per-sample cost. Many filamentous fungi, such as *Aspergillus*, *Fusarium*, *Penicillium* and *Trichoderma*, have been identified by MALDI-TOF [20], where the technique can be used to complement DNA-based identification [60].

We selected 46 strains for analysis using MALDI-TOF, representing at least two of each *Trichoderma* species, previously identified by sequence analysis (Fig 2), with the exception of *T. asperellum*. Distance-based clustering of MALDI TOF spectra produced a dendrogram (Fig 6) with terminal clusters perfectly matching sequence-based identifications (Fig 2). Echoing the AFLP genotyping result (Fig 5), *T. peberdyi* was remarkable for the large phenotypic distance between strains in the MALDI-TOF dendrogram, second only to *T. koningiopsis*. In contrast, the *T. asperelloides* and *T. longibrachiatum* strains, which showed genetic variability in the
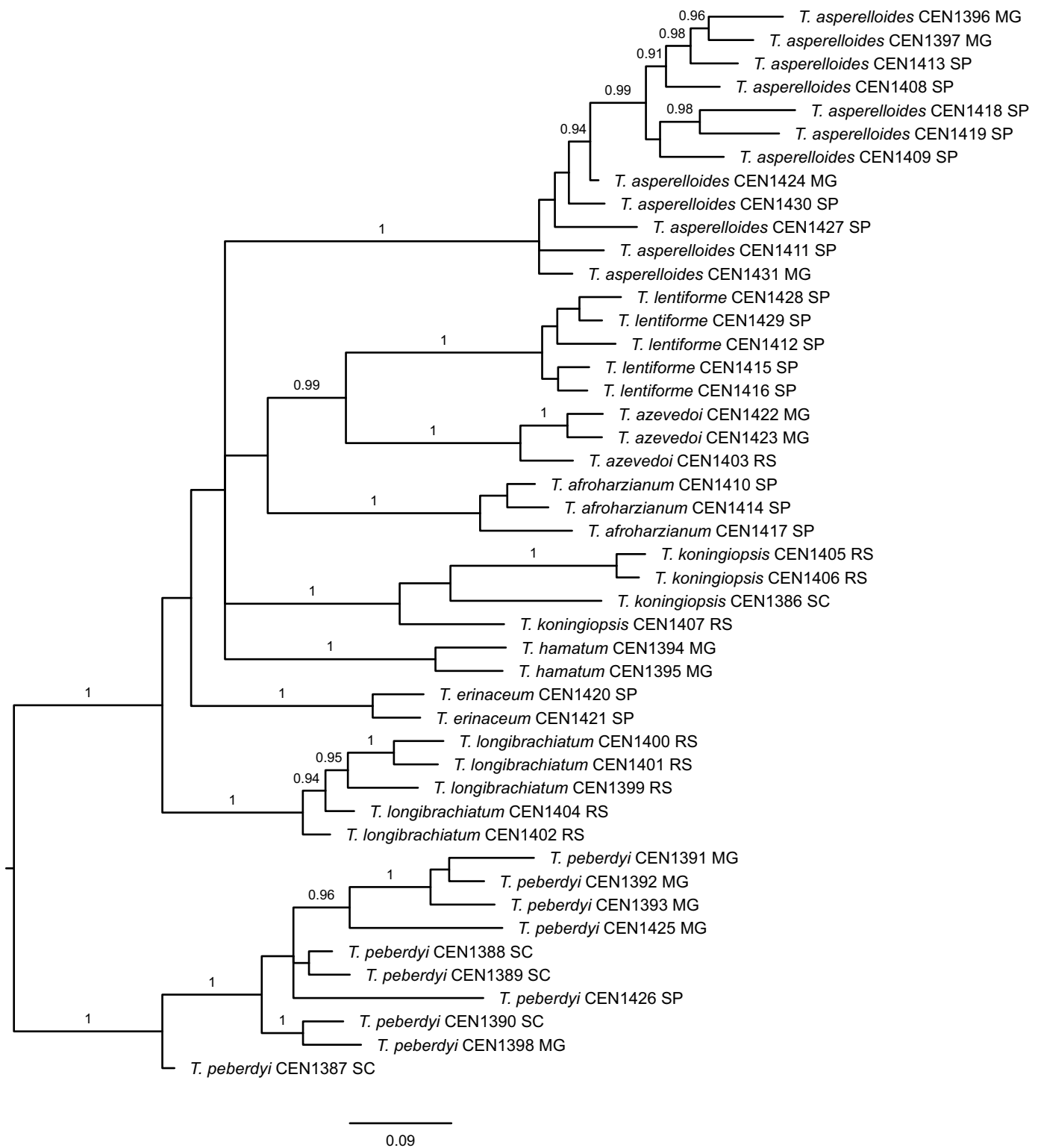
**Fig 5. AFLP midpoint rooted Bayesian phylogram.** Posterior probabilities are given above branches (>0.9) and the scale bar represents expected changes per site. Species names are followed by strain number and collection location by Brazilian state.

**Table 5. Intraspecific genetic distances based on ALFPs.**

| Species | Mean Distance | Maximum Distance |
|---|---|---|
| *T. afroharzianum* | 0.0659 | 0.0797 |
| *T. asperelloides* | 0.0890 | 0.1209 |
| *T. azevedoi* | 0.0623 | 0.0742 |
| *T. erinaceum* | 0.0549 | 0.0549 |
| *T. hamatum* | 0.0769 | 0.0769 |
| *T. koningiopsis* | 0.1447 | 0.1786 |
| *T. lentiforme* | 0.0500 | 0.0659 |
| *T. longibrachiatum* | 0.0703 | 0.0906 |
| *T. peberdyi* | 0.1310 | 0.1978 |

https://doi.org/10.1371/journal.pone.0228485.t005

AFLP analysis, were notably homogenous phenotypically. The most phenotypically diverse species was *T. koningiopsis*, which was sister to *T. erinaceum*, in agreement with the sequence-based phylogeny (Fig 2B). Otherwise, as was the case with AFLP genotyping, the topology of the MALDI-TOF dendrogram was not congruent with the sequence-based phylogeny, where both typing methodologies appear to be unsuitable for establishing deeper phylogenetic relationships in *Trichoderma*. No exclusive location-correlated groupings were observed in the MALDI-TOF species clusters, although some structure was evident in *T. peberdyi*, where a clade containing three strains from SC state was observed, which were joined by a fourth strain
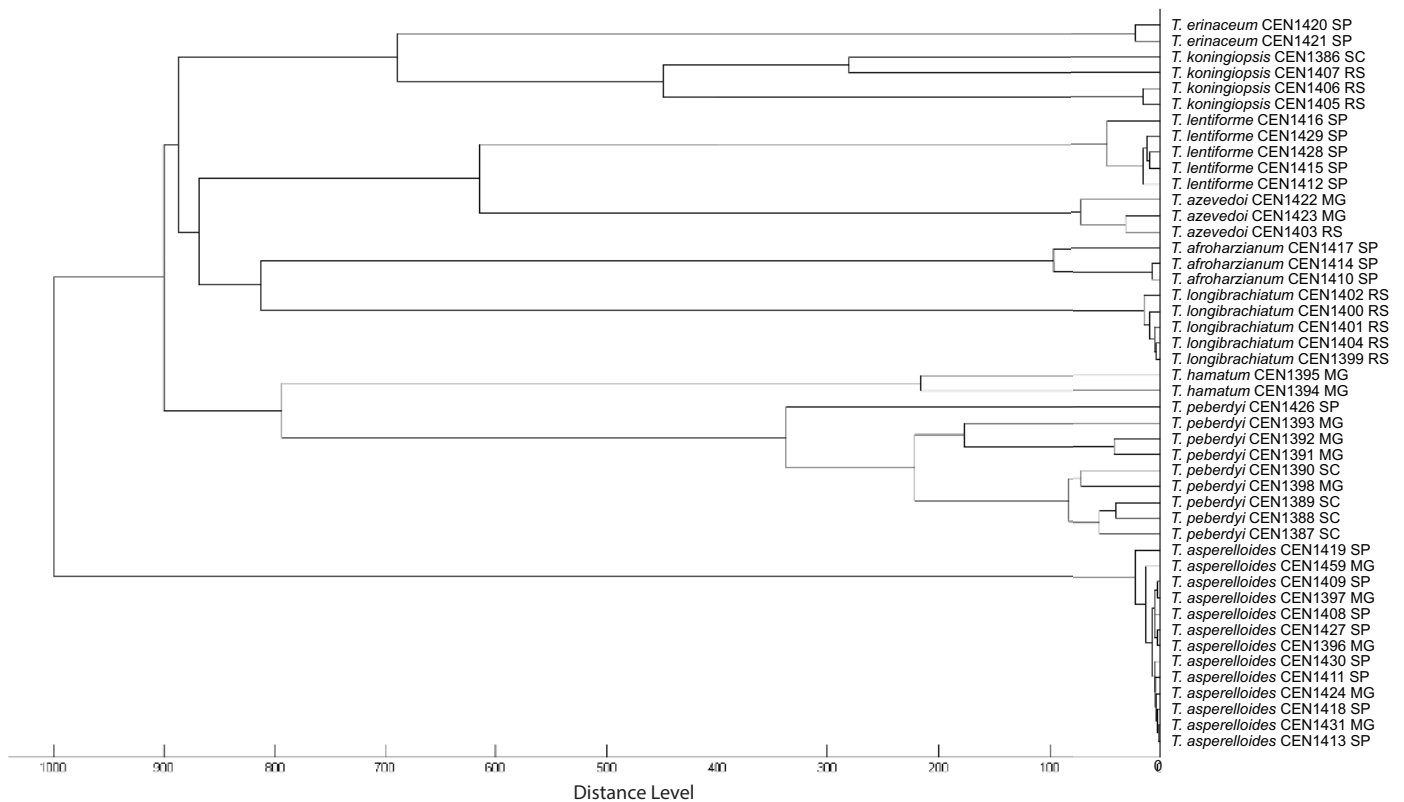


**Fig 6. Dendrogram based on MALDI TOF analysis of *Trichoderma* strains isolated from garlic and onion crop soils.** Species names are followed by strain number and collection location by Brazilian state.

https://doi.org/10.1371/journal.pone.0228485.g006

from MG (CEN1398). Three other *T. peberdyi* strains from MG state formed a sister group and a strain from SP was an outlier in the species. Genetically well-characterized *Trichoderma* species were previously examined using MALDI-TOF, where 129 strains representing 28 species in 8 phylogenetic clades were effectively identified to the species level, providing comparable resolution to ITS sequencing [61]. The authors claimed approximate agreement with the sequence-based phylogeny, which we did not reproduce herein, although this could be due to sampling differences between the two studies.

The major constraint on the use of MALDI-TOF for fungal identification, especially in environmental samples, is the lack of a comprehensive reference spectrum library [62]. Previously, MALDI-TOF was used to identify *Metarhizium* species, where accuracy was progressively improved with the addition of further correctly identified strains to the spectrum library until near perfect matches with DNA-based identifications were obtained [62]. Our analysis was principally directed towards clustering and detection of phenotypic diversity among the onion and garlic-associated strains. However, the technique would appear to be promising for the rapid identification of new *Trichoderma* isolates, since the MALDI-TOF MSP clusters we obtained agreed perfectly with sequence-based identifications. Similarly, MALDI-TOF could be exploited as a fast and economical means of large-scale pre-grouping and triage of anonymous isolates prior to selection of representative individuals for sequence-based phylogenetic identification.

## Conclusions

The large variety of *Trichoderma* species and genotypes identified in a small sample (n = 54) of isolates from garlic and onion crops in South-eastern Brazil represents a considerable resource for the selection of antagonists for biocontrol programs. The biological diversity present is exemplified by the discovery of two new *Trichoderma* species in this sample. While Brazil is among the megadiverse countries, systematic studies on microbial diversity in the range of biomes in the country are currently few [63]. A much larger systematic survey of *Trichoderma* populations associated with both crop and natural soils would enable a clearer picture of the distribution of species in the region. Complimentary sampling of epiphytic and endophytic niches could also broaden the scope for discovery. Such programs also provide the opportunity to preserve distinctive and potentially valuable *Trichoderma* germplasm. Given the laborious nature of pure culture collection and amplicon sequencing for species identification, comprehensive geographical mapping of species could be more efficiently accomplished by metabarcoding, using a sufficiently discriminatory target sequence, such as *tef1-α*.

## Supporting information

**S1 Table. Genbank accession numbers of reference strains used for phylogenetic analysis.**
(PDF)

**S1 Fig. Midpoint rooted maximum likelihood (ML) tree based on the concatenation of *act*, *cal*, ITS, *rpb2* and *tef1-α* matrices.** Ultrafast bootstrap values are given above branches (> = 90%) and the scale bar represents expected changes per site. Strains sequenced in the present study are in bold and are followed by CENxxx numbers. Two new *Trichoderma* species, *T. azevedoi* and *T. peberdyi* are indicated in bold type.
(PDF)

**S2 Fig. Midpoint rooted maximum likelihood (ML) tree based on the *act* matrix.** Ultrafast bootstrap values are given above branches (> = 90%) and the scale bar represents expected

changes per site. Strains sequenced in the present study are followed by CENxxx numbers. (PDF)

**S3 Fig. Midpoint rooted maximum likelihood (ML) tree based on the *cal* matrix.** Ultrafast bootstrap values are given above branches ($>$ = 90%) and the scale bar represents expected changes per site. Strains sequenced in the present study are followed by CENxxx numbers. (PDF)

**S4 Fig. Midpoint rooted maximum likelihood (ML) tree based on the ITS matrix.** Ultrafast bootstrap values are given above branches ($>$ = 90%) and the scale bar represents expected changes per site. Strains sequenced in the present study are followed by CENxxx numbers. (PDF)

**S5 Fig. Midpoint rooted maximum likelihood (ML) tree based on the *rpb2* matrix.** Ultrafast bootstrap values are given above branches ($>$ = 90%) and the scale bar represents expected changes per site. Strains sequenced in the present study are followed by CENxxx numbers. (PDF)

**S6 Fig. Midpoint rooted maximum likelihood (ML) tree based on the *tef1-α* matrix.** Ultra-fast bootstrap values are given above branches ($>$ = 90%) and the scale bar represents expected changes per site. Strains sequenced in the present study are followed by CENxxx numbers. (PDF)

**S1 Dataset. Concatenated data matrix in Nexus format, containing aligned *act*, *cal*, ITS, *rpb2* and *tef1-α* sequences.**
(NEX)

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