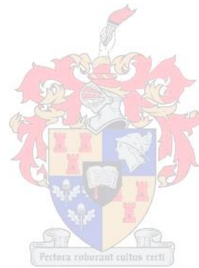
THE DIVERSITY OF *TRICHODERMA* SPP. IN SOUTH AFRICA

Ihan L. du Plessis



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Summary

The genus *Trichoderma* includes economically important species that are routinely applied in fields such as agriculture and biotechnology. Species like *T. harzianum* and *T. asperellum* are commercially applied as biological control agents, whereas other species such as *T. reesei,* are used in the production of industrial enzymes. Few studies have investigated the diversity of this important genus in South Africa. The aim of this study was to investigate the diversity of *Trichoderma* species in South Africa by isolating and identifying strains from soils collected in different parts of the country. In addition, strains were screened for their ability to antagonize plant pathogens *in vitro*, to assess their potential for possible future development as biocontrol agents.

Chapter One explores the history of *Trichoderma* and outlines the various criteria that are used to describe and identify species within the genus. The ecology of *Trichoderma* species, and the manners in which they interact with other organisms, are also discussed. Next, the various impacts of *Trichoderma* species on industry, agriculture and healthcare were outlined to highlight the importance of these organisms. The final section of the chapter reviews the research that has investigated *Trichoderma* strains from South Africa in the past.

A taxonomic inventory of South African *Trichoderma* species is presented in Chapter Two. In total, 161 different *Trichoderma* strains were isolated from 173 sample sites. These were identified based on their morphological characteristics as well as through phylogenetic analyses using ITS and ef1α sequence data. A total of 18 different species were isolated, 14 of which could be identified and six of which are first reports.

Four new *Trichoderma* species were discovered during this study. Two of these species belong to the *T. longibrachiatum* clade and are represented by multiple strains. These species were described in Chapter Three as *T. terrigenum* prov. nom. and *T. vagum* prov. nom. The remaining two species were each represented by a single strain and were described in Chapter Four as *T. restrictum* prov. nom. and *T. undulatum* prov. nom.

Chapter Five investigates the ability of South African *Trichoderma* strains to antagonize the growth of selected plant pathogens *in vitro*. The antagonistic activity of the *Trichoderma* strains were quantified based on their ability to produce volatile and non-volatile antifungal compounds that could inhibit the growth of plant pathogens. The performance of the strains were compared to those of two commercial biocontrol strains that served as positive controls.

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Several of the South African *Trichoderma* strains matched or outperformed commercial biocontrol strains in terms of their ability to antagonise the growth of the plant pathogens and therefore they show potential for possible future development as biocontrol agents.

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Opsomming

Die genus *Trichoderma* bevat ekonomiese belangrike spesies wat toegepas word in landbou en biotegnologie. Spesies soos *T. harzianum* en *T. asperellum* word kommersieel toegedien as biologiese beheer middels waar ander spesies, soos *T. reesei*, gebruik kan word vir die vervaardiging van industriële ensieme. Tot op hede was daar slegs 'n paar studies wat hierdie belangrike groep swamme in Suid Afrika ondersoek het. Die doel van hierdie studie was om die diversiteit van *Trichoderma* spesies in Suid Afrika te bestudeer deur stamme te isoleer en identifiseer vanuit grond monsters wat geneem was regoor die land. Die geïsoleerde stamme was ook ondersoek vir die vermoë om sekere plant patogene se groei te onderdruk onder laboratorium omstandighede. Hierdie was gedoen om hulle potensiaal te meet vir moontlike verdere kommersiële ontwikkeling as biologiese beheer agente.

Hoofstuk Een ondersoek die geskiedenis van *Trichoderma* en beskryf die verskillende kriteria waarvolgens spesies gekarakteriseer word. Die ekologie van *Trichoderma* spesies, en die wyse waardeur hulle ander organismes beïnvloed, word ook bespreek. Die verskeie impakte van *Trichoderma* spesies op die industrie, landbou en gesondheidsorg, word volgende bespreek om die belangrikheid van hierdie groep swamme te beklemtoon. Ten slotte van hierdie hoofstuk word daar 'n oorsig gegee van navorsing wat Suid Afrikaanse *Trichoderma* stamme ondersoek het in die verlede.

Die verskillende *Trichoderma* spesies wat geïdentifiseer was in Suid Afrika tydens hierdie studie word in Hoofstuk Twee gelys en bespreek. In totaal was 161 verskillende *Trichoderma* stamme geïsoleer vanaf 173 grond monsters. Hierdie stamme was geïdentifiseer volgens hulle morfologiese eienskappe sowel as ITS en ef1α DNA basispaaropeenvolging data. In totaal was 18 verskillende *Trichoderma* spesies geïsoleer tydens hierdie studie, 14 van hierdie spesies was geïdentifiseer en ses van die spesies was vir die eerste keer in Suid Afrika aangeteken.

Vier nuwe *Trichoderma* spesies was ontdek tydens hierdie studie. Twee van hierdie spesies behoort aan die *T. longibrachiatum* groep en word verteenwoordig deur meer as een stam. Hierdie spesies was beskryf in Hoofstuk Drie as *T. vagum* prov. nom. en *T. terrigenum* prov. nom. Die ander twee nuwe spesies word elk verteenwoordig deur 'n enkele stam en was beskryf in Hoofstuk Vier as *T. restrictum* prov. nom. and *T. undulatum* prov. nom.

In hoofstuk Fyf word die vermoë van Suid Afrikaanse *Trichoderma* stamme ondersoek om die groei van geselekteerde plant patogene te onderdruk onder laboratorium omstandighede. Die

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antagonistiese aktiwiteit van die *Trichoderma* stamme was gekwantifiseer gebaseer op die mate waarvolgens hulle afgeskeide vlugtige en nie-vlugtige antifungale stowwe die groei van die plant patogene onderdruk het. Die anti-fungus aktiwiteit van die stamme was vergelyk met twee kommersiële biobeheer stamme wat gedien het as positiewe kontrole. Verskeie *Trichoderma* stamme het die kontrole stamme oortref in term van hulle vermoë om die groei van die plant patogene te onderdruk. Hierdie spesies toon potensiaal vir moontlike verdere ontwikkeling as biologiese beheer agente.

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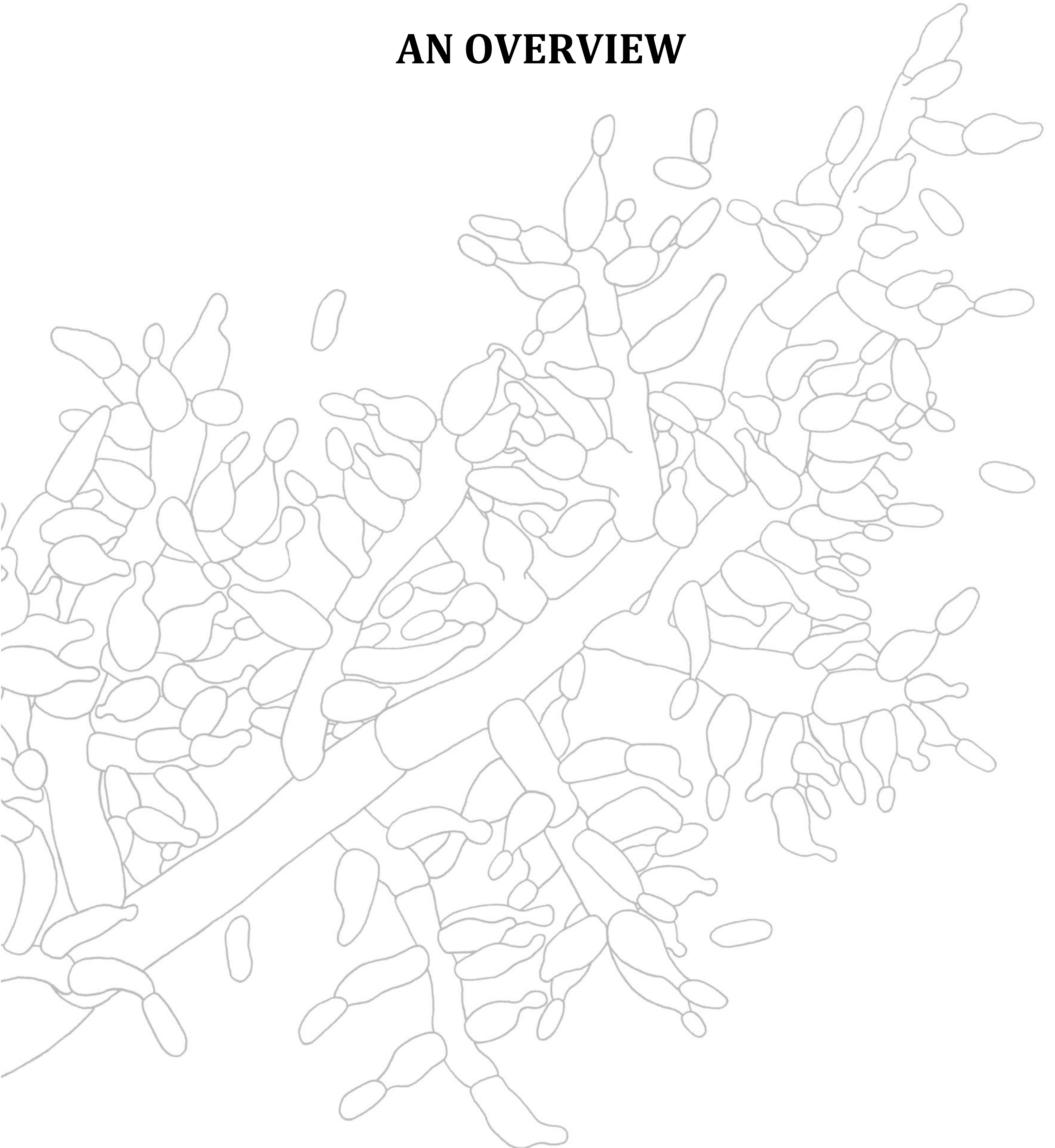
CHAPTER 1

THE GENUS *TRICHODERMA*: AN OVERVIEW



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A brief introduction to *Trichoderma*

The genus *Trichoderma* was first described by Christian Hendrik Persoon more than two centuries ago and more than 170 different species have thus far been described, many of which display cosmopolitan distributions and inhabit diverse ecological niches (Persoon 1794, Klein and Everleigh 1998, Kubicek *et al.* 2008). *Trichoderma* species have been frequently isolated from regions such as Asia (Doi 1972, 1976, Zhang *et al.* 2005), central Europe (Druzhinina and Kubicek 2005, Jaklitsch 2009, 2011) and the Americas (Chaverri and Samuels 2004, Hoyos-Carvajal and Bissett 2011) where the genus has been relatively well documented. However, *Trichoderma* in places such as central Asia and Africa remain understudied (Druzhinina *et al.* 2006). *Trichoderma* species often occur as dominant members of soil fungal communities and were, therefore, primarily regarded in the past as soil fungi (Danielson and Davey 1973, Widden and Abitbol 1980, Nelson 1982, Chaverri and Samuels 2004, Zhang *et al.* 2005, Kubicek *et al.* 2008). However, several studies such as those by Jaklitsch (2009, 2011) and Gal-Hemed *et al.* (2011) have shown that *Trichoderma* species occur in diverse communities outside of the soil environment. Jaklitsch (2009) also suggested that aboveground *Trichoderma* communities may be as diverse as soil-borne communities. New *Trichoderma* species are constantly being added to the genus and this suggests that more species remain to be discovered (e. g. Jaklitch *et al.* 2012, Samuels *et al.* 2012a, Kim *et al.* 2012, 2013, Chaverri *et al.* 2013, Jaklitsch *et al.* 2013, López-Quintero *et al*. 2013).

Trichoderma species fulfil key ecological roles in nature as nutrient cyclers by facilitating processes such as decomposition (Cox *et al.* 2001, Druzhinina *et al.* 2006). These fungi can often be seen growing on decomposing plant materials, on which they form characteristic fertile, shrub-like structures called pustules which bear dark green conidia (asexual state) or stomata (sexual state), which range in colour from brown to yellow with darkly coloured spots (Markovina *et al.* 2005, Jaklitsch 2009). In culture, *Trichoderma* species are easy to recognize based on their fast growth rates and tendencies to form scant aerial mycelium and develop green or white colonies that form compact sporulating pustules (Bissett 1991a, Gams and Bissett 1998). Microscopically *Trichoderma* species display a variety of differently shaped conidiophores, many of which are extensively branched and form hyaline phialides which bear single celled conidia that are green or hyaline (Rifai 1969, Jaklitsch 2009).

The sexual counterparts of *Trichoderma* species (*sensu lato*) were assigned to the genus *Hypocrea* Fries, prior to recent changes made to the International Code of Nomenclature for

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Algae, Fungi and Plants (previously known as the International Code of Botanical Nomenclature) (Samuels 1996, Hawksworth *et al.* 2011, Norvell 2011). Species that were previously assigned to *Hypocrea* now reside in *Trichoderma* because the name was established before *Hypocrea* and was, therefore, granted priority (Rossman *et al.* 2013).

Contemporary *Trichoderma* studies make extensive use of DNA sequence data from several different gene loci in order to identify and describe *Trichoderma* species (Hanada *et al.* 2008, Jaklitsch 2009, Druzhinina *et al.* 2012, Jaklitsch *et al.* 2012, Sun *et al.* 2012). This approach has become widespread amongst *Trichoderma* researchers because of the difficulties associated with characterizing *Trichoderma* species according to their morphologies, which often lead to misidentifications (Samuels 1996, Klein and Everleigh 1998, Lieckfeldt *et al.* 2001, Jaklitsch, 2009).

In the past, a great deal of research has focused on the industrial and agricultural applications of *Trichoderma* strains (Kubicek and Harman 1998, Whipps and Lumsden 2001, Harman *et al.* 2004, Mbarga *et al.* 2012). Species such as *T. harzianum* Rifai are employed as biological control agents in order to counteract plant pathogens (Harman 2000, Hermosa *et al.* 2000, Mbarga *et al.* 2012). Other *Trichoderma* species such as *T. reesei* Simmons produce cellulases that are applied on a commercial scale in the biofuels, food and textiles industries (Galante *et al.* 1998, Hoyos-Carvajal and Bissett 2011, Kubicek 2012).

Not all *Trichoderma* species are considered beneficial though, as some impact on the agricultural industry and healthcare sectors negatively (Kühls *et al.* 1999). *Trichoderma* species such as *T. pleurotum* Yu and Park, *T. pleuroticola* Yu and Park and *T. mienum* Kim cause harm to commercially produced mushroom crops (Komoń-Zelazowska *et. al.* 2007, Sun *et al.* 2012). Other *Trichoderma* species, including *T. longibrachiatum* Rifai*, T. citrinoviride* Bissett and *T. orientalis* Samuels and Petrini, have been identified as opportunistic human pathogens capable of causing life-threatening mycoses in immunocompromised individuals (Guarro *et al.* 1999, Kühls *et al.* 1999, Druzhinina *et al.* 2008, 2011).

Despite the economic and medical importance of *Trichoderma,* only a few studies have investigated the diversity of the genus in South Africa. The majority of studies which reported isolating native *Trichoderma* were restricted to relatively small sampling regions or did not attempt to identify strains to species level (Askew and Laing 1994, Roux and Wingfield 1997, Mouton *et al.* 2012). As a result, we do not currently have a clear understanding of the diversity

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of *Trichoderma* in South Africa and the vast majority of the country has not yet been surveyed for the presence of *Trichoderma* species.

Taxonomic history

Trichoderma Pers. ex Fr. (*Tricho* meaning hair in Greek and *derma* meaning skin in Latin) is an ascomycetous fungal genus from the order *Hypocreales* Lindau. Persoon described four species, with *T. viride* as the generic type (Persoon 1794). The three remaining species, *T. aureum, T. roseum* and *T. nigrescens* were found to be unrelated to *Trichoderma* and were subsequently removed from the genus and assigned to more appropriate genera namely: *Sporotrichum* Pers. ex Fr., *Xylohypha* Pers. ex Fr. and *Trichothecium* Link ex Gray, respectively (Rifai 1969).

During the early 1800's, *Trichoderma* species were recognized primarily based on their colony appearances (Rifai 1969). Detailed studies based on microscopic observations were first conducted during 1865 by the Tulasne brothers, who became some of the first researchers to study the micromorphological characteristics of *Trichoderma* species (Samuels 1996, 2006, Jaklitsch 2009). In addition, the Tulasne brothers made the observation that *T. viride* and the teleomorphic fungus *Hypocrea rufa* Pers. ex Fr. represented two different phases of the same lifecycle (Samuels 1996, Chaverri and Samuels 2004). Brefeld (1891) later confirmed this observation when he was able to prove the connection between these two taxa using culturing techniques (Samuels 1996, Chaverri and Samuels 2004). This was an important milestone in the taxonomy of *Trichoderma* and *Hypocrea* because it established the link between these two genera. Harz (1871) published the first generic delimitation of *Trichoderma* and included information regarding the micromorphological characteristics of its species (Rifai 1969).

An increasing number of *Trichoderma* strains were isolated from soils around the world in the early to mid nineteenth century and it became apparent that *Trichoderma* was a common constituent of soil fungal communities (Goddard 1913, Bisby 1939, Christensen *et al.* 1962). However, relatively few species were added to *Trichoderma* during this time and Samuels (2006) attributed this to the cryptic nature of morphological characteristics displayed by many *Trichoderma* species.

As is the case with many other groups of fungi, the taxonomy of *Trichoderma* has been made problematic in the past due to the fact that it is often difficult to distinguish different taxa from one another based solely on morphological data (Jensen 1931, Kubicek and Harman 1998).

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The difficulties associated with recognizing different *Trichoderma* species based on their morphology was clearly demonstrated in early generic revisions of the genus. Bisby (1939) believed that the morphological variations he observed between different *Trichoderma* species were not evidence enough to justify separate taxa and recognized only the generic type, *T. viride*, as legitimate. For the next 30 years, only this single species was recognized in *Trichoderma* (Samuels 2006a). During the time that this monotypic concept of the genus was implemented, numerous *Trichoderma* strains were misidentified as *T. viride* (Druzhinina and Samuels 2005, Samuels *et al.* 2006, Jaklitsch 2009). Such misidentifications have often been made with economically important *Trichoderma* strains, such as those employed as biocontrol agents (Kullnig *et al.* 1999, Hermosa *et al.* 2000) or industrial enzyme producers (Lieckfeldt *et al.* 2001).

The one species genus proposed by Bisby (1939) was brought into question by Rifai and Webster (1966a, b) who closely studied *Trichoderma* and *Hypocrea* strains that were obtained from different collections around the world. Rifai (1969) reviewed *Trichoderma* and recognized nine separate taxa, producing the first comprehensive monograph of the genus. Rifai (1969) made extensive use of micromorphological data to delineate *Trichoderma* species and also considered the fact that the *Trichoderma* species he recognized associated with distinct teleomorphic states. Interestingly, Rifai (1969) acknowledged that the nine different taxa he recognized most likely constituted several distinct species and referred to them as "species aggregates".

Bissett (1983, 1991a, b) reviewed *Trichoderma* and divided the genus into five different sections based on morphological observations, but founded some of these sections on the species aggregates recognized by Rifai. These included the *Trichoderma* sections: *Longibrachiatum*, *Pachybasium*, *Trichoderma*, *Saturnisporum* and *Hypocreanum* (Bissett 1991a). Subsequent revisions of these sections resulted in the addition of several new species to the genus (Bissett 1991, 1992, Kühls *et al.* 1997, Overton *et al.* 2006, Druzhinina *et al.* 2012).

During the mid to late 19th century, several *Hypocrea* species were described based on strains collected in Asia, New Guinea and South America by Doi and his co-workers (Chaverri and Samuels 2004). Doi (1972) published a detailed monograph of *Hypocrea* that was, until recently, the largest work of its kind addressing *Hypocrea* (Chaverri and Samuels 2004). Impressively, Doi and his co-workers described more than 50 new *Hypocrea* species (Doi 1968, 1971, 1975, 1976, Doi *et al.* 1987). It is, however, unfortunate that the reference material for many of these species have been lost, making it difficult to validate their identity using

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contemporary taxonomic approaches (Jaklitsch 2009). Doi developed species concepts for *Hypocrea* which he based on infrageneric groupings (Doi 1972, Chaverri and Samuels 2004). These infrageneric classifications were founded largely on stroma morphology (Doi 1975). Today, this approach is not frequently employed as contemporary studies have revealed that *Trichoderma* species sharing indistinguishable teleomorphic states do not necessarily group to form monophyletic clades when considering DNA sequence data (Jaklitsch 2009, 2011). For example, certain members of the *H. schweinitzii* complex cannot be identified to species level without considering their anamorphic states or DNA sequence data (Samuels *et al.* 1998, Jaklitsch 2009).

Jaklitsch (2009, 2011) recently investigated the *Trichoderma* community of central Europe. This study was based on phylogenetic analyses using DNA sequence data from Internal Transcribed Spacer (ITS), Translation Elongation Factor 1α (ef1α) and RNA Polymerase subunit B2 (rpb2) loci (Jaklitsch 2009, 2011). In this study, Jaklitsch (2009, 2011) described 25 new species of *Trichoderma* and gave detailed descriptions of the remaining 50 species that were found in Europe, accounting for 75 different species in total.

The history of* Trichoderma *spp. as biological resources

The most important industrial *Trichoderma* species, according to Druzhinina *et al.* (2006), is *T. reesei,* which is commonly employed for the production of industrial enzymes. *T. reesei* was first discovered in the Solomon Islands by Mandels and Reese (1956) during the Second World War (Peterson and Nevalainen 2012). The the United States army became concerned about the high rate at which supplies made from cotton materials were rotting at military bases in the South Pacific (Peterson and Nevalainen 2012). The cause of the rot was a fungus which displayed a remarkable ability to degrade cellulose (Mandels and Reese 1956, Kubicek and Harman 1998, Kubicek *et al.* 2009). Originally the strain was identified as *T. viride.* However, Simmons (1977) recognized the strain to be distinct from *T. viride* and described it as *T. reesei* in honour of its discoverer.

Numerous researchers have investigated the cellulolytic mechanisms of *T. reesei* (Montenecourt and Everleigh 1977, Kubicek-Pranz *et al.* 1991, Tomme *et al.* 1998, Kubicek 2012). Studies such as these have led to the improvement of the original isolate (strain QM6a) to several manipulated strains that show improved capabilities of cellulase production (Petersen and Nevalainen 1995, Kubicek *et al.* 2009). All of the strains of *T. reesei* that are in commercial use today are permutations of the original isolate from the Solomon Islands

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(Kubicek-Pranz *et al.* 1991, Uusitalo *et al.* 1991, Kühls *et al.* 1996). These improved *Trichoderma* strains have allowed for the establishment of *Trichoderma*-based biotechnology enterprises that are centred on the large scale production of cellulases for use in industry (Montenecourt and Eveleigh 1977, Gallo *et al.* 1978, Kubicek *et al.* 2009, Petersen *et al.* 2012).

Other *Trichoderma* species also showed promise for commercial development as biological resources during the early to mid-19th century. Weindling (1932) attributed remarkable biological properties to *Trichoderma* species when he discovered the ability of *Trichoderma lignorum* Tode. ex Harz to grow parasitically on plant pathogens such as *Rhizoctonia solani* Kühn*, Sclerotium rolfsii* Saccardo*,* and *Pythium* spp. Pringsheim. Later, Weindling (1934) also observed that *Trichoderma* species were capable of secreting chemical compounds which he called "lethal principals'' that could inhibit the growth of plant pathogens. Weindling concluded his investigations by speculating that plant pathogens in agriculture could be counteracted on a commercial scale through the application of *Trichoderma* strains (Weindling 1934). Many years later, this vision was realized when the first *Trichoderma*-based commercial biocontrol products were developed in the 1970's and 1980's and were registered with the U. S. environmental protection agency (Hadar *et al.* 1979, Elad *et al.* 1980, Fravel 2005).

***The recent history of* Trichoderma**

In light of the emerging economic importance of *Trichoderma*, a pressing need arose to develop more practical and accurate ways to identify strains to species level (Druzhinina *et al.* 2005). In response to this need, mycologists explored new taxonomic methods, such as the use of DNA sequence data, to characterize *Trichoderma* species. Initially, the ITS region of the ribosomal DNA was used to investigate the phylogenetic relationships between different species (Kühls *et al.* 1997, Kindermann *et al.* 1998, Dodd *et al.* 2000). Such studies revealed that a number of morphological taxa recognized by Bissett and Rifai actually constituted paraphyletic lineages (Samuels 2006b, Kubicek *et al.* 2008). New *Trichoderma* species were recognized based on DNA sequence evidence following such revisions. A new naming scheme was adopted for *Trichoderma* that classified species based on their phylogenetic relationships with one another as opposed to the old system of sectional classification which recognised species based solely on morphological traits (Samuels 2006b, Kubicek *et al.* 2008, Druzhinina *et al.* 2012).

Difficulties associated with accurately identifying *Trichoderma* species meant that a large number of sequences from incorrectly identified *Trichoderma* strains were being submitted to GenBank, decreasing the reliability of routine BLAST searches (Druzhinina *et al.* 2006). The use

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of ribosomal RNA sequence data to quickly identify *Trichoderma* species through barcoding-based approaches was, therefore, considered (Druzhinina *et al.* 2006). The ITS region has been selected as the universal barcoding locus for fungi (Bellemain *et al.* 2010). Druzhinina *et al.* (2006) developed an online *Trichoderma* ITS barcode based identification resource for *Trichoderma* named Trichokey (www.isth.org) that was based on voucher specimens of recognized *Trichoderma* species. This resource allowed query sequences from unidentified *Trichoderma* isolates to be compared to the *Trichoderma* voucher ITS barcode database for fast identifications that were more reliable than BLAST analyses (Druzhinina *et al.* 2006). Trichokey represented a significant step forward in developing a fast and relatively accurate means of species identification that did not demand technical interpretation by the user (Druzhinina *et al.* 2006).

At the time Trichokey v. 1.0 was launched, the phylogenetic relationships between different *Trichoderma* species was investigated to some extent based on sequence data from gene loci other than the ITS region. These include the endochitinase (chi18-5) and RNA polymerase B2 (rpb2) loci (Lieckfeldt *et al.* 2000, Chaverri *et al.* 2004). It was known from such studies that the use of ITS as a barcoding locus for *Trichoderma* would present certain challenges because some species cannot be distinguished confidently from one another based on phylogenetic analyses using ITS sequence data alone (Jaklitsch 2009). Druzhinina *et al.* (2006) considered this when developing Trichokey and argued that ITS based *Trichoderma* barcodes were not intended as phylogenetic tools but would rather serve as diagnostic tools. The ITS sequences between some *Trichoderma* species differ only at a few nucleotide positions and phylogenetic principles are, therefore, not able to distinguish such species. However, species that display ITS sequences which differ consistently at even a few locations can be distinguished from one another diagnostically (Druzhinina and Kubicek 2005). Druzhinina *et al.* (2006) also showed that problematic species identifications using Trichokey can be supplemented to some extent using morphological data. For example, *T. longipile* Bissett and *T. crassum* Bissett share identical ITS sequences but can be easily distinguished from one another based on their morphological traits (Druzhinina *et al.* 2006).

Trichoderma has undergone several important taxonomic changes in January 2011 as a result of changes that occurred at the International Conference of Botanical Nomenclature hosted in Melbourne, Australia. A decision was made to move away from the traditional system of dual nomenclature and embrace the concept of 'one name = one fungus' that was proposed by Hawksworth *et al.* (2011). This was brought about by removing Article 59 from the

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International Code of Nomenclature for Algae, Fungi and Plants that allowed the use of different names for asexual and sexual states of the same fungus (Norvell 2011). Under the new Melbourne code, the younger genus *Hypocrea*, is synonymized with the older genus *Trichoderma,* which now becomes a holomorphic genus as opposed to the asexual genus it represented prior to January 2011 (Rossman *et al.* 2013).

Taxonomic concepts within *Trichoderma*

In the absence of reliable means of species identification it was difficult for early *Trichoderma* researchers to confidently attribute important biological traits to specific taxa. For example, in 1939 Bisby's decision to reduce all of the species in *Trichoderma* to synonymy with the type species, *T. viride*, resulted in some researchers incorrectly believing that *T. viride* was an industrial cellulase producer (Lieckfeldt *et al.* 2001, Druzhinina *et al.* 2008). We now know that this is not the case and that other species are better suited for industrial enzyme production (Tomme *et al.* 1988). Similar misidentifications have also occurred with agriculturally important *Trichoderma* strains, such as those employed as biocontrol agents and crop growth promoters (Kullnig *et al.* 1999, Hermosa *et al.* 2000). This illustrates why accurate *Trichoderma* species identifications are of great importance in applied research.

Species characterization based on morphological observations

Bissett (1983, 1981a, b, c) reviewed *Trichoderma* based on the morphological traits of different species and focused extensively on their micromorphological characteristics. Unfortunately, micromorphological data, even when used in combination with colony morphology data, is still not able to distinguish some *Trichoderma* species from one another (Jaklitsch *et al.* 2013). The difficulty that past investigators experienced in defining *Trichoderma* species can be attributed, in no small part, to the cryptic nature of the morphological characteristics of *Trichoderma* species (Rifai 1979, Kubicek and Harman 1998, Samuels 2006b). Few morphological traits from *Trichoderma* species are of taxonomic importance and these traits often vary to the extent that overlapping occurs between different species, making it difficult to distinguish them from one another (Rifai 1979, Samuels 2006b). This can be clearly demonstrated by the fact that all of the ±100 different *Trichoderma* species that were described by 2006 could at the time only be assigned to roughly 30 different morphogroups (Druzhinina *et al.* 2006).

Samuels *et al.* (2002a) developed an interactive online identification key based on the morphological and phenetic differences between different *Trichoderma* species (http://nt.ars-

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grin.gov/taxadescriptions/keys/*Trichoderma*Index.cfm.) This resource provided many photographic illustrations of the characteristics that were important for distinguishing between different *Trichoderma* species. Unfortunately, only 33 different species were treated by the key, which has not been updated since 2002.

Species characterization based on DNA sequence data

A large number of studies during the past two decades have made use of DNA sequence data to investigate the relationship between *Trichoderma* species (Kühls *et al.* 1997, Kindermann *et al.* 1998, Samuels *et al.* 2006, Jaklitsch 2009, 2011, Druzhinina *et al.* 2010, López-Quintero *et al.* 2013). The relatively weak resolving power of the ITS locus decreases its usefulness in species level identifications of *Trichoderma* strains (Samuels 2006b, Jaklitsch 2009). Contemporary studies have described new species based on the phylogenies derived from other, more resolving, genomic loci such as endochitinase (chi18-5) (Lieckfeldt *et al.* 2000), RNA polymerase subunit B2 (rpb2) (Chaverri *et al.* 2003), actin (Samuels *et al.* 2006), calmodulin (cal1) (Samuels *et al.* 2006), ATP citrate lyase (acl1) (Jaklitsch *et al.* 2013) and translation elongation factor 1-alpha (ef1α) (Samuels *et al.* 2006) genes (Jaklitsch *et al.* 2009, 2013, López-Quintero *et al.* 2013). Samuels *et al.* (2006) compared the species resolving capabilities of ef1α sequence data with actin and calmodulin sequence data and reported that the ef1α sequences provided a strong phylogenetic signal. However, the locus is less suited for determining the phylogenetic relationship between distantly related species (Druzhinina *et al.* 2005). This is because of the divergent nature of ef1α sequences, which causes numerous ambiguously aligned regions to form during multiple sequence alignment (Druzhinina *et al.* 2005). The ef1α region is, therefore, more suited for resolving the terminal nodes of *Trichoderma* clades as opposed to determining genus wide phylogenies (Druzhinina *et al.* 2005). Jaklitsch *et al.* (2009) reported that the intermediate variability and resolving properties of the rpb2 marker make it appropriate for use in determining genus wide phylogenies.

Contemporary studies on the phylogeny of *Trichoderma* make use of Geneological Concordance Phylogenetic Species Recognition (GCPSR) approaches based on sequence data derived from multiple unlinked genomic loci (Taylor *et al.* 2000, Druzhinina *et al.* 2010). This approach may not always be easily implemented in *Trichoderma* because sequence data from reference strains might not be publically available for all the different loci required to perform such analyses.

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Species characterization based on chemotaxonomic data

In addition to conventional DNA sequence and morphology based approaches to species characterization, several alternative chemotaxonomic based tools are also possible. Chemotaxonomy is the use of chemical diversity between different species as a tool to distinguish them (Frisvad *et al.* 1998). In the past, species from several fungal genera such as *Talaromyces* Benjamin and *Fusarium* Link have been shown to produce secondary metabolite profiles that are unique to each species and that these chemical profiles can be used to identify strains to species level (Frisvad*.* 1989, Frisvad *et al.* 2008). Early application of chemotaxonomic tools such as Thin Layer Chromatography (TLC) formed an important part of basidiomycete and lichen taxonomy in the 1970's (Moser 1985, Frisvad *et al.* 2008). Today, techniques such as diode assisted HPLC (DAD-HPLC) are regularly applied to investigate the secondary metabolite profiles of several fungal groups including *Trichoderma* (Thrane *et al.* 2001, Degenkolb *et al.* 2008a, b, Jaklitsch *et al.* 2012). Okuda *et al.* (1982), Stasz *et al.* (1988) and Degenkolb *et al.* (2008b) showed that the secondary metabolite profiles of different *Trichoderma* species show significant variation and could perhaps be employed as a tool by which to identify strains to species level. However, relatively few *Trichoderma* strains have been investigated in this way and so species level identifications based on their secondary metabolite profiles are currently not feasible.

Other means by which to define species, such as Biological Species Recognition based approaches, are difficult to apply in *Trichoderma* due to the impracticalities associated with crossing strains *in vitro* (Druzhinina *et al.* 2005). Contemporary studies describing new *Trichoderma* species consider morphological data, DNA sequence data as well as other sources of relevant information such as ecological data and chemotaxonomic data in a consilient manner in order to define species (Jaklitsch 2006b, Degenkolb *et al.* 2008b, 2009, Atanasova *et al.* 2010, Kraus *et al.* 2012, Samuels *et al.* 2012b).

Morphological characteristics used to describe *Trichoderma* species

The last comprehensive monograph of *Trichoderma* was compiled by Bissett (1983, 1991a, 1991b, 1991c) and treated only a fraction of the species that are known to exist today. As a result, variation occurs between different authors in terms of the morphological traits that have been used to characterise species. It is, therefore, difficult to identify a standard set of morphological traits that should be used to characterise strains. However, several

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contemporary studies have intensely investigated the major clades within the genus, highlighting the important morphological characteristics that need to be considered when identifying and describing *Trichoderma* species (Chaverri and Samuels 2004, Jaklitsch 2009, 2011, Samuels *et al.* 2012a, Jaklitsch *et al.* 2013). These studies include the works of Chaverri and Samuels (2004) (illustrating 40 spp.), Jaklitsch *et al.* (2009, 2011) (illustrating 75 spp.), Samuels *et al.* (2012 a) (illustrating 21 spp.) and Jaklitsch *et al.* (2013) (illustrating 13 spp.).

Trichoderma *strain characterization based on colony traits*

The morphological characterization of *Trichoderma* species have been standardized on three media types namely Corn Meal Dextrose agar (CMD), Synthetic Nutrient poor Agar (SNA) and Potato Dextrose Agar (PDA) (Rifai 1969, Bissett 1991a, b, c, Jaklitsch 2009, 2011). Some authors also study colonies grown on Oatmeal Agar (OA) or Malt Extract Agar (MEA) (Kraus *et al.* 2004, Samuels *et al.* 2012b). Petri dishes should be inoculated using agar plugs cut from the margin of a colonies actively growing on CMD or MEA (Jaklitsch 2009). These inoculation plugs should be placed a few millimetres from the edge of the Petri dish being inoculated with their mycelia side facing the media (Chaverri and Samuels 2004, Jaklitsch 2009). Following inoculation, Petri dishes are incubated at a range of different temperatures including 15°C, 25°C, 30°C and 35°C under 12h day/night lighting regimes using a cool fluorescent light (Jaklitsch 2009). Some authors also incubate Petri dishes at 20°C prior to characterizing species (Chaverri and Samuels 2004). Petri dishes are generally left unwrapped to allow for sufficient aeration. However, those incubated at 35°C are wrapped with Parafilm to prevent desiccation (Jaklitsch 2009). The following characteristics are important to consider when identifying *Trichoderma* species according to Jaklitsch (2009) and are routinely used to characterize species:

Colony growth rate: Colony growth rate data are important to consider when examining *Trichoderma* strains and several contemporary *Trichoderma* species descriptions emphasize this characteristic (Samuels *et al.* 2002b, Hanada *et al.* 2008, Atanasova *et al.* 2010, Kim *et al.* 2012). The growth rate of *Trichoderma* species at certain temperatures and on particular media can be measured by inoculating and incubating fresh media according to methods described above. The colony radius is then measured on a daily basis for seven days or until the colony has overgrown the Petri dish (Jaklitsch 2009). Information, such as the day on which the colony margin reaches the edge of the Petri dish or the day that sporulation first appears, is also recorded (Jaklitsch 2009). Generally, *Trichoderma* strains are fast growing and are able to cover 90 mm Petri dishes in less than a week of incubation under ideal conditions (Jaklitsch 2009).

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Conidium formation: *Trichoderma* colonies produce conidia in a number of different structures depending on the species. These structures influence the appearance and texture of a colony and are, therefore, important to consider when characterising *Trichoderma* species. Often more than one type of these structures can be displayed on the same colony (Jaklitsch 2009).

- **Fertile pustule formation:** Dense bundles of mycelia form (1-5 mm in diameter) which give rise to numerous compact conidiophores (Fig. 1.1).
- **Effuse conidia formation:** Conidia form on aerial mycelia or on the surface layer of mycelium that grows from the substrate. Conidiophores do not clump together to form pustules (Fig. 1.2).
- **Pustule and effuse conidia formation:** Some *Trichoderma* species produce conidia effusely in addition to forming pustules. Upon closer inspection it sometimes becomes apparent that conidia originating from pustules differ morphologically from those that formed at effuse regions (Degenkolb *et al.* 2008a). It has often been recorded whether *Trichoderma* species form such synanamorphs because they are of taxonomic significance (Chaverri and Samuels 2004). (See Fig. 44, page *118* for an illustration of the synanamorphs of *T. spirale*).
- **Granules or shrubs:** These are small sand like conidia forming structures that are comparable to small pustules and give the colony a grainy texture (Jaklitsch 2009) (Fig. 1.3).
- **Conidiophore tufts:** These structures are loosely packed bundles of mycelia that form conidiophores on the outer surface, but are not as densely formed as pustules (Jaklitsch 2009) (Fig. 1.6).

Colony odours: Some *Trichoderma* species that belong to the *T. viride* clade produce distinctive coconut-like odours (Samuels 1996, Jaklitsch 2009). However, few studies mention more than the presence or absence of distinctive odours and this trait is, therefore, of limited taxonomic value to identify species.

Conidium colour: *Trichoderma* species tend to form greyish green to dark green conidia on CMD and SNA. However, some species form hyaline or yellow conidia on other media types such as PDA (Druzhinina and Kubicek 2005, Jaklitsch 2009).

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Exudates: Some *Trichoderma* species produce copious amounts of exudates in colours ranging from hyaline, green or brownish yellow. The presence or absence of such exudates, the quantities in which they are produced, and their colours have been recorded in a number of different *Trichoderma* species descriptions but were omitted in others. This trait is only of limited taxonomic importance (Jaklitsch 2009) (Fig. 1.12).

Reverse colouration: The majority of *Trichoderma* species do not form brightly coloured reverse pigments on CMD or SNA. However, brown or yellow reverse pigmentation can often be seen in colonies grown on PDA. Such observations have often been recorded when *Trichoderma* strains have been characterised (Jaklitsch 2009). (Fig. 1.5).

Zonation: Sporulation can be induced in some *Trichoderma* colonies through exposure to light, particularly light in the blue spectrum (Gupta *et al.* 1997, Betina and Farkas 1998). *Trichoderma* colonies grown under light conditions tend to sporulate significantly more than colonies grown in darkness. This phenomenon is seen in several fungal groups and different *Trichoderma* species display varying degrees of light sensitivity (Jaklitsch 2009). *Trichoderma* colonies are incubated under 12h day/night lighting regimes using cool florescent light to induce the formation of these sporulating and non sporulating zones. This greatly affects the appearance of the colony as well as its texture and many contemporary species descriptions have recorded the extent to which a *Trichoderma* colony forms these zones (Jaklitsch *et al.* 2005, 2009, Atanasova *et al.* 2010, Jaklitsch *et al.* 2012, Kim *et al.* 2012) (Fig. 1.4).

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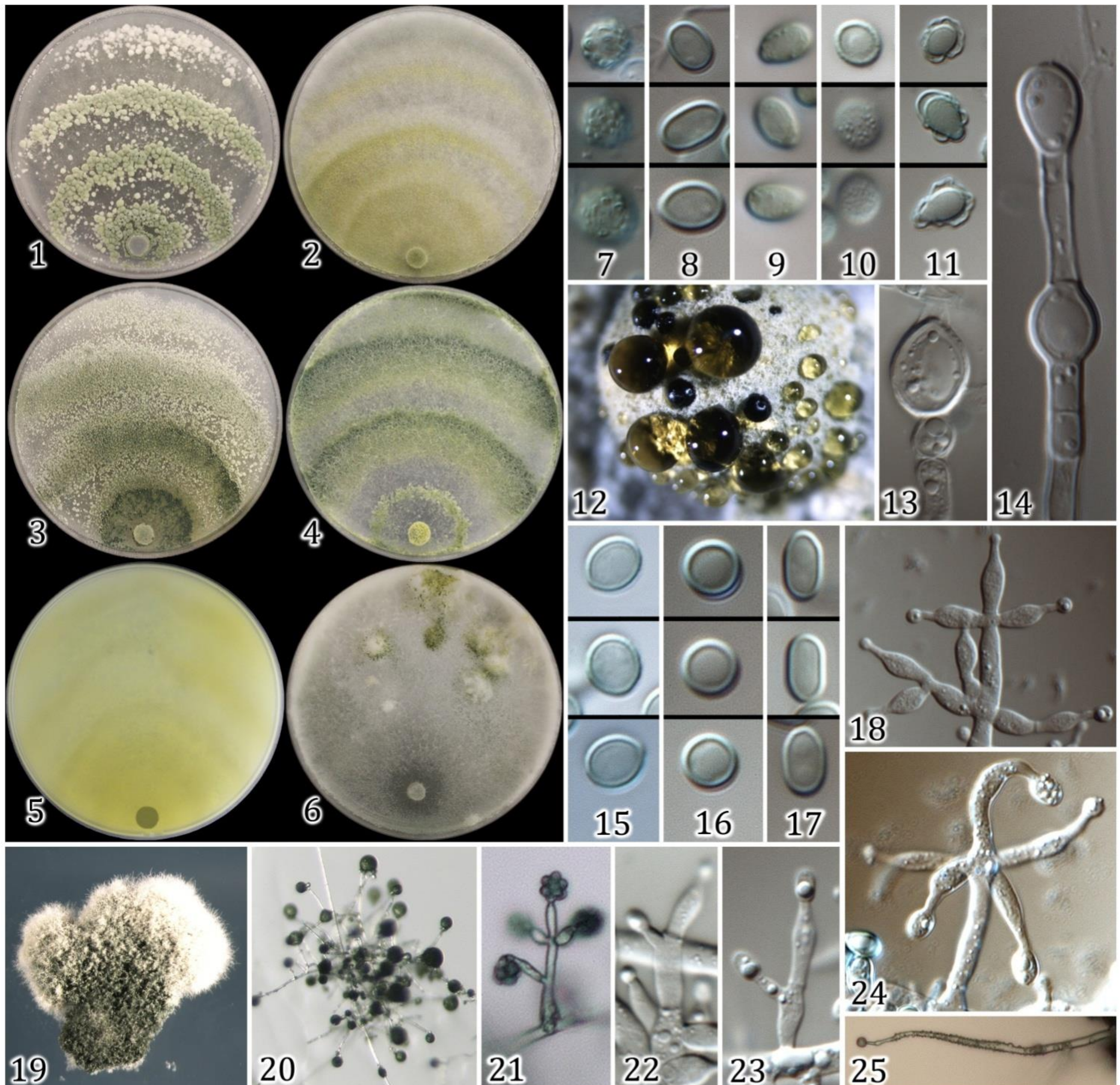

FIGURE 1: Morphological characteristics used to describe *Trichoderma* species: **1. Pustule conidiation** *T. vagum* prov. nom. Strain Tri 13 grown on CMD. **2. Effuse conidiation** *T. harzianum* (Subclade III *T. pseudoharzianum*) Strain Tri 64 grown on PDA. **3. Granular (shrub) conidiation** *T. orientalis* Strain Tri 120 grown on CMD. **4. Zonation** *T. harzianum* (Subclade II *T. afroharzianum*) Strain Tri 79 grown on CMD (under a 12h day/night lighting regime). **5. Reverse colouration** *T. harzianum* (Subclade III - *T. pseudoharzianum*) Strain Tri 64 grown on PDA. **6. Conidiogenous tufts** *T. gamsii* Strain Tri 118 grown on PDA. **7. Warted or echinulate conidia** *T. viride* Strain Tri 66. **8. Smooth conidia** *T. spirale* Strain Tri 160. **9. Slightly roughened conidia** *T. hamatum* Strain Tri 78. **10. Roughened conidia** *T. asperelloides* Strain Tri 150. **11. Bullate or wing-like conidia** *T. saturnisporum* Strain Tri 104. **12. Exudates** *T. saturnisporum* Strain Tri 104. **13. Chlamydospore** *T. koningii* Strain Tri 14. **14.**

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Chlamydospores *T. harzianum* (Subclade III *T. pseudoharzianum*) Strain Tri 64. **15. Subglobose conidia** *T. virens* Strain Tri 101. **16. Spheroid conidia** *T. harzianum* (Subclade II *T. afroharzianum*) Strain Tri 79. **17. Cylindrical conidia** *T. vagum*. Strain Tri 13. **18. Ampuliform phialides** *T. harzianum* (Subclade II *T. afroharzianum*) Strain Tri 79. **19. Pustule** *T. terrigenum* prov. nom. Strain Tri 59. **20. Wet conidial heads** *T. viride* Strain Tri 101. **21. Conidial aggregations** *T. koningii* Strain Tri 14. **22. Intercalary phialide** *T. orientalis* Strain Tri 152. **23. Intercalary phialide** *T. orientalis* Strain Tri 152. **24. Lanceolate phialides** *T. gamsii* Strain Tri 156. **25. Conidiophore elongations** *T. koningii* Strain Tri 159.

Micromorphological characteristics

Micromorphological characterization of *Trichoderma* strains are usually performed using conidiophores taken from colonies grown on CMD or SNA after they have been mounted using a 3% KOH solution (Samuels *et al.* 2002b, Chaverri *et al.* 2004, Jaklitsch 2009). The morphology of conidiophores from colonies grown on CMD or SNA are comparable to those seen in nature (Jaklitsch 2009). Slides should be prepared from the younger parts of the colony that have recently started to sporulate (frequently around Day 3), otherwise conidiophores tend to dry out and become brittle, making it difficult to view intact structures. The following characters are often recorded when the micromorphological characteristics of *Trichoderma* species are described:

Chlamydospores: Many *Trichoderma* species form thick walled chlamydospores either at hyphal terminals (Fig. 1.13) or in between hyphal segments (Fig. 1.14). Shapes range from globose to subglobose, and they commonly form underneath the media surface. Chlamydospores from different *Trichoderma* species also display varying ornamentations and can be smooth or roughened (Jaklitsch 2009). The dimensions of chlamydospores as well as their positioning within hyphae are often recorded.

Conidiophore dimensions: The following conidiophore dimensions are important to consider when characterizing *Trichoderma* species: width of the phialide base, width of the phialide at the widest point, the phialide length, the phialide length/width ratio, the width of the cell from which phialides arise and the length and width of the conidia. These traits were recorded in taxonomic revisions of the genus and are, therefore, important to consider when characterising *Trichoderma* species (Rifai 1969, Bissett 1991a, Jaklitch 2009).

Conidiophore hyphal elongations: Typical of many species from the *T. pachybasium* clade, these hyphal elongations are formed at the ends of conidiophores and are often warted or rough

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walled. Such structures can be sterile or fertile and were extensively used by Bissett (1991) to characterize *Trichoderma* species (Fig. 1.25).

Conidiophore type: *Trichoderma* species display several different types of conidiophores which can be divided into the following categories based on their morphology (Jaklitsch 2009):

- *Trichoderma*-like: Conidiophores are extensively branched which form at right angles relative to the stipe axis, irregular or terminally pyramidal. Phialides arise from branches of the conidiophore as opposed to forming of the stipe (Fig. 2.1).
- *Gliocladium*-like: Densely packed penicilli are formed which develop terminal phialides that run parallel to one another, conidiophores are appressed (Fig. 2.2).
- *Pachybasium*-like: Dense conidiophores which form short ampuliform phialides. Fertile or sterile hyphal elongations sometimes extend from the conidiophore terminus (Fig. 2.3).

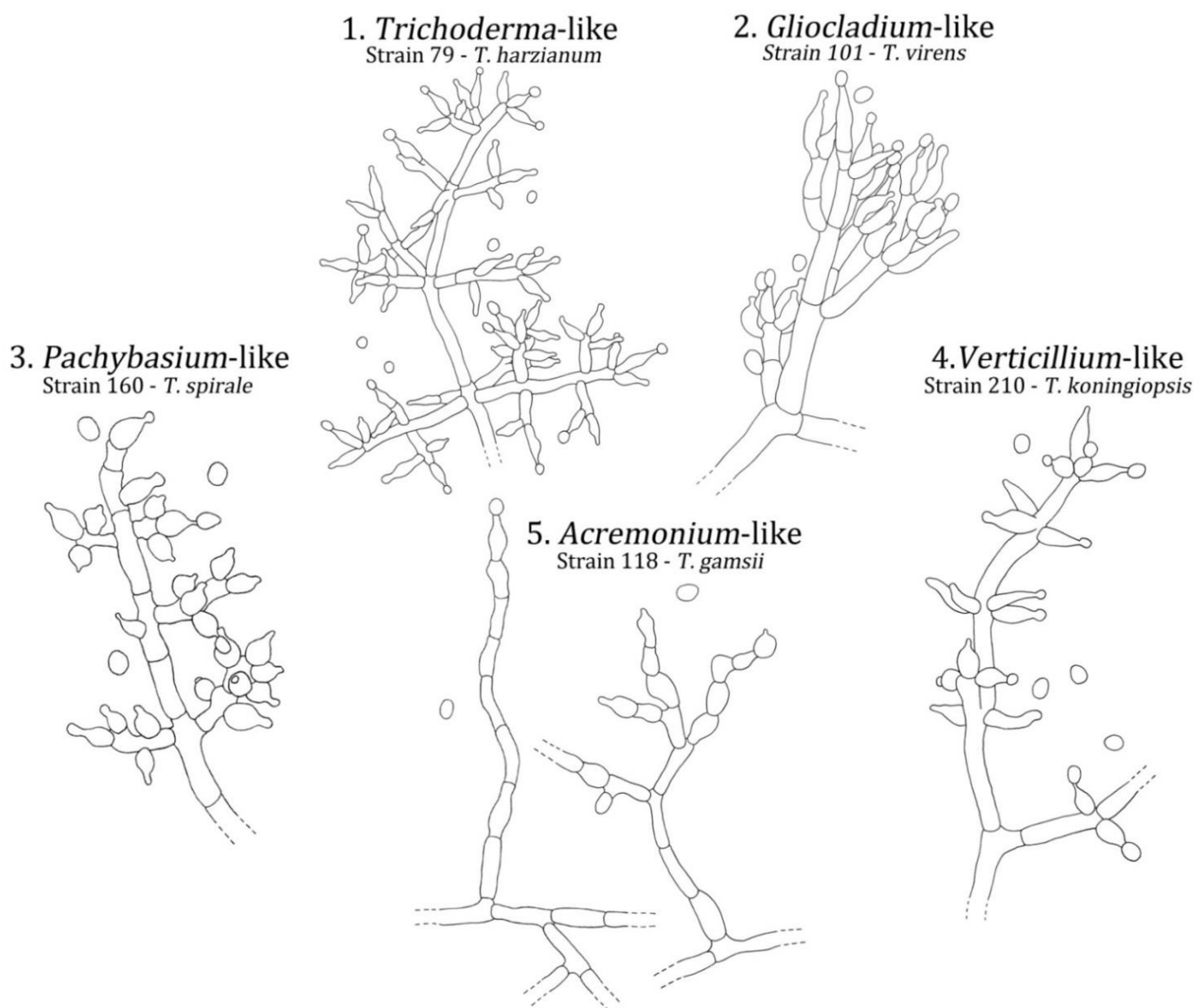

FIGURE 2 The various conidiophore arrangements of *Trichoderma* species (not to scale)

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- *Verticillium*-like: Conidiophores are sparsely branched and form whorls of phialides which originate on the same level, often forming directly from the stipe axis (Fig. 2.4).
- *Acremonium*-like: simple conidiophores, usually only a few phialides which sometimes develop directly from the stipe axis, common amongst members of *T.* sect. *Hypocreanum* (Fig. 2.5).

Phylogenetic studies have shown that *Trichoderma* species forming the same conidiophore types do not necessarily group to form monophyletic clades (Kindermann *et al.* 1998, Kullnig-Gradinger *et al.* 2002).

Conidium ornamentation: *Trichoderma* species display conidia with strikingly different ornamentations including warted (Fig. 1.7), smooth (Fig. 1.8), slightly roughened (Fig 2.9), roughened (Fig. 1.10), or with bullate (wing like) structures (Fig. 1.11). These ornamentations can be observed most effectively when viewing mature conidia.

Conidium shape: *Trichoderma* species most commonly form subglobose (Fig. 1.15), globose (Fig. 1.16) or oval (Fig. 1.17) shaped conidia.

Phialide shape: *Trichoderma* species form ampuliform shaped phialides (Fig. 1.18), lanceolate shaped phialides (Fig. 1.24), or subulate phialides, which are slender or hooked.

Intercalary phialides: Some *Trichoderma* species from the *T. longibrachiatum* clade form unique phialides which produce conidia from a second opening located near the base of the phialide which stems from the supporting cell. The presence or absence of such intercalary phialides are recorded whenever observed (Jaklitsch *et al.* 2006b) (Figs. 1.22 and 1.23).

Regular or irregular conidiophores: This refers to the overall shape of the conidiophore, a regular conidiophore is tree or pyramid like, with several branches at the base and fewer branchings near the apex (See Fig. 2.1 for an example of a regular conidiophore). Irregular conidiophores show unpaired branchings from the stipe and vary in terms of the extent that second degree branches develop. This results in an overall conidiophore shape which appears unbalanced relative to regular conidiophores (See Fig. 5 page *146* for an example of an irregular conidiophore).

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The ecology of *Trichoderma* species and their function in the environment

Trichoderma species are opportunistic and they thrive in various different habitats around the world (Druzhinina *et al.* 2011). These include diverse niches such as leaf litter, termite mounds, Mediterranean sea sponges, stems and roots of plants, water damaged buildings and immunocompromised people and livestock (Sivasithamparam 1991, Fisher *et al.* 1996, Ghisalberti and Bailey *et al.* 2009, Paz *et al.* 2010, Torky and Khalifa 2012).

Trichoderma species fulfil a key role in the environment as decomposers and can often be seen growing on dead plant materials (Jaklitsch 2009, 2011). Some *Trichoderma* species grow parasitically on top of basidiomycetous fungi in tropical regions while others form endophytic relationships within the stems and roots of plants (Zhang *et al.* 2007, Bailey *et al.* 2008). *Trichoderma* species have also been isolated from a variety of different soils ranging from forest soils to deserts and salt marches. In addition, some strains have also been isolated from soils contaminated with oil (Pinholt *et al.* 1979, Roiger *et al.* 1991). *Trichoderma* species employ strikingly different strategies to obtain nutrients within such diverse habitats. Some species are biotrophic and parasitize the intracellular contents of other fungi, whereas others are adapted to saprophytic lifestyles, and some can also break down recalcitrant substances such as cellulose (Kubicek 2012, Atanasova *et al.* 2013).

Researchers have recently begun to assign ecological functions to some *Trichoderma* species through whole genome analyses. Martinez *et al.* (2008) and Kubicek *et al.* (2011) sequenced and compared the genomes of three economically important *Trichoderma* species: *T. virens* Miller, Giddens and Foster, *T. atroviride* Karsten and *T. reesei*. Kubicek *et al.* (2011) found that the ancestral lifestyle of *Trichoderma* was mycoparasitic in nature and not saprophytic as are many extant *Trichoderma* species (Martinez *et al.* 2008, Kubicek *et al.* 2011). This hypothesis was originally proposed by Rossmann and Samuels (1999) who speculated that the ancestors of *Trichoderma* parasitized on wood-degrading basidiomycetes. They argued that *Trichoderma* species developed saprotrophic traits to better inhabit the environments wherein they could find their fungal hosts (Rossmann and Samuels 1999).

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The impacts and applications of *Trichoderma*

Trichoderma species are economically and medically important. Some species are considered beneficial and are routinely applied in fields such as agriculture, biotechnology and restoration ecology (Yedidia *et al.* 1999, Kubicek *et al.* 2009, Chakroun *et al.* 2010). Others are detrimental and may damage commercial mushroom crops or cause respiratory illnesses and mycoses in immunocompromised individuals (Druzhinina *et al.* 2008).

The impacts of **Trichoderma** *spp. in agriculture*

Several *Trichoderma* species such as *T. viride*, *T. harzianum, T. asperellum* Samuels and *T. asperelloides* Samuels have been successfully commercialized as biocontrol agents (Stasz *et al.* 1988, Whipps and Lumsden 2001, Harman *et al.* 2004, Mbarga *et al.* 2012). These *Trichoderma* species interact with plant pathogenic fungi and are able to suppress their growth (Elad *et al.* 1980, Freeman *et al.* 2004). Additionally, some *Trichoderma* species form endophytic relationships with the stems and roots of crop plants and this can lead to improved plant growth rates and yields (Inbar *et al.* 1994, Ousley *et al.* 1994, Harman *et al.* 2000, Yedidia *et al.* 2001). *Trichoderma* species that display these beneficial traits are utilized in commercial agriculture. This enables farmers to counteract harmful plant pathogens and alleviates their dependency on chemical based treatments through integrated pest management systems (Klein and Everleigh 1998, Druzhinina *et al.* 2010).

On the other hand, a number of *Trichoderma* species cause harm to the agriculture industry. Some species such as *T. pleurotum*, *T. pleuroticola* and *T. mienum* aggressively parasitize commercially grown mushroom crops, which result in significant economic losses (Mamoun *et al.* 2000, Komon-Zelazowska *et al.* 2007, Kim *et al.* 2012).

The impact of **Trichoderma** *spp. on human and animal health*

Trichoderma species are generally not regarded as being harmful to human beings. However, some species such as *T. longibrachiatum*, *T. orientalis* and *T. citrinoviride* have been identified as the causative agents of illnesses including asthma and systemic infections (Druzhinina *et al.* 2008).

When fungal spores are inhaled by humans in high concentrations they can illicit immune responses that may lead to the development of fungal allergies (Burrell 1991, Thrane *et al.* 2001, Tang *et al.* 2003, Druzhinina *et al.* 2008). In particular, *T. longibrachiatum* and *T.*

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citroviride are common indoor contaminants and possess significant allergenic capacities (Thrane *et al.* 2001). Fifteen percent of people (from the USA) are said to be clinically atopic in that they show allergic symptoms in response to inhaling high concentrations of airborne fungal spores (Burrell 1991). *Trichoderma* is one of the ten fungal genera most commonly associated with fungal allergies along with others such as *Penicillium* Link, *Aspergillus* Micheli ex Haller and *Cladosporium* Link (Tang *et al.* 2003).

Clinical *Trichoderma* infections, although rare, have been associated with the deaths of infected patients (Guarro *et al.* 1999). Fatalities attributed to *Trichoderma* infections have also been reported in bovine livestock (Torky and Khalifa 2012). Such infections are only known to occur in immunocompromised individuals and are frequently misdiagnosed due to the rarity with which they occur (Druzhinina *et al.* 2008). A number of *Trichoderma* species have in the past been identified as human pathogens, these include: *T. harzianum, T. koningii* Oudemans*, T. pseudokoningii* Rifai and *T. viride* (Guarro *et al.* 1999, De Miguel *et al.* 2005)*.* Today however, it has been established that these species were misidentified and that only three species: *T. longibrachiatum, T. citrinoviride* and *T. oreantalis*, are in fact capable of causing disease in humans (Kühls *et al.* 1999, Tang *et al.* 2003, Druzhinina *et al.* 2008, 2011).

Clinical *Trichoderma* infections are difficult to treat, not only because of the difficulties associated with accurate diagnoses, but because pathogenic *Trichoderma* species are often resistant to many clinical antifungal agents (Kratzer *et al.* 2006). In 1996 two immunocompromised patients undergoing peritoneal dialysis died as a result of *T. longibrachiatum* infections despite extensive treatments with different antifungal medications such as amphotericin B and fluconazole (Campos-Herrero *et al.* 1996, Guiserix *et al.* 1996). Druzhinina *et al.* (2011) proposed that the resistance of *Trichoderma* strains to a wide range of antifungal agents could be attributed to *Trichoderma* strains having adapted to antifungal compounds secreted by their prey in nature. An aggressive *T. longibrachiatum* infection was reported in a patient that received a liver and small bowel transplant (Furukawa *et al.* 1998). The *Trichoderma* infection was, however, successfully treated with the antifungal drug itraconazole (Furukawa *et al.* 1998). Other antifungal drugs that have proven effective in treating *Trichoderma* infections include voriconazole and caspofungin (De Miguel *et al.* 2005, Kratzer *et al.* 2006).

Corley *et al.* (1994) investigated the toxin production of a strain of *T. harzianum* and reported that it produced a novel trichothecene mycotoxin which they named harzianum-A. This finding was concerning in light of the fact that other trichothecenes were known to cause

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mycotoxicoses in farm animals (Grove 1988). In addition, *T. harzianum* had been reported as a biocontrol agent at that time (Elad *et al.* 1980). Degenkolb *et al.* (2008a) investigated the toxin producing *T. harzianum* strain in more detail and found that it was misidentified and actually belonged to *T. strigosum,* which is not a biocontrol strain and belongs to the *T. brevicompactum* clade. They also reported the production of trichothecene-type mycotoxins such as trichodermin and harzianum-A by three other closely related *Trichoderma* species (Degenkolb *et al.* 2008a). These four toxin producing species are currently the only members of *Trichoderma* that are known to produce such toxins (Degenkolb *et al.* 2008a and b). These species are all members of the *T. brevicompactum* clade and are not closely related to any of the species currently used for biocontrol purposes (Degenkolb *et al.* 2008a and b).

Druzhinina *et al.* (2008) reported isolating the opportunistic human pathogen *T. orientalis* from soil in South Africa. This is significant considering that South Africa currently has the largest population of people suffering from HIV in the world (UNAIDS 2009). At the 6th SA AIDS conference in Durban 2013, the Human Science Research Council reported that the estimated number of people living with HIV increased between 2008 and 2013 from 10.6% to 12.3% of the population (Van der Linde 2013). The incidence of *Trichoderma* related illness in South Africa is not currently known. However, it is possible that such cases may have been overlooked due to the difficulties associated with diagnosing *Trichoderma* infections. In light of the growing number of people suffering from HIV in South Africa it is thus possible that opportunistic infections such as those caused by *Trichoderma* spp. may come to light, or become more prevalent, in the future.

The industrial applications of* Trichoderma *spp.

Trichoderma species are metabolically flexible and produce a range of different enzymes and metabolites through several unique biosynthetic pathways (Ghisalberti and Sivasithamparam 1991, Hoyos-Carvajal and Bissett 2011). Whole genome sequence analyses have revealed that approximately one third of all *Trichoderma* genes are unique to the genus and that orthologs of these genes are not known to occur in other fungi (Kubicek *et al.* 2011). This illustrates the vast potential of *Trichoderma* species to serve as source of new and useful metabolites (Ghisalberti and Sivasithamparam 1991). Species such as *T. reesei* produce enzymes that allow them to effectively break down recalcitrant, energy rich substances such as cellulose and xylose (Tomme *et al.* 1988, Kubicek-Pranz 1998). This has made them attractive subjects for research aiming to apply such enzymes in industry (Uusitalo *et al.* 1991, Kubicek and Harman 1998, Kubicek *et al.* 2009, Peterson and Nevalainen 2012). Biofuel industries often make use of

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cellulolytic enzymes originally derived from *T. reesei* (Kubicek *et al.* 2009, Hoyos-Carvajal and Bissett. 2011, Kubicek 2012). Certain food and textiles industries also utilize such enzymes (Galante *et al.* 1998).

Additional applications of *Trichoderma* species, such as their use in bioremediation, have also been developed (Chakroun *et al.* 2010). Laccases from *T. atroviride* have been shown to degrade phenolic compounds such as catechol and o-cresol, which are common pollutants of industrial and agricultural wastewaters (Chakroun *et al.* 2010).

Interactions between *Trichoderma* species and other organisms

Interactions between **Trichoderma** *spp. and plants*

Trichoderma species form endophytic relationships within the roots and stems of certain plants including several crop varieties (Hanada *et al.* 2008, Bailey *et al.* 2009). Such symbiotic interactions are mutually beneficial to both organisms. The *Trichoderma* resident inhabits the root cortex of its plant host and releases metabolites that stimulate root development (Gal-Hemed *et al.* 2011). In addition, *Trichoderma* root symbionts protect their plant hosts directly by competing with pathogenic organisms for the root niche (Gal-Hemed *et al.* 2011).

Trichoderma endophytes can stimulate the systemic immune system of their hosts, which helps these plant to resist attack from a range of different pathogens (Yedidia *et al.* 2000). For example, *Trichoderma* species such as *T. asperelloides* form endophytic relationships with the roots of cucumber plants and are able to induce systemic resistance responses within their hosts (Gal-Hemed *et al.* 2011). This effect is brought about through the increased production of peroxidases and other fungitoxic compounds such as pathogenesis-related proteins by the plant (Yedidia *et al.* 2000). This allows the plant to resist attacks from pathogens even when the location of the attack occurs away from the *Trichoderma* endophyte itself. The *T. asperelloides* induced systemic resistance of cucumber plants enables these plants to more effectively resist attacks from *Pseudomonas syringae* van Hall. in their leaves where the *Trichoderma* endophyte itself is known to be absent (Gal-Hemed *et al.* 2011). In return, the *Trichoderma* symbionts are provided a ready source of nutrients from the plant root exudates and also extends its own niche by promoting the root development of its host (Harman and Shoresh 2007).

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The beneficial interactions between *Trichoderma* biocontrol agents and crop plants are of great importance in the agriculture industry. A number of *Trichoderma* based commercial products have been developed which enable farmers to protect their crops as well as to increase their yields (Benitez *et al.* 2004, Vinale *et al.* 2008, Hoyos-Carvajal *et al.* 2009).

Interactions between **Trichoderma** *spp. and other fungi*

Trichoderma species compete successfully in nature with other groups of fungi to occupy niches and obtain nutrients (Kubicek and Harman 1998). *Trichoderma* species achieve this by antagonizing competing fungi through mechanisms that range from the production of antifungal compounds, parasitizing directly on competing fungi or by outgrowing competitors in a given niche (Harman 2000, Benitez *et al.* 2004, Harman and Shoresh 2007). *Trichoderma* biocontrol strains have been shown to also antagonize plant pathogens through such mechanisms (Benitez *et al.* 2004). Early research investigating the mechanisms driving *Trichoderma*-based biocontrol focused primarily on the direct interactions between *Trichoderma* strains and other organisms such as through mycoparasitism (Weindling 1934, Aluko and Hering 1970). Today it is known that *Trichoderma* biocontrol agents employ a range of direct and indirect mechanisms to interact with other fungi and that these collectively result in the biocontrol effect (Harman 2000, Benitez *et al.* 2004, Harman and Shoresh 2007).

Mycoparasitism

Several *Trichoderma* species are capable of parasitizing plant pathogenic fungi *in vitro* (Chet *et al.* 1981, Elad *et al.* 1983) and *in vivo* (Hubbard *et al.* 1983, Lu *et al.* 2004). Not all *Trichoderma* species display parasitic lifestyles and mycoparasitic strains only parasitize certain hosts (Chet and Inbar 1994). The mechanisms of mycoparasitism have been modeled largely on the study of *T. harzianum* and *T. hamatum* Bainer (Harman 2000, Vinale *et al.* 2008, Druzhinina *et al.* 2011).

During the initial stages of mycoparasitism, the parasitic fungus grows towards target fungi in response to various chemical stimuli (Harman 2006, Druzhinina *et al.* 2011). Specialized coiling and appressoriae structures are then formed on the surface of the host hyphae, which is then penetrated through the release of hydrolytic enzymes (Elad *et al.* 1983, Elad 1996, Druzhinina *et al.* 2011). This allows the mycoparasite to feed on the cellular contents of its hosts (Elad *et al.* 1983, Elad, 1996, Druzhinina *et al.* 2011). Mycoparasitism by *Trichoderma* species is believed to play an important role in the destruction of survival structures, such as sclerotia, formed by some plant pathogens (Verma *et al.* 2007).

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Antibiosis

Trichoderma biocontrol agents secrete numerous antifungal compounds into their environment that are capable of inhibiting the growth of certain plant pathogens (Ghisalberti and Sivasithamparam 1991, Elad 1996, Harman 2006). It has long been known that *Trichoderma* species such as *T. virens* produce antibiotics like gliotoxin that could inhibit the growth of plant pathogens such as *R. solani* and *Sclerotinia americana* Norton and Ezekiel (Weindling 1934, Weindling 1941). The importance of antibiotic production as a mechanism whereby some *Trichoderma* species elicit biocontrol was demonstrated by Howell and Stipanovic (1983). They showed that *T. virens* mutant strains that were unable to synthesize the antibiotic gliovirin also lost their capacity to control *Pythium* damping-off in cotton.

Trichoderma strains vary in their ability to produce antifungal metabolites in response to changing environmental conditions (Howell 2003). Strains that demonstrate good antibiotic production *in vitro* will not necessarily perform the same way under field conditions (Howell 2003). This makes it difficult to determine the biocontrol potential of *Trichoderma* strains based solely on *in vitro* screening experiments. Howell *et al.* (2003) have shown that variables such as the type of substrate, presence of other fungi and incubation temperatures all influence the ability of *Trichoderma* species to produce antifungal compounds.

Competition

Trichoderma biocontrol agents are able to effectively compete against other fungi for space and nutrients within the rhizosphere and soil, and are able to inhabit these niches effectively (Harman 2000, Howell 2003). *Trichoderma* biocontrol agents are well adapted to soil environments and are naturally resistant to fungistatic compounds in soil such as fungicides, herbicides and phenolic compounds that are released by plants (Chet *et al.* 1997). This natural resistance gives *Trichoderma* biocontrol strains a competitive edge over certain plant pathogenic fungi in the soil environment (Chet *et al.* 1997).

In addition, *Trichoderma* biocontrol agents are able to deny certain nutrients to plant pathogens through effective competition (Elad 1996, Harman *et al.* 2004). For example, many filamentous fungi obtain iron through the use of extracellular siderophores which bind to environmental iron deposits (Eisendle *et al.* 2004). Studies by Segarra *et al.* (2010) have shown that certain *Trichoderma* species are able to secrete siderophores that chelate iron to the extent that it becomes unavailable to other fungi. *Trichoderma* species also show excellent capabilities to

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utilize soil nutrients such as cellulose, glucan and chitin, which allows them to thrive within the soil environment where such substances are often abundant (Chet *et al.* 1997).

Trichoderma research in South Africa

Past research

Trichoderma species have been intensively investigated in parts of the world such as central Europe, North America and Japan (Doi 1972, 1976, Druzhinina and Kubicek 2005, Jaklitsch 2009, 2011). However, in places such as central Asia and Africa *Trichoderma* remains relatively understudied (Druzhinina *et al.* 2006), though a few studies have reported *Trichoderma* strains from South Africa.

Bisby (1939) was the first person who reported examining a South African *Trichoderma* strain and identified it as *T. viride*. Many years later, Askew and Laing (1994) isolated and identified seven *Trichoderma* species from the South-eastern parts of South Africa. The primary aims of their study were to investigate possible agricultural applications for these strains and the study was restricted to a relatively small sampling region (Askew and Laing 1994). Similar studies have also been conducted which investigated agricultural aspects of South African *Trichoderma* strains (Askew and Laing 1987, Kotze *et al.* 2011, Mutawila *et al.* 2011). Others reported the isolation of native *Trichoderma* strains but did not attempt identification of these strains beyond the genus level because their studies were focused on other objectives. For example, Roux and Wingfield (1997) reported isolating *Trichoderma* species from *Acacia mearnsii* De Wild*.* trees in the eastern parts of the country but did not report on their identity. Similarly, Mouton *et al.* (2012) isolated *Trichoderma* strains from marine sediments collected off the West Coast of South Africa but also did not attempt species identifications. Other studies have reported small numbers of South African *Trichoderma* strains as part of global biodiversity studies (Chaverri and Samuels 2004, Druzhinina *et al.* 2008, Jaklitsch *et al.* 2006, Kubicek *et al.* 2008, De Respins *et al.* 2010, Druzhinina *et al.* 2010). In total, 13 different *Trichoderma* species were known to reside in South Africa based on the above mentioned studies. However, the majority of these studies were not intended as diversity surveys and were geographically limited to small study areas. In addition, much of the country has not yet been surveyed for the presence of *Trichoderma* species. Interestingly, there is very little overlap between the species reported by these different studies and this might suggest that more *Trichoderma* species could be found in South Africa with continued study.

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Trichoderma *based biocontrol in South Africa*

Trichoderma biological control strategies are routinely employed in South African agriculture by field and greenhouse farmers who seek to protect their crops from fungal pathogens (See: Table 1 on page *199* for a list of *Trichoderma* based biocontrol products that are commercially available in South Africa). Much of the current demand in South Africa for *Trichoderma* biocontrol agents are met by foreign based companies such as Koppert Biological Systems (from the Netherlands) and Agrimm (from Australia) export products such as Vinevax® and Trianum® to the South African market (http://www.agrimm.co.nz /contact.html-Vinevax®, http://www.trianum.com/en/products/trianum-g.html - Trianum®). Such products are sometimes based on *Trichoderma* strains not native to South Africa (for example, Trianum® is based on *T. harzianum* Strain T-22 that was originally isolated in the USA).

Other *Trichoderma* biocontrol products like as Biotricho® and Eco 77® are made by local companies (http://www.agro-organics.co.za/ - Biotricho®, http://www.plant-health.co.za - Eco 77®). Recent studies by Mutawila *et al.* (2011), and Kotze *et al.* (2011) have demonstrated the potential of South African *Trichoderma* strains to be developed as commercial biocontrol agents. The authors did this by proving that two native strains of *T. atroviride* were able to successfully antagonise grapevine pathogens *in vivo* (Mutawila *et al.* 2011, and Kotze *et al.* 2011). This illustrates the potential of native *Trichoderma* strains for biocontrol application and begs the question of whether other native *Trichoderma* strains show similar potential for commercial development.

The potential for further research on Trichoderma *in South Africa*

The South African government recognizes the importance of research which aims to document the country's biodiversity and apply its biological resources (Hamer 2013). This enables researchers from different fields to document South Africa's native biodiversity. From a mycological point of view, South Africa is an ideal location for such studies because of its rich and understudied fungal biodiversity. Crous *et al.* (2006) gave a conservative estimate that 170 000 fungal species exist in South Africa based on local plant: fungal ratios of 1:7. They also commented on how scantily the native fungal biodiversity had been explored in light of the fact that only 800 new fungal species were described from South Africa at the time of their study (Crous *et al.* 2006). It is, therefore, not unfounded to speculate that the vast majority of the fungal diversity of South Africa remains undocumented (Crous *et al.* 2006). This would suggest that a *Trichoderma* diversity survey based in South Africa might yield species that are new to

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science, especially when considering how much of the country has not yet been surveyed. Studies which have in the past investigated fungal diversity in South Africa have revealed that some parts of the country host remarkably diverse fungal communities (Slabbert 2008, Visagie *et al.* 2009, Visagie and Jacobs 2012). Studies by Visagie *et al.* (2009) and Visagie and Jacobs (2012) showed that the communities of other dominant fungal groups of soil fungi, such as *Penicillium* and *Talaromyces*, are indeed remarkably diverse in the south western parts of South Africa and yielded many new species. The plant communities of South Africa are amongst the most diverse in the world and also display high degrees of endemism (Goldblatt 1997, Myers *et al.* 2000, Mucina and Rutherford 2006). Previous studies have shown that a strong positive correlation often exists between plant and fungal diversity (Zak *et al.* 2003, Waldrop *et al.* 2006). Generally, areas showing diverse plant communities also tend to show diverse fungal communities (Zak *et al.* 2003, Waldrop *et al.* 2006). In light of the diverse plant communities that occur in South Africa, it is not surprising that some parts of the country also host diverse fungal communities.

Problem statement

The diversity of *Trichoderma* species in South Africa is largely unknown and no local studies have yet reported novel *Trichoderma* species. This presents a unique opportunity to explore the genus in South Africa. We hypothesize that the *Trichoderma* community found in South Africa will be species rich and that many species remain to be discovered that have not previously been reported in South Africa. This is also based on the fact that very little species overlap occurred between studies that have reported species in the past. The interactions between South African *Trichoderma* strains and plant pathogens are not well studied. *Trichoderma* strains are routinely employed in South African agriculture as biological control agents, however, only a handful of studies have reported on the ability of native *Trichoderma* strains to antagonize selected plant pathogens (Askew and Laing 1987, Kotze *et al.* 2011, Mutawila *et al.* 2011). This study will attempt to address these shortcomings by firstly performing a taxonomic inventory of *Trichoderma* species in South Africa and then to screen selected strains for their ability to antagonize certain plant pathogens *in vitro*.

Research aims

The primary research objective for this study was to investigate the diversity of *Trichoderma* species in South Africa by isolating and identifying strains from different geographic locations

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across the country. In addition, any novel *Trichoderma* species that were discovered during the course of this study were characterised and described.

The secondary research objective was to perform a preliminary assessment of the potential for local *Trichoderma* strains to be developed as commercial biocontrol agents. This was done by investigating their capacity to antagonise the growth of selected plant pathogens *in vitro* through the production of volatile and non-volatile antifungal compounds.

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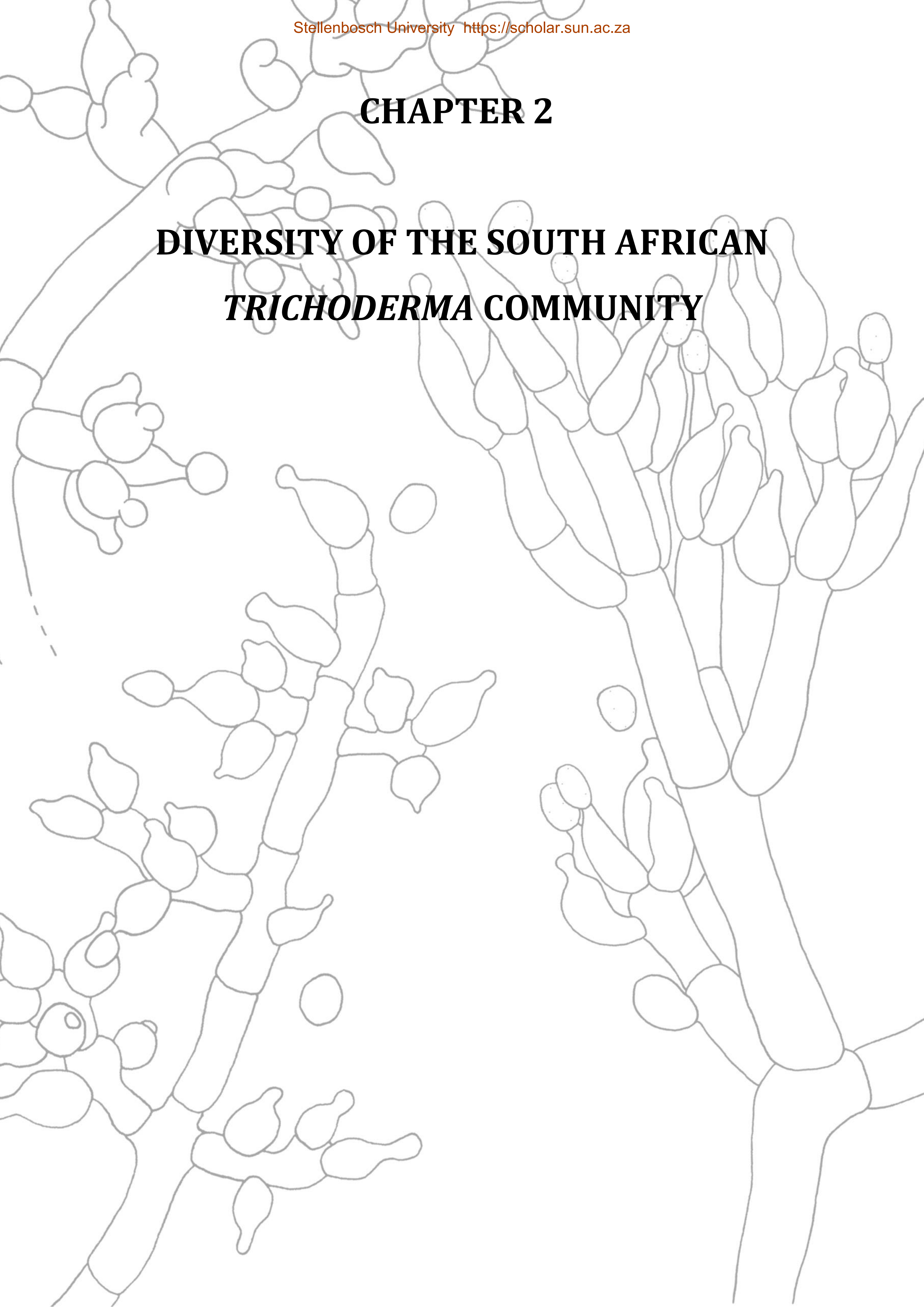

CHAPTER 2

DIVERSITY OF THE SOUTH AFRICAN *TRICHODERMA* COMMUNITY

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Abstract

The genus *Trichoderma* accommodates a large number of species that are economically important. Despite this, few studies have investigated the diversity of the genus in South Africa. During this study 161 different *Trichoderma* strains were isolated from soils collected at 173 sites across South Africa. These strains were identified to species level through phylogenetic analyses using elongation factor DNA sequence data. Morphological analyses were performed in order to validate species identities as far as possible. *Trichoderma* strains were assigned to 18 different groups based on their morphologies. Fourteen of these groups were identified as known *Trichoderma* species and include representatives from several of the major clades currently recognised in the genus. Four additional *Trichoderma* species could not be matched to any known species and are, therefore, considered to be new to science. These species are discussed in Chapters Three and Four respectively. Eight of the *Trichoderma* species identified during this study were known from South Africa based on previous studies and six species are first reports for South Africa. These include: *T. koningii, T. koningiopsis, T. asperelloides, T. lixii, T. virens* and *T. spirale*. The distributions of *Trichoderma* species in South Africa varies to a large extent. Some species, such as *T. atroviride* and *T. virens* show restricted distribution patterns whereas others such as *T. orientalis* and *T. saturnisporum* seem to be more widespread. A large number of strains were identified as the opportunistic human pathogen *T. orientalis,* which can cause life-threatening mycoses in immunocompromised individuals. In addition, this species is widely distributed across the country and was more frequently isolated than any other *Trichoderma* species. This may be of special significance in South Africa considering the large number of people who are immunocompromised. Several *Trichoderma* species that are important biocontrol organisms were also isolated during this study, these include: *T. asperellum, T. asperelloides, T. atroviride, T. hamatum, T. harzianum, T. viride* and *T. virens.*

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Introduction

The fungal genus *Trichoderma* includes several noteworthy species that are commercially employed in agriculture and industry as biological control agents, crop growth promoters and enzyme producers (Yedidia *et al.* 1999, Kubicek *et al.* 2009. Also see pages 20 and 22). Other species are important because of their involvement in causing human illnesses (Burrell 1991, Guarro *et al.* 1999. Also see page 20). Numerous studies investigated the ecology and diversity of *Trichoderma* around the world and more than 170 different species are currently known. The majority of these were described in recent decades following several taxonomic revisions that were largely based on the analysis of DNA sequence data (Chaverri and Samuels 2004, Samuels *et al.* 2006, 2012, Jaklitsch 2009, 2011, Jaklitsch *et al.* 2013).

Many *Trichoderma* species display cosmopolitan distributions and can be isolated from diverse environments such as soil, leaf litter, termite mounds, sea sponges, marine sediments, the stems and roots of plants and water damaged buildings (Ghisalberti and Sivasithamparam 1991, Fisher *et al.* 1996, Bailey 2009, Paz *et al.* 2010, Torky and Khalifa 2012, Mouton *et al.* 2012). *Trichoderma* species employ different strategies to obtain nutrients and display a variety of different lifestyles (e. g. Kubicek 2012, Atanasova *et al.* 2013. Also see page 19). Some species such as *T. harzianum* form endophytic relationships with plant roots and stems while others are parasites of other fungi or act as saprophytes in bulk soils (Vinale *et al.* 2008, Bailey *et al.* 2009).

In contrast to what is known about *Trichoderma* species from other parts of the world, the diversity of *Trichoderma* species in South Africa has only been partially investigated. Previous studies investigating South African *Trichoderma* strains have addressed this shortcoming to some extent and have accounted for thirteen different species. Askew and Laing (1994) reported isolating several *Trichoderma* species from soils, compost and raw pine bark in the Kwazulu-Natal province. These were identified as *T. longibrachiatum* Rifai, *T. polysporum* Rifai, *T. aurioviride* Rifai, *T. hamatum* Bainer, *T. harzianum* Rifai, *T. viride* Persoon and *T. reesei* Simmons (Askew and Laing 1994). Additional studies have resulted in the identification of several South African *Trichoderma* strains as: *T. asperellum* Samuels, Lieckfeldt & Nirenberg, *T. atroviride* Samuels, *T. catoptron* Samuels, *T. gamsii* Samuels and Druzhinina, *T. stromaticum* Costa and *T. orientalis* Samuels and Petrini (Samuels *et al.* 1998, Chaverri and Samuels 2004, Jaklitsch *et al.* 2006, Kubicek *et al.* 2008, Druzhinina *et al.* 2008, De Respins *et al.* 2010, Druzhinina *et al.* 2010, Mutawila *et al.* 2011). Studies such as these represent *Trichoderma*

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strains from relatively small geographic areas in South Africa and a comprehensive understanding of the species diversity in other parts of the country is lacking. The aim of this study was, therefore, to survey the diversity of *Trichoderma* species in South Africa.

Materials and methods

Sampling and strain isolations

To achieve the objective of this study, *Trichoderma* strains were isolated and identified from soil samples that were taken from different parts of South Africa. In total, 173 soil samples were collected and processed in the summer and autumn months of 2012. The GPS locations of the sample sites are listed in Table 1. Soil samples were primarily collected from non-agricultural sites. However, a garden soil sample from site 194 was included in this study as an exception. Samples were collected from the first few centimetres of topsoil and were refrigerated before processing in the lab. *Trichoderma* strains were isolated from soils using standard soil dilution plating techniques, five grams of soil from each site were suspended in 100 ml dH_20 before being diluted 100, 1000 and 10 000 times (Crous *et al.* 2009). These dilutions were then plated onto potato dextrose agar (PDA) (Merck, Germany) supplemented with 50 $mg.l^{-1}$ ampicillin (Applichem, South Africa) and 100 $mg.l^{-1}$ chloramphenicol (Applichem, South Africa) to inhibit bacterial growth. Petri dishes were incubated for 7 days at 25°C under ambient lighting conditions before being inspected under low magnification. *Trichoderma* colonies were then transferred to fresh media and single spore cultures were prepared. This was done by diluting spore suspensions of respective strains and plating them onto water agar (WA). Petri dishes were incubated for 24 hours at 25°C before being inspected under 6× magnification. Germinating spores were cut from the media surface and were transferred to fresh PDA. Following isolation, *Trichoderma* strains were placed into groups based on their colony morphologies. This was done by incubating each *Trichoderma* strain on cornmeal dextrose agar (CMD), supplemented with 2% glucose (Sigma, Germany), for seven days at 25°C under a 12h day night light regime (Jaklitsch 2009). Strains were then divided into groups and representatives from each group were selected for study.

Species identifications

Preliminary identification of the *Trichoderma* group representatives were made through use of the online barcoding based resource, Trichokey V. 2.0, based on ITS sequence data (Druzhinina *et al.* 2006) (Available from: www.isth.info). The phylogenetic relationships between species

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were then inferred based on ef1α sequence data. Finally, morphological analyses were performed in order to confirm species identifications.

DNA extractions, PCR and sequencing

DNA was extracted from *Trichoderma* colonies grown on oatmeal agar using a CTAB protocol (Möller *et al.* 1992). PCRs were performed using primer sets ITS 4 - ITS 5 (White *et al.* 1990) to amplify the ITS1 - 5.8S - ITS2 rDNA gene and EF1F - EF2R (Jacobs *et al.* 2004) to amplify the partial elongation factor 1α gene.

PCRs were set up in 10 µl volumes, which consisted of 5 µl Kapa Taq Ready mix (Kapa Biosystems, Woburn, USA) together with 0.25 µl of each respective primer (0.2 mM concentrations). 0.5 ng of gDNA template was added to the reaction mix along with 4.25 µl H_2O. ITS thermal cycle profiles were set up with an initial denaturing step at 94°C for 5 minutes followed by 29 cycles consisting of 30 seconds denaturing at 94°C, 30 seconds annealing at 56 °C and 45 seconds extending at 72°C and a final elongation step of 5 minutes at 72°C was included. Ef1α thermal cycle profiles were set up with an initial denaturing step at 94°C for 5 minutes followed by 32 cycles consisting of 30 seconds denaturing at 94°C, 30 seconds annealing at 51 °C, and 90 seconds extending at 72°C, with a final elongation step at 72°C for 5 minutes. Sequencing reactions were set up using a BigDye termination cycle premix kit according to the manufacturer's instructions (Applied Biosystems, California, USA). Thermal cycle conditions were set up with an initial denaturing step at 94°C for 5 minutes followed by 25 cycles of denaturing at 94°C for 10 seconds, annealing at 55°C for 10 seconds and extension at 60°C for 4 minutes. Sequencing reaction products were analysed using an ABI Prism 310 genetic analyser.

Phylogenetic analyses

A sequence database consisting of ex-type reference strains were assembled from GenBank. Due to the variability of ef1α sequence data between different *Trichoderma* species, each of the different clades within the genus were analysed separately (Druzhinina *et al.* 2005). Datasets were trimmed using the Geneious v. 4 software package (Kearse *et al.* 2012) (Available from: http://www.geneious.com) and multiple sequence alignments were conducted using Mafft v. 7.023b (Katoh and Standley 2013) (Available from http://mafft.cbrc.jp/alignment/software). The ambiguisly aligned regions within the datasets were identified and removed using the online resource Gblocks (Talavera *et al.* 2007). Phylogenetic analyses were performed with

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MEGA v. 6 (Tamura *et al*. 2013) (Available from: http://www.megasoftware.net/). The purpose of these analyses were to compare the DNA sequence data from the South African *Trichoderma* strains with those of other *Trichoderma* strains in order to identify them. For this reason, neighbour joining analyses were performed and branch strengths were determined through 1000 bootstrap replicates.

Morphological characterizations

Trichoderma strains were cultured under standardized conditions prior to morphological characterization (Jaklitsch 2009). *Trichoderma* strains were pre-grown on CMD for two days at 25°C, 5 mm agar plugs were cut from the margins of the growing colonies and transferred to Petri dishes containing 20 ml of either CMD, PDA or Synthetic Nutrient-poor Agar (SNA). Inoculation plugs were placed within 5 mm of the Perti dish margins with the mycelium side of the plug facing the medium. Petri dishes were incubated under a 12 hour day/night lighting regime using cool fluorescent light, for 21 days, at 5°C, 25°C, 30°C and 35°C, respectively. Colony sizes were recorded daily during the light phase. Petri dishes were examined regularly under low magnification using a dissecting microscope, and the date at which sporulation occurred as well as the arrangement of spore bearing structures were recorded and photographed using a Nicon SMZ 800 Stereo microscope fitted with a Nicon DSfi1 camera. Conidiophores were examined under high magnification using a Nikon Eclipse E800 compound microscope with differential interference contrast capabilities and a CFI plain Apochromat VC 100× lens. Microscope slides were prepared using a 3% KOH solution as mounting fluid (Jaklitsch 2009). Microscopic structures were measured using the Nikon NIS-Elements D software suite (v. 4.0).

Results

Strain isolations

A total of 161 different *Trichoderma* strains were obtained from soils collected at 173 different sites across South Africa. These strains were divided into 21 different groups based on their colony morphologies (Table 2) and representatives from each group were selected for identification (Table 3).

Species identifications and phylogenetic analyses

Amplification of the ITS and ef1α gene regions yielded ±600bp amplicons. Preliminary identifications of the *Trichoderma* group representatives were made based on ITS sequence

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data using the online barcoding resource, Trichokey. The South African *Trichoderma* strains represented five of the 16 different clades in the genus. These include the *T. harzianum* clade, *T. longibrachiatum* clade, *T. pachybasium* 'A' clade, *T. viride* clade and the *T. virens* clade. An additional strain (Tri 81) was isolated which could not be assigned to any of the current clades recognised in the genus.

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TABLE 1 GPS coordinates (DMS) of the 173 sample sites visited during this study

Site number	GPS coordinate S	GPS coordinate E	Site number	GPS coordinate S	GPS coordinate E
1	32°09'26.91"S	18°52'49.79"E	44	33°59'46.29"S	22°33'42.55"E
2	32°03'42.69"S	18°48'47.89"E	45	33°59'29.53"S	22°35'54.41"E
3	32°03'42.30"S	18°49'39.10"E	46	34°01'20.42"S	22°59'38.23"E
4	31°13'48.61"S	18°31'46.32"E	47	34°01'13.61"S	22°58'17.02"E
5	30°45'16.72"S	18°05'14.43"E	48	33°59'28.15"S	22°33'06.47"E
6	31°51'55.87"S	18°40'02.51"E	49	34°03'32.25"S	22°23'03.83"E
7	33°55’12.56"S	18°51’36.12"E	50	33°58'36.96"S	22°37'09.98"E
8	32°09'26.91"S	18°52'49.79"E	51	33°58'36.96"S	22°37'09.98"E
9	32°09'26.91"S	18°52'49.79"E	52	34°10'48.34"S	21°59'21.89"E
10	32°09'26.91"S	18°52'49.79"E	53	34°11'59.37"S	21°40'36.83"E
11	32°09'26.91"S	18°52'49.79"E	54	34°09'04.71"S	21°17'52.66"E
12	33°45'00.63"S	18°28'49.18"E	55	34°05'30.43"S	21°13'15.92"E
13	33°45'00.63"S	18°28'49.18"E	56	34°06'52.29"S	20°54'19.02"E
14	33°45'15.25"S	18°29'41.74"E	57	34°02'35.61"S	20°25'52.23"E
15	33°45'15.25"S	18°29'41.74"E	58	33°49'02.97"S	19°56'57.40"E
16	33°45'49.28"S	18°29'27.27"E	59	33°48'10.64"S	19°48'07.50"E
17	33°45'49.28"S	18°29'27.27"E	60	28°33'06.00''S	19°42'58.00''E
18	33°45'30.62"S	18°28'21.26"E	61	30°59'06.00''S	22°07'36.00''E
19	33°45'49.28"S	18°29'27.27"E	62	24°45’90.09''S	27°56'09.76''E
20	32°29'17.05"S	19°16'15.24"E	63	33°05'22.06"S	18°02'43.31"E
21	32°29'19.27"S	19°16'15.80"E	64	33°03'07.84"S	18°20'30.18"E
22	32°28'24.22"S	19°16'35.93"E	65	33°14'43.55"S	18°34'40.84"E
23	32°28'23.00"S	19°16'35.44"E	66	33°29'55.61"S	18°43'37.74"E
24	24°37'14.80"S	17°57'33.62"E	67	30°59'04.00''S	22°07'36.00''E
25	30°34'19.87"S	17°59'18.87"E	68	28°33'06.00''S	19°42'58.00''E
26	32°10'43.26"S	18°53'28.75"E	69	27°12'38.00''S	22°29'03.00''E
27	27°43'59.99"S	18°22'60.00"E	70	30°41'25.00''S	27°03'09.00''E
28	28°01'18.66"S	18°44'41.90"E	71	30°33'43.00''S	25°31'48.00''E
29	31°46'10.20"S	18°38'15.39"E	72	30°33'43.00''S	25°31’48.00''E
30	28°42'55.81"S	17°37'06.94"E	73	30°57'21.01"S	19°26'33.05"E
31	30°13'10.33"S	17°55'24.84"E	74	31°03'51.59"S	19°46'04.74"E
32	29°39'59.97"S	17°53'00.01"E	75	33°52'52.20"S	18°42'40.10"E
33	31°02'09.99"S	18°15'58.02"E	76	33°52'51.20"S	18°42'44.20"E
34	26°34'28.31"S	18°07'58.69"E	77	33°52'49.90"S	18°42'53.40"E
35	31°36'27.31"S	18°44'30.56"E	78	33°52'48.60"S	18°42'57.40"E
36	32°54'00.00"S	18°46'00.00"E	79	33°52'44.90"S	18°42'59.30"E
37	34°11'57.12"S	24°49'57.00"E	80	33°52'46.10"S	18°42'36.30"E
38	34°11'57.12"S	24°49'56.94"E	81	32°09'56.40"S	23°50'54.60"E
39	34°24'27.56"S	19°10'01.74"E	82	30°20'03.50"S	17°53'40.00"E
40	34°24'27.56"S	19°10'01.74"E	83	29°04'31.90"S	17°51'05.30"E
41	34°24'27.56"S	19°10'01.74"E	84	30°12'53.60"S	17°55'88.00"E
42	34°24'27.56"S	19°10'01.74"E	85	23°56'06.00"S	30°08'13.39"E
43	33°22'14.58"S	22°10'35.34"E	86	31°32'32.20"S	19°48'39.00"E

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TABLE 1 Continued

Site number	GPS coordinate S	GPS coordinate E	Site number	GPS coordinate S	GPS coordinate E
87	23°48'52.0"S	30°01'44.00"E	131	25°29'57.50"S	30°58'17.00"E
88	28°30'43.00"S	28°25'04.58"E	132	25°57'50.00"S	30°32'35.20"E
89	28°30'37.54"S	28°25'03.12"E	133	26°16'52.00"S	30°12'30.20"E
90	28°30'37.09"S	28°25'00.14"E	134	26°38'54.50"S	29°58'43.60"E
91	28°30'38.86"S	28°24'56.40"E	135	27°27'26.00"S	29°52'09.30"E
92	28°30'42.39"S	28°24'44.58"E	136	27°47'29.10"S	29°57'15.80"E
93	28°30'46.02"S	28°24'26.11"E	137	28°11'56.90"S	29°57'43.40"E
94	28°31'4.14"S	28°24'22.10"E	138	28°46'55.60"S	29°26'54.90"E
95	28°15'5.31"S	28°18'40.00"E	139	28°14'06.26"S	29°45'04.90"E
96	28°15'7.02"S	28°18'38.93"E	140	28°58'29.00"S	29°46'21.10"E
97	28°15'10.61"S	28°18'42.27"E	141	30°09'57.50"S	30°49'06.60"E
98	28°15'13.16"S	28°18'44.83"E	142	30°43'08.06"S	30°19'52.20"E
99	28°15'6.38"S	28°18'36.20"E	143	30°31'23.40"S	29°46'21.50"E
100	34°19'35.3"S	18°57'98.80"E	144	30°55'39.80"S	28°58'45.40"E
101	33°59'49.7"S	18°39'20.00"E	145	31°50'00.70"S	28°33'10.80"E
102	34°17'46.3"S	18°52'90.70"E	146	32°23'25.00"S	27°59'51.00"E
103	33°17'34.5"S	18°54'31.40"E	147	32°45'51.40"S	28°14'53.70"E
104	34°19'08.30"S	18°57'82.60"E	148	33°09'56.80"S	27°29'49.20"E
105	34°17'14.20"S	19°00'06.80"E	149	33°35'24.20"S	26°54'04.60"E
106	34°17'14.20"S	19°00'06.80"E	150	33°36'37.80"S	25°58'11.30"E
107	34°18'19.90"S	18°49'33.30"E	151	33°58'22.70"S	24°56'31.50"E
108	33°22'53.80"S	18°40'08.50"E	152	33°58'04.20"S	23°55'50.00"E
109	33°36'56.10"S	19°30'12.30"E	153	33°59'55.50"S	22°37'18.70"E
110	33°28'14.10"S	19°41'23.50"E	154	34°10'11.20"S	21°20'18.10"E
111	33°13'8.30"S	20°36'15.30"E	155	34°00'51.00"S	20°23'44.90"E
112	32°58'36.60"S	21°41'31.40"E	156	33°48'15.90"S	19°48'59.30"E
113	32°22'11.20"S	22°32'05.90"E	157	25°55'32.30"S	30°05'17.50"E
114	31°53'2.70"S	23°07'28.80"E	158	33°59'06.70"S	20°49'46.00"E
115	31°14'35.60"S	24°13'36.10"E	159	33°59'48.80"S	20°46'54.49"E
116	30°43'34.10"S	25°05'28.30"E	160	34°04'37.40"S	19°28'09.90"E
117	25°55'40.60"S	30°04'59.40"E	161	33°59'23.60"S	20°49'41.90"E
118	30°06'55.60"S	25°45'37.40"E	162	33°55'00.40"S	19°53'17.20"E
119	28°57'53.90"S	26°18'07.20"E	163	33°57'35.98"S	18°52'29.53"E
120	28°17'14.90"S	26°45'41.00"E	164	30°39'60.00"S	18°42'60.00"E
121	27°34'52.80"S	26°41'53.40"E	165	29°07'60.00"S	19°23'60.00"E
122	26°50'17.50"S	26°38'37.10"E	166	33°49'08.90"S	23°10'47.40"E
123	26°18'19.50"S	26°49'36.30"E	167	33°34'27.00"S	22°42'36.40"E
124	25°45'15.80"S	27°18'50.70"E	168	33°40'31.30"S	21°53'14.50"E
125	26°1'31.90"S	28°08'53.40"E	169	33°34'50.40"S	22°35'28.80"E
126	25°47'51.50"S	28°34'07.50"E	170	33°33'14.00"S	22°58'36.00"E
127	25°41'20.30"S	30°12'45.10"E	171	33°58'00.50"S	23°23'53.90"E
128	25°23'27.50"S	27°28'09.20"E	172	33°49'08.90"S	23°10'47.40"E
129	25°26'2.00"S	30°51'19.30"E	173	33°38'28.29"S	18°59'45.40"E
130	25°29'12.30"S	30°58'58.20"E			

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TABLE 2 Isolation locations of each *Trichoderma* species that was isolated during this study. The GPS coordinates for all sample sites are listed in table one.

Species	**Site numbers for each isolation locations**
T. asperelloides	126
T. asperellum	2, 3, 5, 6, 7, 19, 47, 50
T. afroharzianum prov. nom. (Subclade III)	20
T. atroviride	82, 75
T. gamsii	17, 75, 98, 128, 129, 140
T. hamatum	26, 51, 57, 46, 50, 49, 63, 159
T. harzianum (Subclade II)	45, 49, 72, 122, 135
T. harzianum (Subclade V-X)	133
T. harzianum (unknown group)	163
T. koningii	20, 26,54, 128
T. koningiopsis	117, 135, 137, 138, 145, 159, 163
T. lixii	28, 42, 57, 87, 108, 111, 126, 130, 131, 161
T. orientalis	14, 19, 20, 48, 59, 62, 66, 75, 96, 126, 137, 138, 140, 143, 144, 161
T. saturnisporum	4, 7, 18, 20, 29, 54, 65, 71, 78, 82, 93, 131, 154
T. spirale	97, 146
T. virens	42
T. viride	22, 102, 103
T. vagum prov. nom.*	8
T. terrigenum prov. nom.*	29
T. undulatum prov. nom.*	57
T. restrictum prov. nom.*	130

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TABLE 3 List of *Trichoderma* species isolated during this study. * *Trichoderma* species discussed in Chapters Four or Five

Clade	Species name	Number of strains	Group representative	Potential importance of species	First report in South Africa?
***T. viride* clade**	*T. viride*	3	Tri 66	Biocontrol species	No
	T. atroviride	3	Tri 219	Biocontrol species	No
	T. gamsii	11	Tri 156	Biocontrol species	No
	T. koningii	5	Tri 14		Yes
	T. koningiopsis	12	Tri 214	-	Yes
***T. longibrachiatum* clade**	*T. vagum* prov. nom.*	1	Tri 13	-	Yes
	T. terrigenum prov. nom.*	1	Tri 59	-	Yes
	T. saturnisporum	27	Tri 100	-	No
	T. orientalis	35	Tri 120	Human pathogen	No
***T. pachybasium* 'A' clade**	*T. hamatum*	11	Tri 78	Biocontrol species	No
	T. asperelloides	2	Tri 150	Biocontrol species	Yes
	T. asperellum	12	Tri 10	Biocontrol species	No
	T. restrictum prov. nom.*	1	Tri 144	-	Yes
***T. harzianum* clade**	*T. lixii*	18	Tri 139	-	Yes
	T. harzianum (Subclade III)	1	Tri 64	Biocontrol species	No
	T. harzianum (Subclade V-X)	2	Tri 191	Biocontrol species	No
	T. harzianum (unknown group)	1	Tri 216	Biocontrol species	No
	T. harzianum (Subclade II)	10	Tri 79	Biocontrol species	No
***T. virens* clade**	*T. virens*	2	Tri 101	Biocontrol species	Yes
	T. spirale	3	Tri 160	-	Yes
Novel lineage	*T. undulatum* prov. nom.*	1	Tri 81	-	Yes

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Taxonomy

Phylogenetic species identifications were confirmed by comparing the morphologies of *Trichoderma* group representatives with their close relatives. Each group representative was characterized and illustrated for comparison to other *Trichoderma* species. Nineteen *Trichoderma* species were identified in total and are discussed below.

The *Trichoderma viride* clade

This study isolated five different species which belong to the *T. viride* clade from 20 different sample sites across South Africa (Fig. 1 and Table 2). These species were identified through a phylogenetic analysis based on ef1α sequence data as *T. viride, T. atroviride, T. gamsii, T. koningii* and *T. koningiopsis* (Fig. 2, Table 4). Three of these species are used commercially as biocontrol organisms (Harman *et al.* 2004, Anees *et al.* 2010).

***Trichoderma viride* Pers., Neues Mag. Bot. 1: 92. 1794: Fries, Syst. Mycol. 3:215. 1832.**

Trichoderma viride is the type species of the genus and was described by Persoon (1794). Unfortunately none of the original material examined by Persoon still remains and Jaklitsch (2006), therefore, designated a neotype for the species from the Czech Republic. Later, Jaklitsch (2011) provided a detailed description of the neotype (Jaklitsch 2011). *T. viride* is found across Europe, the Americas and parts of southern Asia and Australia.

This study isolated a total of three strains of *T. viride* from three different sites in South Africa. These were represented by Strain Tri 66 (Fig. 1 and Table 2). Phylogenetic analysis based on ef1α sequence data showed that Strain Tri 66 did not form a clear group with the ex-type sequence of *T. viride* and a second *Trichoderma* species, *T. martiale* Samuels, was found to also exhibit ef1α sequences that was similar to that of Strain Tri 66 (Fig. 2 and table 4). A morphological analysis was performed to investigate the identity of Strain Tri 66 further (Figs. 3 and 4). This revealed that Strain Tri 66 matched *T. viride* based on its morphology and could be distinguished from *T. martiale* most notably by its growth rate on PDA, which was much slower than that of *T. martiale* (Hanada *et al.* 2008).

Strain Tri 66 displayed morphological traits that were similar to the description of the neotype of *T. viride* (Jaklitsch 2011).

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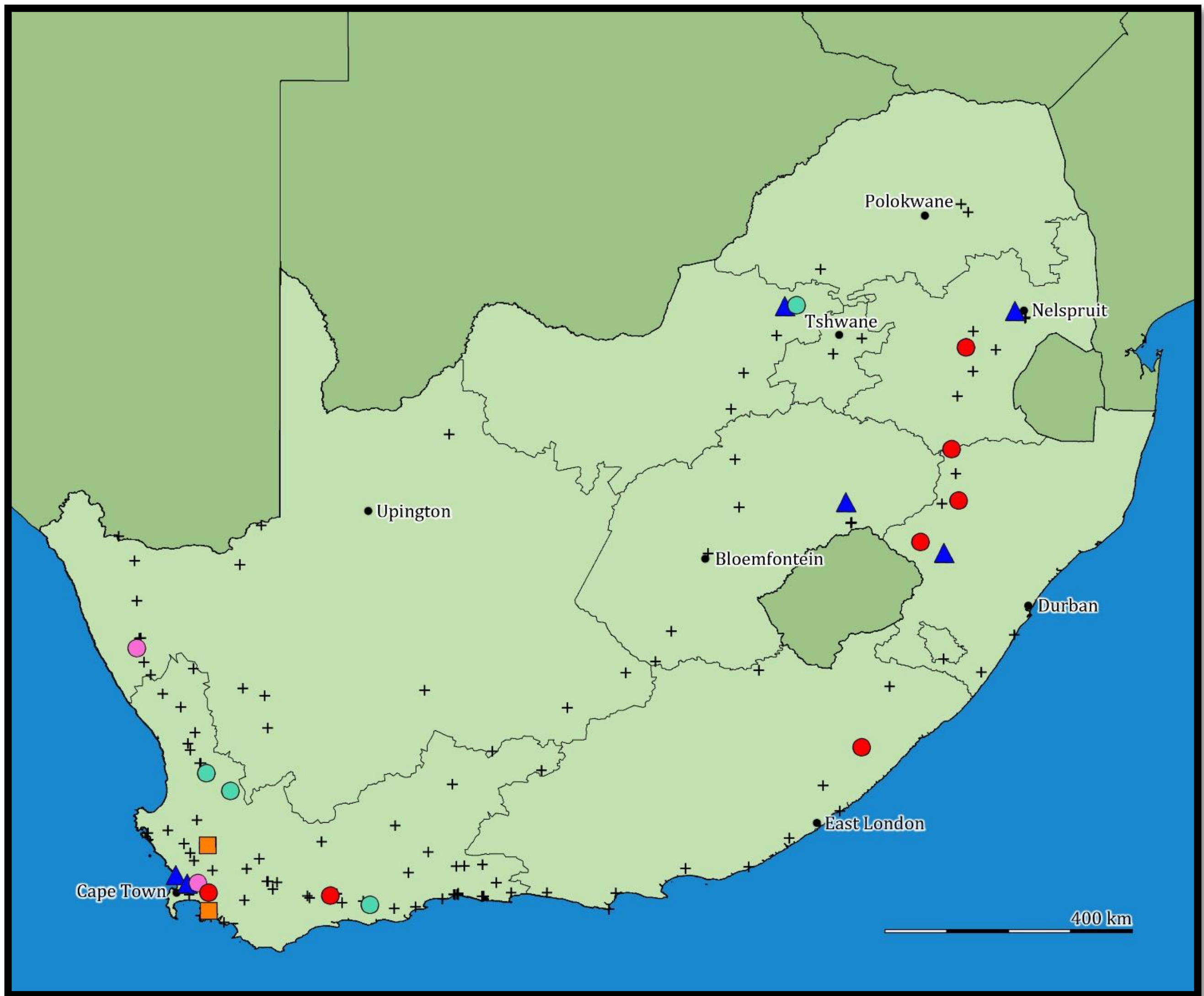

FIGURE 1 Distribution of the *Trichoderma viride* clade in South Africa, *T. koningiopsis* and *T. koningii* were isolated from soils collected across the country whereas the remaining species displayed distributions that were restricted to the Cape. Black crosses indicate sample sites. ***T. atroviride,*** **(pink circles)** isolated from 2 different sites. ***T. koningii,*** **(teal circles)** isolated from 4 different sites. ***T. gamsii,*** **(blue triangle)** isolated from 6 different sites. ***T. koningiopsis,*** **(red circle)**: isolated from 7 different sites. ***T. viride,*** **(orange squares)** isolated from 3 different sites. Note that some sites are located close to one another and cannot be visualised at this scale. Scale bar = 400km.

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TABLE 4 *Trichoderma* strains from the *T. viride* clade used for phylogenetic comparisons are listed along with their respective GenBank accession numbers for ef1α sequence data. Ex-type strains are indicated in bold and with a *'T'*.

GenBank	Taxon name	Strain number	Reference
AF456882	*H. atroviridis*	G.J.S. 96-200	Dodd *et al.* 2003
FJ860611	*H. atroviridis*	CBS 119499	Jaklitsch 2009
AF456886	*H. atroviridis*	C.T.R. 81-50	Dodd *et al.* 2003
AF456902	*H. atroviridis*	G.J.S 91-87	Dodd *et al.* 2003
AF456900	*H. atroviridis*	C.T.R. 68-1	Dodd *et al.* 2003
AF456887	***H. atroviridis T***	G.J.S. 98-134	Jaklitsch *et al.* 2012
JN715659	***H. hispanica T***	S 453	Jaklitsch *et al.* 2012
EU248630	*H. intricata*	G.J.S. 02-78	Hanada *et al.* 2008
AY376060	***H. intricata T***	G.J.S. 97-88	Holmes *et al.* 2004
JN715611	*H. koningii*	C.P.K. 1937	Jaklitsch *et al.* 2012
DQ288994	*H. koningii*	CBS 979.70	Samuels *et al.* 2006
DQ109548	*H. koningii*	G.J.S. 96-120	Samuels *et al.* 2006
AF456909	*H. koningii*	CBS 457.96	Dodd *et al.* 2003
DQ307571	*H. koningii*	G.J.S. 00-168	Samuels *et al.* 2006
AY376045	***H. koningii T***	G.J.S. 89-122	Holmes *et al.* 2004
FJ860659	*H. ochroleuca*	CBS 119502	Jaklitsch 2009
AY376056	*H. rogersonii*	G.J.S 94-115	Holmes *et al.* 2004
FJ860691	*H. rogersonii*	C.P.K. 2420	Jaklitsch 2009
AY376054	*H. rufa*	ATCC 28038	Holmes *et al.* 2004
AY376052	*H. rufa*	CBS 240.63	Holmes *et al.* 2004
DQ841723	*H. rufa*	G.J.S 05-463	Jaklitch *et al.* 2006
AF456904	*H. rufa*	CBS 101526	Dodd *et al.* 2003
AY376053	*H. rufa*	CBS 101526	Holmes *et al.* 2004
AY937449	*H. rufa*	ATCC 28020	Samuels *et al.* 2006
FJ860692	*H. rufa*	C.P.K. 2867	Jaklitsch 2009
DQ307555	*H. rufa*	G.J.S 04-372	Druzhinina *et al.* 2006
DQ672615	***H. rufa T***	CBS119325	Jaklitsch 2009
FJ860703	*H. stilbohypoxyli*	CBS 119501	Jaklitsch 2009
HM535606	*H. stilbohypoxyli*	G.J.S 05-474	Samuels *et al.* 2006
AY376063	***H. stilbohypoxyli T***	G.J.S. 96-30A	Holmes *et al.* 2004
FJ860717	***H. valdunensis T***	CBS 120923	Jaklitsch 2009
AY376048	*H. viridescens*	CBS 433.34	Holmes *et al.* 2004
DQ672610	***H. viridescens T***	CBS 119321	Jaklitsch *et al.* 2006
DQ307561	***T. austrokoningii T***	G.J.S. 99-146	Samuels *et al.* 2006
DQ307562	*T. austrokoningii*	G.J.S. 99-147	Samuels *et al.* 2006
DQ284976	***T. caribbaeum T***	G.J.S 98-43	Samuels *et al.* 2006
DQ284977	*T. caribbaeum*	G.J.S 97-3	Samuels *et al.* 2006
AY376040	*T. dingleyae*	G.J.S. 99-203	Holmes *et al.* 2004
DQ284974	*T. dorotheae*	G.J.S. 99-194	Samuels *et al.* 2006
DQ307536	***T. dorotheae T***	G.J.S. 99-202	Samuels *et al.* 2006

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TABLE 4 Continued

GenBank accession	Taxon name	Strain number	Reference
EU280025	*T. erinaceum*	DAOM 237546	Hoyos-Carvajal *et al.*
AY750880	***T. erinaceum*** [T]	DAOM 230019	Samuels et al. 2006
DQ307529	*T. gamsii*	G.J.S. 92-60	Samuels *et al.* 2006
DQ841722	*T. gamsii*	G.J.S. 05-111	Samuels *et al.* 2006
DQ307541	*T. gamsii*	G.J.S. 04-09	Samuels *et al.* 2006
DQ790647	***T. gamsii*** [T]	UNISS 4.2	Migheli *et al.* 2009
DQ289006	*T. koningiopsis*	G.J.S. 04-373	Samuels *et al.* 2006
DQ284981	*T. koningiopsis*	G.J.S 04-10	Samuels *et al.* 2006
FJ463269	*T. koningiopsis*	G.J.S 04-314	Chaverri *et al.* 2008
FJ463275	*T. koningiopsis*	G.J.S 05-204	Chaverri *et al.* 2008
AF456910	*T. koningiopsis*	G.J.S. 95-175	Dodd *et al.* 2003
FJ467647	*T. koningiopsis*	G.J.S. 06-263	Chaverri *et al.* 2008
DQ284969	*T. koningiopsis*	G.J.S. 91-7	Samuels *et al.* 2006
DQ284966	***T. koningiopsis*** [T]	G.J.S. 98-43	Samuels *et al.* 2006
DQ307534	***T. martiale*** [T]	G.J.S. 04-40	Samuels *et al.* 2006
EU280004	*T. ovalisporum*	DAOM 232078	Hoyos-Carvajal *et al.*
DQ307540	*T. ovalisporum*	DIS 203C	Samuels *et al.* 2006
AY376037	***T. ovalisporum*** [T]	DIS 70A	Holmes *et al.* 2004
DQ289004	*T. petersenii*	G.J.S. 04-164	Samuels *et al.* 2006
DQ289000	*T. petersenii*	DAOM 165782	Samuels *et al.* 2006
DQ284980	***T. petersenii*** [T]	G.J.S. 04-355	Samuels *et al.* 2006
DQ307563	***T. rogersonii*** [T]	G.J.S. 04-158	Samuels *et al.* 2006
JQ425717	*T. strigosum*	ATCC 28031	Chaverri *et al.* 2003
JQ425704	*T. strigosum*	CPK 3602	Chaverri *et al.* 2003
AY937442	***T. strigosum*** [T]	DAOM 166121	Samuels *et al.* 2006
DQ284973	***T. taiwanenense*** [T]	G.J.S. 95-93	Samuels *et al.* 2006

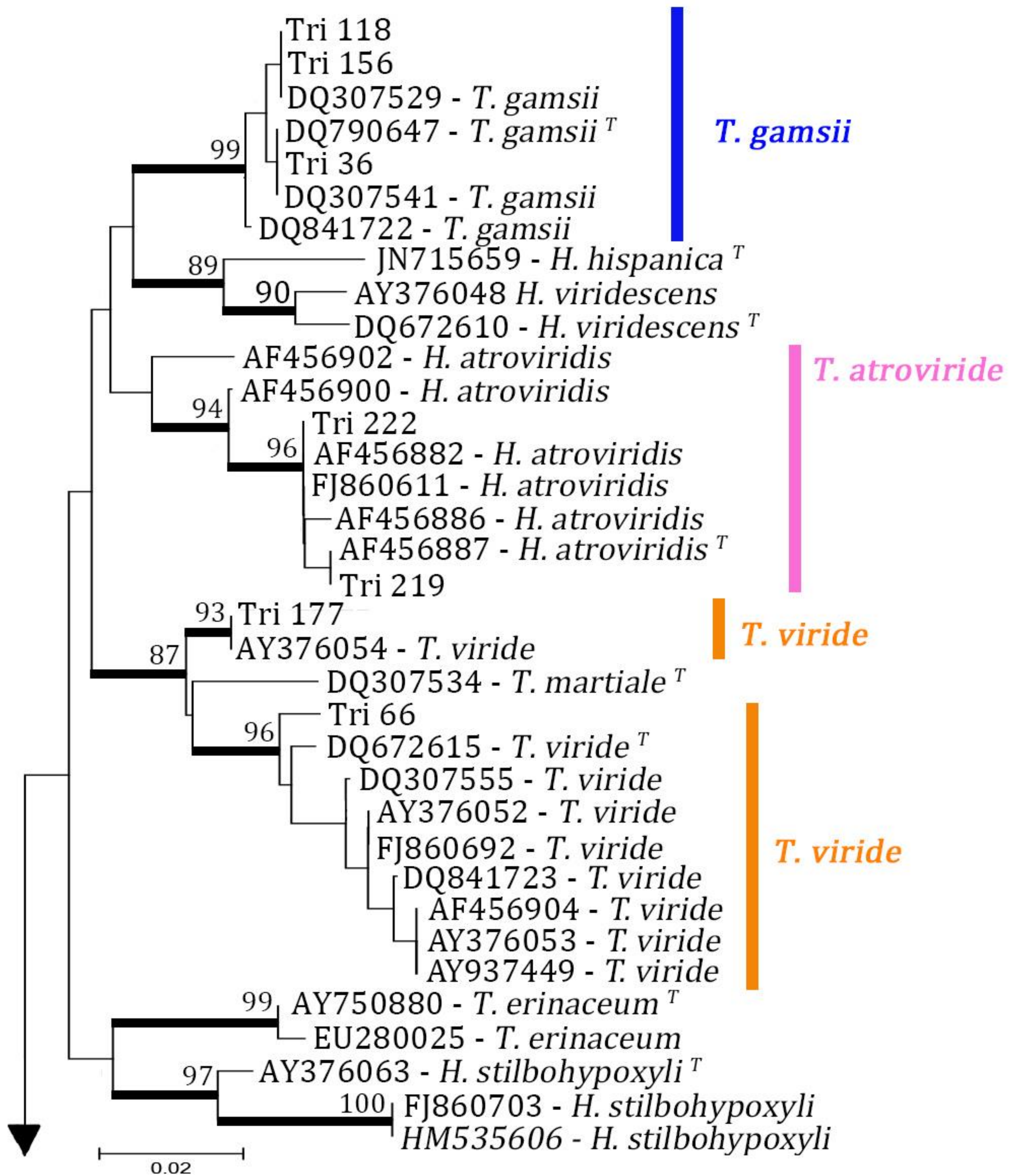

FIGURE 2. Neighbour joining tree based on ef1α sequence data indicating the phylogenetic relationships between members of the *Trichoderma viride* clade and South African *Trichoderma* strains belonging to the same clade. Nodes which are supported by bootstrapping values that exceed 80% are indicated by thickened branches. Ex-type culture sequences are indicated with a '*T*'.

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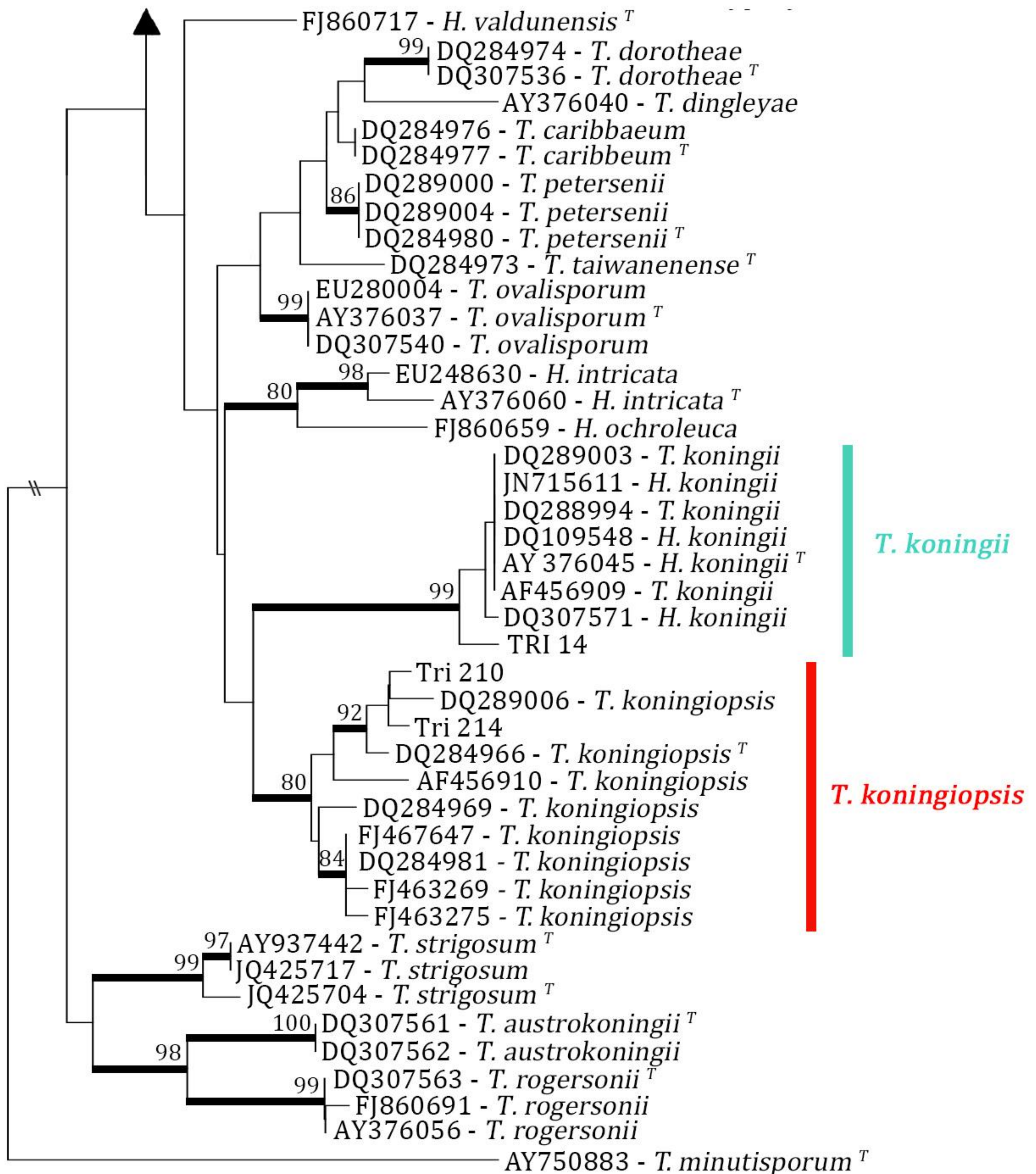

FIGURE 2 continued

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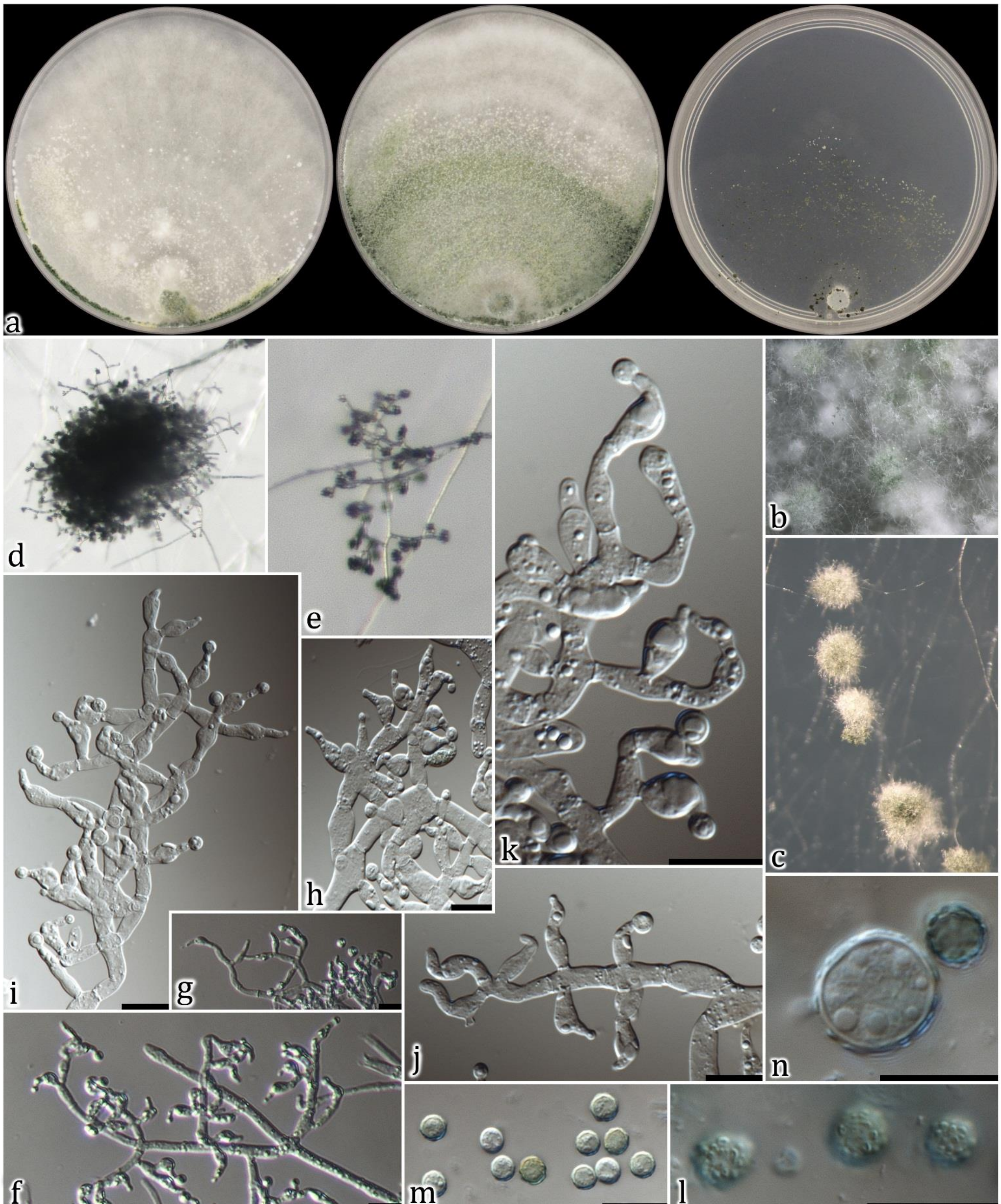

FIGURE 3. Morphological features characteristic of *Trichoderma viride*, Strain Tri 66. a. Colonies of *T. viride* incubated on CMD for 14 days (left), PDA for 14 days (middle) and SNA for 24 days (right). b, c. Stereo microscope images, from PDA (top) and SNA (bottom). d, e. Stereo microscope images from SNA. f, g. Conidiophores on CMA 400x magnification. h, i, j, k. Conidiophores formed on CMD 1000x. l, m. Conidia 1000x. n. Chlamydospore 1000x. All scale bars are 10μm in length.

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FIGURE 4. *Trichoderma viride*. Line drawings from strain Tri 66. Scale bar = 10µm

Similar to *T. viride*, Strain Tri 66 sporulated primarily within a broad band of fused, cottony pustules around the margin on CMD (Fig. 3 a). In addition, whitish downy colonies were produced on PDA, which sporulated effusely (Fig. 3 a, c). Micromorphologically Strain Tri 66 could not be distinguished from *T. viride*. Ill-defined conidiophores developed bearing curved or sinuous phialides with long necks (Fig. 3 j, k, i). Coarsely tuberculate, subglobose conidia were also observed from Strain Tri 66 (Fig. 3 l, m).

***Trichoderma atroviride* Karst, Bidr. Känn. Finl. Nat. Folk 51: 363. 1892.**

Trichoderma atroviride belongs to the the *T. viride* clade and is known to occur in Europe, Central- and North America (Jaklitsch 2011). A neotype was designated for the species by Samuels and Candoussau (1998) from France and Jaklitch (2011) provided a detailed description of its anamorphic state. During this study three strains of *T. atroviride* were isolated from 2 different sites (Fig. 1 and Table 2). This species was represented by Strain Tri 219. Phylogenetically Strain Tri 219 and an additional strain, Tri 222, grouped with the ex-type sequence of *T. atroviride* based on ef1α sequence data (Fig. 2 and Table 4). Strain Tri 219 was characterized morphologically to confirm phylogenetic identification (Figs. 5 and 6).

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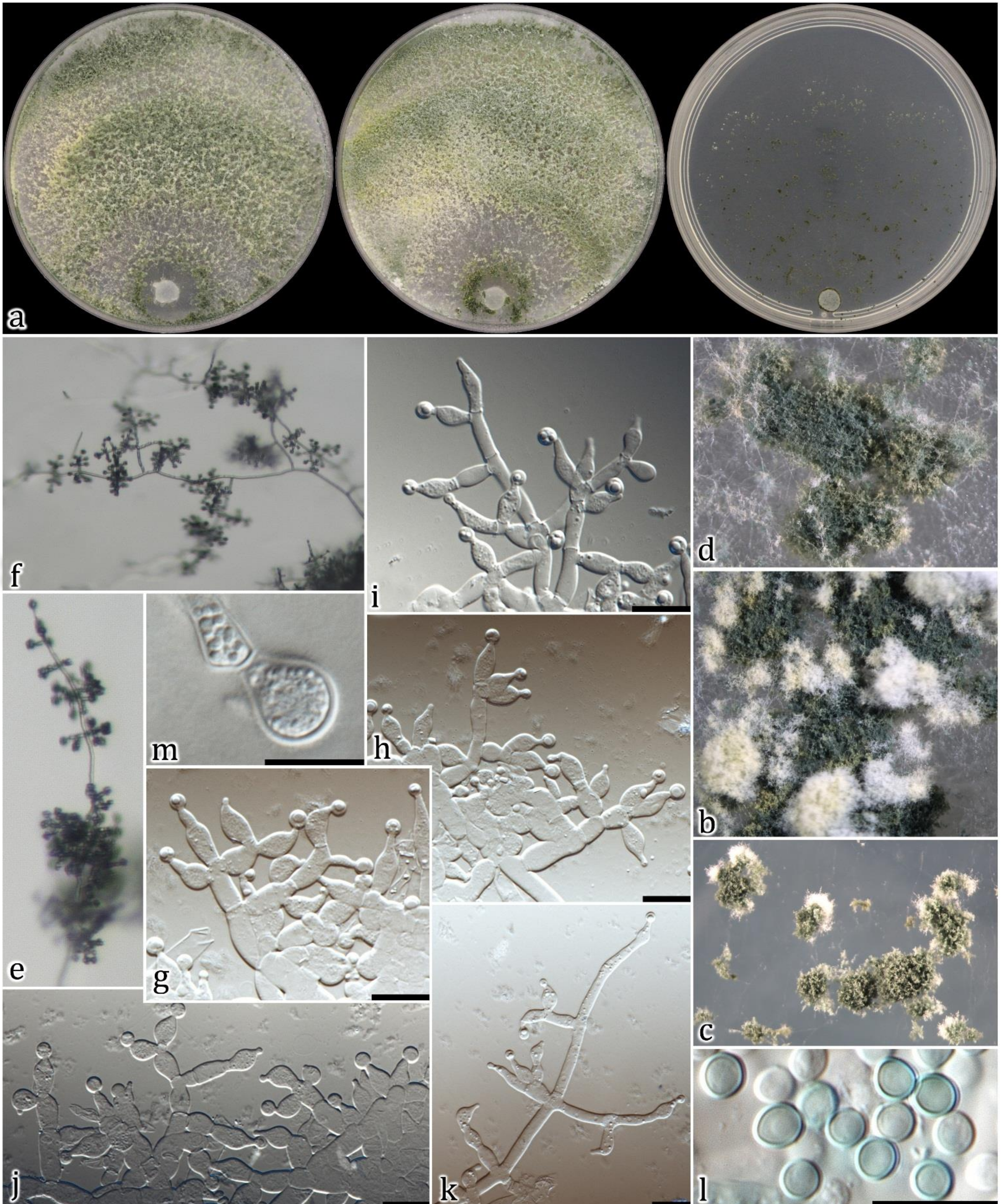

FIGURE 5. Morphological features characteristic of *Trichoderma atroviride*, Strain Tri 219. a. colonies of *T. atroviride* incubated on CMD for 7 days (left), PDA for 7 days (middle), SNA for 21 days (right). b, c, d. Stereo microscope images, from CMD (top), PDA (middle) and SNA (bottom). e, f. Conidiophores formed on SNA 400x magnification. g, h, i, j, k. Conidiophores formed on CMD 1000x. l. Conidia 1000x. m. Chlamydospore 1000x. All scale bars are 10μm in length.

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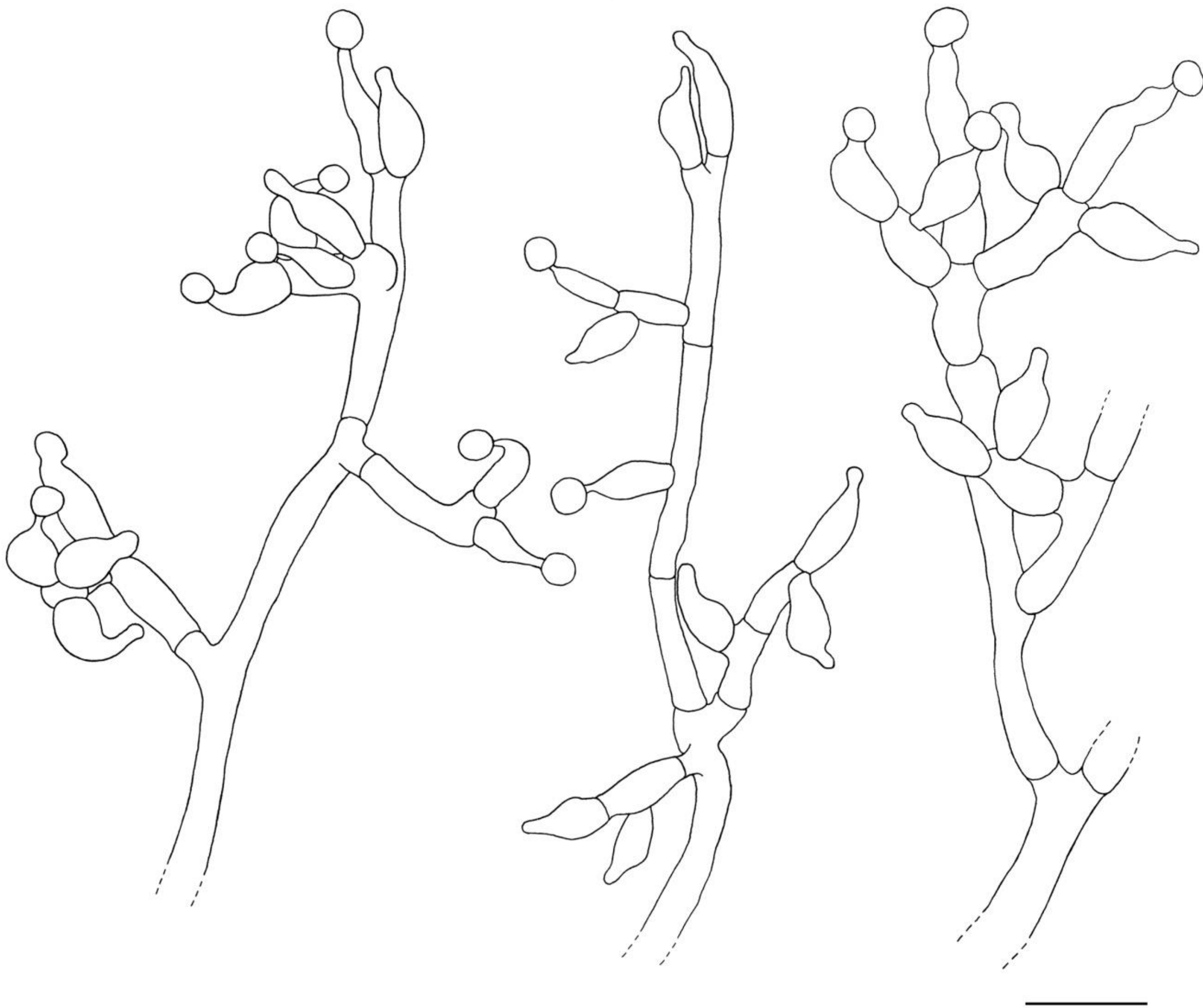

FIGURE 6. *Trichoderma atroviride.* Line drawings from Strain Tri 219. Scale bar = 10µm

Morphologically strain Tri 219 is similar *T. atroviride* according to the descriptions of the neotype (Jaklitsch 2011). On CMD, Tri 219 developed thin hyaline colonies that formed indistinct concentric zones (Fig. 5 a) and produced a noticeable coconut odour. Colonies of Tri 219 grew slightly faster than *T. atroviride* on CMD, forming 54 mm colonies after three days, whereas the ex-neotype was reported to form 45 - 48 mm colonies under the same conditions, although this difference was inconspicuous. The colony morphologies of Strain Tri 219 on CMD and SNA matched the descriptions of the ex-neotype and displayed typical downy tufts (Fig. 5 a, b) (Jaklitsch 2011).

Micromorphologically Strain Tri 219 matched the descriptions of *T. atroviride* (Jaklitch 2011). Conidiophores form long axes (Fig. 5 e, f) and smooth, green subglobose conidia were formed (Fig. 5 l). In addition, Tri 219 displayed straight or slightly curved ampuliform shaped phialides (Fig. 5 g, i). Phialides were sometimes solitary or in small whorls, and were widely spaced (Fig. 5 k). Smooth, terminal chlamydospores were observed on CMD (Fig. 5 g).

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***Trichoderma gamsii* Samuels & Druzhin., *Studies in Mycology* 56: 168. 2006.**

Trichoderma gamsii was first described by Samuels and Druzhinina (2006) from soil and displays a cosmopolitan distribution. *T. gamsii* is a member of the *T. viride* clade. The species has been shown to antagonize certain plant pathogens *in vitro* and *in vivo* and is known to form endophytic relationships with some plants (Jaklitsch *et al.* 2006, Anees *et al.* 2010).

During this study 11 strains were isolated from 6 sites (Fig. 1 and Table 2) that were identified as *T. gamsii* based on ef1α sequence data (Fig. 2 and Table 4). This group, represented by Strain 156, was characterized morphologically in order to confirm the identity of the species (Figs. 7 and 8).

On PDA, Strain Tri 156 displayed growth rates that matched *T. gamsii* (Samuels and Druzhinina 2006). Strain Tri 156 also formed a dense layer of white mycelium on CMD that did not sporulate profusely (Fig. 8 a). The growth rate on SNA was slightly slower than expected, colonies only reached 48 mm in radius when incubated at 25°C for 7 d relative to *T. gamsii* which usually forms 70 mm colonies under the same conditions. Strain Tri 156 sporulated abundantly on SNA and produced a coconut odour which is characteristic of *T. gamsii* (Fig. 8 a, d).

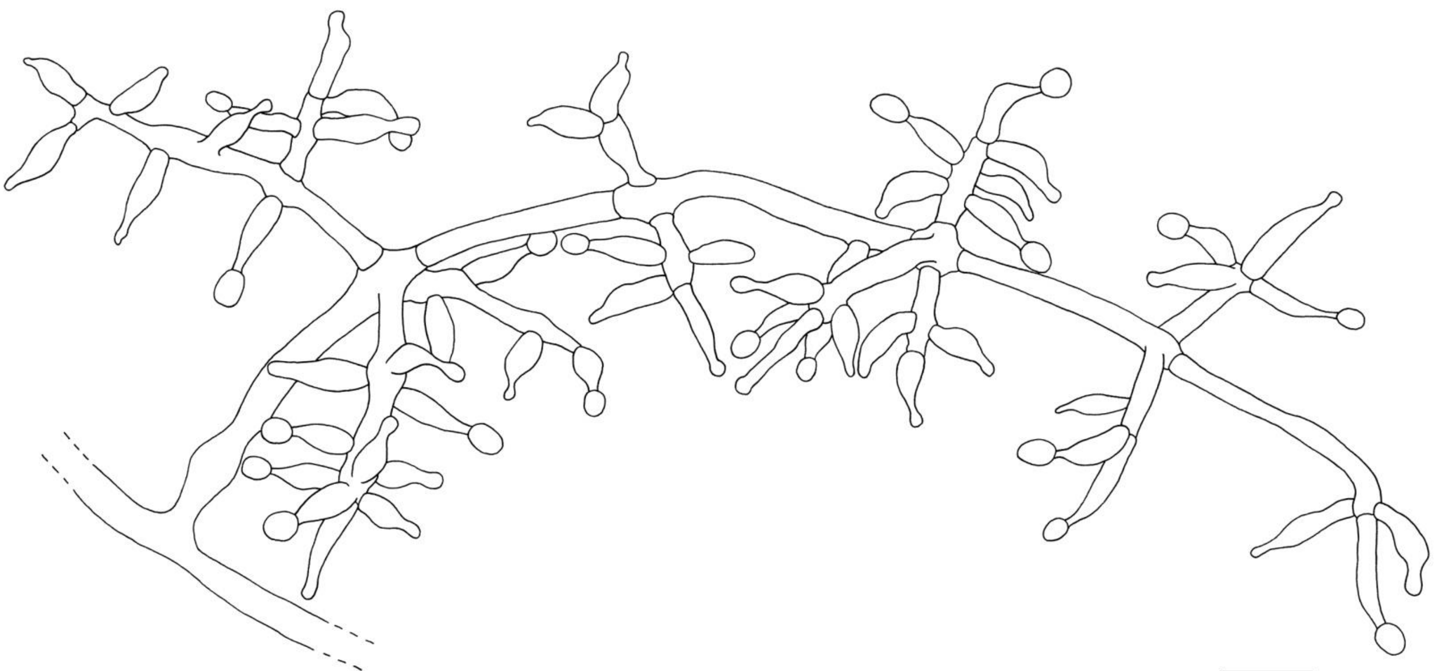

FIGURE 7. *Trichoderma gamsii.* Line drawings of Strain Tri 156. Scale bar = 10μm

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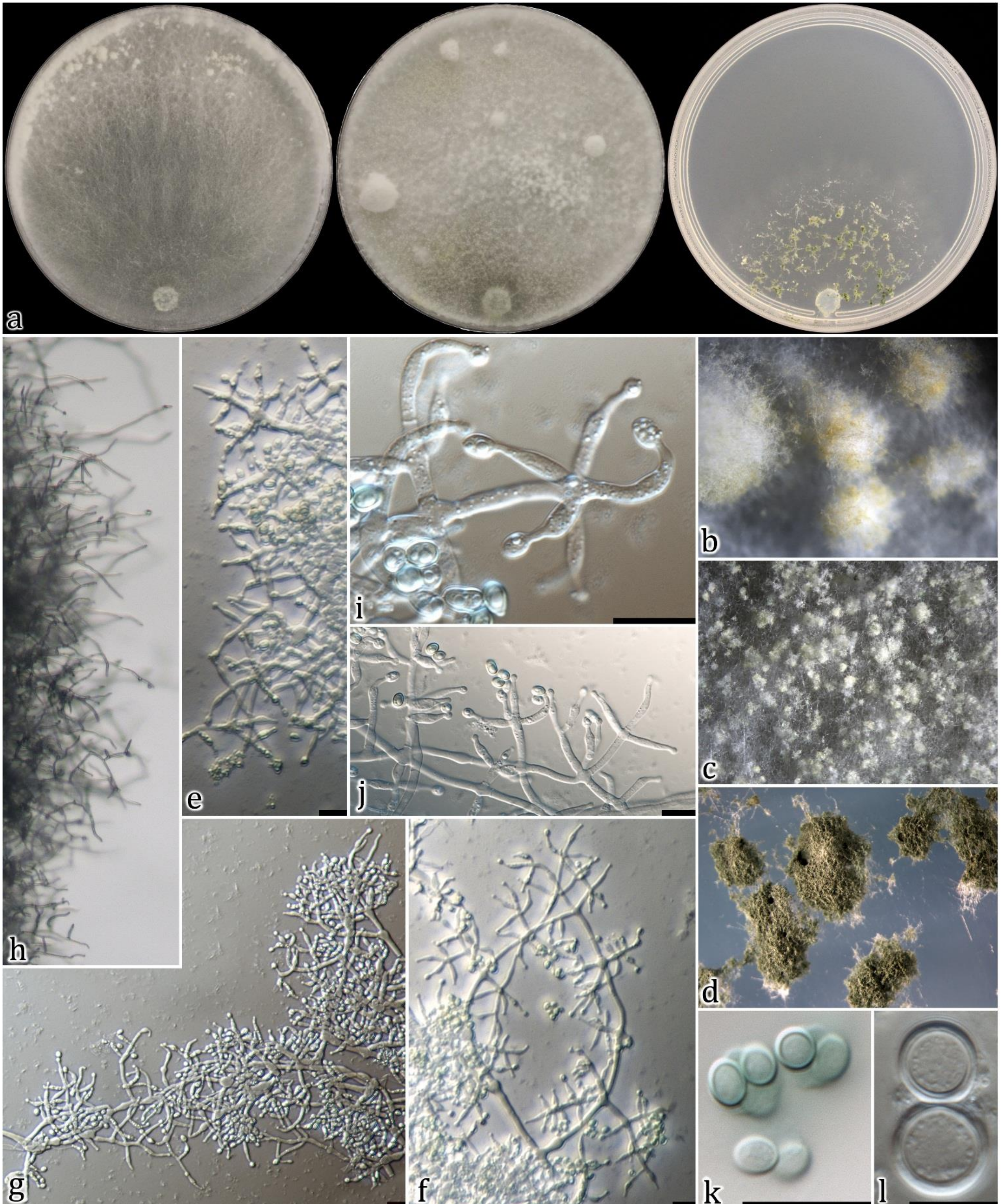

FIGURE 8. Morphological features characteristic of *Trichoderma gamsii* Strain Tri 156. a. Colonies of *T. gamsii* incubated on CMD for 14 days (left), PDA for 14 days (middle), SNA for 7 days (right). b, c, d. Stereo microscope images, from CMD (top) and PDA (middle) and SNA (bottom). e, f. Conidiophores formed on SNA 400x. g. Conidiophores formed on SNA 200x. h. Stereomicroscope image on SNA. i, j. Conidiophores formed on CMD 1000x. k. Conidia 1000x. l. Chlamydospores 1000x. All scale bars are 10μm in length.

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Micromorphologically, Strain Tri 156 was similar to *T. gamsii* in that conidiophore branchings occurred at swollen nodes along the stipe axis (Fig. 8 f, j). In addition, lageniform phialides were observed bearing smooth, ellipsoidal to subglobose conidia (Fig. 8 k). Intercalary phialides and terminal chlamydospores were formed by Strain Tri 156, which is also consistent with the morphology of *T. gamsii* (Fig. 8 l).

***Trichoderma koningii* Oudem., Archives Néerlandaises 7: 291. 1902**

Trichoderma koningii forms part of the *T. viride* clade. The species was described by Oudemans in 1902 and was re-described by Samuels *et al.* (2006), who determined that *T. koningii s. l.* consisted of 12 separate phylogenetic lineages that share similar morphologies. *T. koningii* is known to occur in the USA, Canada and Europe.

Five different *Trichoderma* strains were isolated from four different sites (Fig. 1 and table 2) during this study that were identified as *T. koningii* based on ef1α sequence data (Fig. 2 and table 4). A morphological analysis was performed to confirm this identification (Figs. 9 and 10).

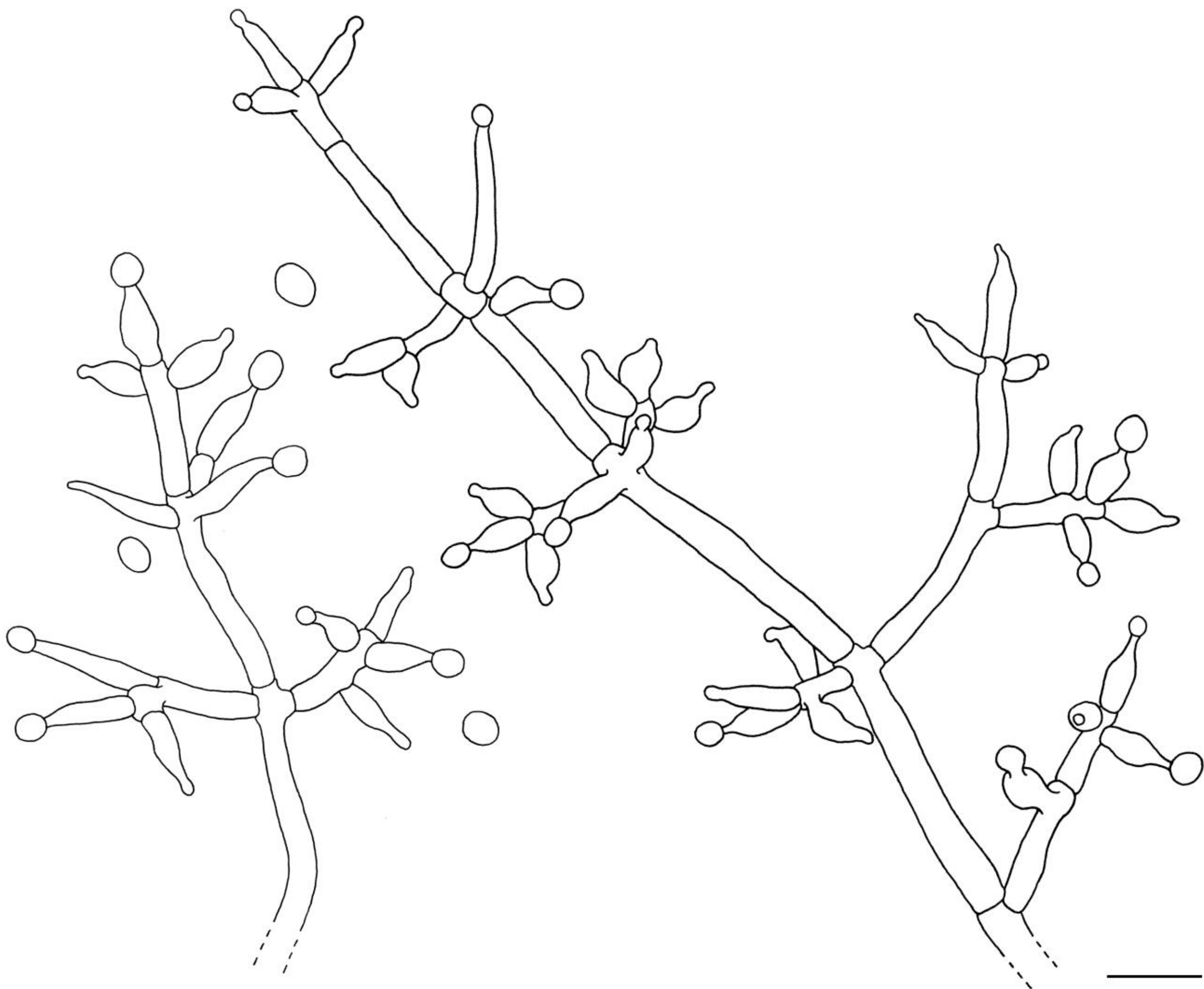

FIGURE 9. *Trichoderma koningii*. Line drawings from Strain Tri 14. Scale bar = 10µm

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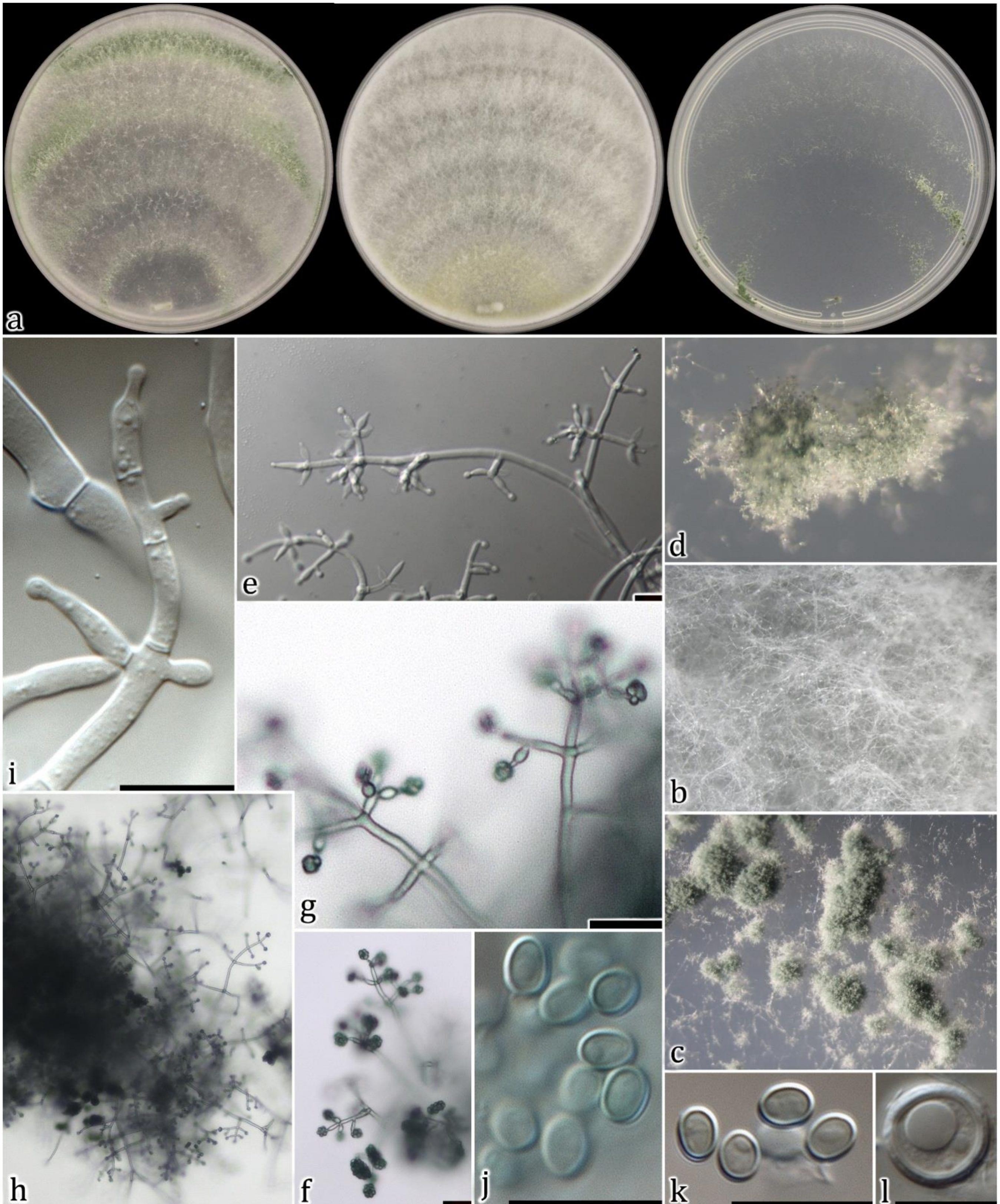

FIGURE 10. Morphological features characteristic of *Trichoderma koningii*, Strain Tri 14. a. Colonies of *T. koningii* incubated on CMD for 7 days (left), PDA for 7 days (middle) and SNA for 7 days (right). b, c, d. Stereo microscope images, from CMD (top), PDA (middle) and SNA (bottom). e. Conidiophore on CMA 400x magnification. f, g. Conidiophores on SNA 400x. h. Stereo microscope image from SNA. i. Conidiophores formed on CMD 1000x. j, k. Conidia 1000x. l. Chlamydospore 1000x. All scale bars are 10μm in length.

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Strain Tri 14 conformed to morphological descriptions provided by Samuels *et al.* (2006). Colonies grown on PDA produced abundant white mycelia and conidiation started in the centre of the colony and radiated outwards in faint concentric rings (Fig. 10 a). On CMD, colonies sporulated in a band along the colony periphery (Fig. 10 a).

Micromorphologically, Strain Tri 14 also matched the descriptions of *T. koningii* (Samuels *et al.* 2006). Conidiophores formed a distinguished main axis and branching occurred at nodes which diverged from the stipe at approximately right angles. Second degree branching were also observed bearing lageniform phialides (Fig. 10 e, i). Conidia were oblong and smooth-walled (Fig. 10 j, k). Chlamydospores formed on CMD (Fig. 10 l).

***Trichoderma koningiopsis* Samuels Suárez & Evans, Studies in Mycology 56: 117. 2006**

Trichoderma koningiopsis forms part of the *T. viride* clade and is known to occur in the Americas, Europe and North Africa. This study isolated 12 strains from seven different sites (Fig. 1 and table 2) that were identified as *T. koningiopsis* based on ef1α sequence data (Fig. 2 and table 4). This group was represented by Strain Tri 214 and a morphological analysis was performed to confirm the identification (Figs. 11 and 12).

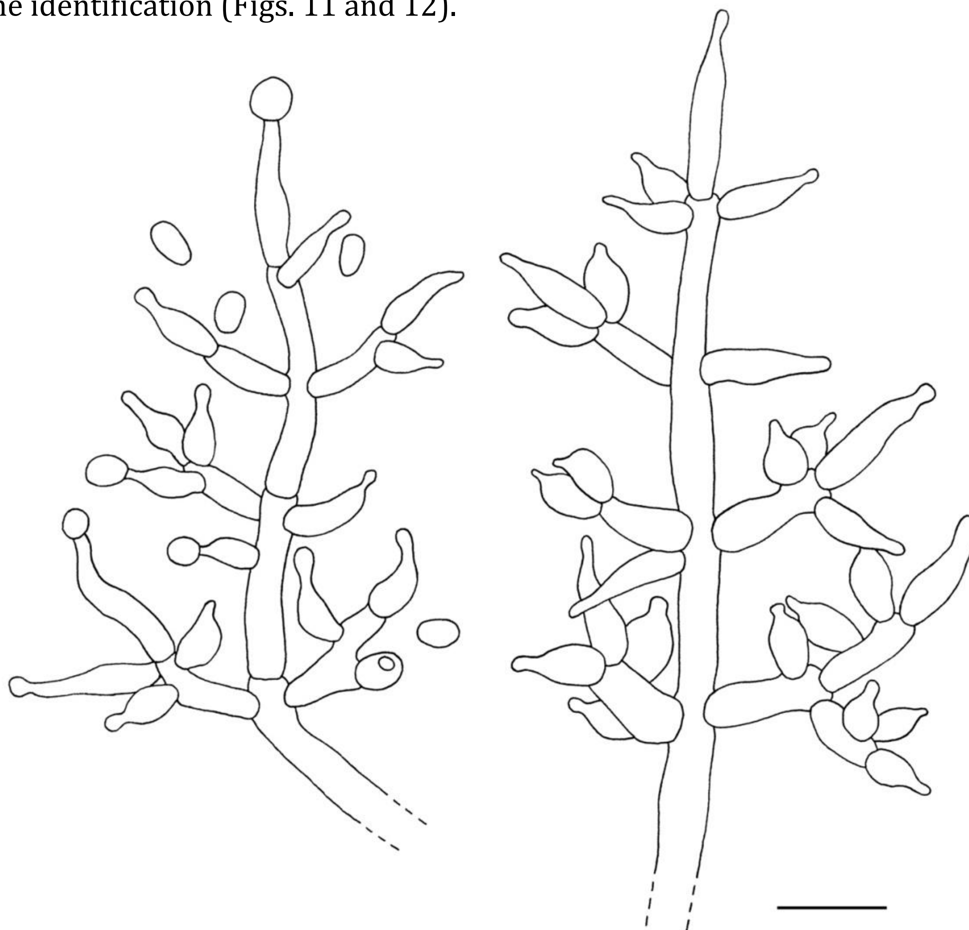

FIGURE 11. *Trichoderma koningiopsis*. Line drawings from strain Tri 214. Scale bar = 10μm

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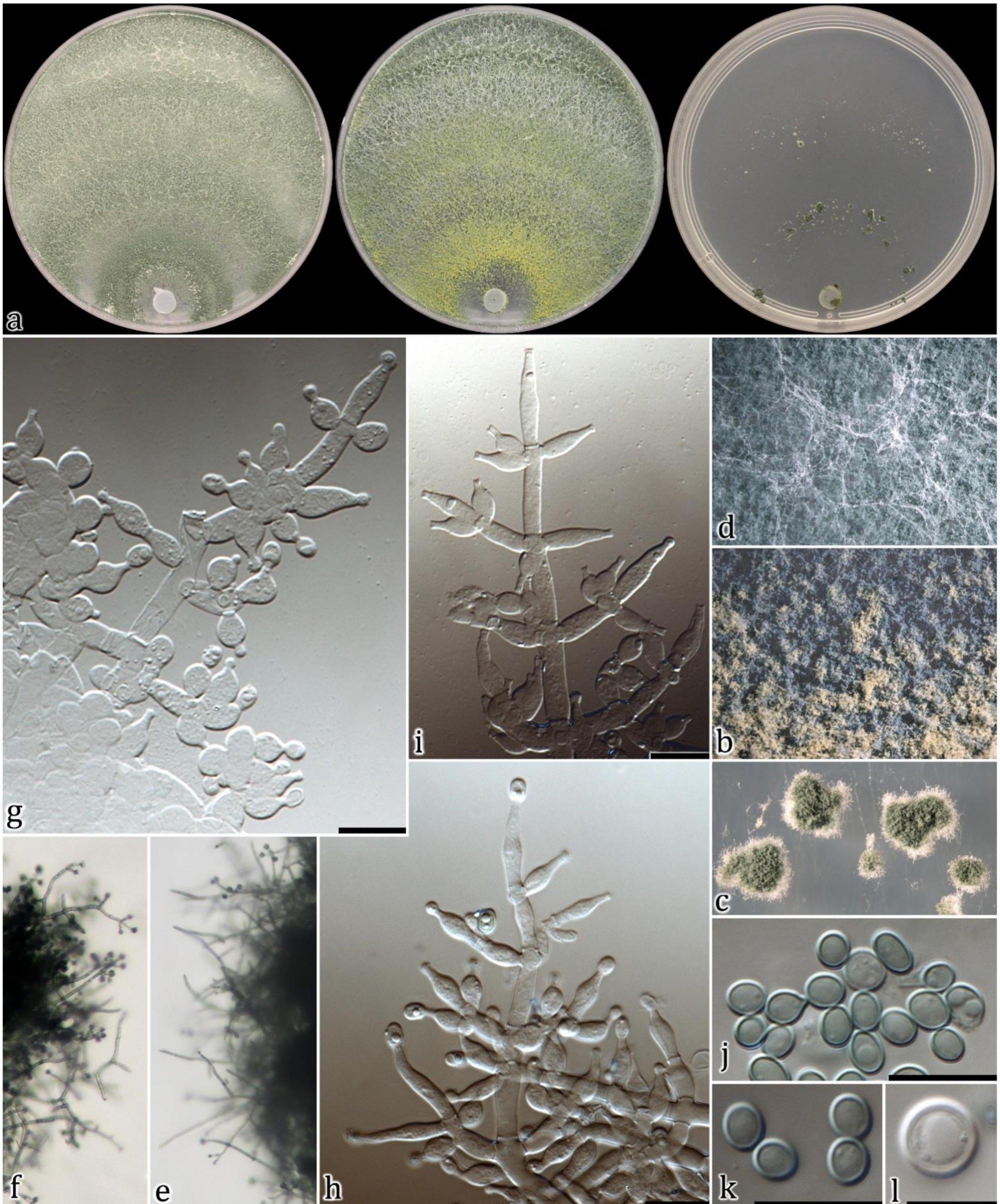

FIGURE 12. Morphological features characteristic of *Trichoderma koningiopsis*, Strain Tri 214. a. Colonies of *T. koningiopsis* incubated on CMD for 7 days (left), PDA for 7 days (middle) and SNA for 14 days (right). b, c, d. Stereo microscope images, from CMD (top), PDA (middle) and SNA (bottom). e, f. Stereo microscope images from SNA. g, h, i. Conidiophores formed on CMD 1000x magnification. j, k. Conidia 1000x. l. Chlamydospore 1000x. All scale bars are 10µm in length.

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Strain Tri 214 matched the original descriptions of *T. koningiopsis* (Samuels *et al.* 2006). Colonies on PDA sporulated heavily and formed a dense fertile lawn which made up three poorly defined concentric rings (Fig. 12 a). Colonies on CMD also formed a continuous lawn of conidia (Fig. 12 d). Strain Tri 214 did, however, deviate from the descriptions of *T. koningiopsis* by sporulating effusely as opposed to producing compact pustules (Fig. 12 a) (Samuels *et al.* 2006). Despite this, Strain Tri 214 matched the concept of *T. koningiopsis* in all regards.

Micromorphologically, Strain Tri 214 matched the descriptions of *T. asperelloides* by Samuels *et al.* (2006) in that conidiophores formed a distinguished central axis that branched slightly less than 90° relative to the central axis, with branches tended to orientate towards the conidiophore apex (Fig. 12 h, i). Several other traits of Strain Tri 214 was also consistent with those of *T. asperelloides*: Phialides varied in shape from short and swollen in the middle (Fig. 12 g) to lageniform (Fig. 12 h). In addition, second degree branches were observed to form in pairs (Fig. 12 h, i) and conidia were ellipsoidal and smooth (Fig. 12 j, k). Finally, globose chlamydospores formed on CMD (Fig. 12 l).

The *Trichoderma longibrachiatum* clade

Four different species belonging to the *T. longibrachiatum* clade were isolated from 30 different sites during this study (Fig. 13 and Table 2). Two of these species were identified as *T. saturnisporum* and *T. orientalis* based on a phylogenetic analysis using ef1α sequence data (Fig. 14, table 5). The remaining two species, *T. vagum* prov. nom. and *T. terrigenum* prov. nom. were discovered to be novel and are discussed in Chapter Three. Members of the *T. longibrachiatum* clade are not utilised commercially as biocontrol agents and a number of species from this clade, such as *T. longibrachiatum, T. citrinoviride* and *T. orientalis,* are opportunistic human pathogens (Guarro *et al.* 1999, De Miguel *et al.* 2005).

***Trichoderma saturnisporum* Hammill, Mycologia 62: 112. 1970**

Trichoderma saturnisporum belongs to the *T. longibrachiatum* clade based on ef1α, cal1 and chi18-5 sequence data (Samuels *et al.* 2012). Hammill (1970) originally described the species from Georgia but the ex-type has since been re-described in greater detail (Samuels *et al.* 1998, Gams and Bissett 1998). *T. saturnisporum* has been recorded from all over the world, including the Americas, Europe, Asia, Australia and South Africa (Samuels *et al.* 2012). It has

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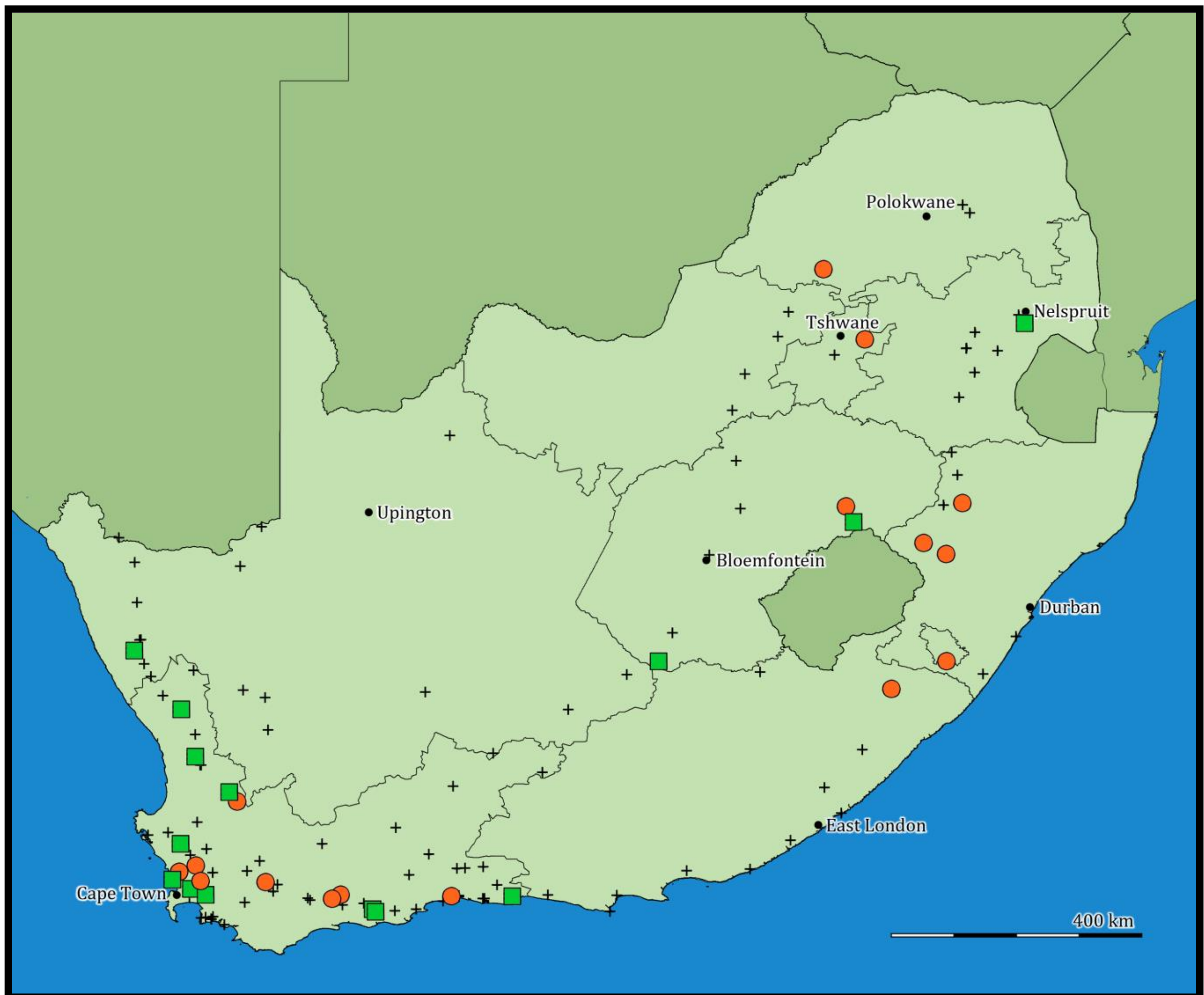

FIGURE 13. Distribution of the *Trichoderma longibrachiatum* clade in South Africa, *T. orientalis* and *T. saturnisporum* were isolated from soils collected across the country. Black crosses indicate sample sites. ***T. orientalis,*** **(orange circle)** isolated from 16 different sites. ***T. saturnisporum,*** **(green squares)** isolated from 13 different sites. Note that some sites are located close to one another and cannot be visualised at this scale. Scale bar = 400km.

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TABLE 5 *Trichoderma* strains from the *T. longibrachiatum* clade used for phylogenetic comparisons are listed along with their respective GenBank accession numbers for ef1α sequence data. Collection numbers for the strains that were used are listed along with their respective GenBank accession numbers. Ex-type strains are indicated in bold and with a *'T'*.

GenBank accession	Taxon name	Strain number	References
AY956321	*H. andinensis*	GJS 90-140	Samuels 2006
EU280042	*H. andinensis*	DAOM 220821	Hoyos-Carvajal *et al.* 2009
JN133570	*H. andinensis*	G.J.S. 09-62	Chaverri *et al.* 2013
AY857282	*H. orientalis*	TUB F-837	Druzhinina *et al.* 2005
EU401593	*H. orientalis*	G.J.S. 91-157	Druzhinina *et al.* 2008
AY937421	*H. orientalis*	CBS 243.63	Samuels 2006
EU280038	*H. orientalis*	DAOM 220863	Hoyos-Carvajal *et al.* 2009
EU401581	***H. orientalis*** ***T***	G.J.S. 88-81	Druzhinina *et al.* 2008
FJ860666	*H. parestonica*	C.P.K. 2427	Jaklitch 2009
FJ860667	*H. parestonica*	CBS 120636	Jaklitch 2009
FJ860705	*H. subalpina*	CBS 119128	Jaklitch 2009
FJ860706	*H. subalpina*	C.P.K.3126	Jaklitch 2009
EU401616	*T. aethiopicum*	PPRC H5	Druzhinina *et al.* 2008
EU401614	*T. aethiopicum*	PPRC J11	Druzhinina *et al.* 2008
JN175591	*T. citrinoviride*	G.J.S. 90-111	Druzhinina *et al.* 2012
EU338334	*T. citrinoviride*	DAOM 139758	Degenkolb *et al.* 2008
AY937419	***T. effusum*** ***T***	DAOM 230007	Samuels 2006
FJ763149	*T. flagellatum*	PPRC ET7	Samuels *et al.* 2012
FJ763184	***T. flagellatum*** ***T***	PPRC ET58	Samuels *et al.* 2012
AY937423	*T. ghanense*	G.J.S. 95-137	Samuels 2006
EU280043	*T. ghanense*	DAOM 220800	Hoyos-Carvajal *et al.* 2009
JN175583	***T. gillesii*** ***T***	G.J.S. 00-72	Druzhinina *et al.* 2012
JN175598	***T. gracile*** ***T***	G.J.S. 10-263	Druzhinina *et al.* 2012
AY937425	*T. konilangbra*	G.J.S. 96-147	Samuels 2006
JN175569	*T. longibrachiatum*	G.J.S. 07-21	Druzhinina *et al.* 2012
AY937412	***T. longibrachiatum*** ***T***	ATCC18648	Samuels 2006
GQ354351	*T. parareesei*	TUB F-430	Druzhinina *et al.* 2010
GQ354353	***T. parareesei*** ***T***	TUB F-1066	Druzhinina *et al.* 2010
GQ354373	*T. parareesei*	G.J.S. 07-26	Druzhinina *et al.* 2010
JN175572	*T. pinnatum*	G.J.S. 02-120	Druzhinina *et al.* 2012
JN175571	***T. pinnatum*** ***T***	G.J.S. 04-100	Druzhinina *et al.* 2012
AY937429	*T. pseudokoningii*	G.J.S. 81-300	Samuels 2006
JN175588	***T. pseudokoningii*** ***T***	G.J.S. NS-19	Druzhinina *et al.* 2012
EU280037	*T. pseudokoningii*	DAOM 167678	Hoyos-Carvajal *et al.* 2009
GQ354347	*T. reesei*	G.J.S. 97-177	Druzhinina *et al.* 2010
Z23012	*T. reesei*	QM9414	Nakari *et al.* 1993

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TABLE 5 continued

GenBank accession	Taxon name	Strain number	References
JN175601	*T. reesei*	G.J.S. 09-74	Druzhinina *et al.* 2012
AY937441	*T. rossicum*	DAOM 230011	Samuels 2006
EU280062	*T. rossicum*	DAOM 233977	Hoyos-Carvajal
JN182279	*T. saturnisporum*	C.P.K. 1268	Druzhinina *et al.* 2012
JN258682	*T. saturnisporum*	C.P.K. 3406	Druzhinina *et al.* 2012
AY937414	*T. saturnisporum*	CBS 886.72	Samuels 2006
EU595046	*T. saturnisporum*	SF21	Sadfi-Zouaou *et al.* 2009
AY865642	*T. saturnisporum*	IMI 146852	Druzhinina *et al.* 2005
JN388897	*T. saturnisporum*	ATCC 28023	Druzhinina *et al.* 2012
JN182273	*T. sinensis*	C.P.K. 530	Druzhinina *et al.* 2012
AY750889	*T. sinensis*	DAOM 230004	Samuels 2006
JN175597	***T. solani*** [T]	G.J.S. 08-81	Druzhinina *et al.* 2012
AY937441	*T. virens*	DAOM 230011	Samuels 2006
FJ463366	*T. virens*	G.J.S 95-78	Chaverri *et al.* 2008

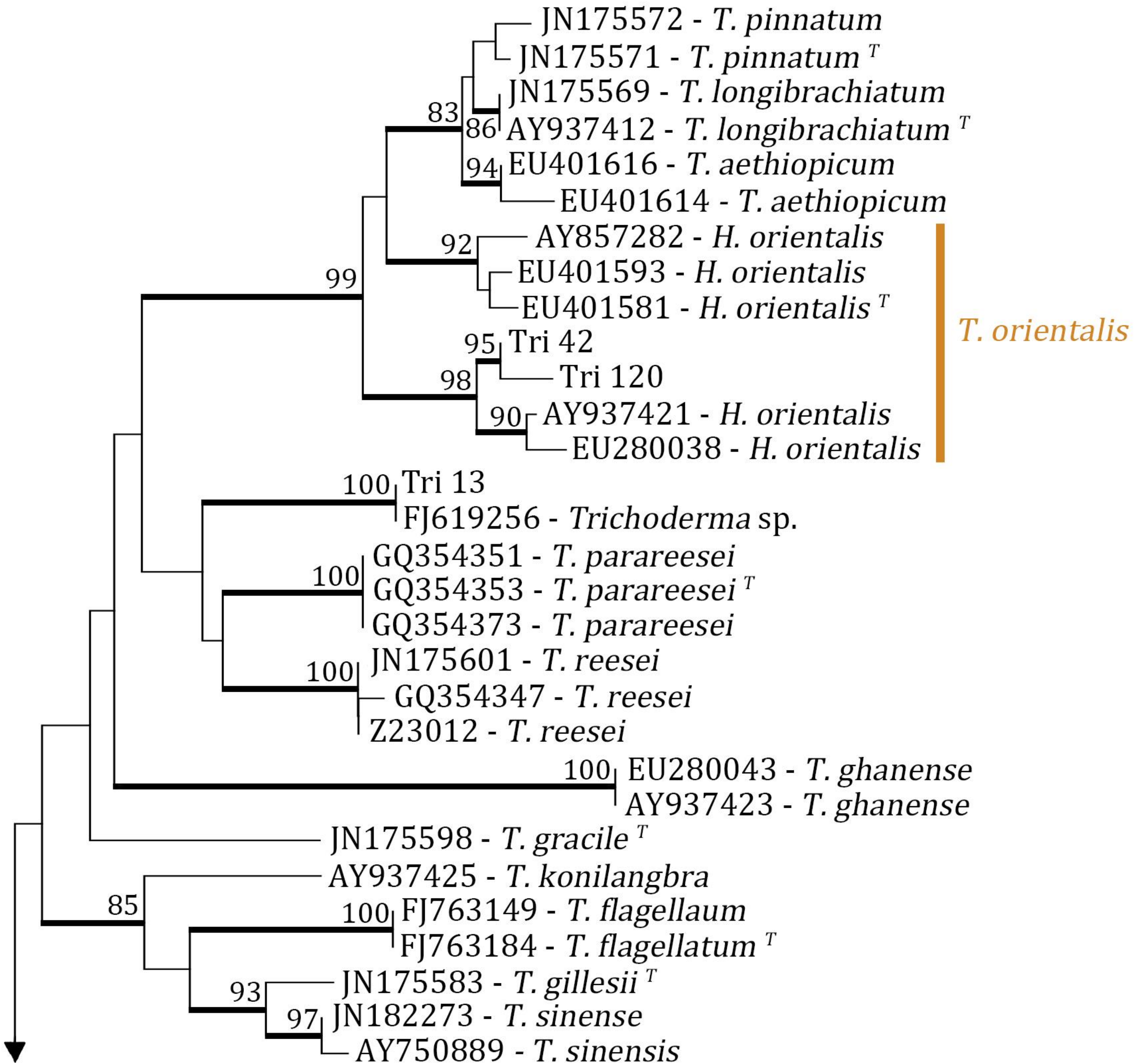

FIGURE 14. Neighbour joining tree based on ef1α sequence data indicating the phylogenetic relationships between accepted members of the *Trichoderma longibrachiatum* clade and South African *Trichoderma* strains belonging to the same clade. Nodes which are supported by bootstrapping values that exceed 80% are indicated by thickened branches. Ex-type culture sequences are indicated with a '*T*'.

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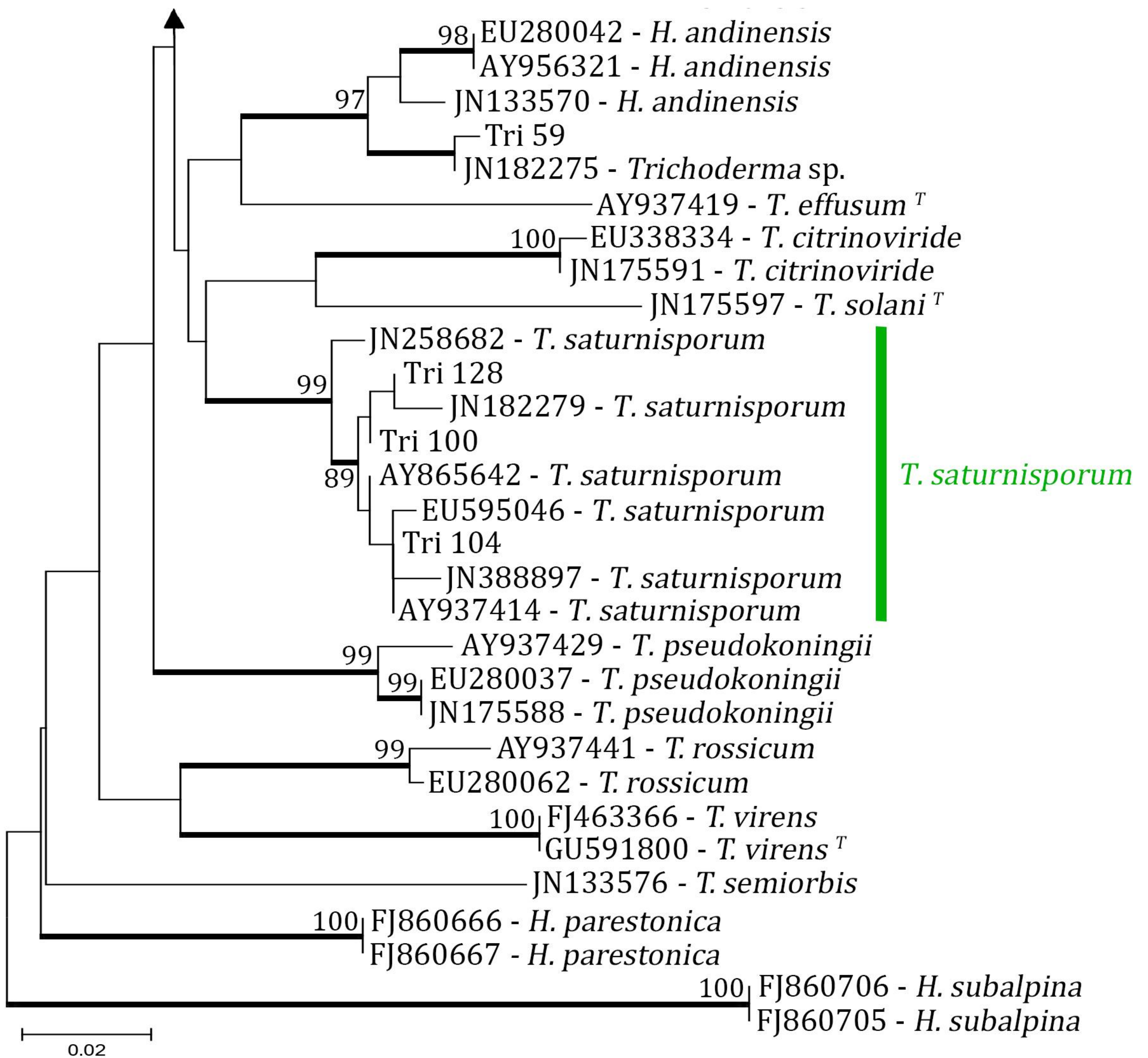

FIGURE 14 Continued

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previously been reported that *T. saturnisporum* is rarely isolated, despite its widespread distribution (Samuels *et al.* 2012). This was not found to be the case in this study, as *T. saturnisporum* was the second most common *Trichoderma* species isolated (Table 3).

Twenty seven strains of *T. saturnisporum* were isolated from 13 sites during this study (Fig. 13 and table 2). These strains were identified as *T. saturnisporum* based on ef1α sequence data (Fig. 14 and table 5). Strain Tri 100 served as the group representative and was characterized morphologically in order to confirm phylogenetic identification (Figs. 15 and 16). Morphologically, strain Tri 100 resembled *T. saturnisporum* in that colonies on SNA are slow to produce conidia, small pustules only becoming visible in small aggregates (<1 mm) following more than a week of incubation (Samuels *et al.* 1998). Colonies on CMD also produced small, dispersed conidiophore aggregates (Fig. 16 a). On PDA, colonies did not form abundant white aerial hyphae and sporulated more heavily than would be expected from a strain of *T. saturnisporum* (Samuels *et al.* 1998) (Fig. 16 a). However, micromorphologically Strain Tri 100 matched the descriptions of the ex-type (Samuels *et al.* 1998), with conidiophores branching asymmetrically up to two times and forming hooked phialides in small whorls (2-3 phialides) (Fig. 16 g, i). Intercalary phialides were not observed to form on CMD.

FIGURE 15. *Trichoderma saturnisporum*. Line drawings from Strain Tri 100. Scale bar =10μm

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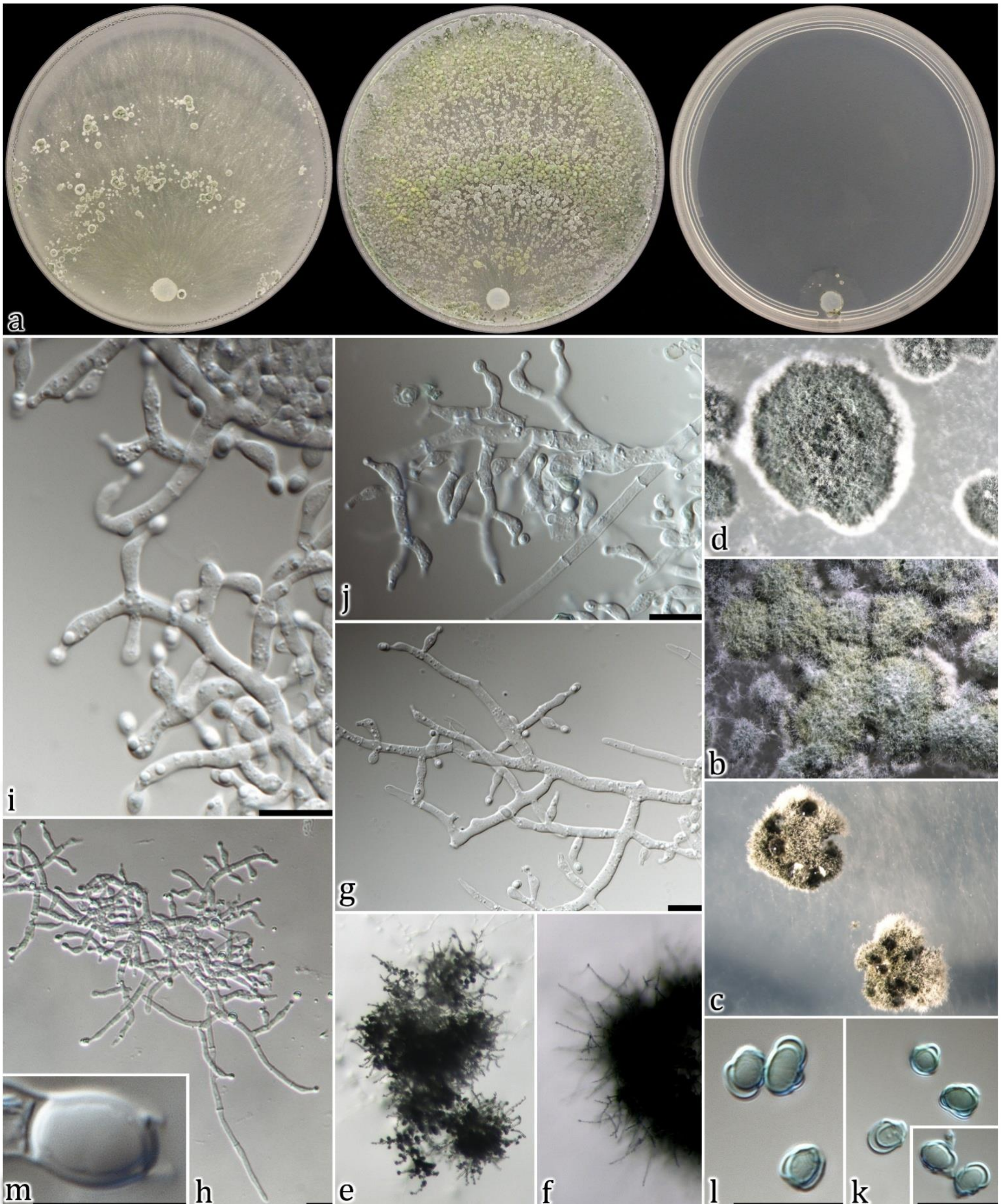

FIGURE 16. Morphological features characteristic of *Trichoderma saturnisporum*, Strain Tri 100. a. Colonies of *T. saturnisporum* incubated on CMD for 7 days (left), PDA for 7 days (middle) and SNA for 14 days (right). b, c, d. Stereo microscope images, from CMD (top), PDA (middle) and SNA (bottom). e, f. Stereo microscope images from SNA. g, h. Conidiophores on CMA 400x magnification. i, j. Conidiophores formed on CMD 1000x. k, l. Conidia 1000x. m. Chlamydospores 1000x. All scale bars are 10µm in length.

Strain Tri 100 formed ellipsoidal conidia that developed wing-like ornamentations (Fig. 16 k, l). Samuels *et al.* (1998) noted that this trait is uncommon among *Trichoderma* spp. and that other *Trichoderma* species do not form wing-like ornamentations as conspicuously as *T. saturnisporum*. Chlamydospores were also observed to form abundantly on CMD (Fig. 16 m).

***Trichoderma orientalis* Samuels & Petrini, Studies in Mycology 41: 30. 1998.**

Trichoderma orientalis forms part of the *T. longibrachiatum* clade (Samuels *et al.* 2012). The species is known to display a cosmopolitan distribution and has previously been reported from South Africa by Druzhinina *et al.* (2008). The ex-type of *T. orientalis* was recently re-described in greater detail by Samuels *et al.* (2012) when the *T. longibrachiatum* clade was reviewed.

This study isolated 35 strains of *T. orientalis* from 16 sites (Fig. 13 and Table 2), these were identified based on ef1α sequence data (Fig. 14 and Table 5) and a morphological analysis of the group representative, Strain Tri 120, confirmed this identification (Figs. 17 and 18).

Strain Tri 120 displayed morphological characteristics that resembled those of *T. orientalis* (Samuels *et al.* 2012). Colonies formed concentric rings on PDA and SNA along with yellow pigments (Fig. 18 a) and small granular pustules (Fig. 18 b, c, d). The only difference between Strain Tri 120 and the description of *T. orientalis* was that the colonies on SNA displayed a slightly slower growth rates than was expected (Samuels *et al.* 2012).

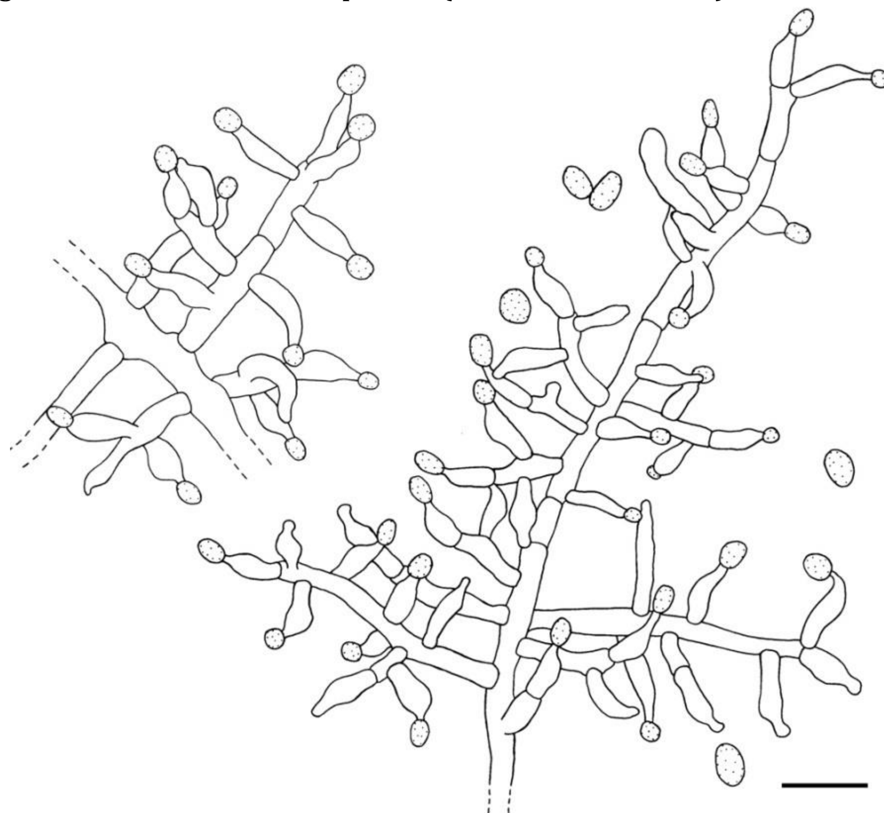

FIGURE 17. *Trichoderma orientalis*. Line drawings from Strain Tri 120. Scale bar = 10μm

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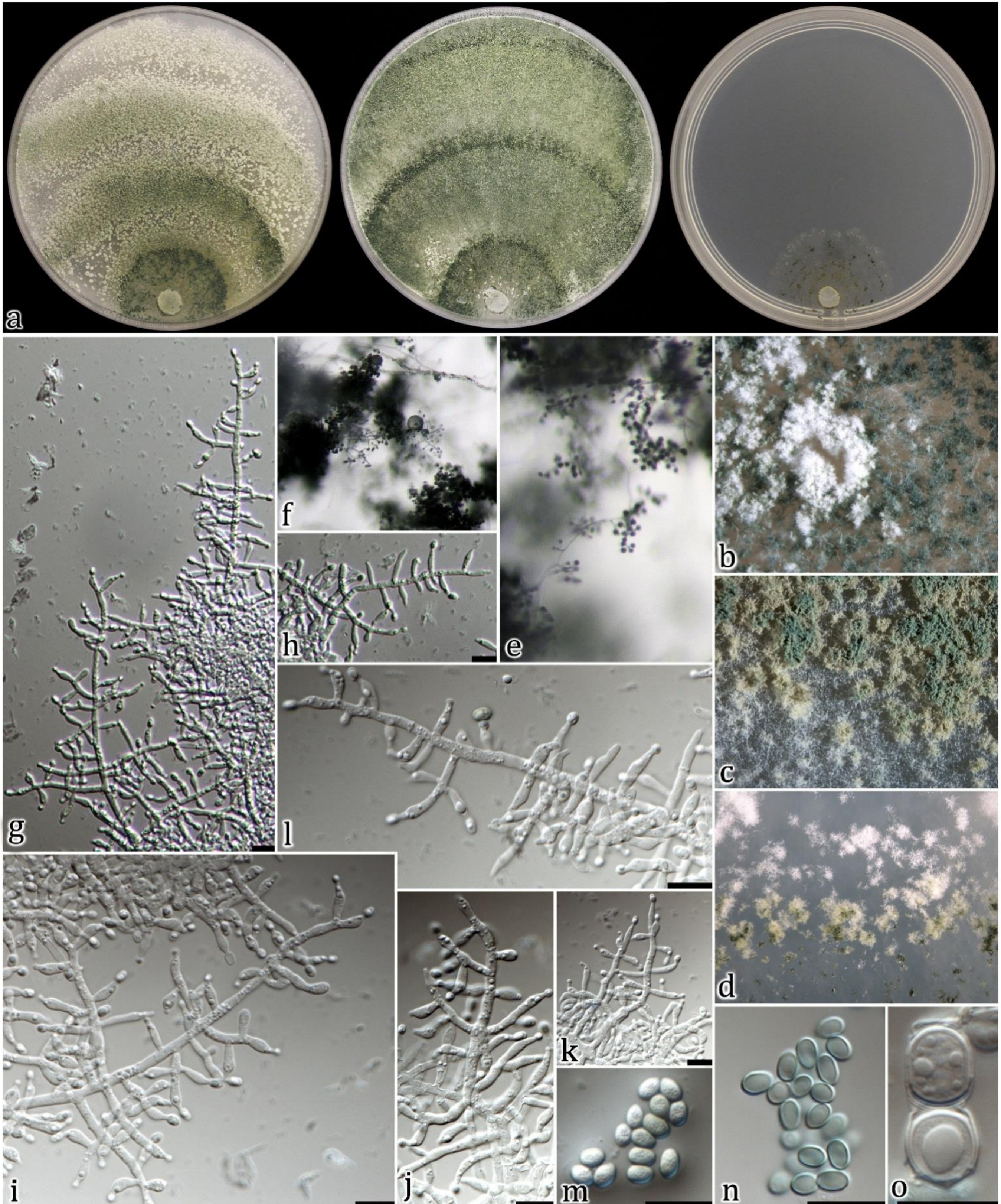

FIGURE 18. Morphological features characteristic of *Trichoderma orientalis*, Strain Tri 120. a. Colonies of *T. orientalis* incubated on CMD for 7 days (left), PDA for 7 days (middle), SNA for 21 days (right). b, c, d. Stereo microscope images, from CMD (top), PDA (middle) and SNA (bottom). e, f. Stereo microscope images from PDA. g, h. Conidiophores from CMA 400x magnification. i, j, k, l. Conidiophores from CMD 1000x. m, n. Conidia 1000x. o. Chlamydospores 1000x. All scale bars are 10μm in length.

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Micromorphologically Strain Tri 120 also resembled *T. orientalis* (Samuels *et al.* 2012). Conidiophores displayed long central axes that formed 5-8 layers of phialides from the apex before the first branching (Fig. 18 g, h, i, l). The phialides of Strain Tri 120 were also lageniform and often solitary (Fig. 18 g, h, i, l). The only micromorphological traits of Strain Tri 120 that deviated from the description of *T. orientalis* was that the conidia displayed finely roughened ornamentations, as opposed to the smooth conidia generally formed by *T. orientalis* , although this difference was not conspicuous (Fig. 18 m, n).

The *Trichoderma pachybasium* 'A' clade

Four different species belonging to the *T. pachybassium* 'A' clade were isolated from 18 different sites during this study (Fig. 19 and Table 2). Three of these were identified as *T. asperellum, T. asperelloides* and *T. hamatum* based on a phylogenetic analysis using ef1α sequence data (Fig. 20, Table 6). The remaining species, *T. restrictum* prov. nom., was discovered to be novel and is discussed in Chapter 4. The *T. pachybassium* 'A' clade includes a number of important species such as *T. asperellum* and *T. asperelloides* that are used as biocontrol organisms (Segarra *et al.* 2010).

***Trichoderma hamatum* Bonord. ex Bainier, Bulletin de la Société Mycologique de France 22: 131. 1906**

Trichoderma hamatum was first described by Bonorden in 1851. Bainer re-described the species in 1906 and provided detailed illustrations. However, Bainer was not able to examine the material originally collected by Bonorden (Bissett 1991a). Bissett neotypified the species in 1991 and Chaverri *et al.* (2003) provided a detailed description of the neotype. *T. hamatum* belongs to the *T. pachybasium* 'A' clade (also known as the *T. hamatum* clade) of *Trichoderma* and is known to display a cosmopolitan distribution. During this study, 11 strains of *T. hamatum* were isolated from eight different sites (Fig. 19 and Table 2). These strains were represented by Strain Tri 78 and were identified based on ef1α sequence data (Figs. 20 and 6). A morphological analysis confirmed this result (Figs. 21 and 22).

Strain Tri 78 matches the description of *T. hamatum* in that colonies grow rapidly on CMD and PDA, and display submerged mycelia (Chaverri *et al.* 2003). Small droplets of exudates were also formed on PDA (Fig. 22 c). However, the sporulation of Strain Tri 78 differed from the

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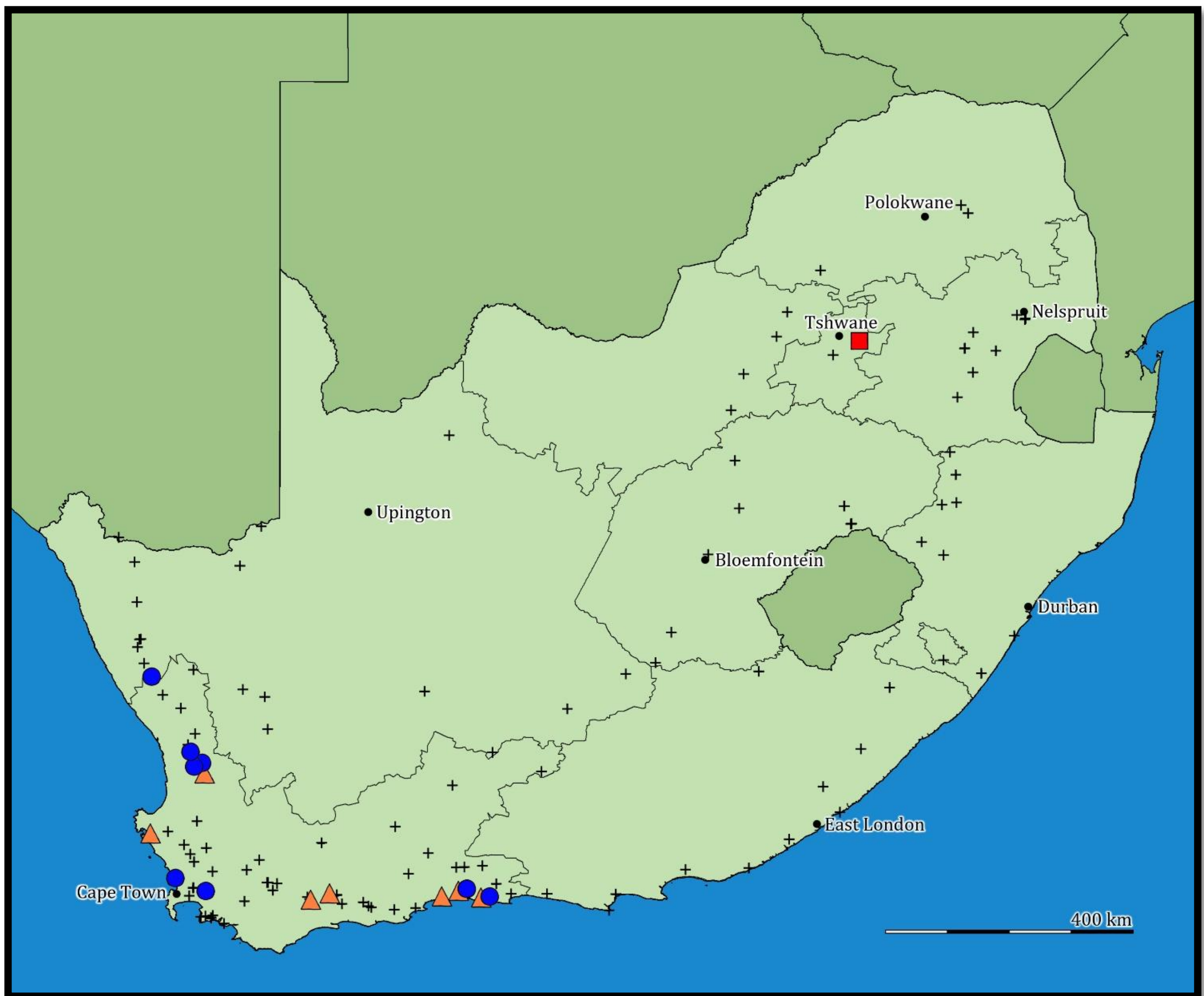

FIGURE 19. Distribution of the *Trichoderma pachybasium* "A" *clade* in South Africa*. T. asperellum* and *T. hamatum* were isolated from soils collected in the cape whereas *T. asperelloides* was only isolated in the northern parts of South Africa. Black crosses indicate sample sites. ***T. asperelloides,* (red square)** isolated from 1 site. ***T. hamatum,* (orange triangles)** isolated from 8 different sites. ***T. asperellum,* (blue circles)** isolated from 8 different sites. Note that some sites are located close to one another and cannot be visualised at this scale. Scale bar = 400km.

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TABLE 6 *Trichoderma* strains from the *T. pachybasium* A clade used for phylogenetic comparisons are listed along with their respective GenBank accession numbers for ef1α sequence data. Ex-type strains are indicated in bold and with a *'T'*.

GenBank accession	Taxon name	Strain number	Reference
AY665710	*H. flavoconidia*	CPK 454	Druzhinina *et al.* 2005
DQ020001	***H. flavoconidia*** *T*	G.J.S. 99-49	Samuels 2006
AF348115	***H. neorufa*** *T*	G.J.S. 96-132	Samuels *et al.* 2002
AF487670	***H. neorufa*** *T*	G.J.S. 96-135	Dodd *et al.* 2002
AY937438	***H. pezizoides*** *T*	G.J.S. 01-257	Samuels 2006
JN133571	*T. asperelloides*	G.J.S. 04-187	Chaverri and Samuels 2013
DQ109550	*T. asperelloides*	G.J.S. 99-6	Samuels *et al.* 2006
GU198236	*T. asperelloides*	G.J.S. 99-6	Samuels *et al.* 2010
GU248412	***T. asperelloides*** *T*	G.J.S. 04-116	Samuels *et al.* 2010
EU856323	*T. asperellum*	EU 856323	Samuels and Ismaiel 2009
EF186000	*T. asperellum*	G.J.S. 02-63	Begoude *et al.* 2007
FJ463285	*T. asperellum*	G.J.S. 91-162	Samuels 2006
EU248629	*T. asperellum*	G.J.S. 05-226	Hanada *et al.* 2008
GU198290	*T. asperellum*	G.J.S. 04-15	Samuels *et al.* 2010
AY376058	***T. asperellum*** *T*	G.J.S. 01-294	Holmes *et al.* 2004
JX684011	***T. eijii*** *T*	TUF C100002	Kim *et al.* 2013
EU856320	*T. evansii*	DIS 380A	Samuels and Ismaiel 2009
EU883566	***T. evansii*** *T*	DIS 341HI	Samuels and Ismaiel 2009
EU883565	*T. hamatum*	G.J.S. 04-203	Samuels and Ismaiel 2009
DQ109544	*T. hamatum*	G.J.S. 98-170	Samuels *et al.* 2006
EU856315	*T. hamatum*	G.J.S. 05-18	Samuels and Ismaiel 2009
EU856317	*T. hamatum*	G.J.S. 05-262	Samuels and Ismaiel 2009
EU279966	*T. hamatum*	DAOM 237553	Hoyos-Carvajal *et al.* 2009
AY665702	*T. hamatum*	CPK 1214	Druzhinina *et al.* 2005
EU856318	*T. hamatum*	G.J.S. 04-325	Samuels and Ismaiel 2009
AF456911	***T. hamatum*** *T*	DAOM 167057	Dodd *et al.* 2003
EU856324	*T. lieckfeldtiae*	DIS 376F	Samuels and Ismaiel 2009
EU856328	*T. lieckfeldtiae*	G.J.S. 05-01	Samuels and Ismaiel 2009
DQ109541	*T. paucisporum*	G.J.S. 03-69	Samuels *et al.* 2006
DQ109540	***T. paucisporum*** *T*	G.J.S. 01-13	Samuels *et al.* 2006
AY665704	*T. pubescens*	CPK 489	Druzhinina *et al.* 2005
AY750887	***T. pubescens*** *T*	DAOM 166162	Samuels *et al.* 2006 b
EU856322	*T. theobromicola*	DIS 376F	Samuels and Ismaiel 2009
EU856321	***T. theobromicola*** *T*	DIS 85F	Samuels and Ismaiel 2009
GU198243	***T. yunnanense*** *T*	CBS 121219	Samuels *et al.* 2010

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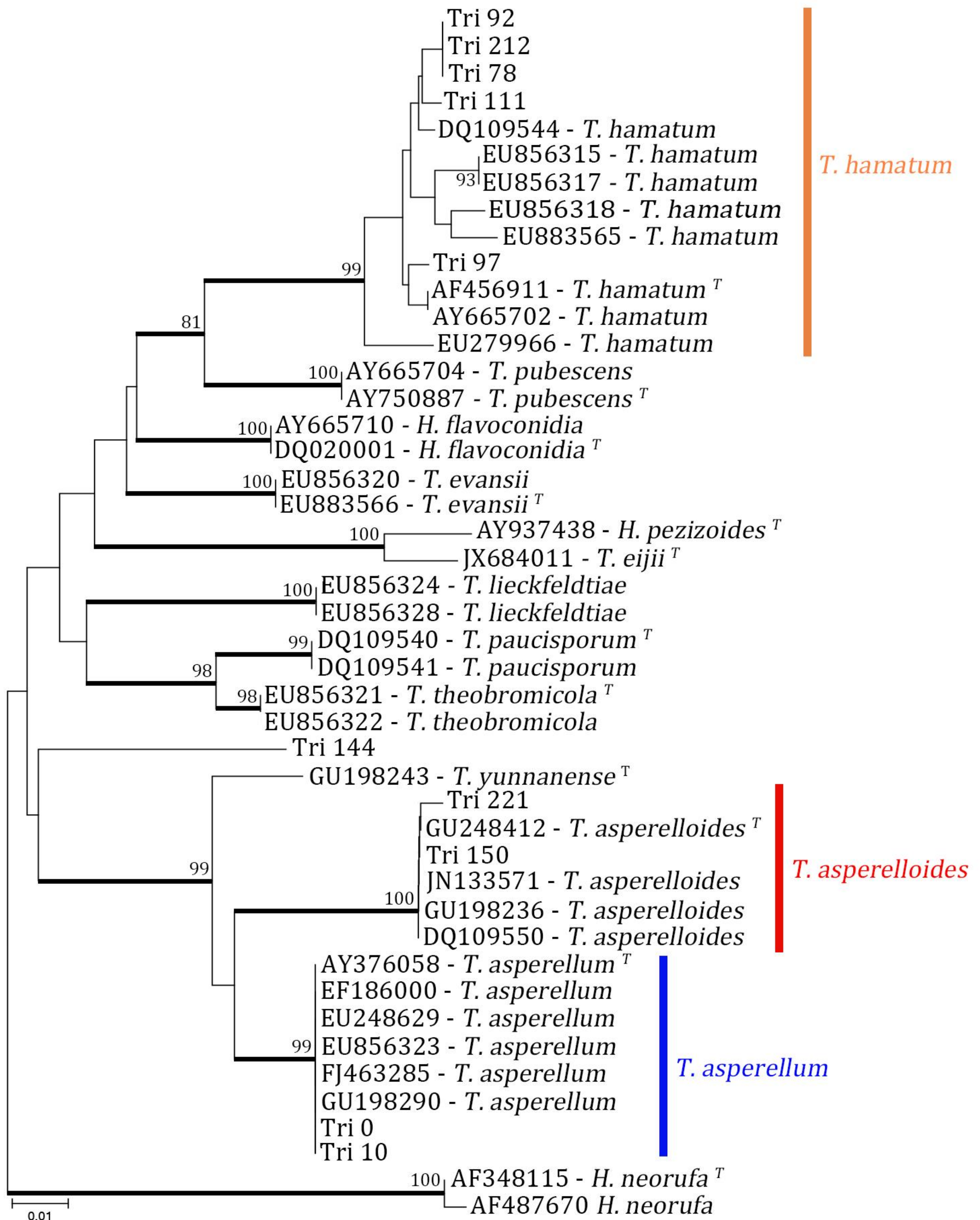

FIGURE 20. Neighbour joining tree based on ef1α sequence data indicating the phylogenetic relationships between accepted members of the *T. hamatum* clade and South African *Trichoderma* strains belonging to the same clade. Nodes which are supported by bootstrapping values that exceed 80% are indicated by thickened branches. Ex-type culture sequences are indicated with a '*T*'.

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descriptions of *T. hamatum* in that pustules fused to form continuous fertile layers as opposed to forming pustules (Fig. 22 a)(Chaverri *et al.* 2003).

Micromorphologically Strain Tri 78 matched *T. hamatum* in that conidiophores appeared straight and rigid. Basal hyphae were also conspicuously broad and display thickened walls (Figs. 22 f, g) (Chaverri *et al.* 2003). The mature conidia were held in droplets of green liquid (Fig. 20 e) and were oblong and smooth-walled, forming slightly pointed apexes (Fig. 22 j). Subglobose chlamydospores were also observed on CMD (Fig. 22 k). Phialides are ampuliform shaped (Fig. 22 i). However, some appeared to be elongated and more closely resembled lageniform phialides. The type of *T. hamatum* has not reported to display lageniform shaped phialides. Despite this, Strain Tri 78 still seems to conform to the concept of *T. hamatum* (Chaverri *et al.* 2003).

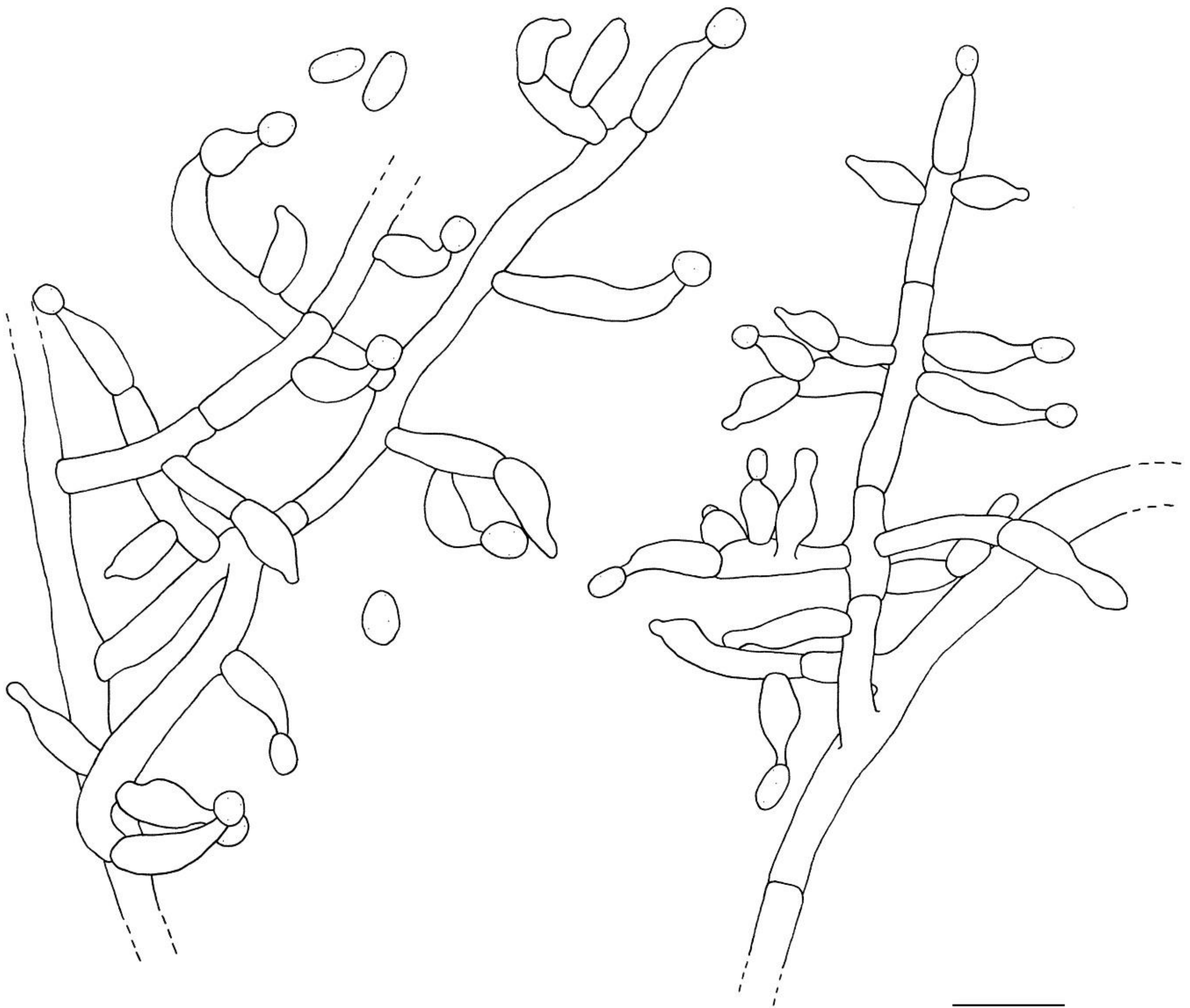

FIGURE 21. *Trichoderma hamatum.* Line drawings from strain Tri 78. Scale bar = 10μm

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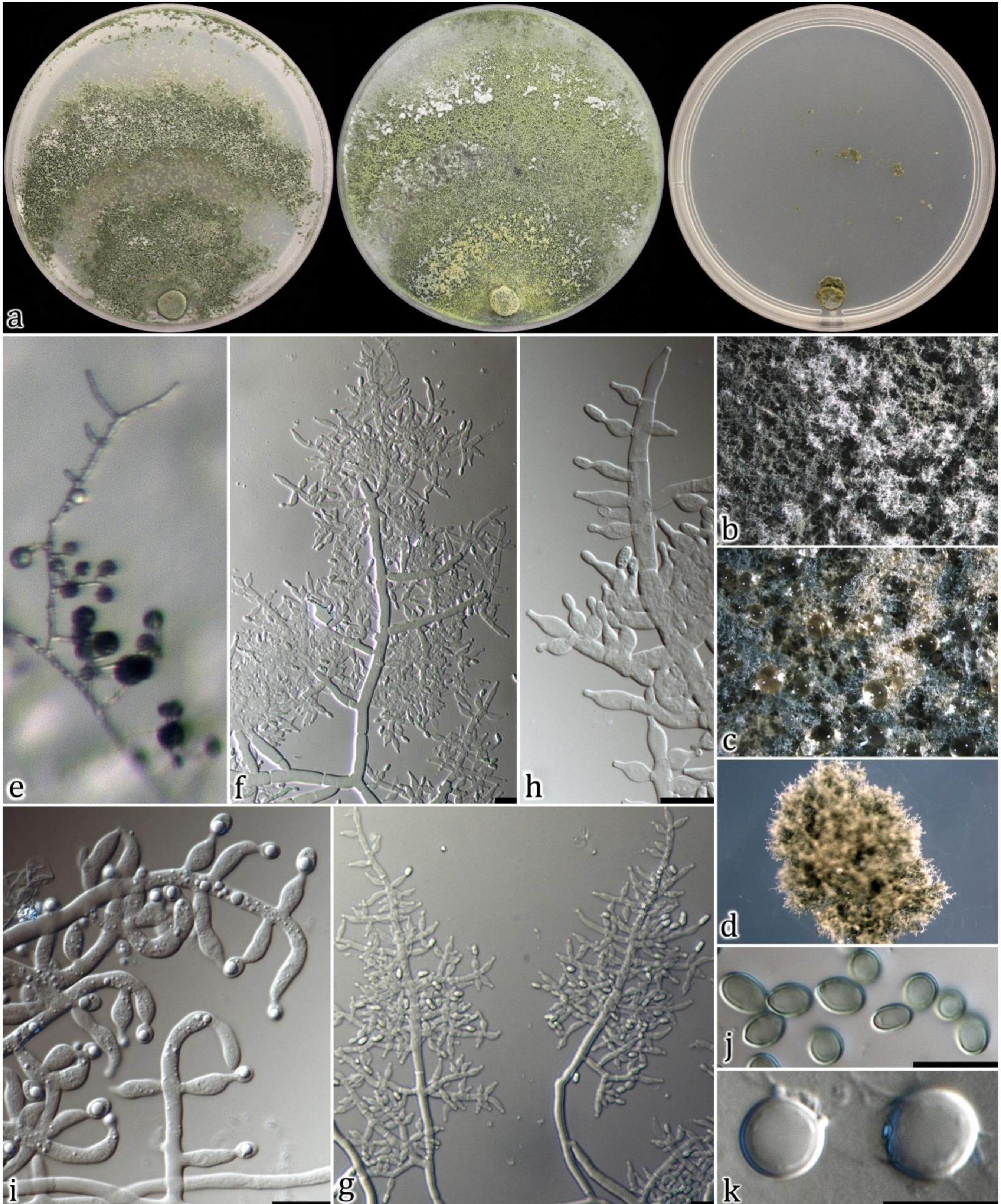

FIGURE 22. Morphological features characteristic of *Trichoderma hamatum*, Strain Tri 78. a. colonies of *T. hamatum* incubated on CMD for 7 days (left), PDA for 7 days (middle), SNA for 21 days (right). b, c, d. Stereo microscope images, from CMD (top) PDA (middle) and SNA (bottom). e. Stereo microscope image from CMA. f, g. Conidiophores formed on SNA 400x magnification. h, i. Conidiophores formed on CMD 1000x. j. Conidia 1000x. k. Chlamydospores 1000x. All scale bars are 10μm in length.

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***Trichoderma asperelloides* Samuels, Mycologia 102: 961. 2010**

Trichoderma asperelloides forms part of the *T. pachybasium* 'A' or *hamatum* clade of *Trichoderma*. The species displays a worldwide distribution and cannot be morphologically distinguished from *T. asperellum* (Samuels and Petrini 2010). These two species can only be distinguished from one another based on ef1α, rpb2, act and ITS sequence data or proteomic analysis using MALDI-TOF MS (Samuels and Petrini 2010).

During this study two strains that were isolated from a single site (Fig. 19 and Table 2) were identified as *T. asperelloides* based on ef1α sequence data (Fig. 20 and Table 6). This identification was confirmed through a morphological analysis (Figs. 23 and 24).

Morphologically Strain Tri 150 matches *T. asperelloides* (Samuels and Petrini 2010). Colonies covered the Petri dishes within one week with a continuous lawn of fertile conidiophores (Fig. 24 a). On SNA, colonies develop small, scattered pustules which lack protruding conidiophores or sterile hairs (Fig. 24 a, c).

Micromorphologically, the conidiophores of Strain Tri 150 form distinguishable central stipes which branch in pairs (Fig. 24 b, f). Secondary branches were also observed but no third stage

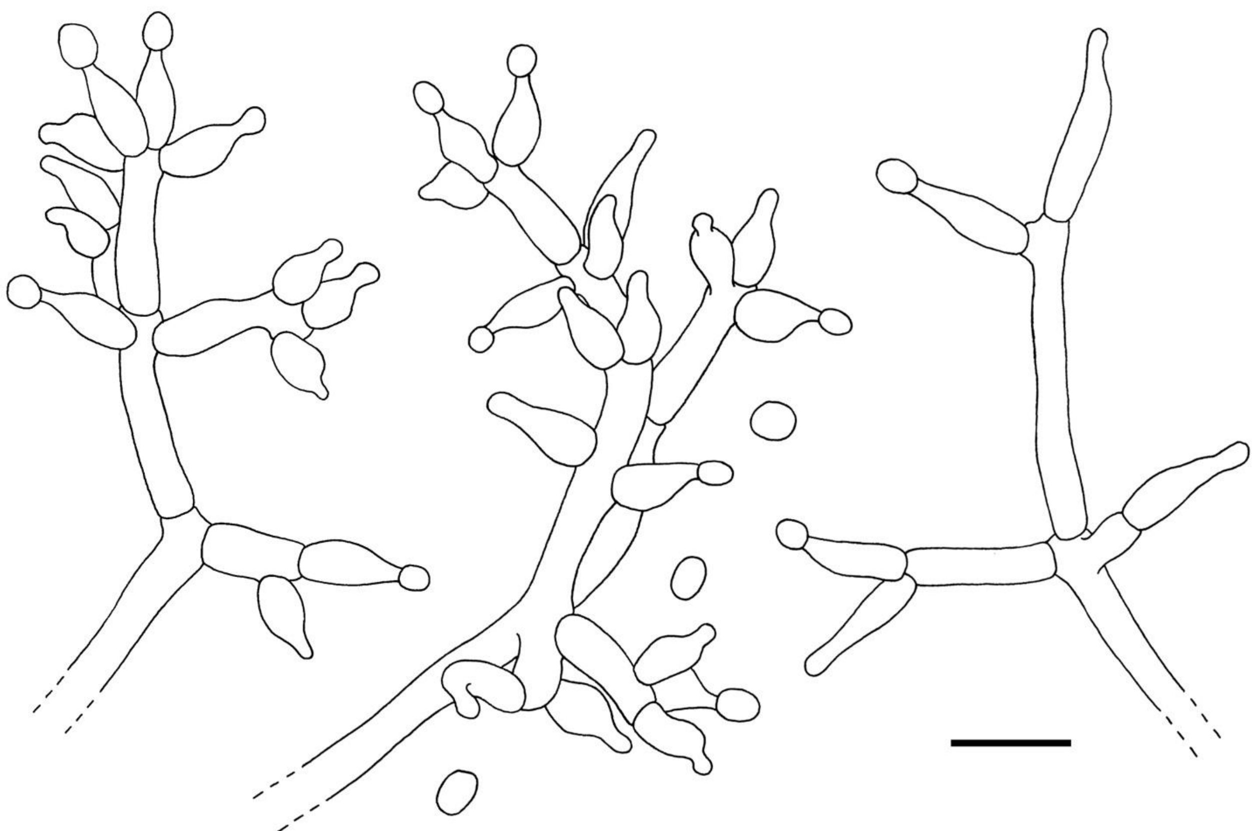

FIGURE 23. *Trichoderma asperelloides.* Line drawings from Strain Tri 150. Scale bar = 10 µm

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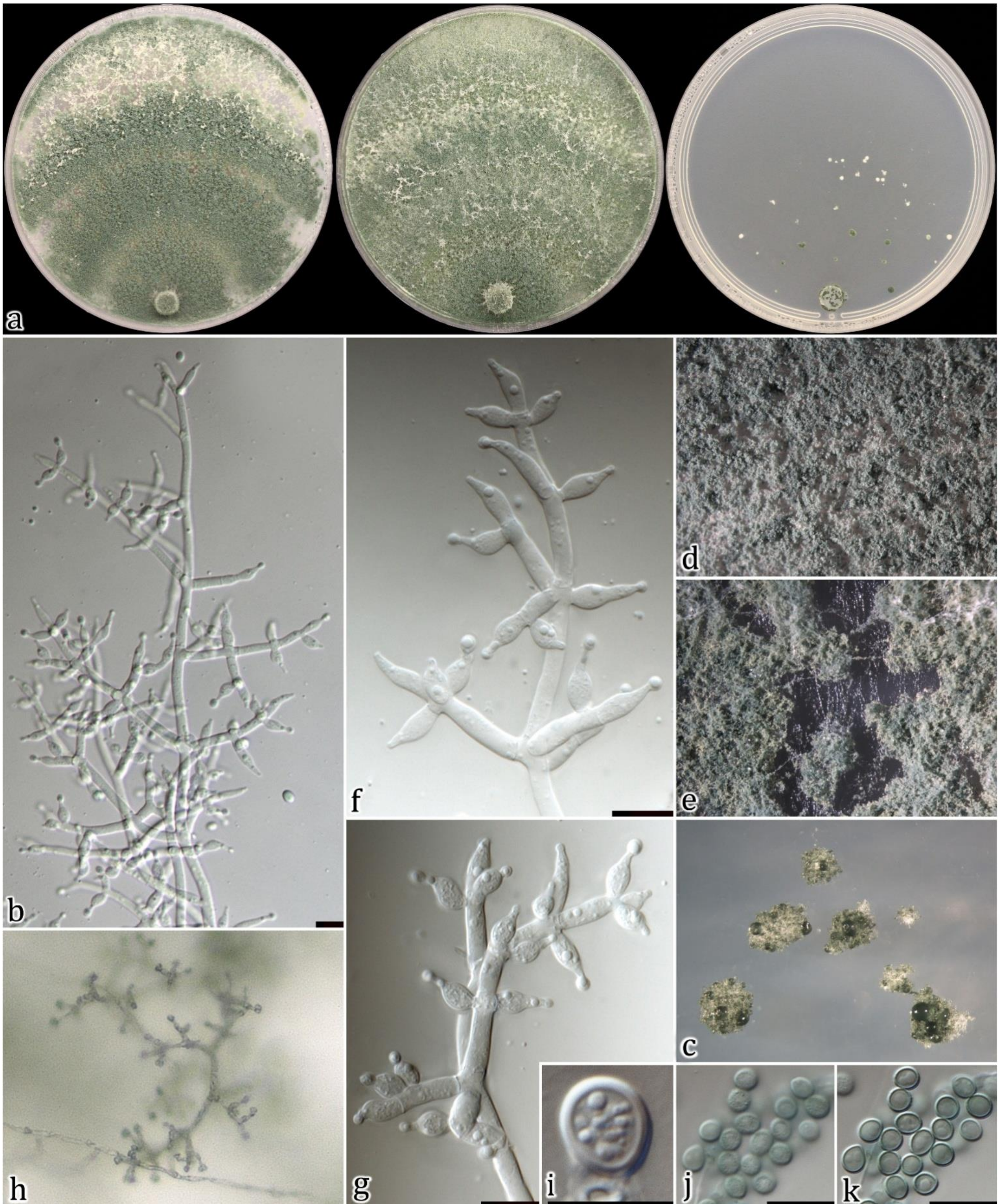

FIGURE 24. Morphological characteristic of *T. asperelloides*, Strain Tri 150. a. Colonies of *T. asperelloides* grown on CMD for 7 days (left), PDA for 7 days (middle), SNA for 7 days (right). b. Conidiophores formed on CMD 400x magnification. c, d, e. Stereo microscope images, from CMD (top), PDA (middle) and SNA (bottom). f, g. Conidiophores formed on CMD 1000x magnification. h. Stereo microscope image of conidiophores from CMD i. Chlamydospore, 1000x. j, k. conidia 1000x. All scale bars are 10μm in length.

branchings were observed (Fig. 24 b). Branches terminated in small whorls of phialides (1-4). Conidia displayed subsphearoidal shapes and slightly warted ornamentations (Fig. 24. j, k). On CMD, chlamydospores were formed on the termini of branches (Fig. 24 i).

***Trichoderma asperellum* Samuels, Lieckf. & Nirenberg, Sydowia 51: 81. 1999**

Trichoderma asperellum was first described by Samuels, Lieckfeldt & Nirenberg in 1999 and displays a worldwide distribution (Samuels and Petrini 2010). Several strains have been developed as biocontrol agents in commercial agriculture (Watanabe *et al.* 2007, Sagarra *et al.* 2010).

This study resulted in the isolation of 12 strains of *T. asperellum* from eight different sites (Fig. 19 and Table 2) that were identified based on ef1α sequence data (Fig. 20 and Table 6). A morphological analysis confirmed the identity of the species (Figs. 25 and 26).

Strain Tri 10 matched the colony descriptions of *T. asperellum* with the exception of one trait (Samuels *et al.* 1999). Strain Tri 10 was observed to sporulate heavily on CMD whereas the type strain of *T. asperellum* was reported to display only lightly sporulating regions on this medium (Samuels *et al.* 1999). Despite this, Strain Tri 10 strongly resembled *T. asperellum* and

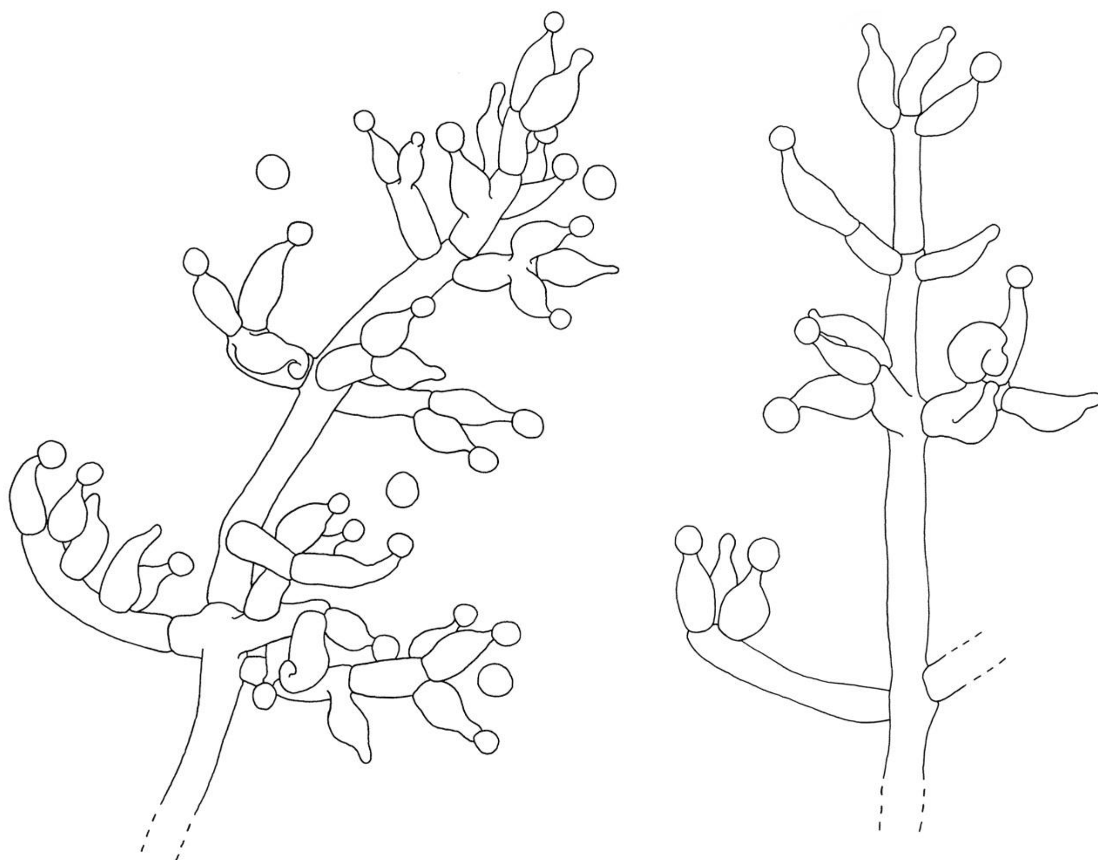

FIGURE 25. *Trichoderma asperellum.* Line drawings from strain Tri 10. Scale bar = 10 μm

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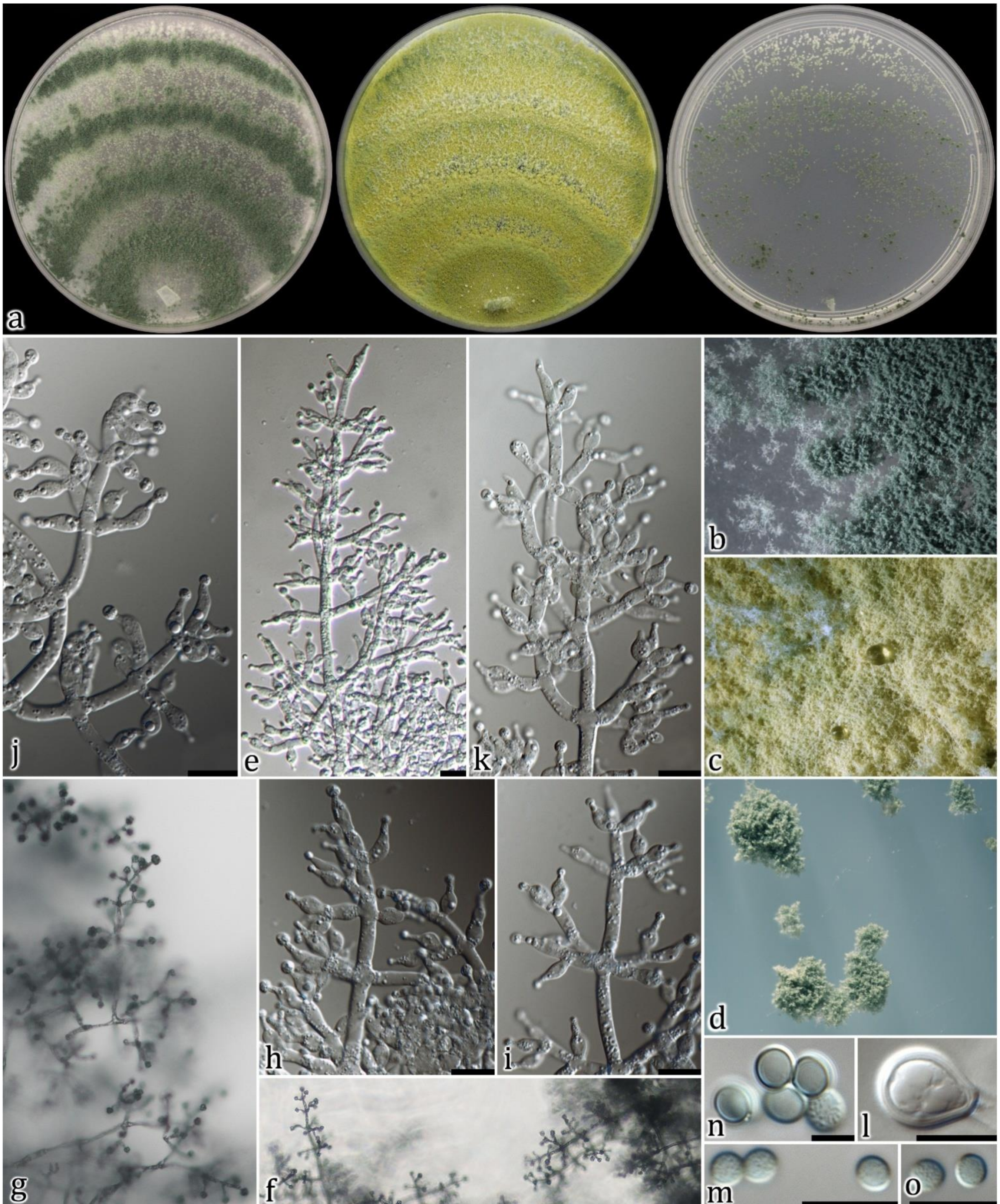

FIGURE 26. Morphological features characteristic of *Trichoderma asperellum*, strain Tri 10. a. Colonies of *T. asperellum* grown on CMD for 7 days (left), PDA for 7 days (middle), SNA for 7 days (right). b, c, d. Stereo microscope images, from CMD (top), PDA (middle) and SNA (bottom). e. Conidiophore on CMA 400x magnification. f, g. Stereo microscope images of conidiophores formed on SNA. h, i, j, k. Conidiophores formed on CMD 1000x. l. Chlamydospore 1000x. m, n, o. Conidia 1000x. All scale bars are 10μm in length.

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displayed colonies which did not produce, soluble pigments, odours or aerial mycelia on PDA and formed dark green sporulating regions on CMD and SNA (Fig. 26 a).

Micromorphologically Strain Tri 10 also matched *T. asperellum* by developing roughly symmetrical conidiophores that branched extensively, often forming secondary branches in roughly perpendicular orientations relative to the central axis (Fig. 26 h, e) (Samuels *et al.* 1999). In addition, tertiary branchings were observed which bore ampuliform shaped phialides (Fig. 26 h, i, j) with slightly warted subglobose conidia (Fig. 26 m, n, o). Small, terminal chlamydospores were also observed on CMD (Fig. 26 l).

Samuels and Petrini (2010) reported that *T. asperellum* and *T. asperelloides* are morphologically indistinguishable from one another. Despite this, South African strains from these two species seemed to display discernible morphological variations. Most noticeable were the formation of yellow colonies on PDA by *T. asperellum* (Fig. 26 a) as opposed to green colonies formed by *T. asperelloides* (Fig. 24 a). Additionally, *T. asperelloides* formed abundant white aerial mycelium on both CMD and PDA (Fig. 24 a), whereas *T. asperellum* did not form noticeable aerial mycelium on any medium (Fig. 26 a). *T. asperellum* also seemed to sporulate more heavily on SNA (Fig. 26 a) relative to *T. asperelloides* (Fig. 24 a).

The *Trichoderma harzianum* clade

This study isolated 2 different species from the *T. harzianum* clade from 18 different sites (Fig. 27 and table 2). These were identified through phylogenetic analyses as *T. harzianum* and *T. lixii* (Fig. 28, Table 7). The *T. harzianum* clade consists of a small number of species, some of which, like *T. harzianum*, are well known for their role as biocontrol agents (Elad *et al.* 1980, Yedidia *et al.* 1999, O'Neill *et al.* 1996, Roco and Luz 2001, John *et al.* 2004). Rifai (1969) described *T. harzianum* as a 'species aggregate'. This still seems to be the case today because the precise species composition of the clade remains unresolved despite extensive investigation into the phylogeny of the clade through GCPSR-based approaches (Chaverri *et al.* 2003, Druzhinina *et al.* 2010). Chaverri and Samuels (2002) reported *H. lixii* to be the sexual state of the fungus *T. harzianum.* However, Chaverri *et al.* (2003) later showed that the ex-types of *T. harzianum* and *T. lixii* did not group together based on phylogenetic analyses. Druzhinina *et al*. (2010) confirmed this finding and also recognised a distinct phylogenetic lineage within the *T. harzianum* species complex, which is currently awaiting formal description.

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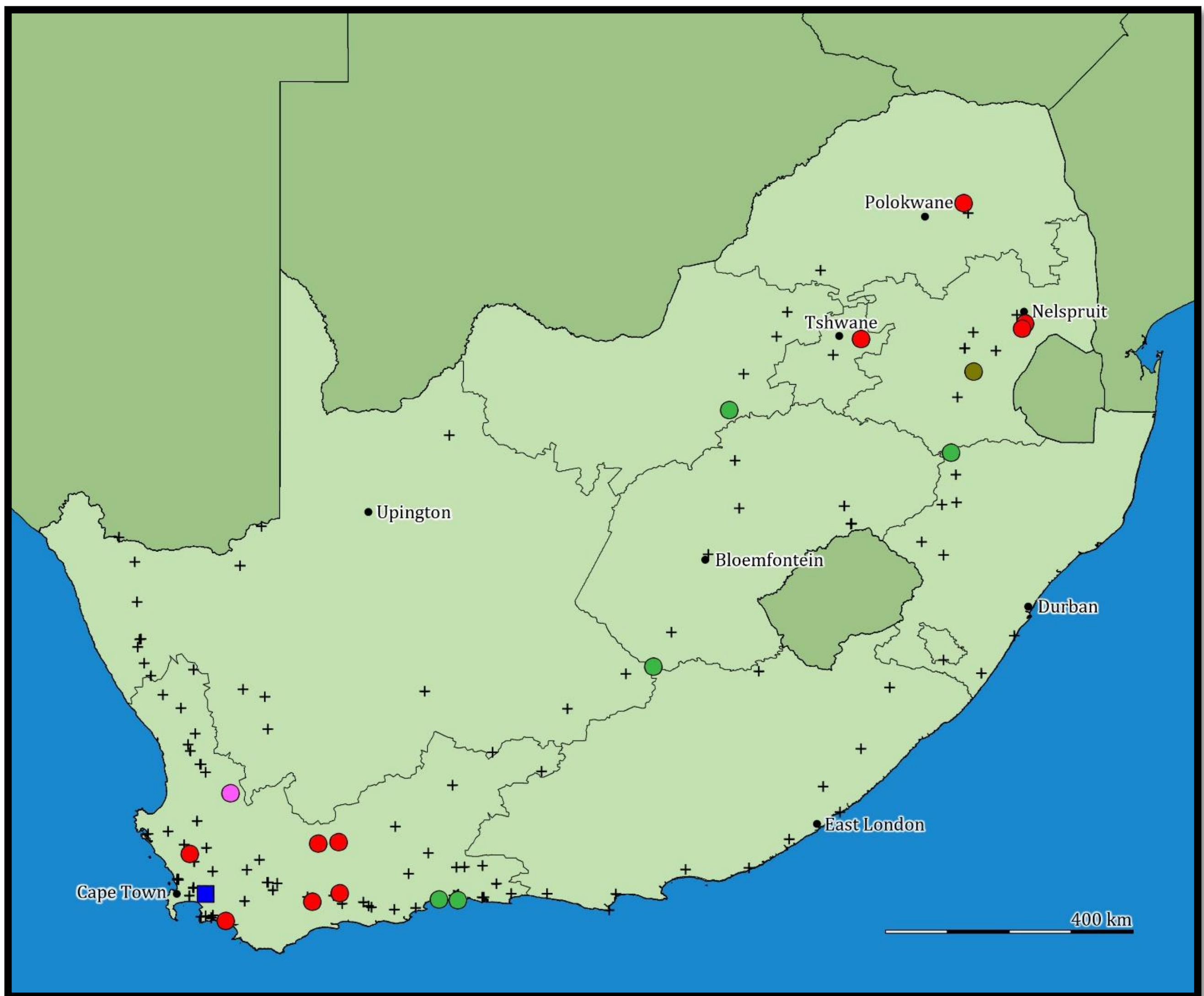

FIGURE 27. Distribution of the *Trichoderma harzianum clade* in South Africa*.* *T. lixii* was isolated from northern and southern sites only. *T. harzianum* (unknown clade) and *T. harzianum* (Subclade III) displayed distributions restricted to the Cape whereas *T. harzianum* (Subclade II) was distributed across South Africa. *T. harzianum* (subclade V or X) was only isolated from a single northern site. Black crosses indicate sample sites. ***T. lixii,* (red circles)** isolated from 10 different sites. ***T. afroharzianum* prov. nom., (Subclade II) (green circles)** isolated from 5 different sites. ***T. harzianum,* (Subclade III) (pink circle)** isolated from 1 site. ***T. harzianum,* (Subclade V or X) (brown circle)** isolated from 1 site. ***T. harzianum,* (lone lineage) (blue square)** isolated from 1 site. Note that some sites are located close to one another and cannot be visualised at this scale. Scale bar = 400km.

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TABLE 7 *Trichoderma* strains from the *T. harzianum* clade used for phylogenetic comparisons are listed along with their respective GenBank accession numbers for ef1α sequence data. Ex-type strains are indicated in bold and with a *'T'*.

GenBank accession	Taxon name	Strain number	Reference
AY937440	*H. albocornea*	G.J.S 97-28	Samuels 2006
EU498313	*H. alni*	CPK 2494	Jaklitsch *et al.* 2008
EU498314	*H. alni*	CPK 2854	Jaklitsch *et al.* 2008
EU498312	***H. alni*** ***T***	CBS 120633	Jaklitsch *et al.* 2008
AY937417	*H. atrogelatinosa*	G.J.S. 00-162	Samuels 2006
AY937431	*H. cornea*	G.J.S. 97-75	Samuels 2006
AY937426	*H. cornea*	G.J.S. 97-90	Samuels 2006
EU498321	*H. epimyces*	CPK 2417	Jaklitsch *et al.* 2008
EU498320	***H. epimyces*** ***T***	CBS 120534	Jaklitsch *et al.* 2008
AY605775	*H. lixii*	DAOM 231402	Druzhinina *et al.* 2010
AY605773	*H. lixii*	DAOM 231408	Druzhinina *et al.* 2010
AY605774	*H. lixii*	DAOM 231405	Druzhinina *et al.* 2010
EF191333	*H. lixii*	JBNZ 111	Druzhinina *et al.* 2010
EF191331	*H. lixii*	JBNZ 24	Druzhinina *et al.* 2010
EF191327	*H. lixii*	G.J.S. 05-22	Druzhinina *et al.* 2010
EF191328	*H. lixii*	G.J.S. 05-32	Druzhinina *et al.* 2010
EF191326	*H. lixii*	G.J.S. 05-82	Druzhinina *et al.* 2010
FJ577777	*H. lixii*	G.J.S. 98-64	Druzhinina *et al.* 2010
FJ577776	*H. lixii*	G.J.S. 98-65	Druzhinina *et al.* 2010
AY605784	*H. lixii*	JBT 1244	Druzhinina *et al.* 2010
EF116558	*H. lixii*	PPRC J12	Nagy *et al.* 2007
EF116562	*H. lixii*	SZMC 3203	Nagy *et al.* 2007
EF113554	*H. lixii*	TUB F-750	Nagy *et al.* 2007
FJ577784	*H. lixii*	UNISS 10.5M	Druzhinina *et al.* 2010
EF392742	*H. lixii*	W.J. 2786	Jaklitsch 2009
EF191329	*H. lixii*	JBGA 3804	Druzhinina *et al.* 2010
EF191338	*H. lixii*	JBRSA 122	Druzhinina *et al.* 2010
EF191339	*H. lixii*	JBSERB 24	Druzhinina *et al.* 2010
FJ577788	*H. lixii*	PPRC RW-14	Druzhinina *et al.* 2010
EF191321	*H. lixii*	DAOM 231435	Druzhinina *et al.* 2010
FJ577787	*H. lixii*	PPRC RW6	Druzhinina *et al.* 2010
EF113551	*H. lixii*	PPRI 3909	Nagy *et al.* 2007
EF191330	*H. lixii*	JBNZ 12	Druzhinina *et al.* 2010
FJ577789	*H. lixii*	PPRC R12	Druzhinina *et al.* 2010
AF348101	***H. lixii*** ***T***	CBS 226.95	Samuels *et al.* 2002
FJ860665	*H. parepimyces*	CBS 122768	Jaklitsch 2009
FJ860665	***H. parepimyces*** ***T***	CBS 122768	Jaklitsch 2009
EU279972	*H. tawa*	DAOM 232841	Hoyos-Carvajal *et al.* 2009
FJ463313	***H. tawa*** ***T***	G.J.S. 97-174	Chaverri and Samuels 2004
AF348097	*T. aggressivum*	CBS 433.95	Samuels *et al.* 2002

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TABLE 7 continued

GenBank accession	*Taxon name*	Strain number	References
AF348098	***T. aggressivum*** [T]	DAOM 222156	Samuels *et al.* 2002
AF348102	*T. pleurotica*	G.J.S. 95-81	Samuels *et al.* 2002
EU918160	*T. pleurotica*	CPK 3196	Laszlo *et al.* 2009
FJ870906	*T. tomentosum*	CPK 2563	Jaklitsch 2009
AY937446	*T. velutinum*	DAOM 230014	Samuels 2006
AY937415	***T. velutinum*** [T]	DAOM 230013	Samuels 2006

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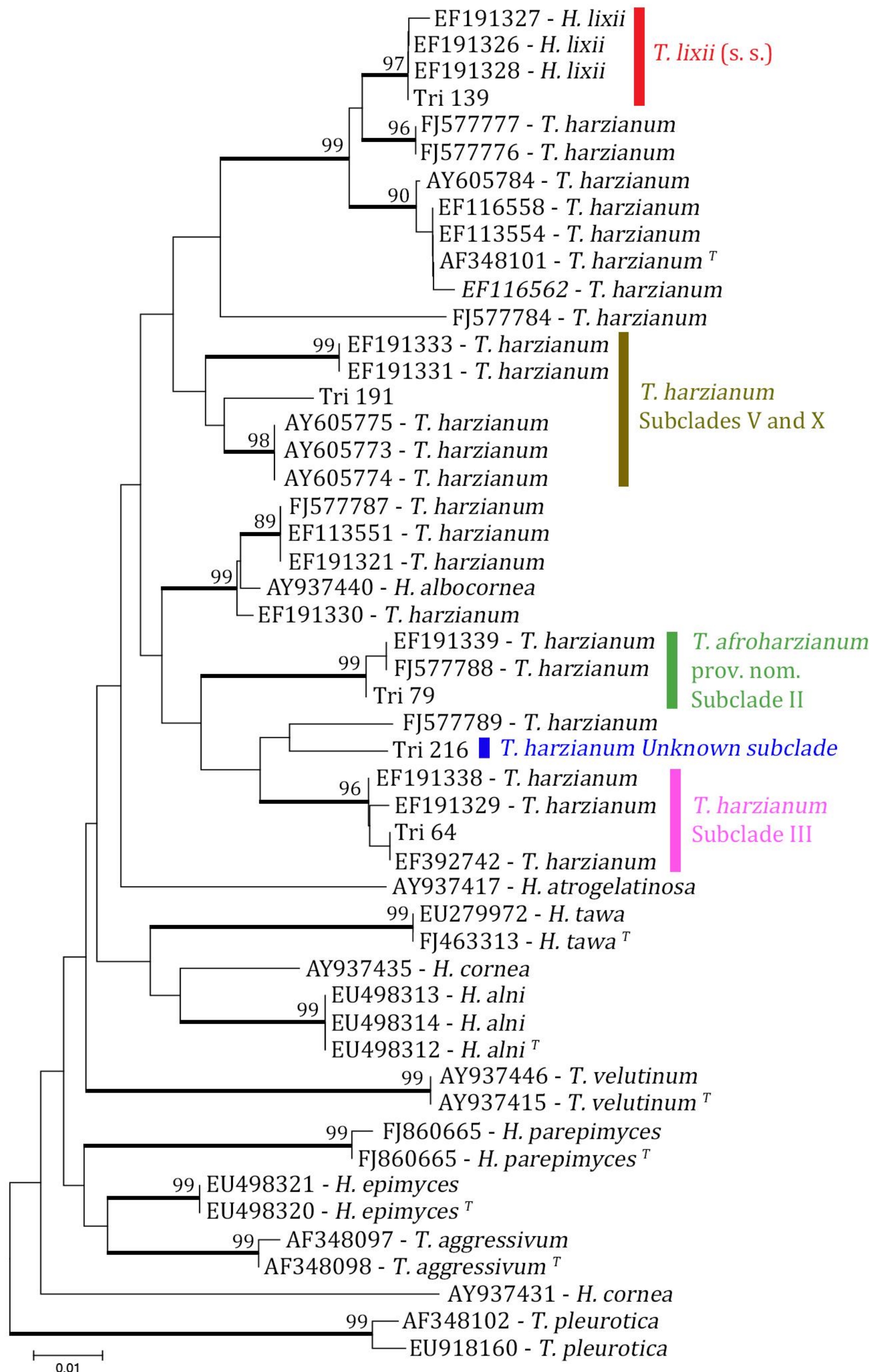

FIGURE 28 Neighbour joining tree based on ef1α sequence data indicating the phylogenetic relationships between accepted members of the *Trichoderma harzianum clade* and South African *Trichoderma* strains belonging to the same clade. Nodes which are supported by bootstrapping values that exceed 80% are indicated by thickened branches. Ex-type culture sequences are indicated with a *'T'.*

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***Trichoderma lixii* Pat., Revue Mycologique Toulouse 51: 138, 1891**

Trichoderma lixii (formerly *Hypocrea lixii*) was first described in 1891 by Patoullard. Unfortunately the material that Patoullard examined no longer exists and Chaverri and Samuels (2002) therefore epitypified *H. lixii* and described its sexual state. They also recognized *H. lixii* to be the sexual state of *T. harzianum s. l.* (Chaverri and Samuels 2002). Following revision of the *T. harzianum* clade by Druzhinina *et al.* (2010), it was concluded that *H. lixii* and *T. harzianum* are genetically isolated from one another and, therefore, constitute separate taxa. Jaklitch 2009 provided a detailed description of the asexual state of the ex-epitype *T. lixii* as a member of the *T. harzianum* clade and displays a cosmopolitan distribution (Druzhinina *et al.* 2010).

During this study, 18 different strains of *T. lixii* were isolated from 10 different sites (Fig. 27 and Table 2). These strains were identified based on their ef1α sequence data (Fig. 28 and Table 7) and a morphological analysis confirmed these results (Figs. 29 and 30).

Morphologically Strain Tri 139 displayed several characteristics which match the ex-epitype of *T. lixii* (Jaklitsch 2009). Strain Tri 139 forms colonies that show broad, ill defined, concentric zones which sporulate effusely (Fig. 30 a). Aerial hyphae were not conspicuous and the surface texture of the colony

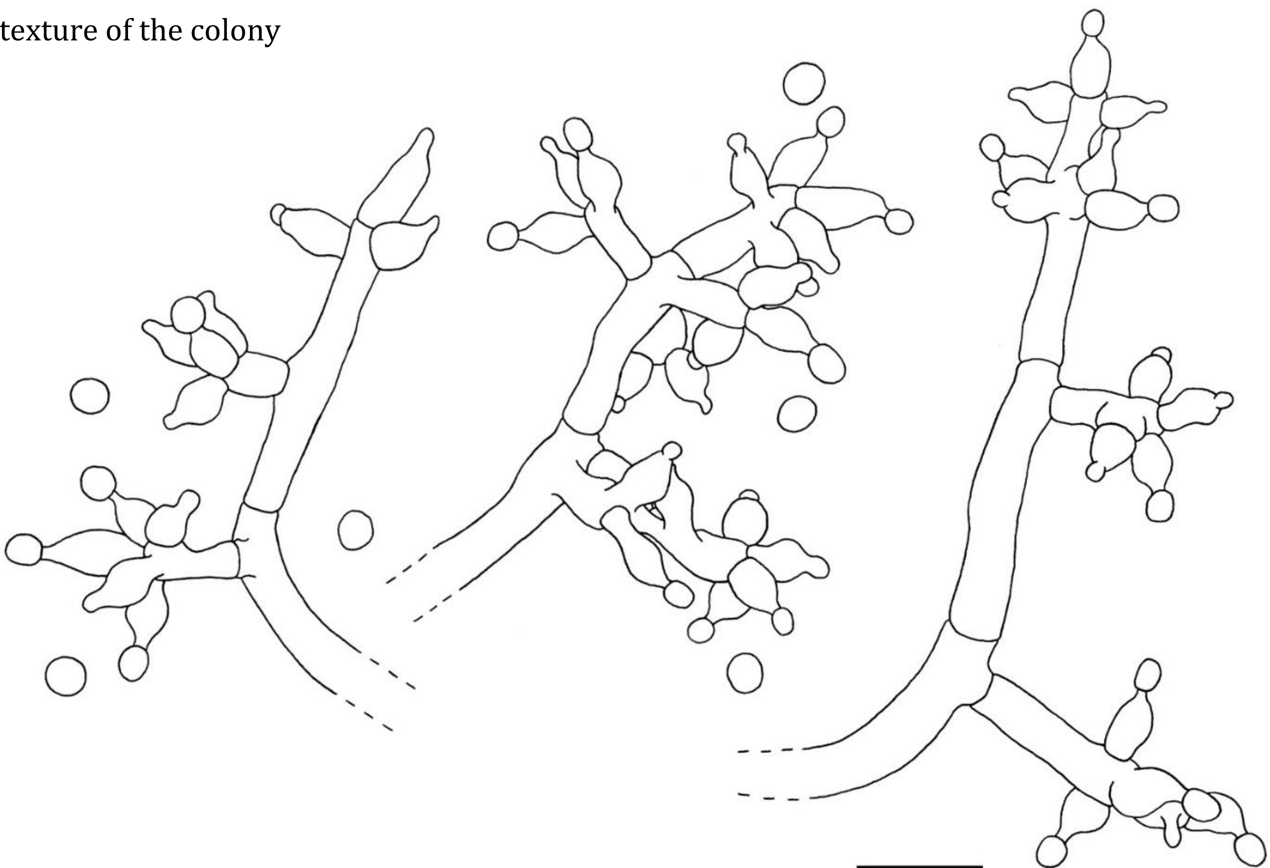

FIGURE 29. *Trichoderma lixii*. Line drawings from Strain Tri 139. Scale bar = 10µm

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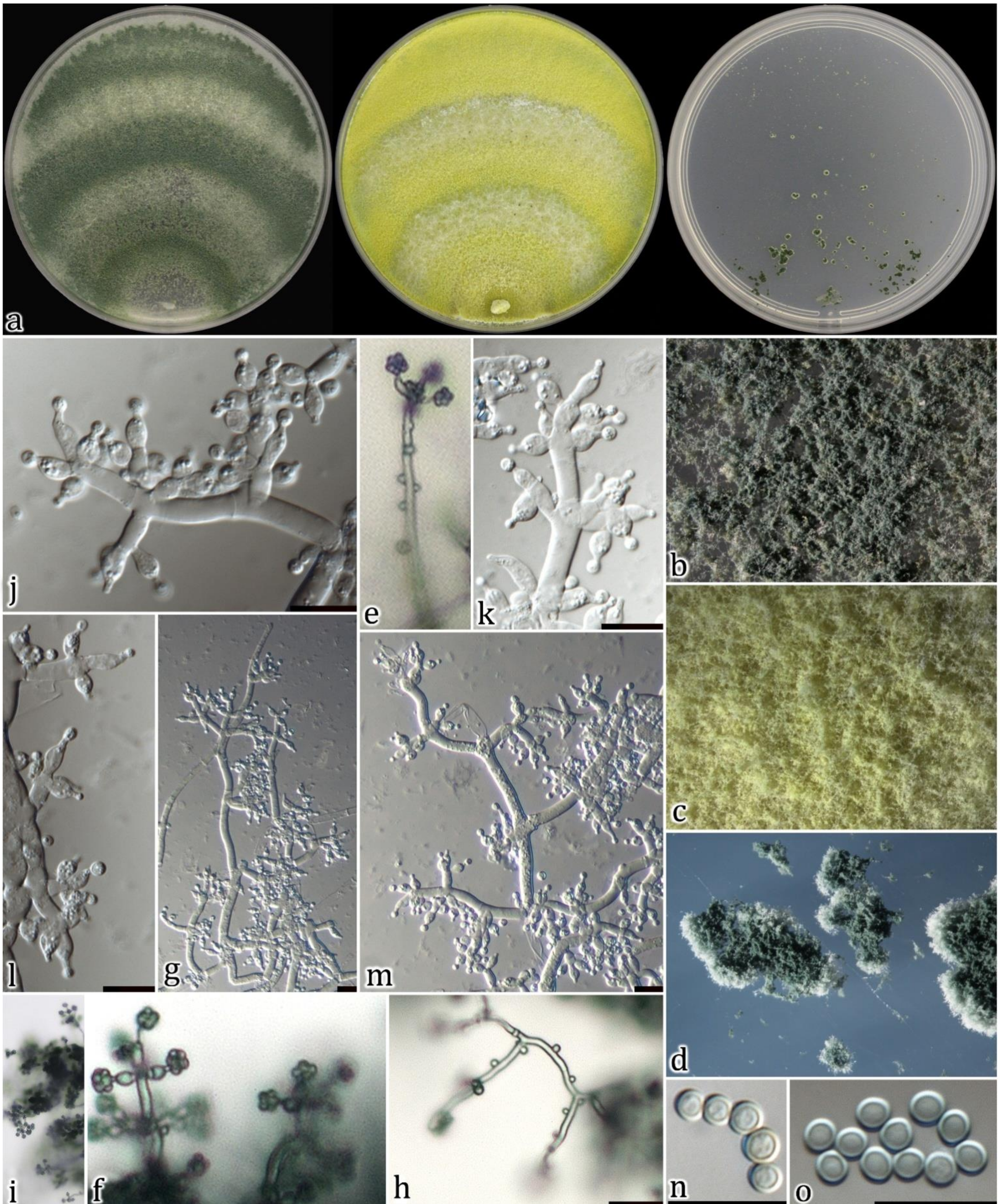

FIGURE 30. Morphological features characteristic of *Trichoderma lixii*, Strain Tri 139. a. Colonies of *T. lixii* incubated on CMD for 7 days (left), PDA for 7 days (middle) and SNA for 7 days (right). b, c, d. Stereo microscope images, from CMD (top), PDA (middle) and SNA (bottom). e, f, g, h. Conidiophores on CMA 400x magnification. i. Stereo microscope image from SNA. j, k, l, m. Conidiophores formed on CMD 1000x. n, o. Conidia 1000x. All scale bars are 10µm in length.

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appeared granulose due to the formation of small fertile tufts. Colonies did not, however, display yellow reverses on CMA as would normally be expected from a strain of *T. lixii* (Jaklitsch 2009). However, colonies on PDA fitted the description of *T. lixii* well by appearing yellow and forming ill defined concentric zones that sporulated abundantly (Fig. 30 a). On SNA, Strain Tri 139 also matched the descriptions of *T. lixii* and produced colonies that were thin and hyaline and that formed inconspicuous aerial hyphae (Fig. 30 a).

Micromorphologically Strain Tri 139 also matched the descriptions of *T. lixii* (Jaklitsch 2009). Conidiophores displayed thick central axes (Fig. 30 j, k). Phialides were mostly ampuliform shaped bearing smooth subglobose conidia in dry heads (Fig. 30 l, n, o, j).

***Trichoderma harzianum Rifai, Mycological Papers 116: 38. 1969* (*T. pseudoharzianum nom. prov. dub. sensu Druzhinina et al. 2010*)**

T. harzianum s. l. is the most frequently isolated *Trichoderma* species worldwide (Jaklitch 2009). Species within the *T. harzianum* clade are particularly difficult to characterize based on their morphology (Druzhinina *et al.* 2010). A recent study by Druzhinina *et al.* (2010) revealed that at least three monophyletic lineages, representing distinct species, exist within the *T. harzianum* complex. These closely related species are reportedly similar to one another based on their morphologies (Druzhinina *et al.* 2010). These include *T. harzianum s. s*. and *T. lixii,* which are currently the only species that are formally recognised within the *T. harzianum* complex, as well as *T. afroharzianum* prov. nom. which is awaiting formal description and has been named provisionally. (Druzhinina *et al.* 2010). A large number of strains belonging to the *T. harzianum* complex do not form clear phylogenetic groupings with any of the three above mentioned species and are referred to by Druzhinina *et al.* (2010) as: '*T. pseudoharzianum* nom. prov. dub.' in order to distinguish them from phylogenetically recognised species (Druzhinina *et al.* 2010). *Trichoderma pseudoharzianum* nom. prov. dub. has itself been divided into several different subclades based on ef1α, cal1 and chi18-5 sequence data (Druzhinina *et al.* 2010). These include Subclades II, III, IV, V and X. The different subclades of *T. pseudoharzianum* nom. prov. dub. are morphologically indistinguishable from *T. harzianum s. s.* (Druzhinina *et al.* 2010). In terms of colony morphology, the South African strains conformed to the descriptions of Jaklitsch (2009).

The subclades of T. pseudoharzianum nom. prov. dub. isolated during this study

Four *Trichoderma* strains belonging to the *T. harzianum* complex were identified as *T. pseudoharzianum* nom. prov. dub based on a phylogenetic analysis using ef1α sequence data

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(Fig. 28 and Table 7). No formal descriptions for *T. pseudoharzianum* nom. prov. dub., or the different subclades, are currently published.

This study isolated a single strain, Tri 64, which was recognised as a member of Subclade III of *T. pseudoharzianum* nom. prov. dub. Two additional strains, represented by Strain 191, belong to either Subclade V or X (Fig. 28). Phylogenetic analysis was not able to place this strain confidently in either subclade. Lastly, a single strain, Tri 216, could not be placed within any of the current subdivisions of *T. pseudoharzianum* nom. prov. dub. and is believed to constitute a lone lineage (Fig. 28). These strains were isolated from three different sites (Fig. 27 and Table 2). During this study it was observed that the strains of *T. pseudoharzianum* nom. prov. dub. from different subclades display colony morphologies that varied from one another. For this reason, representatives from each subclade were illustrated to highlight these morphological differences: Subclade III - Figs. 31 and 32, Subclade V or X - Figs. 33 and 35, lone lineage - Figs. 34 and 36.

Strain Tri 64 (Subclade III) varied most notably from the other two subclades based on its colony morphology on CMD and formed colonies which did not display signs of zonation (Fig. 32a). Strain 191 (Subclade V or X) and Strain 216 (lone lineage), on the other hand, formed colonies which showed prominent signs of zonation (Figs. 35 and 36). In addition, Strain Tri 64 displayed micromorphological characteristics that also distinguished it from other subclades of *T. pseudoharzianum* prov. nom. dub. Strain Tri 64 displayed long, slender, lanceolate phialides (Fig. 31) relative to the short, wide, ampuliform phialides formed by strains Tri 191 and 216 (Figs 33 and 34). Strain Tri 64 also sporulated poorly on SNA (Fig. 32 a), whereas Strains Tri 191 and 216 produced numerous fertile pustules (Figs. 35 and 36). Strain Tri 216 can be differentiated from the other subclades of *T. pseudoharzianum* based on its colony appearance on PDA. Strain Tri 216 forms green colonies with downy white areal mycelium (Fig. 32), compared to Strains Tri 191 and 64 which both display yellow colonies that do not form white areal mycelium as conspicuously as Strain Tri 216 (Figs. 35 and 36).

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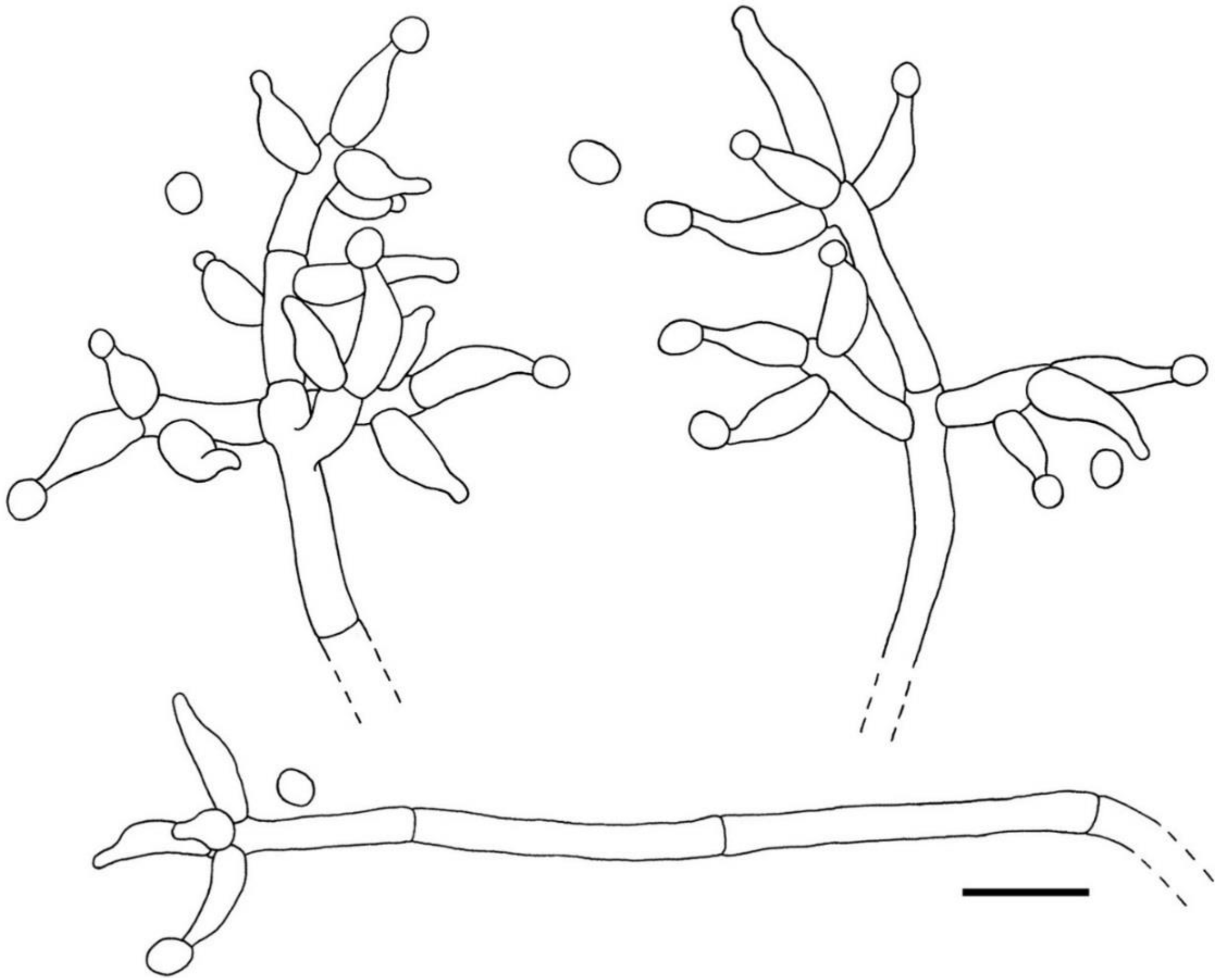

FIGURE 31. *Trichoderma harzianum* (*T. pseudoharzianum* nom. prov. dub.) (Subclade III). Line drawings from Strain Tri 64. Scale bar = 10µm.

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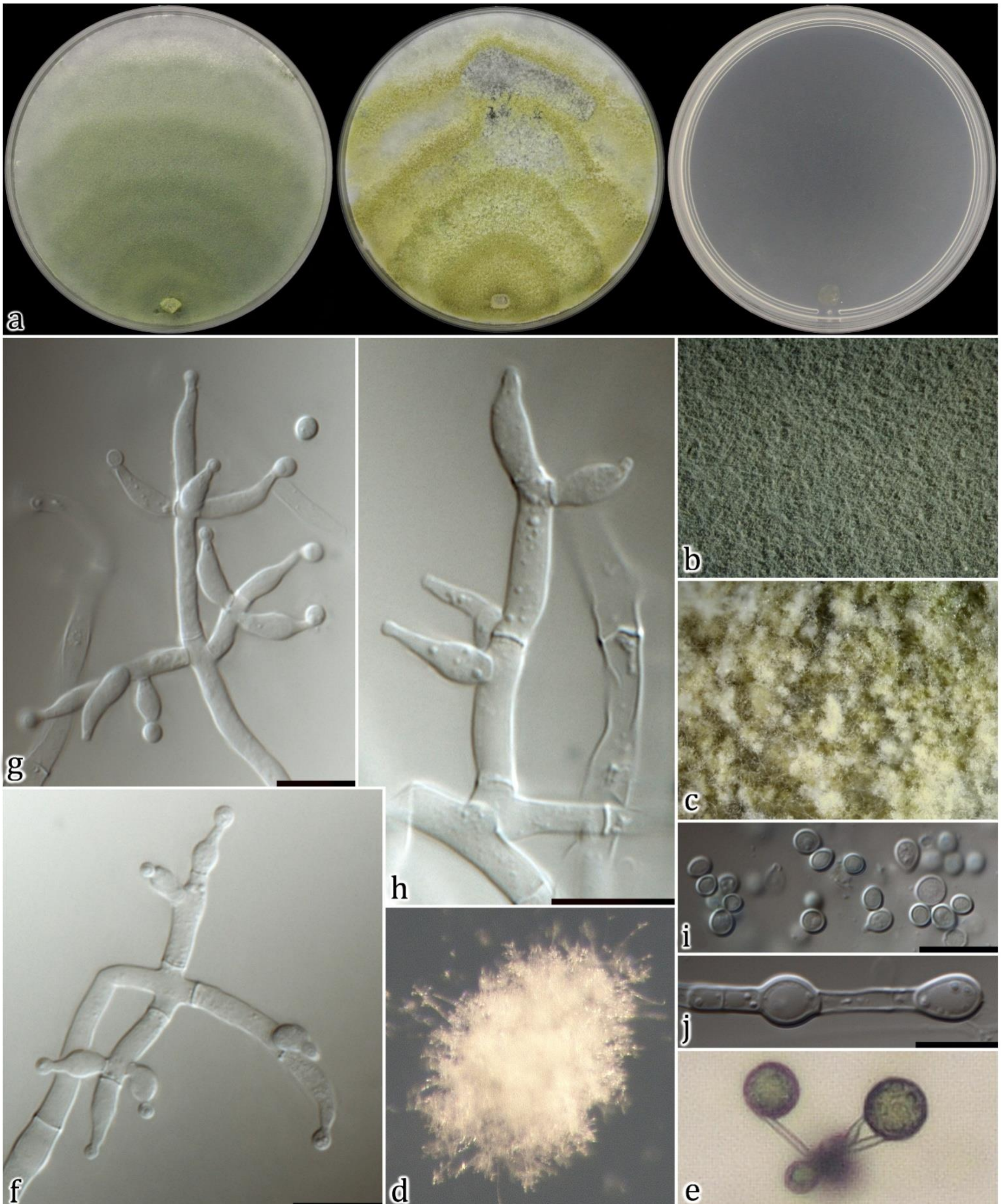

FIGURE 32. Morphological features characteristic of *T. harzianum* (*T. pseudoharzianum* nom. prov. dub.) (Subclade III), Strain Tri 64. a. Colonies of *T. pseudoharzianum* nom. prov. dub. incubated on CMD for 7 days (left), PDA for 7 days (middle), SNA for 7 days (right). b, c. Stereo microscope images, from CMD (top) and PDA (middle). d, e. Stereo microscope image from SNA. f, g, h. Conidiophores formed on CMA 1000x magnification. i. Conidia 1000x. j. Chlamydospores 1000x. All scale bars are 10µm in length.

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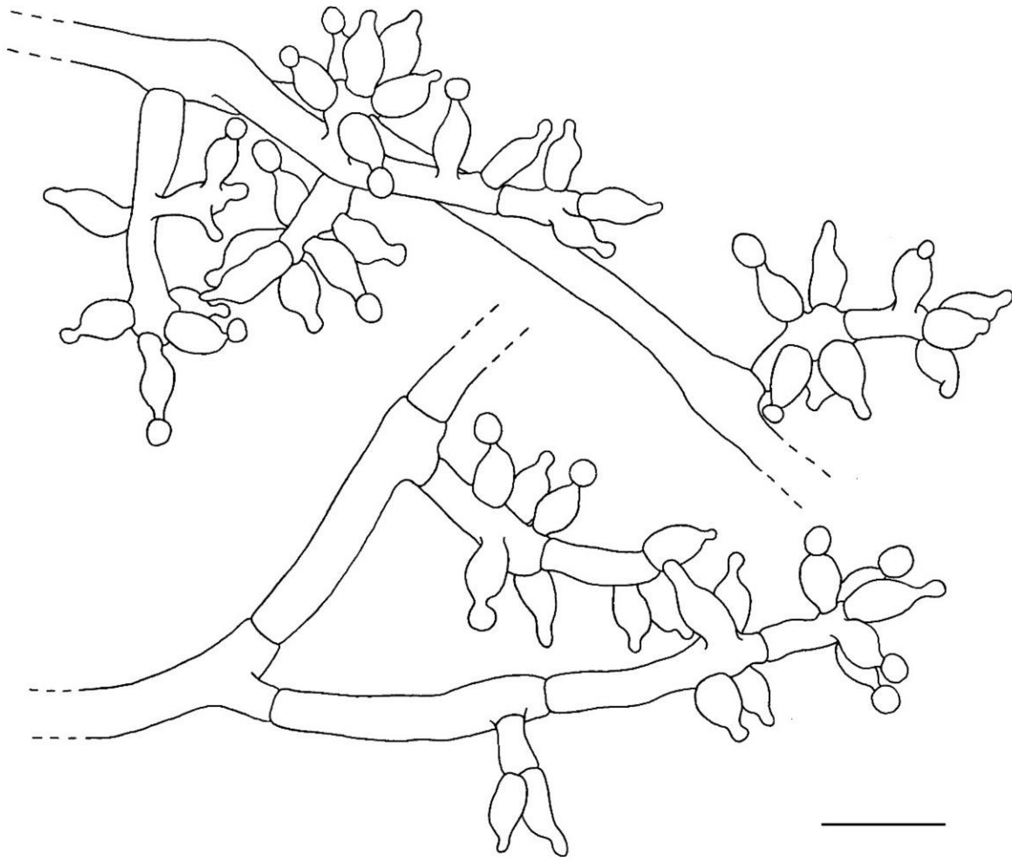

FIGURE 33. *Trichoderma harzianum* (*T. pseudoharzianum* nom. prov. dub.) (Subclade V or X). Line drawings from Strain Tri 191. Scale bar = 10µm

FIGURE 34. *Trichoderma harzianum* (*T. pseudoharzianum* nom. prov. dub.) (lone lineage). Line drawings from Strain Tri 216. Scale bar = 10µm.

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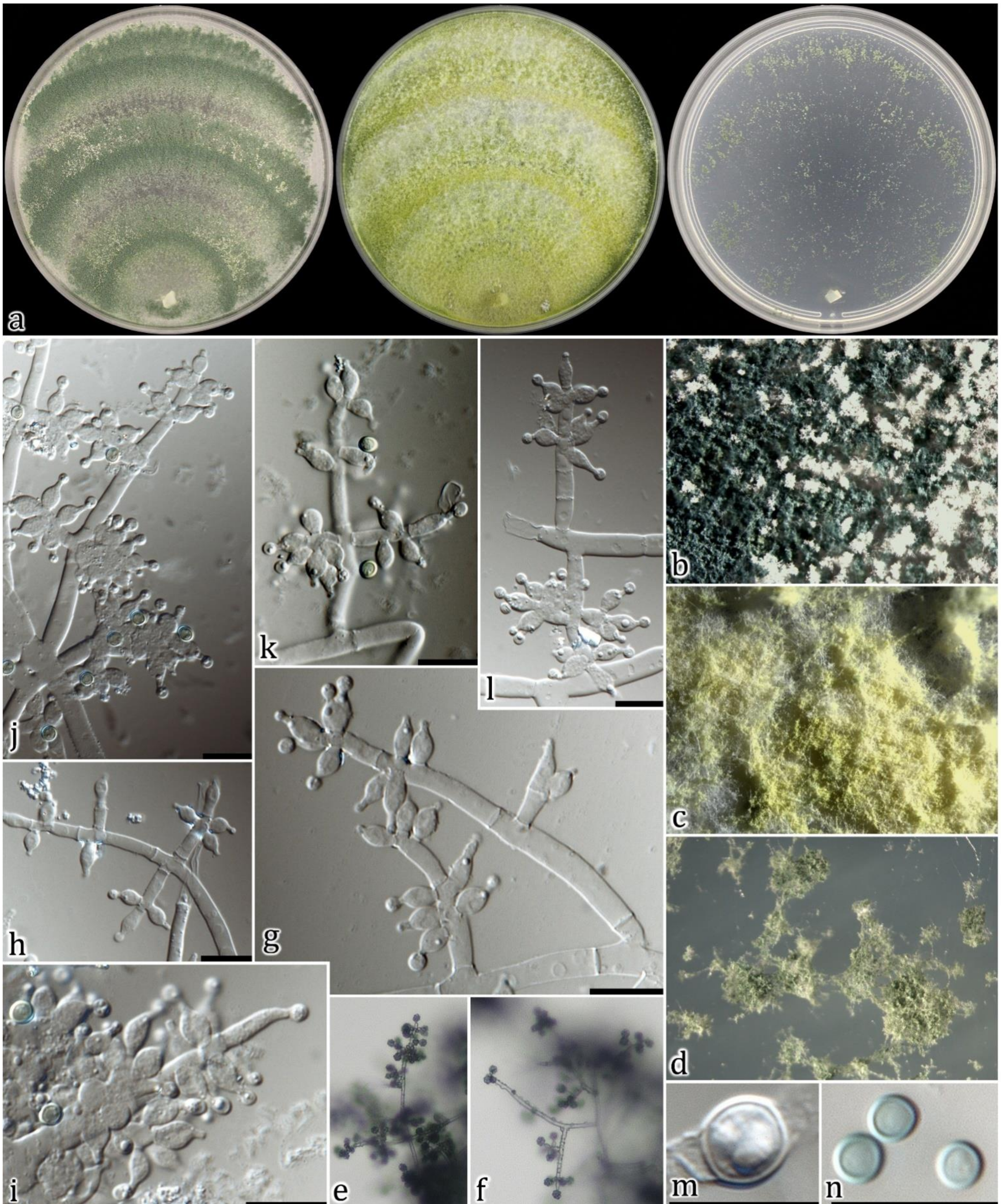

FIGURE 35. Morphological features characteristic of *Trichoderma harzianum* (*T. pseudoharzianum* nom. prov. dub.) (Subclade V or X), Strain Tri 191. a. Colonies of *T. harzianum* incubated on CMD for 7 days (left), PDA for 7 days (middle) and SNA for 7 days (right). b, c, d. Stereo microscope images, from CMD (top), PDA (middle) and SNA (bottom). e. f. Stereo microscope images from SNA. g, h, i, j, k, l. Conidiophores formed on CMD 1000x. m. Chlamydospore 1000x. n. Conidia 1000x. All scale bars are 10μm in length.

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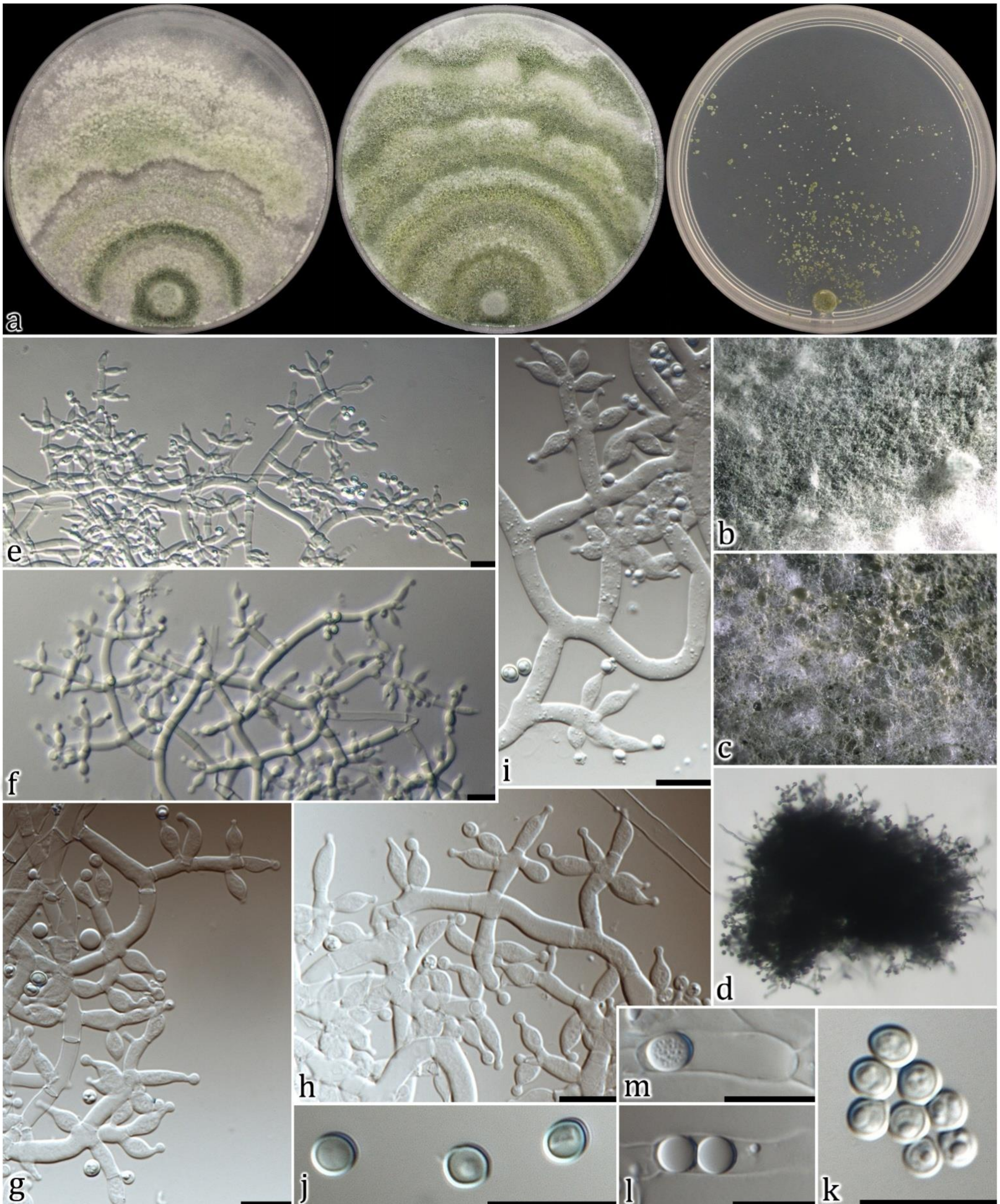

FIGURE 36. Morphological features characteristic of *Trichoderma harzianum* (*pseudoharzianum* nom. prov. dub.) (lone lineage), Strain Tri 216. a. Colonies of *T. harzianum* incubated on CMD for 7 days (left), PDA for 7 days (middle) and SNA for 7 days (right). b, c, d. Stereo microscope images, from CMD (top), PDA (middle) and SNA (bottom). e, f. Conidiophores on CMA 400x magnification. g, h, i. Conidiophores formed on CMD 1000x. j, k. Conidia 1000x., l, m. Chlamydospores 1000x. All scale bars are 10µm in length.

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***Trichoderma harzianum* Rifai, *Mycological Papers 116: 38. 1969* (*T. afroharzianum nom. prov. sensu Druzhinina et al. 2010*)**

Trichoderma afroharzianum nom. prov. is not a formally recognized name and is currently awaiting description (Druzhinina *et al.* 2010). The species forms part of the *T. harzianum* complex and was recognized by Druzhinina *et al.* (2010) to constitute a monophyletic lineage based on phylogenetic analyses using ef1α, cal1 and chi18-5 sequence data.

This study isolated 10 different strains from 5 different sites (Fig. 27 and Table 2) that were identified as *T. afroharzianum* nom. prov. based on a phylogenetic analysis using ef1α sequence data (Fig. 28 and Table 7). This group of strains was represented by Strain Tri 79 and was characterised morphologically (Figs. 37 and 38).

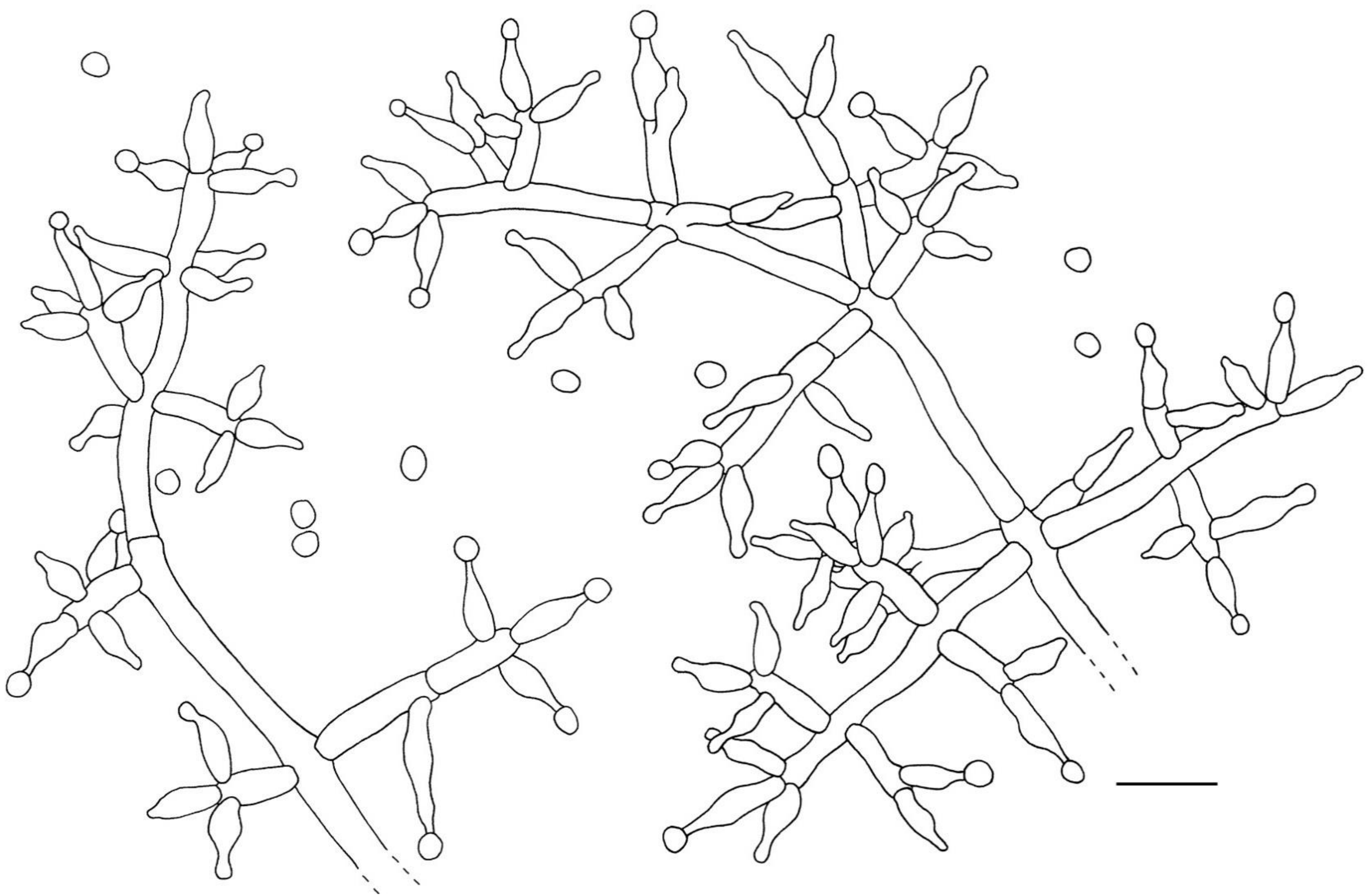

FIGURE 37. *Trichoderma afroharzianum* nom. prov. Line drawings from Strain Tri 79. Scale bar = 10µm

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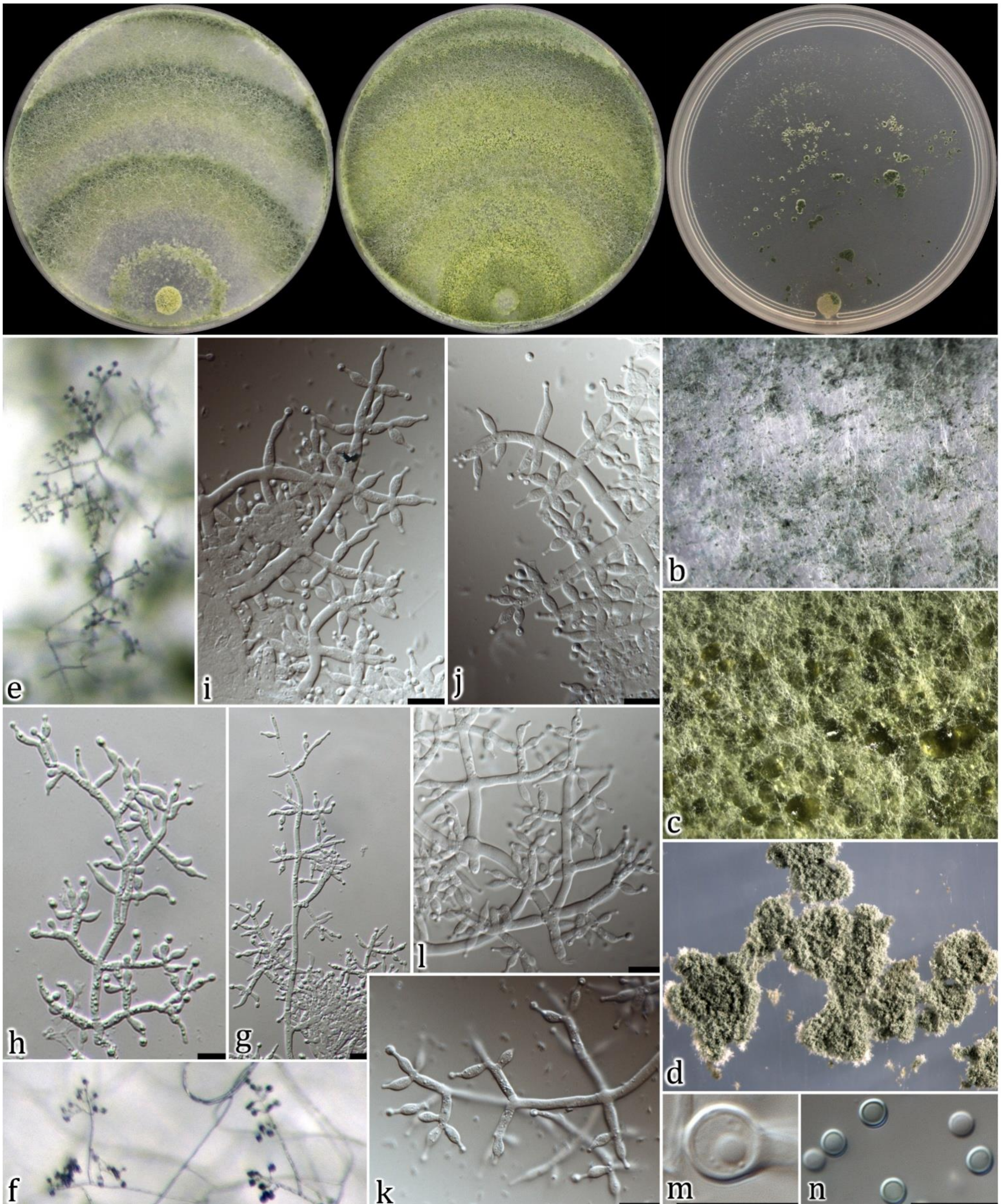

FIGURE 38. Morphological features characteristic of *Trichoderma afroharzianum* prov. nom., Strain Tri 79. a. colonies of *T. afroharzianum* incubated on CMD for 7 days (left), PDA for 7 days (middle) and SNA for 14 days (right). b, c, d. Stereo microscope images, from CMD (top) PDA (middle) and SNA (bottom). e, f. Stereo microscope image from CMA. g, h. Conidiophores formed on CMA 400x magnification. i, j, k, l. Conidiophores formed on CMD 1000x. m. Chlamydospore 1000x. n. Conidia 1000x. All scale bars are 10µm in length.

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The *Trichoderma virens* clade

This study isolated two different *Trichoderma* species, from three different sites, that belong to the *T. virens* clade (Fig. 39 and Table 2). These were identified as *T. virens* and *T. spirale* through phylogenetic analyses (Fig. 40, Table 8). *T. virens* is commercially used as a biocontrol organism (Baek *et al.* 1999)

***Trichoderma virens* Mill., Giddens and Foster ex Arx, Beihefte zur Nova Hedwigia 87: 288 (*1987*)**

Trichoderma virens was originally described as *Gliocladium virens* due to the *Gliocladium*-like branching patterns of its conidiophores (Miller *et al.* 1957). Rifai (1969) noted that some of the conidiophores produced by *G. virens* did in fact, resemble *Trichoderma* but still supported the placement of *G. virens* within *Gliocladium,* based on its phialide disposition. In contrast to this, Seiffert *et al*. (1985) reported that the *Gliocladium*-like anamorphs of *Hypocrea* species are distinct from *Gliocladium,* a view that was shared by Von Arx (1987) who formally transferred *G. virens* to *Trichoderma.* Investigations into the phylogenetic placement of *T. virens* based on 28S and ITS sequence data revealed that the species was, in fact, distinct from *Gliocladium* and grouped with *Trichoderma* species (Rehner and Samuels 1994, 1995). Bissett (1991) reviewed *Trichoderma* and re-described the anamorphs of *T. virens* in detail, based on the ex-type culture. *T. virens* is known to occur in Canada, New Zealand, Europe and the USA but has not been previously reported from South Africa. This study isolated two *Trichoderma* strains from a single site (Figs. 39 and Table 2) that were identified as *T. virens* based on ef1α sequence data (Fig. 40 and Table 8). A morphological analysis confirmed this result (Figs. 41 and 42).

Morphologically, Strain Tri 101 matched the descriptions of *T. virens* (Bissett 1991) and displayed fast growth rates on PDA and CMD, forming colonies of more than 70 mm in radius after 4 days of growth. Colonies on PDA and CMD also formed white floccose aerial mycelium as well as effuse fertile regions (Fig 41 a). Micromorphologically Strain Tri 101 also matched the descriptions of *T. virens* (Bissett 1991). Conidiophores were appressed with primary and secondary stage branches forming at sharp angles from of the central axis, whereas branches were orientated towards the conidiophore apex (Figs. 41 h, i, j, k). The phialides and conidia of Strain 101 were also consistent with *T. virens*, phialides developed in closely appressed whorls at the termini of branches and were mostly ampuliform or lageniform shaped

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FIGURE 39. Distributions of South African members of the *Trichoderma virens clade. T. virens* was only isolated in the southwestern parts of the country whereas *T. spirale* was isolated from soils collected near the central parts of South Africa. Black crosses indicate sample sites. ***T. spirale,*** **(red circles)** isolated from 2 different sites. ***T. virens,*** **(purple square)** isolated from 1 site. Note that some sites are located close to one another and cannot be visualised at this scale. Scale bar = 400km.

TABLE 8 *Trichoderma* strains from the *T. virens* clade used for phylogenetic comparisons are listed along with their respective GenBank accession numbers for ef1α sequence data.. Ex-type strains are indicated in bold and with a *'T'*.

GenBank	Taxon name	Strain number	References
EU280048	*H. crassa*	DAOM 164916	Hoyos-Carvajal *et al.* 2009
AY737727	***H. cuneispora* T**	G.J.S. 91-93	Samuels 2006
FJ179569	*H. gelatinosa*	C.P.K. 1618	Jaklitsch *et al.* 2008
AY737740	*H. gelatinosa*	G.J.S. 88-17	Samuels 2006
FJ860643	***H. longipilosa* T**	CBS 120953	Jaklitsch 2009
AY937430	*H. longipilosa*	DAOM 177227	Samuels 2006
AY937451	***H. strictipilis* T**	DAOM 172827	Samuels 2006
AY937450	*H. strictipilis*	DAOM 167072	Samuels 2006
AY750895	*H. strictipilis*	DAOM 167646	Samuels 2006
AY737745	***H. phyllostachydis* T**	G.J.S. 92-123	Samuels 2006
FJ860673	*H. phyllostachydis*	CBS114071	Jaklitch 2009
AY750894	*H. virens*	G.J.S. 01-287	Samuels 2006
FJ463366	*H. virens*	G.J.S. 95-78	Direct submission
FJ463365	*H. virens*	G.J.S. 95-80	Direct submission
EU280065	*H. virens*	DAOM 237548	Hoyos-Carvajal *et al.* 2009
FJ463364	*H. virens*	G.J.S. 06-114	Direct submission
FJ463362	*H. virens*	G.J.S. 01-201	Direct submission
GU591800	***H. virens* T**	GLI 39	Chaverri and Samuels
AY750881	***T. fertile* T**	DAOM 167161	Samuels 2006
EU280055	*T. helicum*	DAOM 230016	Hoyos-Carvajal *et al.* 2009
AY937433	*T. helicum*	DAOM 230017	Samuels 2006
EU280051	*T. longipile*	DAOM 1772271A	Hoyos-Carvajal *et al.* 2009
FJ463291	*T. spirale*	CBS 120963	Direct submission
EU280050	*T. spirale*	DAOM 229883	Hoyos-Carvajal *et al.* 2009
AY750896	*T. spirale*	DAOM 177714	Samuels 2006
EU280049	***T. spirale* T**	DAOM 183974	Hoyos-Carvajal *et al.* 2009

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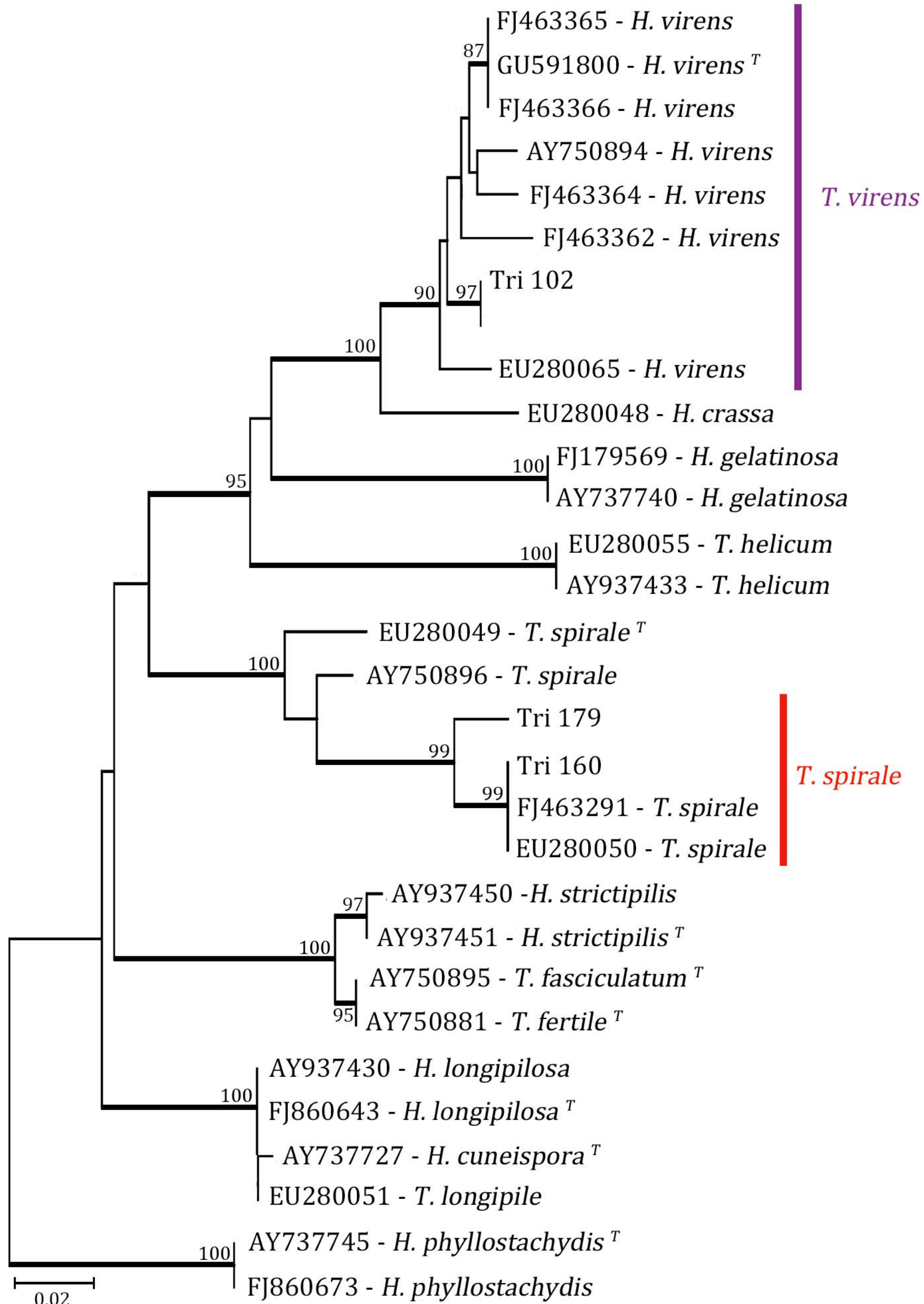

FIGURE 40. Neighbour joining tree based on ef1α sequence data indicating the phylogenetic relationships between accepted members of the *T. virens* clade and South African *Trichoderma* strains belonging to the same clade. Nodes which are supported by bootstrapping values that exceed 75% are indicated by thickened branches. Ex-type culture sequences are indicated with a *'T'*.

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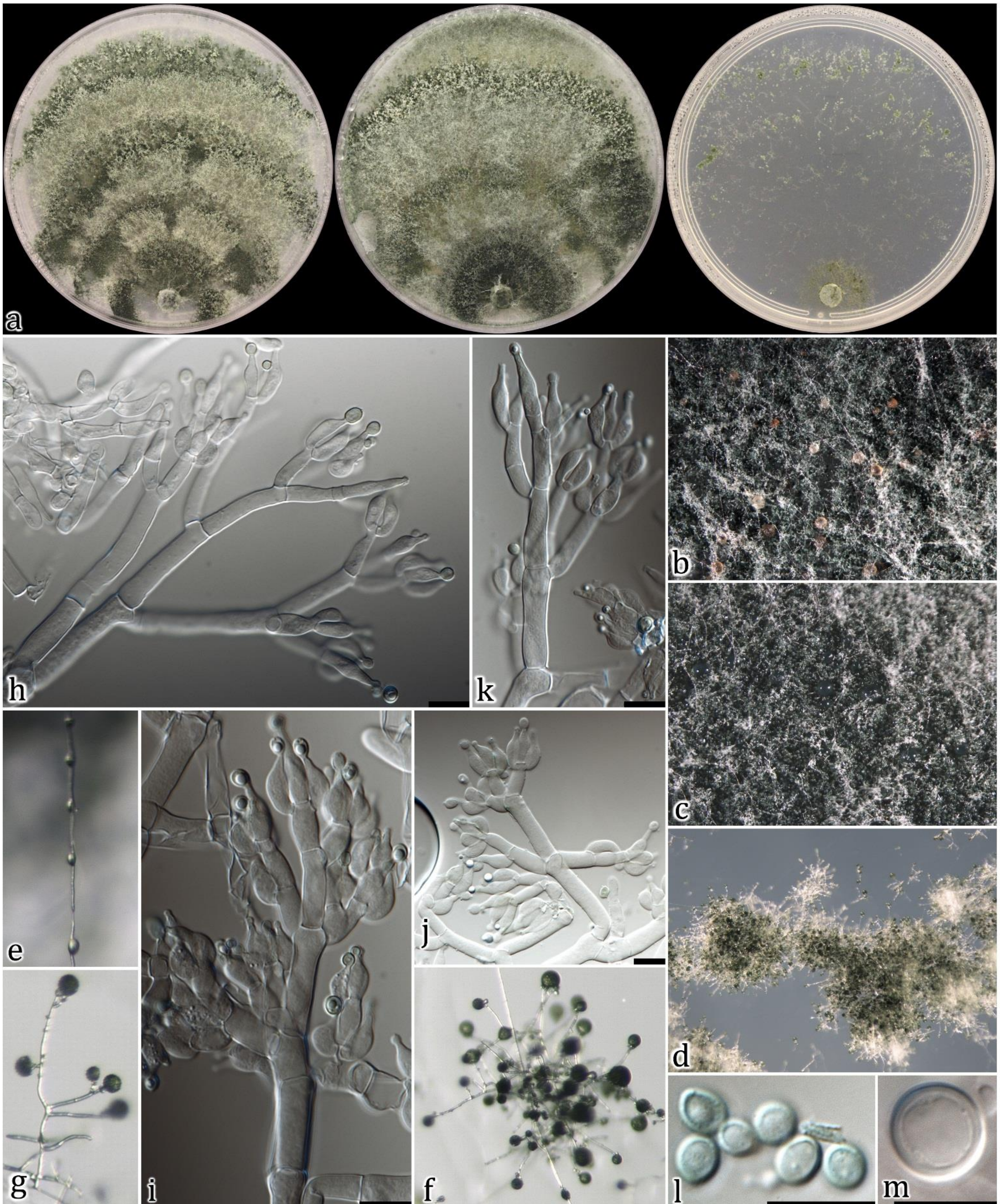

FIGURE 41. Morphological features characteristic of *Trichoderma virens*, Strain Tri 101. a. Colonies of *T. virens* incubated on CMD for 7 days (left), PDA for 7 days (middle) and SNA for 7 days (right). b, c, d. Stereo microscope images, from CMD (top), PDA (middle) and SNA (bottom). e, f, g. Stereo microscope images from SNA. h, i, j, k. Conidiophores formed on CMD 1000x magnification. l. Conidia 1000x. m. Chlamydospore 1000x. All scale bars are 10µm in length.

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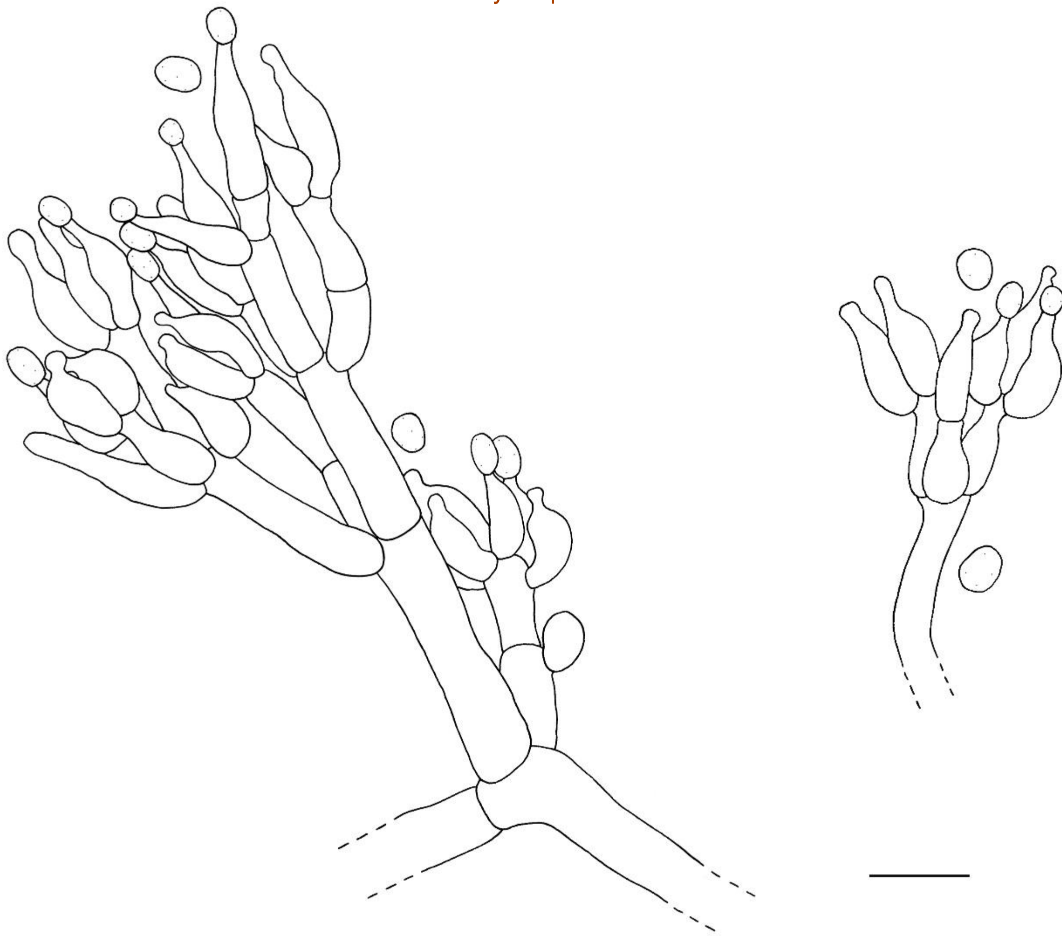

FIGURE 42. *Trichoderma virens*. Line drawings from Strain Tri 101. Scale bar = 10µm

(Figs. 44 h, i, j, k). Conidia were ellipsoidal and displayed slightly warted ornamentations (Fig. 41 l). Chlamydospores were formed on CMD (Fig. 41 m).

***Trichoderma spirale* Bissett, Can. J. Bot. 69: 2408. 1992**

Trichoderma spirale is morphologically associated with *T.* sect. *Pachybasium* (*sensu* Bissett 1991) based on its conidiophore appearance and the presence of sterile conidiophore elongations which sometimes extend from the conidiophore main axis (Bissett 1991). Phylogenetically though, this species belongs to the *T. virens* clade and is unrelated to the *Pachybasium* clade based on rbp2 sequence data (Samuels *et al.* 2006). *T. spirale* was re-described by Chaverri *et al.* (2003) in greater detail using the same ex-type strain as was used by Bissett in his original description (DAOM 183974). The species is known to display a cosmopolitan distribution (Chaverri *et al.* 2003). During this study, three *Trichoderma* strains, isolated from two different sites (Fig. 39 and Table 2), were identified as *T. spirale* based on ef1α sequence data (Fig. 40 and Table 8). A morphological analysis of Strain Tri 160 confirmed this result (Figs. 43 and 44).

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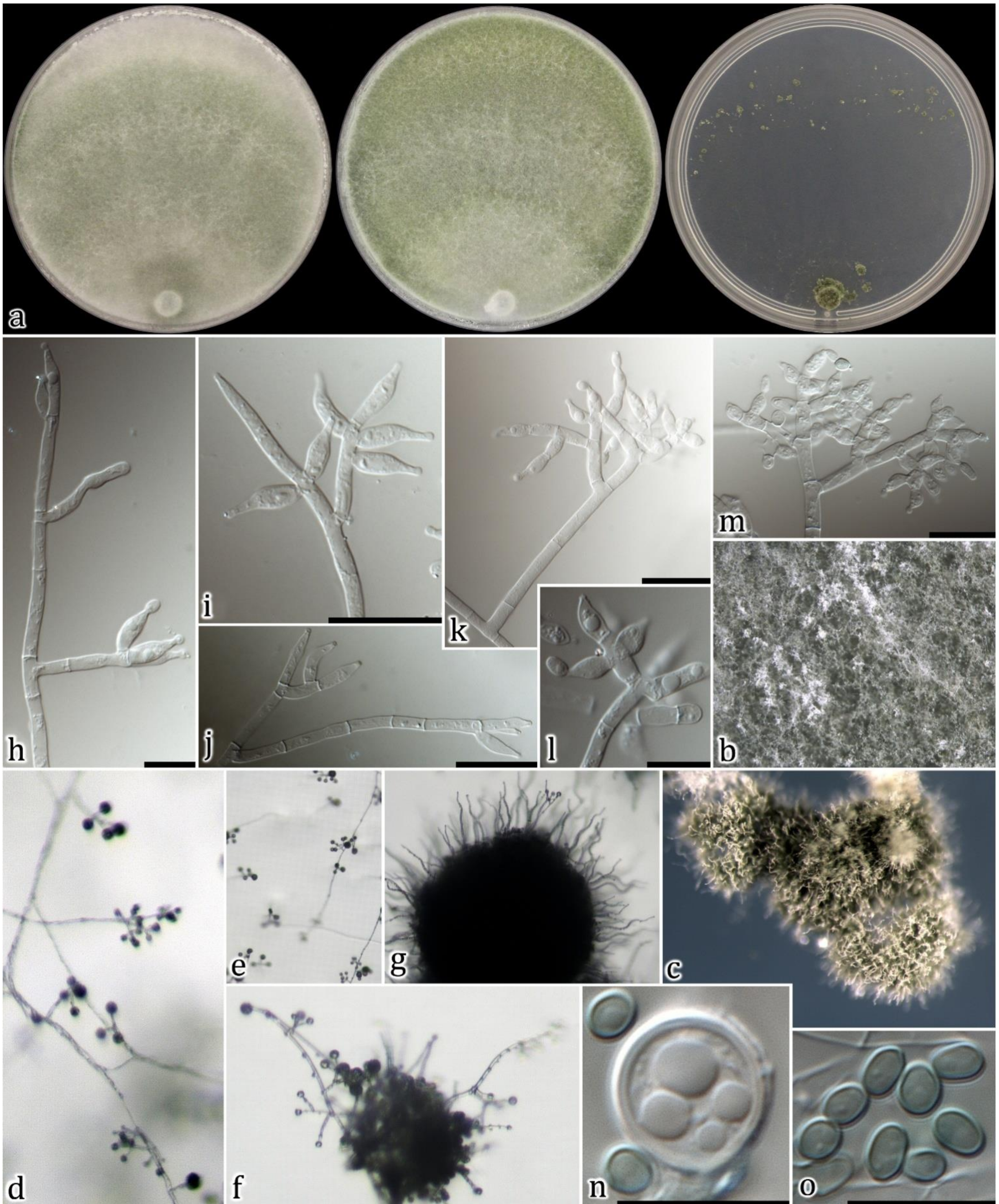

FIGURE 43. Morphological features characteristic of *Trichoderma spirale*, Strain Tri 160. a. Colonies of *T. spirale* incubated on CMD for 7 days (left), PDA for 7 days (middle) and SNA for 21 days (right). b, c. Stereo microscope images, from CMD (top) and SNA (bottom). d, e, f, g. Stereo microscope images from SNA. h, i, j, k, l, m. Conidiophores on CMA 1000x magnification. n. Chlamydospore 1000x. o. Conidia 1000x. All scale bars are 10µm in length.

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FIGURE 44. *Trichoderma spirale*. Line drawings from strain Tri 160. Conidiophores formed within pustules are illustrated on the right and conidiophores formed in effuse regions, on the left. Scale bar = 10μm

Morphologically, Strain Tri 160 fitted the description of *T. spirale* (Bissett 1991) and displayed rapidly growing colonies on CMA that reached 67 mm in three days when incubated at 25°C. Colonies formed dull yellow reverses in addition to forming indistinct mouldy aromas. On CMD and SNA colonies formed abundant sterile, white aerial hyphae that were convoluted or curved, appearing rugose under the dissecting microscope (Fig. 43 b, g). Micromorphologically Strain Tri 160 also matched the descriptions of *T. spirale* (Bissett 1991). Smooth, ellipsoid conidia (Fig. 43 o) developed, along with large thick walled chlamydospores (Fig. 43 n). Phialides were ampuliform in conidiophores developing in pustules (Figs. 43 m, l and 44), and lageniform when formed at effusely sporulating regions (Figs. 43 h, I and 44).

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Discussion

Few studies have identified *Trichoderma* species from the African continent (Druzhinina *et al.* 2006). Studies that have reported on South African species were mostly aimed at biotechnological or agricultural applications of the strains and were generally not focused on the diversity or ecology of local species (Chapter 1, page 26). Only 13 different *Trichoderma* species had been identified from South Africa prior to this study (Bisby 1939, Askew and Laing 1994, Samuels *et al.* 1998, Druzhinina *et al.* 2008, Kotze *et al.* 2011). Eight of these species were re-isolated during this study and 10 additional species were identified that were not previously known to occur in South Africa (Table 3). Some of these first reports, including *T. virens* and *T. asperelloides,* are recognised biocontrol organisms (Baek *et al.* 1999, Howell *et al.* 1999, Samuels *et al.* 2010). It would be interesting to know whether commercial biocontrol strains that are imported to South Africa persist in the natural environment following their application. Such biocontrol organisms are selected based on their abilities to interact with other organisms in their environment in a number of different ways (Chapter 1, page 23). The impact of *Trichoderma* biocontrol agents on non-target organisms is largely unknown and few studies have thus far investigated their secondary impacts (Brimner and Boland 2003).

The *Trichoderma* species isolated during this study represent five of the 12 major clades recognised in the genus (*sensu*: Druzhinina *et al.* 2005). In total, 19 different *Trichoderma* species were identified, four of which are believed to be new to science. Two of these new species are described in Chapter two and the remaining two are described in Chapter three

Several of the *Trichoderma* species identified during this study, such as *T. saturnisporum*, *T. harzianum, T. koningiopsis* and *T. gamsii*, are known to be cosmopolitan species that have been isolated from many different parts of the world (Jaklitch *et al.* 2006, López-Quintero *et al.* 2013). It is, therefore, not surprising to learn that these species display widespread distribution ranges within South Africa (Figs. 1, 13 and 27).

It is interesting to note that the most common *Trichoderma* species isolated in South Africa is the opportunistic human pathogen, *T. orientalis.* Thirty five different strains of *T. orientalis* were isolated at 16 different sites from around the country located in several different biomes including the Savannah, Fynbos and Nama Karoo (Fig. 13 and Table 2). This was not unexpected considering that the species is known to display cosmopolitan distributions and inhabits diverse niches around the world (Druzhinina *et al.* 2008). Druzhinina *et al.* (2008) previously

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reported identifying a single South African *Trichoderma* strain as *T. orientalis* but the extent to which the species was distributed within the country was not known. Other local species, such as *T. asperelloides* and *T. virens,* displayed restricted distributions in South Africa and were isolated from few sample sites relative to other species (Figs. 19 and 39 and Table 2). Through isolating and identifying *Trichoderma* strains from various different parts of the South Africa, this study has contributed to our current understanding of the diversity and distribution of this economically important genus within South Africa.

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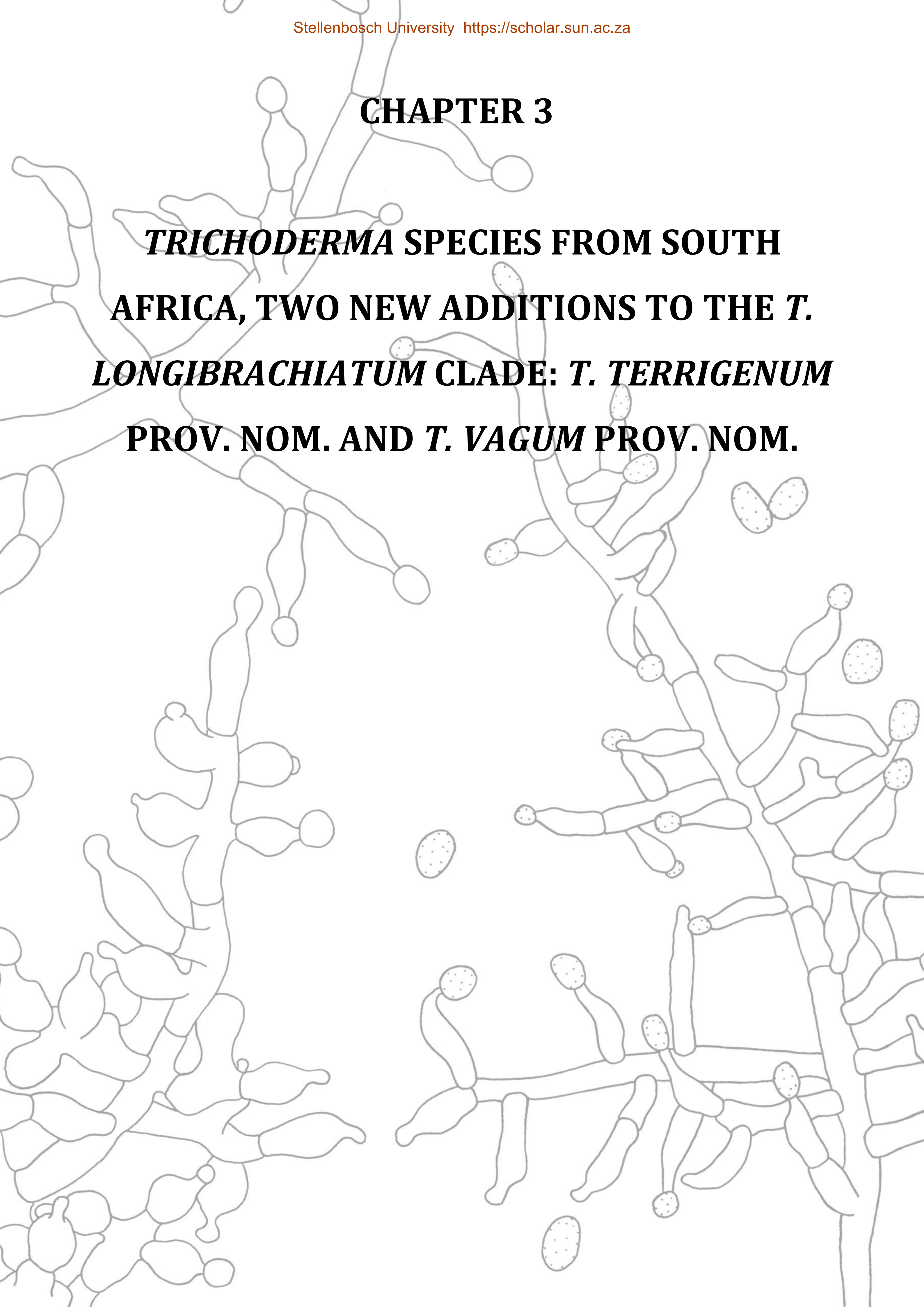

CHAPTER 3

TRICHODERMA SPECIES FROM SOUTH AFRICA, TWO NEW ADDITIONS TO THE *T. LONGIBRACHIATUM* CLADE: *T. TERRIGENUM* PROV. NOM. AND *T. VAGUM* PROV. NOM.

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Abstract

Two new *Trichoderma* species were discovered during a recent survey of the genus in South Africa and are described here as *T. vagum* prov. nom. and *T. terrigenum* prov. nom. A phylogenetic analysis based on elongation factor 1α sequence data grouped the new species with the *T. longibrachiatum* clade and showed that they did not match any of the existing species within this group. Morphologically, these species display traits that are unusual for members of the *T. longibrachiatum* clade. These include ampuliform shaped phialides and a weak tendency to form intercalary phialides. The two new species seem to be widely distributed and are represented by strains that were isolated from different locations around the world including Hawaii and the west coast of Isreal. *T. vagum* was isolated from soil in Clanwilliam. This species is closely related to *T. reesei* and *T. parareesei* and does not sporulate on SNA at 25°C. *T. vagum* can be distinguished from its relatives by its tendency to develop pustules on CMD. In addition, *T. vagum* forms phialides that are less broad than those of *T. reesei* and *T. parareesei*. *T. terrigenum* was isolated from soil in Klaver and is closely related to *T. andinensis*. These two species can be differentiated from one another based on their different growth rates on PDA and SNA.

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Introduction

Trichoderma Persoon is one of the most commonly isolated genera of fungi. Its members display cosmopolitan distributions and are known to inhabit diverse ecological niches (Harman *et al.* 2004, Samuels 2006). *Trichoderma* species have been reported from soils in tropical and Mediterranean climates as well as desert soils and salt marshes (Klein and Everleigh 1998, Migheli *et al.* 2009, Hoyos-Carvajal and Bissett 2011). They also inhabit niches such as water damaged buildings and marine environments and form endophytic relationships with plants (Thrane *et al.* 2001, Evans *et al.* 2003, Paz *et al.* 2010). The genus currently accommodates approximately 170 accepted species, the majority of which were described during the past two decades (e.g. Chaverri and Samuels 2004, Samuels *et al.* 2006, 2012, Jaklitsch 2009, 2011, Jaklitsch *et al.* 2013). *Trichoderma* species in culture are typically fast growing fungi which form sparse aerial mycelium and green or white pustules (Bissett 1991a). Microscopically, *Trichoderma* species display highly ramified conidiophores that bear slimy, single celled conidia that are green or hyaline (Rifai 1969).

The *T. longibrachiatum* clade is one of several monophyletic lineages currently recognized within the genus (Druzhinina *et al.* 2011). Kubicek *et al.* (2011) determined that it is also one of the youngest lineages in *Trichoderma* and is phylogenetically the most distinct from other clades in the genus (Druzhinina and Kubicek 2005). Members of the *T. longibrachiatum* clade generally show high optimal and maximum growth temperatures and colonies with yellowish reverse pigmentations (Jaklitsch 2009, Samuels *et al.* 2012). Microscopically, members of the *T. longibrachiatum* clade typically display conidiophores that are sparingly branched with distinct central axes bearing lageniform shaped phialides singly or in whorls along the axis or branches (Samuels *et al.* 2012). Conidia are mostly smooth-walled with ellipsoidal or oblong dimensions (Samuels *et al.* 2012).

Rifai first described *Trichoderma longibrachiatum* in his review of the genus in 1969. He referred to *T. longibrachiatum* as a 'species aggregate', recognizing that it probably represented a complex of several distinct taxa (Rifai 1969). Bissett (1984, 1991a) later reviewed the genus and elevated some of the aggregate species recognized by Rifai, including *T. longibrachiatum*, to sectional status. During subsequent revisions, Bissett (1991b) refined *T.* sect. *Longibrachiatum,* largely based on micromorphological observations, and recognized a total of five different species. Later, Kühls *et al.* (1997) used ITS 1 and 2 sequence data to infer the phylogenetic relationships between a number of *Trichoderma* species and found that *T.* sect.

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Longibrachiatum constituted a monophyletic clade whereas other sections such as *T.* sect*. Pachybasium* and *T.* sect*. Trichoderma* were paraphyletic. In light of these findings, Samuels *et al.* (1998) reviewed *T.* sect*. Longibrachiatum* and recognized most of the species described by Bissett. They also described several new taxa, raising the total number of accepted species in *T.* sect*. Longibrachiatum* to ten (Samuels *et al.* 1998). Bissett *et al.* (2003) studied *Trichoderma* strains that were collected in Asia and added two species to the *T. longibrachiatum* clade. This study was based on the combined use of physiological, morphological and phylogenetic data and illustrated the advantages of employing a multidisciplinary approach to define species in *Trichoderma* (Bissett *et al.* 2003). Similarly, Atanasova *et al.* (2010) made use of carbon source utilization profiling to prove the novelty of *T. parareesei,* which is morphologically similar to *T. reesei* Simmons. Druzhinina *et al.* (2012) and Samuels *et al.* (2012) reviewed the *T. longibrachiatum* clade based on phylogenetic analyses using tef1, cal1 and chi8-5 sequence data. They added eight new species to the *T. longibrachiatum* clade while several other new species were also recognized and are awaiting formal description (Druzhinina *et al.* 2012). The total number of *Trichoderma* species currently recognized within the *T. longibrachiatum* clade is twenty one (Samuels *et al.* 2012).

A number of *Trichoderma* species from the *T. longibrachiatum* clade are of economic and medical importance. Some species, such as *T. reesei,* are producers of cellulolytic and hemicellulolytic enzymes that are used in the biofuel, food and textile industries (Zeilinger *et al.* 1998, Galante *et al.* 1998). Other members of the *T. longibrachiatum* clade, such as *T. citrinoviride* (Bissett 1983)*, T. longibrachiatum* (Rifai 1969) and *T. orientalis* (Samuels and Petrini 1998) have the capacity to cause respiratory illnesses as well as invasive mycoses in immunocompromised people (Burrell 1991, Samuels *et. al.* 1998, Kratzer 2006, Druzhinina *et al.* 2008, 2011, Torky 2012. Also see page 20). Members of the *T. longibrachiatum* clade are not commercially applied in the agricultural sector as biocontrol agents or crop growth promoters like some other *Trichoderma* species. They are also not normally known to cause harm to commercially produced mushroom crops (Samuels *et al.* 1998).

Despite the importance of *Trichoderma* species in industry, agriculture and healthcare the diversity of the genus in South Africa has not been comprehensively investigated. Studies that report on native strains have identified a total of thirteen different species from a restricted number of locations across South Africa and many parts of the country remain unstudied (Samuels *et al.* 1998, Chaverri and Samuels 2004, Jaklitsch *et al.* 2006, Druzhinina *et al.* 2008, Kubicek *et al.* 2009, De Respins *et al.* 2010, Druzhinina *et al.* 2010, Mutawila *et al.* 2011). Two

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species isolated from the botanically diverse Fynbos biome in the southwestern parts of the country were found to constitute new species within the *T. longibrachiatum* clade and are described here. The objectives of this study were, therefore, to characterize and describe *T. vagum* prov. nom. and *T. terrigenum* prov. nom. and to compare these new species to other *Trichoderma* species in the *T. longibrachiatum* clade through phylogenetic and morphological analyses.

Materials and methods

Soil sampling and Trichoderma *isolations*

Soils were collected from 173 different sites across South Africa. Standard soil dilution plating protocols were used to isolate *Trichoderma* strains from soil samples (Crous et al. 2009). Five grams of soil were suspended in 100ml of sterilized distilled water, this was then diluted 100, 1000 and 10 000 times before being plated onto potato dextrose agar (PDA) (Merck, Germany) supplemented with 100 $mg.l^{-1}$ chloramphenicol (Applichem, South Africa) and 50 $mg.l^{-1}$ streptomycin (Applichem, South Africa) to inhibit bacterial growth. Petri dishes were incubated for one week at 25°C before being inspected under low magnification. Fungal colonies that displayed morphological characteristics typical of *Trichoderma* were transferred onto fresh oatmeal agar.

For morphological identification, strains were grown on corn meal dextrose agar (CMD) for 7 days at 25°C under a 12h day/night lighting regime (Jaklitsch 2009). Strains were then divided into morphological groups based on their colony morphologies and representatives from each group were selected for identification.

Phylogenetic analysis

DNA was extracted from *Trichoderma* group representatives grown on CMD for 7 days using a CTAB protocol described by Möller *et al.* (1992). The Internal Transcribed Spacer region (ITS) and elongation factor 1-α (ef1α) loci were amplified using primer sets ITS4 - ITS5 and EF1F (White *et al.* 1990)- EF2R (Jacobs *et al.* 2004) respectively (Thermal cycle profiles are described elsewhere, see page 52). The resulting amplicons were sequenced using a BigDye termination cycle premix kit (Applied Biosystems, California, USA) and sequencing reaction products were analysed using an ABI Prism 310 genetic analyser.

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ITS sequence data were submitted to the online *Trichoderma* barcode based identification resource Trichokey V. 2.0 for preliminary identifications (Available from: http://www.isth.info). *Trichoderma* species in the *T. longibrachiatum* clade were investigated further through phylogenetic analyses based on ef1α sequence data. A dataset was assembled from the NCBI and the ISTH websites that consisted of sequence data from representative strains of the *T. longibrachiatum* clade and included ex-type strains (Table 1). Sequence data from South African *Trichoderma* strains were then added to the dataset for comparison.

Sequence datasets were trimmed using the software suite Geneious v. 4 (Kearse *et al.* 2012) (Available from: http://www.geneious.com) and were aligned using MAFFT v. 7.023b (Katoh and Standley 2013) (Available from http://mafft.cbrc.jp/alignment/software). The ambiguously aligned regions within the dataset were identified through the use of the Gblocks server available online (http://molevol.cmima.csic.es/castresana/ Gblocks_server.html). These ambiguous regions were then excluded from the phylogenetic analyses. The phylogenetic relationship between *Trichoderma* strains represented in the datasets were inferred through neighbor joining analyses using MEGA v. 6 (Tamura *et al.* 2013) (Available from: http://www.megasoftware.net). Node assurances were calculated by performing a bootstrap analysis with 1000 replications.

Morphological characterization

The new species were cultured on CMD supplemented with 2% glucose (Sigma, Germany), PDA (Merck, Germany) and synthetic nutrient poor agar (SNA), formulated according to Crous *et al.* (2009) in 90 mm Petri dishes. Inoculation materials were prepared by growing *Trichoderma* strains on CMD for three days before removing 5 mm agar plugs from the growing edges of the colonies (Jaklitsch 2009). Plugs were transferred to the various different media with the mycelia side facing the media (Jaklitch 2009). Petri dishes were incubated at 15°C, 25°C, 30°C and 35°C under 12h day/night lighting regimes using cool white fluorescent light (Jaklitsch 2009). Colonies were measured daily and were photographed every seven days.

Results

Species identifications

Preliminary attempts to identify native *Trichoderma* strains were based on ITS sequence data using the online resource Trichokey v. 2.0. This did not result in a match being found between

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Strain Tri 13 any of the voucher sequences represented in the ISTH database. Strain Tri 59, on the other hand, was identified by Trichokey as *H. andinensis* Samuels & Petrini, although the identification reliability was marked as 'low' in light of the fact that this species is represented by only a single voucher strain. The two new species were isolated from the Fynbos biome in the southwest parts of the country at two different locations: Klawer (*T. vagum*, 31°46'10.20"S 18°38'15.39"E) and Clanwilliam (*T. terrigenum*, 32°9'23.85"S 18°52'56.10"E) (Fig. 1). A phylogenetic analysis based on ef1α sequence data was conducted to further investigate the identity of the native strains (Fig. 2 and Table 1). *T. vagum* seemed to cluster together with *T. reesei* Simmons and *T. parareesei* Atanasova. *T. terrigenum* clustered with *T. andinensis* Samuels and Petrini (Fig. 2).

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FIGURE 1 Isolation locations of the two new *Trichoderma* species that belong to the *T. longibrachiatum* clate which were isolated during this study. The new species ***T. vagum*** **prov. nom. (purple triangle)** and ***T. terrigenum*** **prov. nom. (red triangle)** seemed to show restricted distributions in the south-western parts of the country. Black crosses indicate sites sampled which did not yield representatives from the *T. longibrachiatum* clade. Scale bar = 400km.

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TABLE 1 *Trichoderma* strains from the *T. longibrachiatum* clade used for phylogenetic comparisons are listed along with their respective GenBank accession numbers for ef1α sequence data. Ex-type strains are indicated in bold and with a *'T'*.

GenBank accession	Taxon name	Strain number	References
AY956321	*H. andinensis*	GJS 90-140	Samuels 2006
EU280042	*H. andinensis*	DAOM 220821	Hoyos-Carvajal *et al.* 2009
JN133570	*H. andinensis*	G.J.S. 09-62	Chaverri *et al.* 2013
AY857282	*H. orientalis*	TUB F-837	Druzhinina *et al.* 2005
EU401593	*H. orientalis*	G.J.S. 91-157	Druzhinina *et al.* 2008
AY937421	*H. orientalis*	CBS 243.63	Samuels 2006
EU280038	*H. orientalis*	DAOM 220863	Hoyos-Carvajal *et al.* 2009
EU401581	***H. orientalis*** ^{T}	G.J.S. 88-81	Druzhinina *et al.* 2008
FJ860666	*H. parestonica*	C.P.K. 2427	Jaklitch 2009
FJ860667	*H. parestonica*	CBS 120636	Jaklitch 2009
FJ860705	*H. subalpina*	CBS 119128	Jaklitch 2009
FJ860706	*H. subalpina*	C.P.K.3126	Jaklitch 2009
EU401616	*T. aethiopicum*	PPRC H5	Druzhinina *et al.* 2008
EU401614	*T. aethiopicum*	PPRC J11	Druzhinina *et al.* 2008
JN175591	*T. citrinoviride*	G.J.S. 90-111	Druzhinina *et al.* 2012
EU338334	*T. citrinoviride*	DAOM 139758	Degenkolb *et al.* 2008
AY937419	***T. effusum*** ^{T}	DAOM 230007	Samuels 2006
FJ763149	*T. flagellatum*	PPRC ET7	Samuels *et al.* 2012
FJ763184	***T. flagellatum*** ^{T}	PPRC ET58	Samuels *et al.* 2012
AY937423	*T. ghanense*	G.J.S. 95-137	Samuels 2006
EU280043	*T. ghanense*	DAOM 220800	Hoyos-Carvajal *et al.* 2009
JN175583	***T. gillesii*** ^{T}	G.J.S. 00-72	Druzhinina *et al.* 2012
JN175598	***T. gracile*** ^{T}	G.J.S. 10-263	Druzhinina *et al.* 2012
AY937425	*T. konilangbra*	G.J.S. 96-147	Samuels 2006
JN175569	*T. longibrachiatum*	G.J.S. 07-21	Druzhinina *et al.* 2012
AY937412	***T. longibrachiatum*** ^{T}	ATCC18648	Samuels 2006
GQ354351	*T. parareesei*	TUB F-430	Druzhinina *et al.* 2010
GQ354353	***T. parareesei*** ^{T}	TUB F-1066	Druzhinina *et al.* 2010
GQ354373	*T. parareesei*	G.J.S. 07-26	Druzhinina *et al.* 2010
JN175572	*T. pinnatum*	G.J.S. 02-120	Druzhinina *et al.* 2012
JN175571	***T. pinnatum*** ^{T}	G.J.S. 04-100	Druzhinina *et al.* 2012
AY937429	*T. pseudokoningii*	G.J.S. 81-300	Samuels 2006
JN175588	***T. pseudokoningii*** ^{T}	G.J.S. NS-19	Druzhinina *et al.* 2012
EU280037	*T. pseudokoningii*	DAOM 167678	Hoyos-Carvajal *et al.* 2009
GQ354347	*T. reesei*	G.J.S. 97-177	Druzhinina *et al.* 2010
Z23012	*T. reesei*	QM9414	Nakari *et al.* 1993
AY956321	*H. andinensis*	GJS 90-140	Samuels 2006
EU280042	*H. andinensis*	DAOM 220821	Hoyos-Carvajal *et al.* 2009
JN133570	*H. andinensis*	G.J.S. 09-62	Chaverri *et al.* 2013

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TABLE 1 Continued

GenBank accession	Taxon name	Strain number	References
JN175601	*T. reesei*	G.J.S. 09-74	Druzhinina *et al.* 2012
AY937441	*T. rossicum*	DAOM 230011	Samuels 2006
EU280062	*T. rossicum*	DAOM 233977	Hoyos-Carvajal
JN182279	*T. saturnisporum*	C.P.K. 1268	Druzhinina *et al.* 2012
JN258682	*T. saturnisporum*	C.P.K. 3406	Druzhinina *et al.* 2012
AY937414	*T. saturnisporum*	CBS 886.72	Samuels 2006
EU595046	*T. saturnisporum*	SF21	Sadfi-Zouaou *et al.* 2009
AY865642	*T. saturnisporum*	IMI 146852	Druzhinina *et al.* 2005
JN388897	*T. saturnisporum*	ATCC 28023	Druzhinina *et al.* 2012
JN182273	*T. sinensis*	C.P.K. 530	Druzhinina *et al.* 2012
AY750889	*T. sinensis*	DAOM 230004	Samuels 2006
JN175597	***T. solani*** [T]	G.J.S. 08-81	Druzhinina *et al.* 2012
AY937441	*T. virens*	DAOM 230011	Samuels 2006
FJ463366	*T. virens*	G.J.S 95-78	Chaverri *et al.* 2008
JN182275	*T. sp.*	C.P.K. 667	Druzhinina *et al.* 2012
FJ619256	*T. sp.*	OY14707	Paz *et al.* 2010

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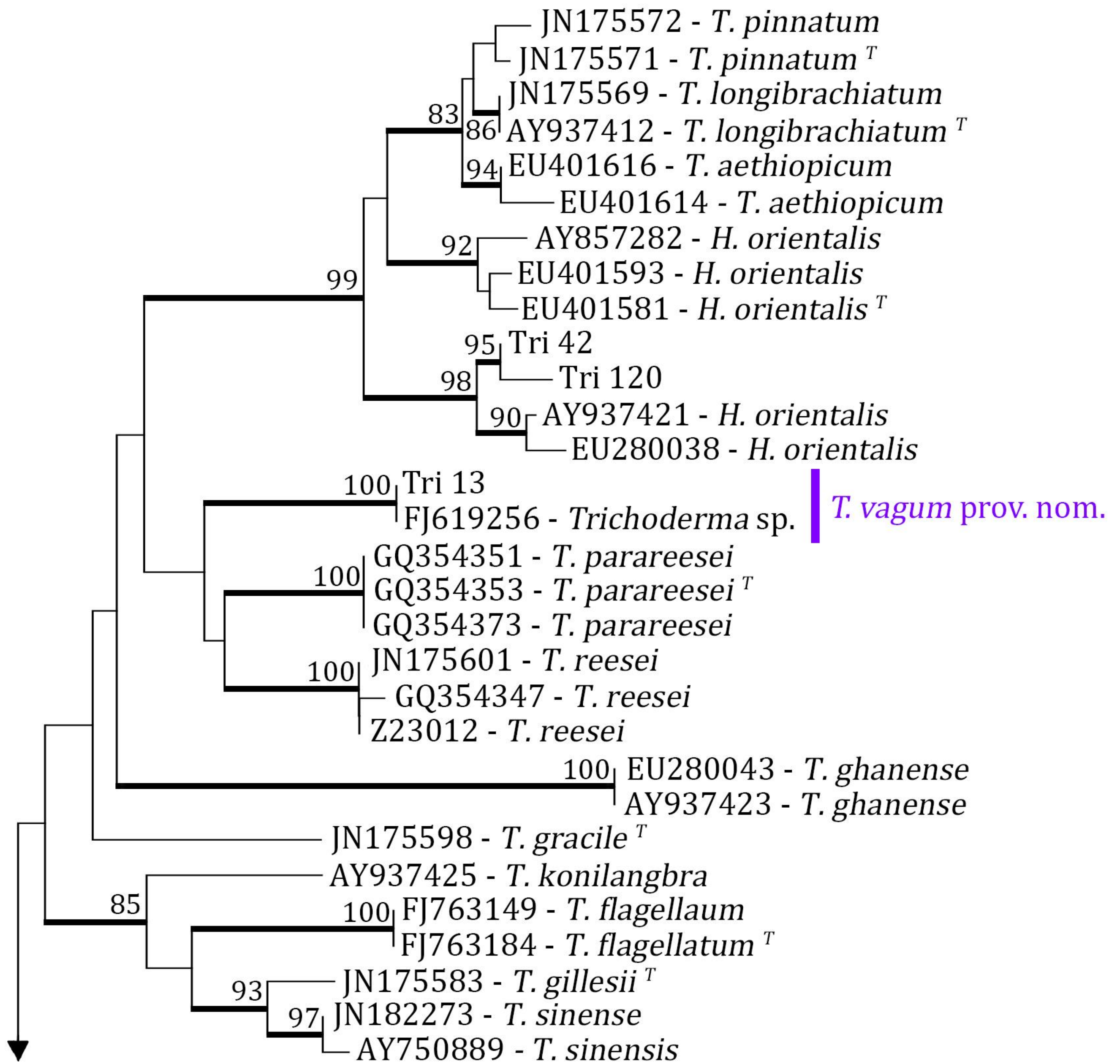

FIGURE 2. Neighbour joining tree based on ef1α sequence data indicating the phylogenetic relationships between accepted members of the *Trichoderma longibrachiatum* clade and the new *Trichoderma* species. Nodes which are supported by bootstrapping values that exceed 80% are indicated by thickened branches. Ex-type culture sequences are indicated with a '*T*'.

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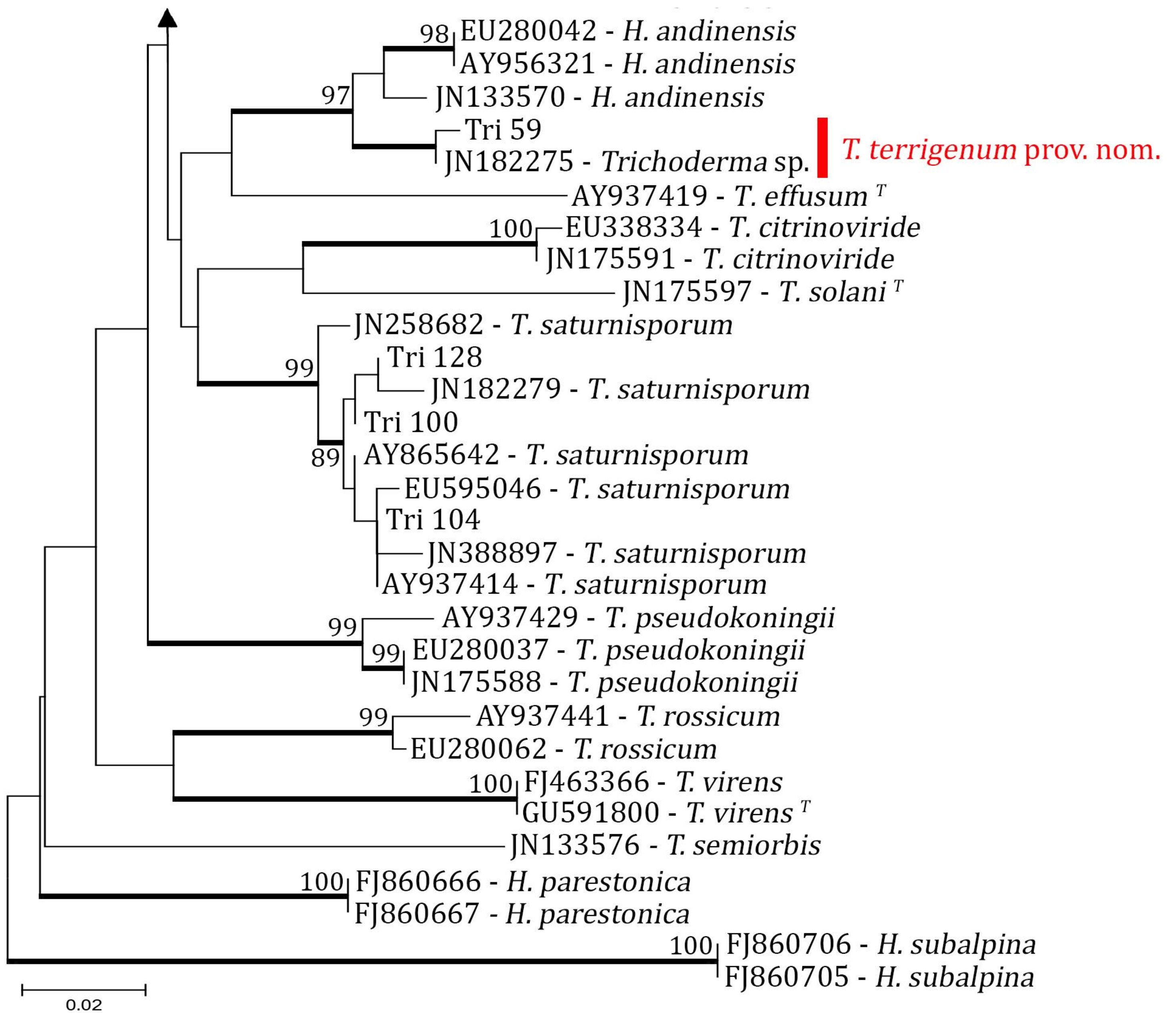

FIGURE 2 continued

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Taxonomy

Trichoderma vagum I. L. du Plessis, I. S. Druzhinina, O. Yarden and K. Jacobs prov. sp. nov.* **- Figs. 3, 4**

Etymology. Latin, *vagum:* meaning wanderer, in reference to the fact that the species has been isolated from terrestrial as well as marine environments from different parts of the world.

CMD; optimum growth temperature at 25°C. Colony radius after 72h when grown at 25°C = 53 mm. Colonies cover the Petri dish on day four, at 15°C colonies reach 17 mm radius after 72h incubation and 46 mm on day seven, at 30°C colonies reach 39 mm radius after 72h and cover the Petri dish on day five. At 35°C colonies reach 8 mm radius after 7 days. CMD at 25°C; Colony hyaline in sterile zones, thin, prominent concentric zones formed as a result of photo

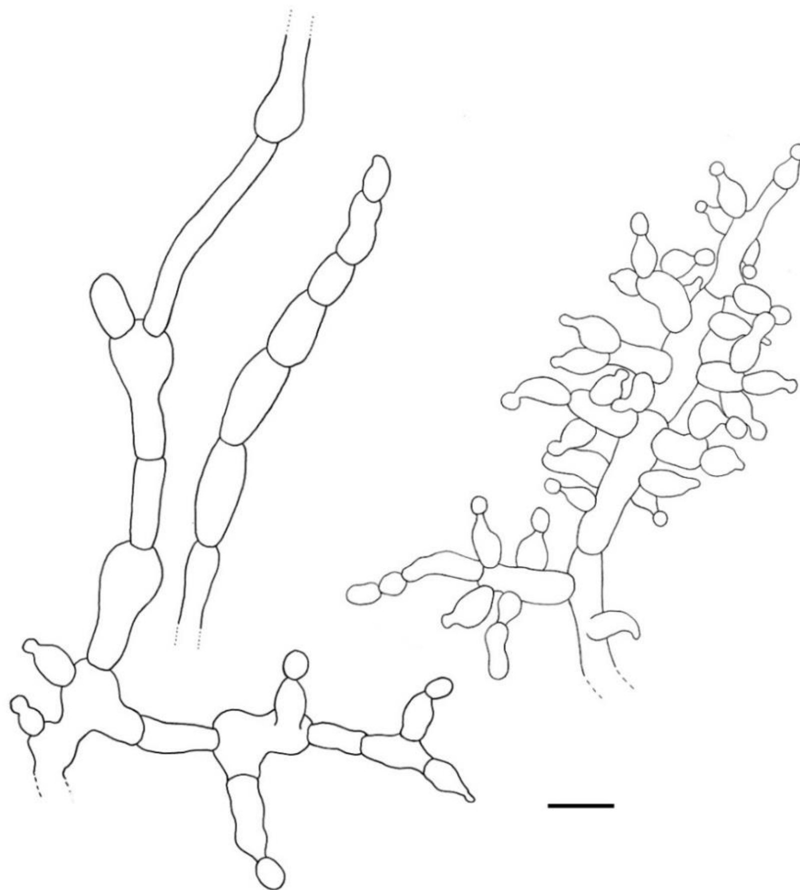

FIGURE 3. *Trichoderma vagum* prov. nom. line drawings from Strain Tri 13 (holotype) cultured on CMD. Scale bar is 10 µm in length

* Please note that the descriptions provided here are preliminary and should not be cited as they will be published formally elsewhere

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induced sporulation brought about by the 12 h day/night lighting regime during incubation. Aerial hyphae scant, no coilings produced, autolytic excretions visible at 25°C. No distinct odours or diffusible pigments produced. Colourless exudates produced in small quantities at mature areas of the colony which sporulate heavily. Conidiospore formation starts on day three, fertile pustules develop which bear light green conidia, becoming darker as the pustules mature. Pustules mostly 2-3 mm in diameter. Smaller conidiation structures, <0.5 mm in diameter, are also present and give some colony zones a grainy texture.

Conidiophores appear *Verticillium*-like. However, phialides are not born in regular levels around the axis but rather form in unpaired arrangements. Conidiophores branch asymmetrically from the supporting hyphae which often forms irregular thickenings throughout the length of the conidiophore, becoming as thick as 10 µm at some parts before tapering off towards the terminus. Phialides commonly borne directly from the stipe or on primary branches, secondary branches are not produced. Conidiophores are straight or curved. Branches from the stipe are unpaired and occur at irregular intervals. Such branches are most commonly at right angles relative to the conidiophores axis, or, are slightly oriented towards the conidiophore terminus. Conidiophore elongations smooth and often reach ± 200 µm in length, the terminus often bearing fertile phialides. Phialides solitary or in whorls of 2-3(-5), mostly divergent, or inclining towards the conidiophore terminus. Phialides $(4.5-)6.0 \pm 8.5(-10.5) \times (3.0-)3.5 \pm 4.5(-5.0)$ µm (n=50), l/w ratio$: 1.5 \pm 2.5(-3.0)$. Phialide base: $(1.5-)2.0 \pm 2.5(-3.5)$ µm wide, phialides ampuliform with symmetrical or slightly bent necks, thickest most around the middle. Conidia $4.0 \pm 5.0(-6.0) \times (2.0-)2.5 \pm 3.0$ µm, l/w ratio: $1.5 \pm 2.0\ (-2.5)$, borne in wet heads, smooth, oval, green, spores contain a small number of large guttules or a single large guttule. Round, smooth-walled chlamydospores are formed on CMD in mature colonies.

On CMD at 15°C: colony hyaline, regular margin. Colonies grown in darkness do not sporulate and also do not form hyphal coilings or display autolytic excretions. Colonies grown at 30°C similar to those formed at 25°C in many regards. However, pustules tend to be more loosely arranged within tufts and are not as compact as can be seen at 25°C. Colonies grown at 35°C appearing strikingly different than those grown at 25°C: growth rate much slower at 35°C, colonies are not zonate in response to day/night lighting incubation regimes whereas

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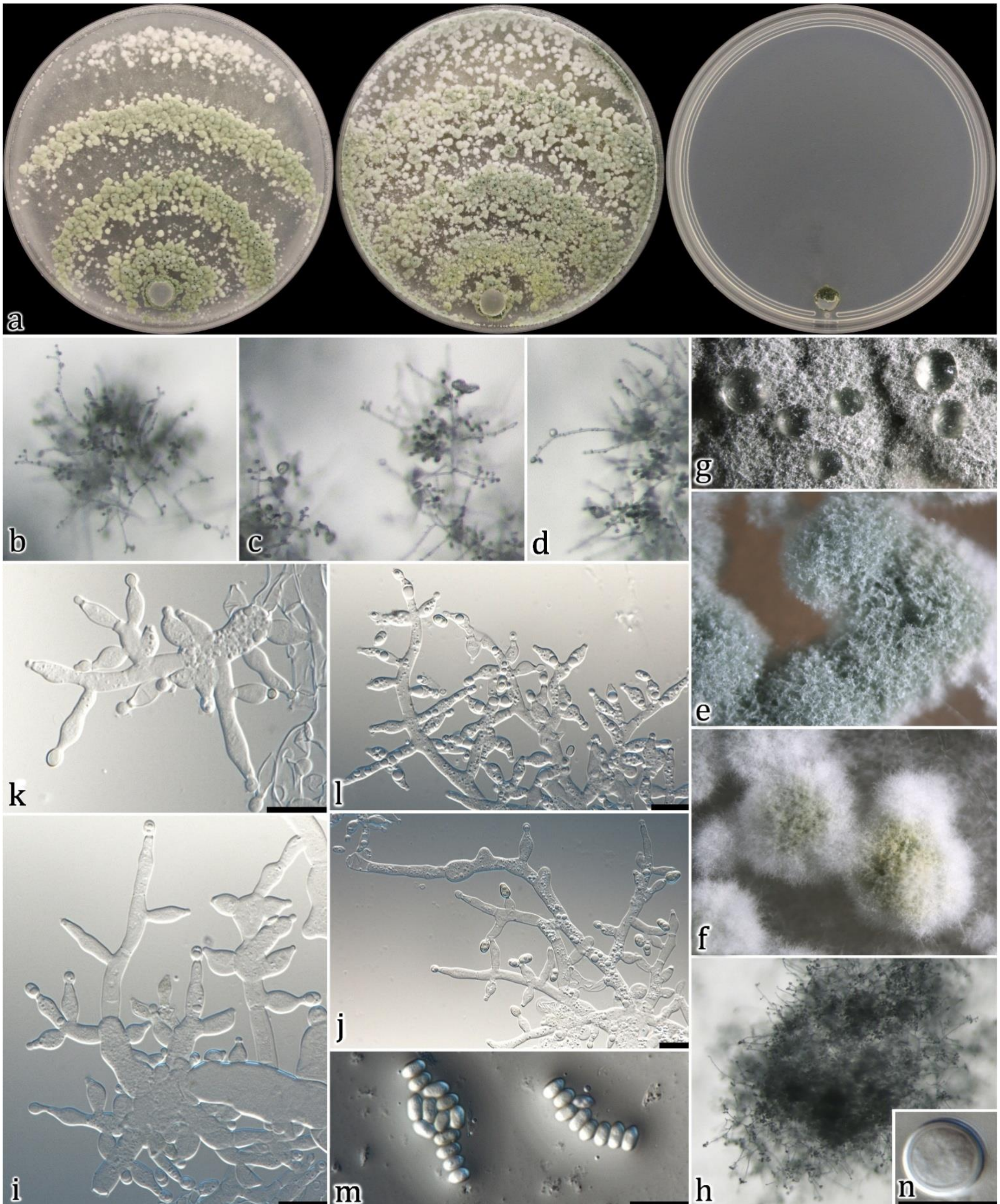

FIGURE 4. Morphological features characteristic of *Trichoderma vagum* prov. nom., (holotype strain Tri 13). a. Colonies of *T. vagum* incubated on CMD for 7 days (left), PDA for 7 days (middle) and SNA for 7 days (right). b, c, d, e, f. Stereo microscope images from PDA. g, h. Stereo microscope images, from CMD. i, j, k, l. Conidiophores formed on CMD 1000x magnification. m. Smooth oblong conidia 1000x. n. Chlamydospore 1000x . All scale bars are 10µm in length.

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this effect is conspicuous at 25°C, pustules are much smaller forming granular textures relative to the much larger pustules formed at 25°C. No exudates can be seen at 35°C, whereas they do form at 25°C, yellow soluble pigments clearly visible at 35°C whereas this is not the case at 25°C.

PDA: optimum growth temperature at 30°C when grown on PDA. Colony radius after 72h when grown on PDA at 30°C = 71mm, colonies cover the Petri dish on Day Four, at 15°C colonies reach 18 mm radius after 72h and 54 mm at Day Seven. At 25°C colonies reach 59 mm radius after 72h and cover the Petri dish on Day Four. At 35°C colonies reach 18 mm radius after Day Seven. PDA at 25°C: Colony hyaline at sterile zones, zonation less conspicuous than can be seen on CMD, sporulation occurring in pustules at first, light shades of green, later forming much darker conidia in effuse arrangements between pustules as the colony matures, no exudates produced. Pale orange soluble pigment produced, not very conspicuous. No distinct odours produced. Sporulation starts on day four. At 30°C colonies resemble those formed at 25°C. However, pustules tend to be more loosely arranged, sometimes forming on top of downy regions of the colony with no contact to the surface of the media.

SNA: optimum growth temperature at 30°C when grown on SNA. Colony radius after 72h when grown on SNA at 30°C = 15 mm, colonies reach 35 mm on Day Seven. At 15°C colonies reach 3 mm in radius after 72h and 18 mm at Day Seven. At 25°C colonies reach 5 mm radius after 72h and 19 mm on Day Seven. At 35°C colonies reach 12 mm radius after 7 days. SNA at 25°C; Colony hyaline, no sporulation occurs except around the CMD inoculation plug, sterile surface mycelia with no aerial hyphae. Mycelia growing subsurface, no soluble pigments or distinct odours produced. At 30°C the colony forms a small number of scattered, fertile, 2 mm dark green pustules.

Specimens examined: South Africa, Western Cape Province, Clanwilliam (32°9'23.85"S 18°52'56.10"E). Isolated from bulk soils, Jul 2012, collected by I. L. du Plessis, ex-type culture Tri 13 (HOLOTYPE); *Additional specimen examined*: Israel, 200 m off-shore at Sedot-Yam (32° 29′.00"N, 34°53′.00"E). Isolated from marine sea sponges (*Psammocinia sp*.), Jan 2007, collected by Z. Paz *et al.*, Strains OY 14707 and O.Y.7107.

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Trichoderma terrigenum I. L. du Plessis, I. S. Druzhinina, O. Yarden and K. Jacobs, prov. sp. nov.* **- Figs 5, 6.**

Etymology. Named in reference to soil, the environment from which the type strain of the species was first isolated.

CMD: optimum growth temperature at 30°C for CMD, colony radius after 48h when grown on CMD at 30 °C = 54 mm, the colony covers the plate on Day Three. At 25°C colonies reach 69 mm in radius after 72h incubation and cover the Petri dish on Day Four. At 15°C colonies reach 19 mm radius after 72h and 55 mm on Day Seven. At 30°C colonies reach 54 mm radius after 72h. At 35°C colonies reach 32 mm radius after 7 days. CMD at 25°C; Colonies hyaline in non-sporulating regions, concentric zones visible but inconspicuous, aerial hyphae inconspicuous.

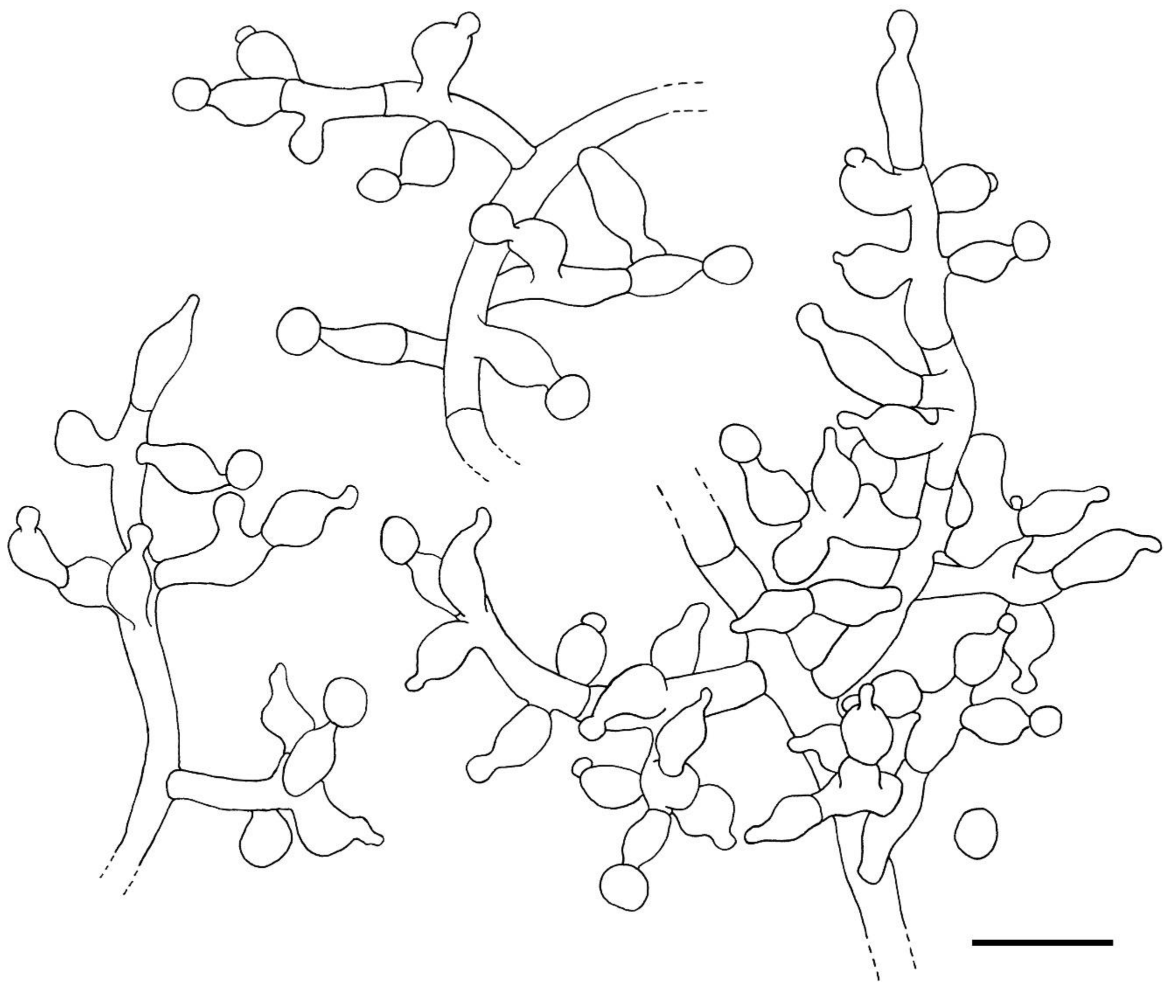

FIGURE 5. *Trichoderma terrigenum* prov. nom. line drawings from Strain Tri 59 (holotype) cultured on CMD. Scale bar is 10 μm in length

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No hyphal coilings, exudates, autolytic excretions, distinct odours or diffusing pigments produced. Conidiospore formation starts on Day Three. Dark green conidia develop on pustules (1-2 mm wide) that later fuse to form extended fertile regions, distal regions of the colony display limited effusely sporulating regions or small fertile granules.

Conidiophores appear *Verticillium*-like. However, phialides do not form at regular levels around the axis but rather develop in solitary arrangements, consisting of a straight or bent stipe from which multiple branches arise unpaired, at irregular intervals, along the central axis. Stage Two branches also present. However, no Stage Three branches are produced. Branching occurs at right angles relative to the stipe axis, some orientating slightly towards the conidiophores apex. Conidia forming in small wet heads, phialides solitary or in whorls of 2-4, divergent. Phialides: $(4.0-)4.5 \pm 6.5(-9.5) \times 2.5 \pm 3.5$ µm (n=50), l/w ratio: $1.5 \pm 2.0(-3.0)$. Phialide base: $(1.0-)1.5 \pm 2.0(-2.5)$ µm wide (n=50), phialides ampuliform with symmetrical or slightly bent necks, thickest most around the middle. Conidia: $(3.0-)3.5 \pm 4.5(-5.0) \times 2.0 \pm 3.0(-3.5)$ µm, l/w ratio: $(1.0-)1.5 \pm 2.0(-9.5)$ smooth, oval, less commonly subspheroidal, containing few, small guttules or one large guttule. Round, smooth-walled chlamydospores are formed on CMD in mature colonies. At 15°C: colonies show poor growth and do not sporulate. Some hyphal coilings form on short stalks extending from the media surface. At 30°C: sterile pustules (2-3mm) can be seen forming near the central regions of the colony, continuous fertile zones form nearer to the periphery. At 35°C: the colony appearance changes compared to that at 25°C, the colony does not sporulate so profusely and pustules remain small, forming a grainy texture.

PDA: optimum growth temperature at 30°C when grown on PDA. Colony reaches 58 mm radius after 48h and covers the Petri dish on Day Three at 30°C. At 15°C colonies reach 20 mm radius after 72h and 45 mm on Day Seven. At 25°C colonies reach 73 mm radius after 72h and cover the plate on Day Four. At 35°C colonies reach 31 mm radius on Day Seven. Colonies on PDA at 25°C: Colony dense, inconspicuously zonate, pustules develop which fuse to form broad fertile regions alternating with hyaline zones. Autolytic excretions scant, hyphal coilings absent. No distinct odours or exudates produced. Reverse colouration of te colony is pale orange. Conidiospore formation noted on day three, dense, heavily sporulating regions form near the point of inoculation bearing dark green conidia. At 15°C: Colonies show restricted growth and do not sporulate, hyphal coils form abundantly on aerial stalks.

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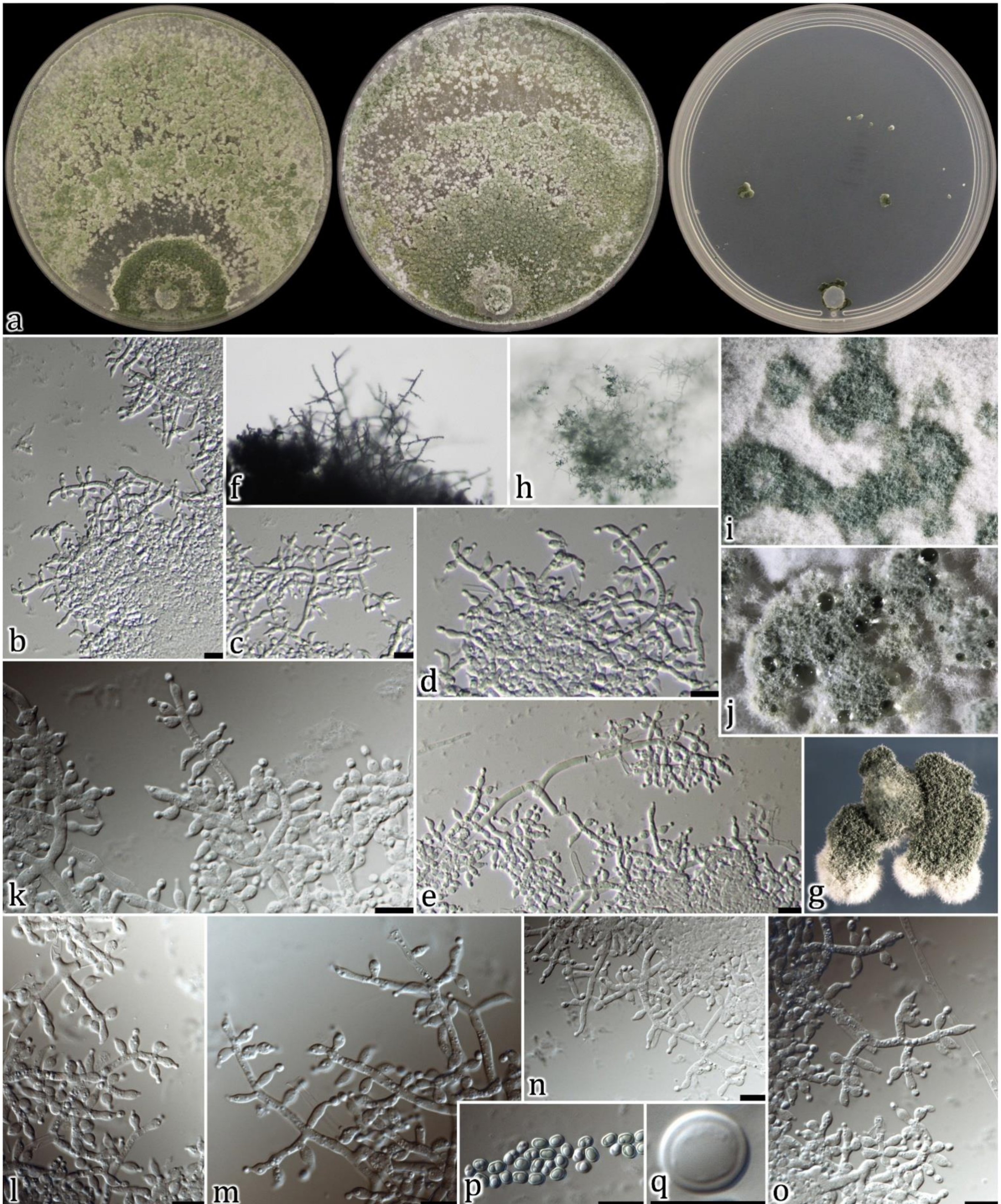

FIGURE 6. Morphological features characteristic of *Trichoderma terrigenum* prov. nom., (holotype Strain Tri 59). a. Colonies of *T. terrigenum* incubated on CMD for 7 days (left), PDA for 7 days (middle), SNA for 7 days (right). b, c, d, e. Conidiophores formed on CMD 400x magnification. f, g. Stereo microscope images from SNA. h, i. Stereo microscope images, from CMD. j. Stereo microscope images, from PDA. k, l, m, n, o. Conidiophores formed on CMD 1000x. p. Conidia 1000x. q. Chlamydospore 1000x. All scale bars are 10μm in length.

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At 30°C: mycelia appearing more yellowish than can be seen at 25°C, fertile regions are dense. At 35°C: colony displays abundant aerial mycelia that develop into small fertile pustules that are loosely arranged, conidia colours are lighter shades of green compared to what can be seen at 25°C.

SNA: optimum growth temperature at 30°C when grown on SNA. Colony radius after 72h when grown on SNA at 30°C = 32mm, colonies cover the plate on Day Five. At 15°C colonies reach 7 mm radius after 72h and 25 mm on Day Seven. At 25°C; Colonies reach 24 mm radius after 72h and cover the plate on Day Seven. At 35°C: Colonies reach 57 mm radius by Day Seven. SNA at 25°C; colony hyaline, mycelial growth occurs mostly below the surface of the medium. Autolytic excretions and coilings inconspicuous. No diffusible pigments or distinguishing odours noted. Pustules develop, 2-4 mm in diameter, bearing dark green conidia and forming sterile white mycelia at the margins. At 30°C: growth rate improves, pustules develop as described for 25°C. At 35°C: colonies grow slower than at 30°C, small, granular, fertile pustules develop abundantly.

Specimens examined: South Africa, Western Cape province, Klawer, (31°46'10.20"S 18°38'15.39"E). Isolated from bulk soil, Jul 2012, collected by I. L. du Plessis, ex-type culture, Strain Tri 59 (HOLOTYPE).

Discussion

The two new species, *T. vagum* and *T. terrigenum,* are members of the *T. longibrachiatum* clade based on a phylogenetic analysis using ef1α DNA sequence data and can be distinguished from other *Trichoderma* species in this clade (Fig. 2). Morphologically, these species display a limited number of characteristics that confirm their affiliation with the *T. longibrachiatum* clade (Samuels *et al.* 2012). *Trichoderma vagum* grows restrictedly at 35°C and does not form yellow reverse colourations. Similarly, *T. terrigenum* also shows restricted growth at 35°C, although this species does secrete yellow pigment into its substrate. In addition, both new species form mostly ampuliform as opposed to lageniform shaped phialides and do not produce intercalary phialides as abundantly as some of the other *T. longibrachiatum* clade members. However, similar to other species in the *T. longibrachiatum* clade, *T. vagum* and *T. terrigenum* display conidiophores that are sparingly branched and form a central axis from which phialides arise in unpaired whorls or singly along the stipe.

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Morphological analyses were performed to investigate the relationship between the new *Trichoderma* strains and their closest phylogenetic relatives. The new species were found to display morphological characteristics that distinguished them from their close relatives.

T. terrigenum is phylogenetically closely related to *T. andinensis* Samuels and Petrini (Fig. 2). These two species can readily be distinguished from one another based on their growth rates. On PDA, *T. andinensis* typically does not grow fast enough to cover a 90 mm petri dish when incubated for 72h at 25°C whereas *T. terrigenum prov. nom,* on the other hand, is able to do so (Samuels *et al.* 2012). On SNA, *T. andinensis* typically covers a 90 mm petri dish when grown for 72h at 25°C, whereas *T. terrigenum* only produces colonies that are 24 mm in radius after seven days (Samuels *et al.* 2012). These two species also differ from one another in terms of their colony morphologies: *T. andinensis* is nearly sterile when grown on PDA at 25°C whereas *T. terrigenum* sporulates profusely at this temperature (Samuels *et al.* 1998). In addition, *T. andinensis* does not secrete any pigment into its substrate at any temperature whereas *T. terrigenum* secretes pigment to some extent at 25°C, 30°C and 35°C, resulting in substrate discolouration (Samuels *et al.* 2012). These species do, however, display indistinguishable conidial structures.

T. vagum is phylogenetically closely related to *T. parareesei* Jaklitsch and Atanasova and *T. reesei* Simmons (Fig. 2). The new species can be distinguished from its close relatives based on several different morphological characteristics. *Trichoderma reesei* grows well on SNA and colonies reach a radius of 32-40 mm after two days of incubation, whereas *T. vagum* does not grow at this rate and only forms colonies that are 19 mm in radius after seven days of incubation (Atanasova *et al.* 2010). In addition, *T. reesei* is not known to form well defined pustules on CMD, whereas *T. vagum* forms numerous fertile pustules on this medium (Atanasova *et al.* 2010). Micromorphologically, these two species also differ in some regards. *T. reesei* typically forms lageniform phialides with straight necks that are rarely bent, while *T. vagum* forms ampuliform phialides that are often asymmetrical and bent in different orientations (Atanasova *et al.* 2010). Intercalary phialides are readily formed by *T. reesei.* However, such phialides are not abundantly produced by *T. vagum,* although they have been observed (Atanasova *et al.* 2010). These two species share indistinguishable conidial morphologies and both form smooth-walled oblong conidia with parallel sides (Atanasova *et al.* 2010).

Trichoderma parareesei grows well on SNA and can form colonies with a radius of 40-43 mm after two days of incubation at 25°C (Atanasova *et al.* 2010). In comparison, *T. vagum* does not grow as quickly and only produces colonies that are 19 mm in radius following seven days of

incubation. In addition, these species display different growth rates on CMD. *T. parareesei* can cover a 90 mm Petri dish in three days when incubated at 25°C, whereas *T. vagum* colonies reach 53 mm under the same conditions (Atanasova *et al.* 2010). These species can also be distinguished based on their colony morphologies, *T. vagum* forms conspicuously zonate colonies on CMD whereas *T. parareesei* does not display zonate colonies (Atanasova *et al.* 2010). In addition, colonies of *T. parareesei* grown on CMD sporulate after one day of incubation at 25°C whereas colonies from *T. vagum* only start to sporulate on Day Three (Atanasova *et al.* 2010). Micromorphologically, these species differ from one another in terms of their phialide morphologies. *T. parareesei* produces phialides that are 2.5 - 3.5 µm in width, which is thinner that those of *T. vagum* at 3.5 - 4.5 µm (Atanasova *et al.* 2010). Lastly, these organisms differ in terms of the ecological niches they inhabit. *T. parareesei* has been isolated several times from different locations around the world, all from tropical environments (Atanasova *et al.* 2010). *Trichoderma vagum,* in contrast, was isolated from marine sea sponges as well as from soil in the Fynbos biome that has a Mediterranean climate.

Trichoderma species are known to inhabit soil environments where they contribute to key ecological processed such as nutrient cycling (Kubicek-Pranz 1998, Jaklitsch 2009). Information regarding the ecology of *T. vagum* and *T. terrigenum* and their function in nature is limited due to fact that only a handful of strains representing these species have thus far been identified. *Trichoderma vagum* is represented by three different strains that were isolated from strikingly different environments around the world. The holotype, Strain *Tri 13,* was isolated from a terrestrial site in the Fynbos biome of South Africa whereas the remaining two strains, O.Y.14707 and O.Y.7107, were isolated by Paz *et al.* (2010) from marine sponges in the Mediterranean sea. This shows that *T. vagum* is not confined to the soil environment and suggests an interesting capacity for the species to inhabit diverse ecological niches, a trait displayed by many *Trichoderma* species (Klein and Everleigh 1998, Druzhinina *et al.* 2005, Kubicek *et al.* 2009). *T. terrigenum* is represented by two different strains, the holotype, Strain Tri 59, was isolated from soil in the Fynbos biome of South Africa whereas the second Strain, C.P.K 667, was isolated from soil in tropical Hawaii. Unfortunately, Strain C.P.K 667 has been lost since its isolation and it could not be morphologically characterised (Samuels *et al.* 2012). The only data available for this strain is in the form of DNA sequence data.

Gal-Hemed *et al.* (2011) previously showed that Strains O.Y.14707 and O.Y.7107 (*T. vagum*) were phylogenetically distinct from other members of the *T. longibrachiatum* clade through applying the concepts of GCPSR based on chi18-5, rbp2 and cal1 sequence data. In addition, Gal-

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Hemed *et al.* (2011) investigated the potential usefulness of these strains as biocontrol agents by determining their capacity to antagonise plant pathogens *in vitro*. They concluded that these strains were capable of producing volatile and non-volatile antibiotics *in vitro* that showed inhibitory affects against *Alternaria alternata* Keissler and *Rhizoctonia solani* Kühn (Gal-Hemed *et al.* 2011).

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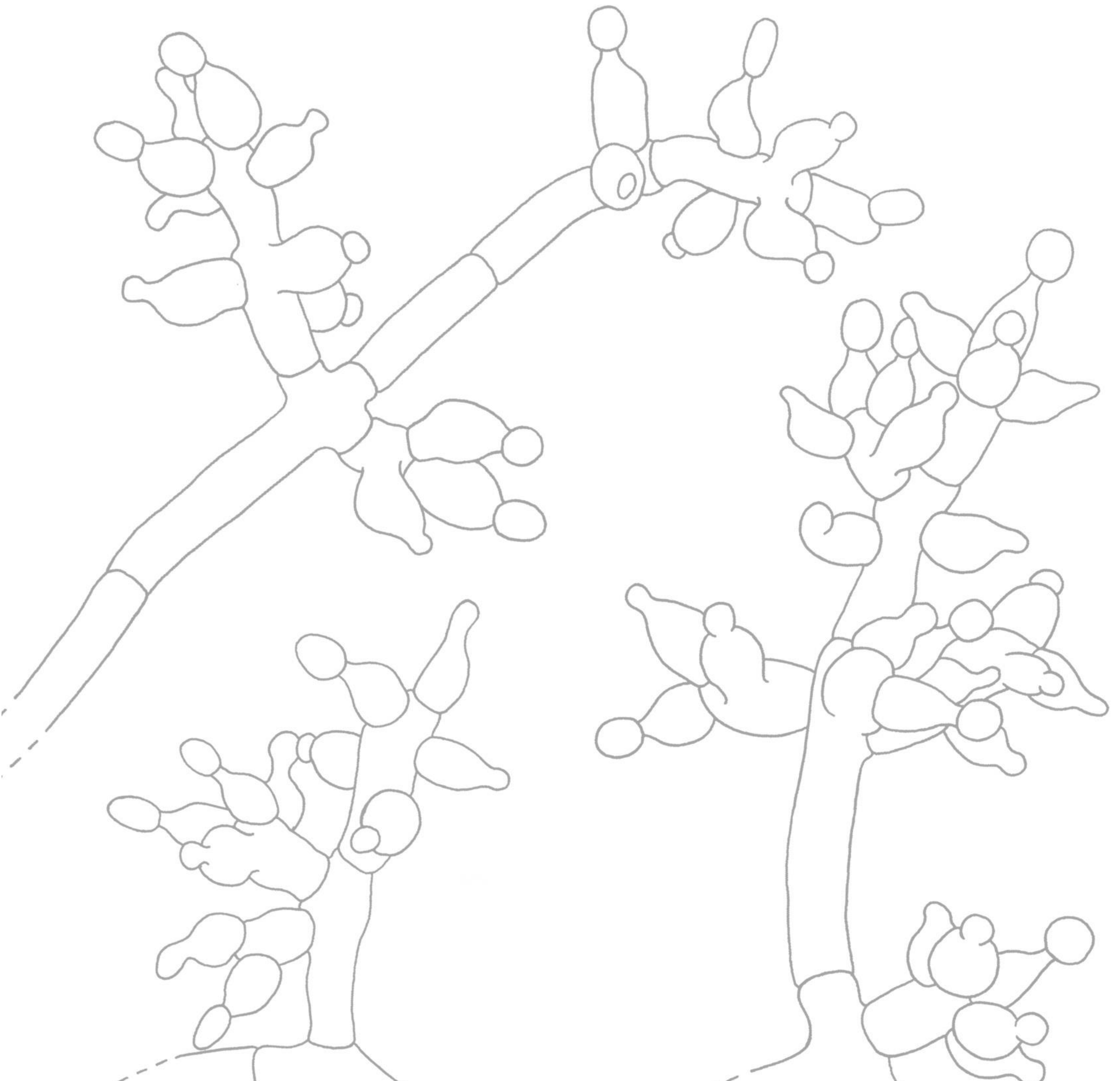
CHAPTER 4

TRICHODERMA UNDULATUM PROV. NOM., AND *T. RESTRICTUM* PROV. NOM., TWO NEW SPECIES ISOLATED FROM SOIL IN SOUTH AFRICA



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Abstract

The genus *Trichoderma* includes more than 170 different species that can be divided into 16 separate clades based on DNA sequence data. During a recent survey of the diversity of *Trichoderma* species in South Africa, nineteen different species were isolated and identified from soil. Two of these species could not be matched to known *Trichoderma* species based on ef1α and ITS sequence data and are described here as *T. undulatum* prov. nom. and *T. restrictum* prov. nom. *Trichoderma restrictum* displays morphological traits that suggest its affinity to the *T. pachybasium* 'A' clade. This is further supported by a phylogenetic analysis based on ef1α sequence data. However, the ef1α sequence data from Strain Tri 144 does not match any of the other members of the clade. The *T. pachybasium* 'A' clade is known for hosting economically important *Trichoderma* species that are routinely employed in agriculture as biocontrol agents and crop growth promoters. The new species grows slowly on SNA, reaching only 6 mm in radius on Day Seven, *T. restrictum* also displays *Pachybasium*-like conidiophores with smooth, oval conidia. *Trichoderma undulatum* displays morphological traits that are typical of *Trichoderma* species. However, ITS and ef1α sequence data from *T. undulatum* did not resemble those of any currently described *Trichoderma* species. A genus wide phylogeny based on ITS sequence data was, therefore, performed to determine the phylogenetic placement of *T. undulatum* within the genus. The species was found to constitute a unique lineage within *Trichoderma* and could not be grouped with any of the other clades currently recognized within the genus. *T. undulatum* displays a slow growth rate on CMA and SNA, which is unusual of a *Trichoderma* species. In addition, *T. undulatum* displays wavelike mycelia on PDA as well as *Trichoderma*-like conidiophore branching patterns with oblong, smooth-walled conidia.

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Introduction

The genus *Trichoderma* displays a cosmopolitan distribution and includes more than 170 different species from diverse habitats and substrates (Samuels 2006, Chaverri and Samuels 2013. Also see page 19). *Trichoderma* species are typically fast growing fungi that display saprobic lifestyles and form green or white colonies with sparse aerial mycelium and green or white pustules (Bissett 1991a). Microscopically species display highly branched conidiophores with hyaline phialides and single celled conidia (Rifai 1969, Jaklitsch 2009).

A recent diversity survey of *Trichoderma* in South Africa has revealed a number of species that are new to science (See Table 3. Page 58). One of these new species is a member of the *T. pachybassium* clade which is known for hosting economically important *Trichoderma* species such as *Trichoderma asperellum* Samuels and *T. hamatum* Bonorden (Lew *et al.* 1990, Inbar *et al.* 1994, Harman 2006, Segarra *et al.* 2010, Peterson and Nevalainen 2012. Also see pages 23 and 24).

The genus *Pachybasium* was described by Saccardo in 1885 in order to accommodate the fungus *Verticillium hamatum* Bonorden, which he believed to be distinct from the genus *Verticillium* Nees. Later, Hughes (1958) recognised *Pachybasium* to be a synonym of *Trichoderma* and synonymised the two genera, preserving the older name *Trichoderma.* Thirty three years later, Bissett reviewed *Trichoderma* and divided the genus into five different sections based on the morphological variations he observed between different species (Bissett 1991a). Species that were previously assigned to *Pachybasium* were now allocated to a morphologically defined section within *Trichoderma* that Bissett named *T.* sect*. Pachybasium* (Bissett 1991a). The *Pachybasium* section included *T. hamatum,* which previously represented the type of *Pachybasium*. Bissett characterised *T. sect Pachybasium* as having complexly branched conidiophores that formed branches with crowded verticils of short, wide, ampuliform phialides and smooth, ellipsoid conidia (Bissett 1991b). Later, Kindermann *et al.* (1998) investigated the phylogenetic relationships of species within the *T. pachybasium* section based on rDNA sequence data. They found that the species from *T. sect pachybasium* did not form a monophyletic clade and that some species aggregated into a separate clade. These results were later confirmed by Kullnig-Gradinger *et al.* (2002), who conducted phylogenetic analyses on members of *T.* sect*. Pachybasium* based on multiple genomic loci including tef1, chi18-5, mitSSU*,* and D1, D2 28s rDNA. The study showed that *T.* sect*. Pachybasium* (*sensu* Bissett 1991a) can be phylogenetically divided into two distinct groups that Kindermann *et al.*

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(1998) named *T. pachybasium* Clade 'A' and Clade 'B' (Kullnig-Gradinger *et al.* 2002). The larger of these two groups, Clade 'B' is phylogenetically distinct from the sectional type *T. hamatum*. The species that belong to this group were subdivided into multiple clades namely, the *T. harzianum*, *T. virens*, *T. semiorbis*, *T. strictipilis*, *T. stromaticum* and *T. pachybasioides* clades (Kullnig-Gradinger *et al.* 2002, Samuels *et al.* 2006). The second group, Clade 'A', included *T. hamatum* along with relatively few other species and clustered together with species from *T.* sect. *Trichoderma* (Druzhinina *et al.* 2005).

This study isolated two new *Trichoderma* species as part of a greater undertaking that aimed to document the diversity of the genus in South Africa. One of these species belongs to the *T. pachybasium* 'A' clade and the other constitutes a novel lineage within the genus and is not closely related to any of the *Trichoderma* clades currently recognised. The aim of this study was, therefore, to characterise and describe these species as *T. restrictum* prov. nom. and *T. undulatum* prov. nom.

Materials and methods

Soil sampling, strain isolations and group allocations

Trichoderma strains were isolated from soils collected at 173 different locations across South Africa (See page 55). In total, 161 *Trichoderma* strains were isolated according to standard soil dilution plating techniques that were described elsewhere (See page 51). These strains were found to represent 19 different species, most of which were identified and are discussed in Chapter 2. The two new *Trichoderma* species are each represented by a single strain, *T. restrictum* is represented by Strain Tri 144 and *T. undulatum* is represented by Strain Tri 81. Strain Tri 144 was isolated from garden soil in the city of Nelspruit (25°29'12.30"S 30°58'58.20"E) and Strain Tri 81 was isolated from the town of Swellendam (34°02'35.61"S 20°25'52.23"E) (Fig. 1). The techniques used to characterize strains as well as to measure the growth of the strains were described elsewhere (See page 53).

DNA extractions, PCR and sequencing

The molecular techniques used during this study are described elsewhere (See page 52). Briefly, DNA was extracted from fungal cultures according to a modified CTAB protocol described by Möller *et al.* (1992). PCRs were then set up to amplify the ITS and ef1α genomic loci and the resulting amplicons were sequenced as were described in Chapter two.

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FIGURE 1 Isolation location of the new species ***T. restrictum* prov. nom. (red circle)** and ***T. undulatum* prov. nom. (blue circle)**. These species showed restricted distributions to the north-east and south-western parts of the country, respectively. Black crosses indicate sites sampled that did not yield *Trichoderma* strains belonging to these species. Scale bar = 400km.

Species identifications and phylogenetic analyses

Preliminary *Trichoderma* species identifications were done through NCBI BLAST as well as the online *Trichoderma* barcoding resource, Trichokey (Druzhinina *et al.* 2006. http://www.isth.info). Phylogenetic analyses were performed to investigate the relationship between the isolated *Trichoderma* species and their relatives more closely. For such analyses, the same methodological approaches were used as were described elsewhere (See page 52). Two separate sequence datasets were assembled from GenBank in order to investigate the phylogenies of each of the two strains.

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The first dataset, used to investigate the phylogeny of *T. restrictum*, consisted of a combined set of ITS and ef1α sequence data from reference ex-type strains of species within the *T. pachybasium* 'A' clade (Tables 1 and 2). *T. strigosum* Bissett was selected as an outgroup for the analysis because this species is a member of the neighboring *T. viride* clade (Jaklitsch 2009).

The second *Trichoderma* strain, Tri 81, did not associate with species from any of the clades currently recognized in the genus based on sequence homology searches through NCBI BLAST analyses. Therefore, phylogenetic analyses based on high resolution loci, such as ef1α sequence data, would not be possible as the variability of the sequences would not permit meaningful multiple sequence alignments. For this reason a genus wide phylogeny based on ITS sequence data was performed in order to investigate the phylogenetic placement of Strain Tri 81 within *Trichoderma*. The dataset for this analysis consisted of representatives from each of the clades that are recognized in *Trichoderma* according to Druzhinina *et al.* (2005).

Multiple sequence alignments of the datasets prior to phylogenetic analysis resulted in a number of ambiguously aligned basepairs. These ambiguous regions were identified through the use of the online resource Gblocks (v. 1.0) and were excluded from subsequent phylogenetic analyses because such ambiguously aligned regions would have lead to a loss of phylogenetic signal (Lutzoni *et al.* 2000).

Results

Species identifications

Preliminary attempts to identify Strains Tri 144 and Tri 81 through the online barcoding-based resource Trichokey revealed that these two strains did not match any of the *Trichoderma* species represented in the database and were marked as 'unknown'. NCBI BLAST analysis using sequence data from Strain Tri 144 showed a 95.1% identity match with its nearest relatives based on ITS sequence data as well as a 87.3% identity match based on ef1α sequence data. The nearest BLAST hit results for strain Tri 144, based on ITS sequence data, belonged to the *T. pachybasium* 'A' clade (*T. hamatum* - 95.1% identity, *T. asperellum* (95.1% identity and *T. pubescens*, 95.1% identity). A phylogenetic analysis of the combined ITS and ef1α sequence dataset (Tables 1 and 2) was performed to further investigate the position of Strain Tri 144 in the *T. pachybasium* 'A' clade (Fig. 2).

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NCBI BLAST analysis revealed that Strain Tri 81 only shared 90.0% sequence homology, based on ITS sequence data, with its nearest relatives. Similarly, the BLAST analysis based on ef1α sequence data yielded only an 88.1% match with its nearest relatives. The nearest relatives for Strain Tri 81, based on ITS sequence data, were from a range of different clades within *Trichoderma*. For example *T. brevicompactum* (90.0% homology) belongs to the *T. lutea* clade and *T. virens* (89.5% homology) belongs to the *T. virens* clade. A phylogenetic analysis of the ITS region was performed to determine the placement of Strain Tri 81 within *Trichoderma* (Figure 3, Table 3).

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TABLE 1 *Trichoderma* strains from the *T. pachybasium* 'A' clade used for phylogenetic comparisons through a combined dataset of ITS and ef1α sequence data. Collection numbers for the strains that were used are listed along with their respective GenBank accession numbers for ITS sequence data. Ex-type strains are indicated in bold and with a *'T'*.

GenBank accession - ITS	Taxon name	Strain	References
DQ023301	***H. flaviconidia* *T***	G.J.S. 99-49	Samuels 2006
AF487654	*H. neorufa*	G.J.S. 96 132	Samuels *et al.* 2002
NR077132	***H. neorufa* *T***	G.J.S. 96135	Dodd *et al.* 2002
JN133553	*T. asperelloides*	G.J.S. 04187	Chaverri *et al.* 2013
DQ315464	*T. asperelloides*	G.J.S. 996	Samuels *et al.* 2006
GU198301	***T. asperelloides* *T***	G.J.S. 04116	Samuels *et al.* 2010
EU856297	*T. asperellum*	G.J.S. 01294	Samuels and Ismaiel 2009
AY380912	***T. asperellum* *T***	CBS 433 97	Holmes *et al.* 2004
JX238476	***T. eijii T***	TUFC 100002	Kim *et al.* 2013
EU856295	*T. evansii*	DIS 380a	Samuels and Ismaiel 2009
EU856294	*T. evansii*	DIS 282d	Samuels and Ismaiel 2009
EU883568	***T. evansii* *T***	DIS 341HI	Samuels and Ismaiel 2009
EU280136	*T. hamatum*	DAOM 237553	Hoyos-Carvajal *et al.* 2009
DQ109530	*T. hamatum*	G.J.S. 98170	Samuels *et al.* 2006
EU883567	*T. hamatum*	G.J.S. 04203	Samuels and Ismaiel 2009
EU856292	*T. hamatum*	G.J.S. 05262	Samuels and Ismaiel 2009
EU280124	***T. hamatum* *T***	DAOM 167057	Hoyos-Carvajal *et al.* 2009
EU856301	*T. lieckfeldtiae*	G.J.S. 05-01	Samuels and Ismaiel 2009
EU856299	*T. lieckfeldtiae*	G.J.S. 04196	Samuels and Ismaiel 2009
DQ109527	*T. paucisporum*	G.J.S. 0369	Samuels *et al.* 2006
DQ109526	***T. paucisporum* *T***	G.J.S. 0113	Samuels *et al.* 2006
DQ000632	***T. pezizoides* *T***	G.J.S. 01-257	Samuels 2006
EU856280	*T. pubescens*	G.J.S. 01207	Samuels and Ismaiel 2009
EU280121	***T. pubescens* *T***	DAOM 166162	Hoyos-Carvajal *et al.* 2009
DQ109531	*T. strigosum*	DIS 173k	Samuels *et al.* 2006
DQ083027	***T. strigosum* *T***	DAOM 166121	Holmes *et al.* 2004
EU856296	*T. theobromicola*	DIS 376f	Samuels and Ismaiel 2009
DQ109525	***T. theobromicola* *T***	DIS 85f	Samuels *et al.* 2006
GU198302	***T. yunnanense* *T***	CBS 121219	Samuels *et al.* 2010

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TABLE 2 *Trichoderma* strains from the *T. pachybasium* 'A' clade used for phylogenetic comparisons through a combined dataset of ITS and ef1α sequence data. Collection numbers for the strains that were used are listed along with their respective GenBank accession numbers for ef1α sequence data. Ex-type strains are indicated in bold and with a '*T*'.

GenBank accession - ef1α	Taxon name	Strain	References
DQ020001	***H. flaviconidia* *T***	G.J.S. 99-49	Samuels 2006
AF348115	*H. neorufa*	G.J.S. 96 132	Samuels *et al.* 2002
AF487670	***H. neorufa* *T***	G.J.S. 96135	Dodd *et al.* 2002
JN133571	*T. asperelloides*	G.J.S. 04187	Chaverri *et al.* 2013
DQ109550	*T. asperelloides*	G.J.S. 996	Samuels *et al.* 2006
GU248412	***T. asperelloides* *T***	G.J.S. 04116	Samuels *et al.* 2010
EU856323	*T. asperellum*	G.J.S. 01294	Samuels and Ismaiel 2009
AY376058	***T. asperellum* *T***	CBS 433 97	Holmes *et al.* 2004
JX684011	***T. eijii* *T***	TUFC 100002	Kim *et al.* 2013
EU856320	*T. evansii*	DIS 380a	Samuels and Ismaiel 2009
EU856319	*T. evansii*	DIS 282d	Samuels and Ismaiel 2009
EU883566	***T. evansii* *T***	DIS 341HI	Samuels and Ismaiel 2009
EU279966	*T. hamatum*	DAOM 237553	Hoyos-Carvajal *et al.* 2009
DQ109544	*T. hamatum*	G.J.S. 98170	Samuels *et al.* 2006
EU883565	*T. hamatum*	G.J.S. 04203	Samuels and Ismaiel 2009
EU856317	*T. hamatum*	G.J.S. 05262	Samuels and Ismaiel 2009
AF456911	***T. hamatum* *T***	DAOM 167057	Dodd *et al.* 2003
EU856328	*T. lieckfeldtiae*	G.J.S. 05-01	Samuels and Ismaiel 2009
EU856324	*T. lieckfeldtiae*	G.J.S. 04196	Samuels and Ismaiel 2009
DQ109541	*T. paucisporum*	G.J.S. 0369	Samuels *et al.* 2006
DQ109540	***T. paucisporum* *T***	G.J.S. 0113	Samuels *et al.* 2006
AY937438	***T. pezizoides* *T***	G.J.S. 01-257	Samuels 2006
EU856304	*T. pubescens*	G.J.S. 01207	Samuels and Ismaiel 2009
AY750887	***T. pubescens* *T***	DAOM 166162	Samuels 2006
FJ463284	*T. strigosum*	DIS 173K	Samuels *et al.* 2006
AY937442	***T. strigosum* *T***	DAOM 166121	Samuels 2006
EU856322	*T. theobromicola*	DIS 376f	Samuels and Ismaiel 2009
EU856321	***T. theobromicola* *T***	DIS 85f	Samuels and Ismaiel 2009
GU198243	***T. yunnanense* *T***	CBS 121219	Samuels *et al.* 2010

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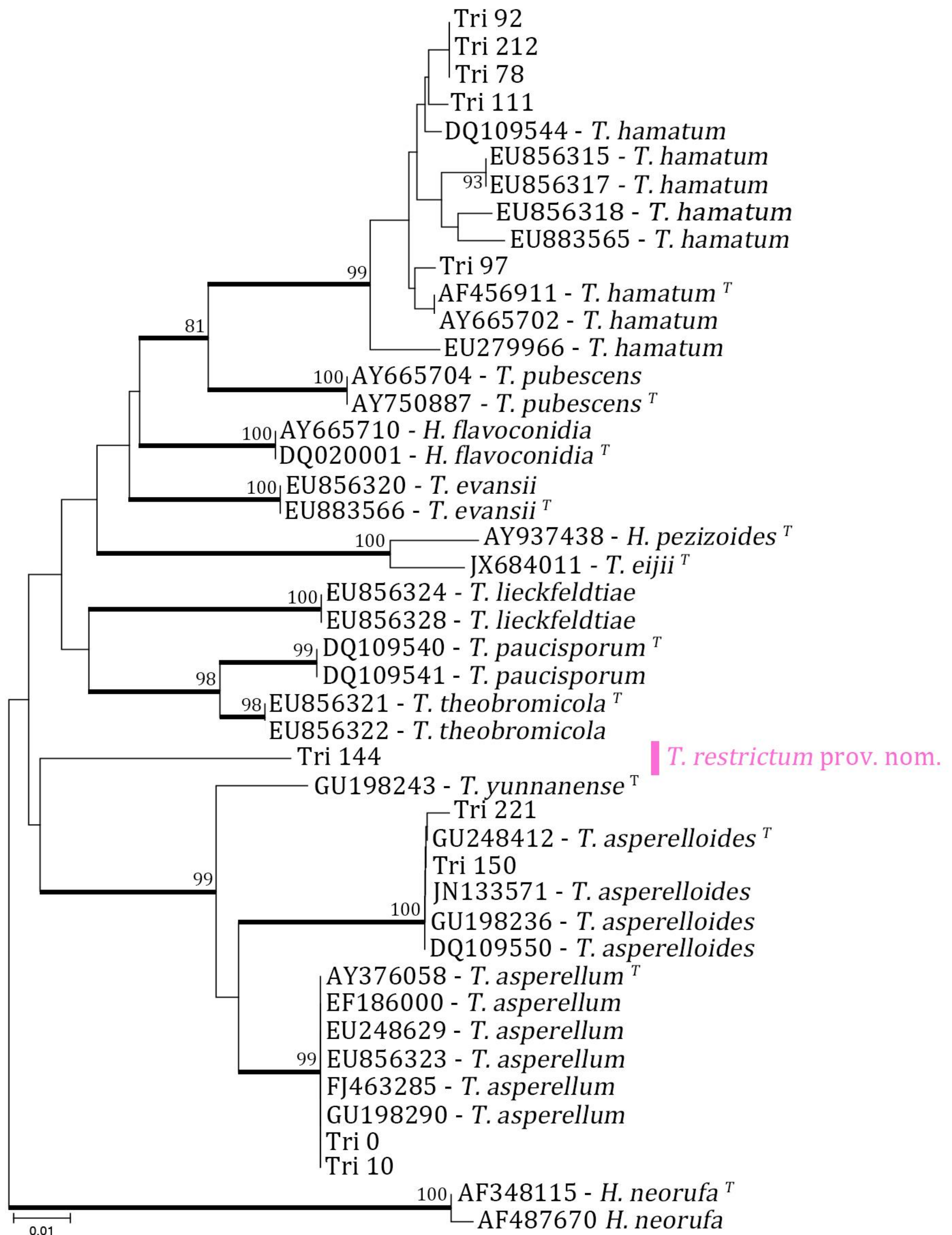

FIGURE 2 Neighbour joining tree indicating the phylogenetic relationships between accepted members of the *Trichoderma pachybasium* 'A' clade and *T. restrictum* prov. nom. based on a combined ef1α and ITS sequence dataset. Nodes which are supported by bootstrapping values that exceed 75% are indicated by thickened branches. GenBank accession numbers on the left-hand side indicate ef1α sequences and those on the right indicate ITS sequences. Ex-type culture sequences are indicated with a *'T'*.

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TABLE 3 *Trichoderma* strains used for phylogenetic comparison with Strain Tri 81. Each of the species within this analysis represents a different clade in *Trichoderma sensu* Druzhinina *et al.* 2005. GenBank accession numbers are indicated for each strain included in the analysis.

GenBank accession	Taxon name	Strain number	References
AY737764	*H. ceramica*	G.J.S. 88-70	Samuels 2006
FJ860743	*H. ceramica*	CBS 114576	Jaklitsch 2009
AY737762	*H. chlorospora*	G.J.S. 98-1	Samuels 2006
AY737774	*H. chromosperma*	G.J.S. 94 67	Samuels 2006
DQ835413	*H. citrina*	G.J.S. 95-183	Overton *et al.* 2006
DQ835417	*H. citrina*	CBS 894.85	Overton *et al.* 2006
AY737775	*H. gelatinosa*	G.J.S. 88 17	Samuels 2006
FJ860760	*H. gelatinosa*	C.P.K 1930	Jaklitsch 2009
FJ860771	*H. lutea*	CBS 1211.31	Jaklitsch 2009
AF487662	*H. lutea*	G.J.S. 89-129	Dodd *et al.* 2002
AY240841	*H. pachybasioides*	G.J.S. 89-135	Lu *et al.* 2004
FJ860794	*H. pachybasioides*	CBS 121277	Jaklitsch 2009
EU330959	*H. phyllostachydis*	G.J.S. 92 123	Degenkolb *et al.* 2008
FJ860809	*H. phyllostachydis*	CBS 114071	Jaklitsch 2009
AY865635	*H. psychrophila*	CBS 343.71	Druzhinina *et al.* 2005
FJ860818	*H. psychrophila*	C.P.K. 1602	Jaklitsch 2009
DQ677655	*H. rufa*	CBS 119325	Jaklitsch *et al.* 2006
DQ838532	*H. rufa*	C.P.K. 1006	Jaklitsch *et al.* 2006
AY737758	*H. semiorbis*	DAOM 167636	Samuels 2006
HM466664	*H. semiorbis*	G.J.S. 99-108	Direct submission
FJ860848	*H. strictipilosa*	C.P.K. 2034	Jaklitsch 2009
FJ860850	*H. strictipilosa*	C.P.K. 3135	Jaklitsch 2009
AY737753	*H. sulawesensis*	G.J.S. 85-228	Samuels 2006
FJ442676	*H. virens*	G.J.S. 95-78	Direct submission
DQ086142	*H. voglmayrii*	C.P.K. 948	Jaklitsch *et al.* 2005
DQ086143	*H. voglmayrii*	C.P.K. 941	Jaklitsch *et al.* 2005
EU280124	*T. hamatum*	DAOM 167057	Hoyos-Carvajal *et al.* 2009
FJ411988	*T. hamatum*	C.P.K. 2669	Belayneh *et al.* 2010
AF057606	*T. harzianum*	CBS 226 95	Ospina-Giraldo *et al.* 1998
AY857234	*T. harzianum*	TUB F 1005	Druzhinina *et al.* 2005
DQ083022	*T. helicum*	DAOM 230016	Samuels 2006
KC171341	*T. helicum*	ATCC MYA 4845	Samuels 2006
EU401556	*T. longibrachiatum*	CBS 816.68	Druzhinina *et al.* 2008
NR120298	*T. longibrachiatum*	ATCC 18648	Kühls *et al.* 1996
EU280068	*T. spirale*	DAOM 183974	Hoyos-Carvajal *et al.* 2009
FJ442608	*T. spirale*	CBS 120963	Direct submission
AF097912	*T. stromaticum*	G.J.S. 97-182	Samuels *et al.* 2000
AF097913	*T. stromaticum*	G.J.S. 97-183	Samuels *et al.* 2000
AF099005	*T. virens*	GLi 39	Samuels *et al.* 2000

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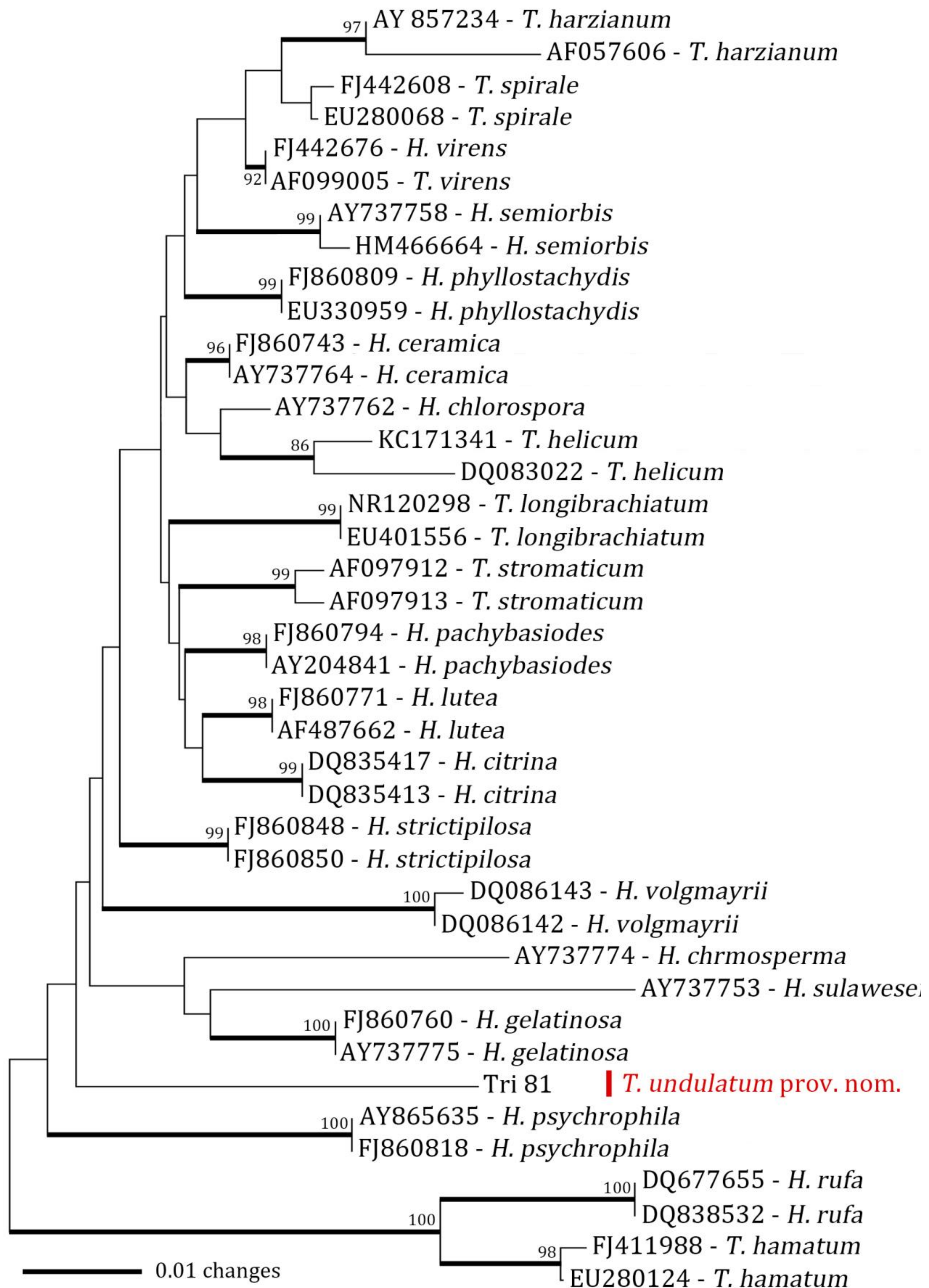

FIGURE 3 Neighbour joining tree indicating the phylogenetic relationships between accepted members of the *T. pachybasium* 'A' clade and Strain Tri 81 based on ef1α sequence data. Nodes which are supported by bootstrapping values that exceed 75% are indicated by thickened branches. Ex-type culture sequences are indicated with a '*T*'.

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Taxonomy

Trichoderma restrictum I. L. du Plessis and K. Jacobs, prov. sp. nov.* **- Figs. 4, 5**

Etymology. Named in reference to the slow growth rate of this species on SNA

CMD: optimum growth temperature at 25°C. Colony radius after 72h when grown at 25°C = 32 mm, colonies cover the Petri dish by Day Seven. At 15°C colonies reach 5 mm radius after 72h and 18 mm on Day Seven. At 30°C colonies reach 15 mm radius after 72h and 35 mm on Day Seven. At 35°C no growth occurs. CMD at 25°C: colonies hyaline at non-sporulating zones, forming a soft velvety texture at the growing margins as well as in zones adjacent to fertile regions as a result of hair like hyphae projecting from the media surface. Zonation is apparent as a result of day/night incubation regimes but the effect is not conspicuous. Fertile radial bands are composed of several fused pustules, these fertile zones alternate with regions containing fewer pustules that are also smaller. Hyphal coilings form along the periphery of the colony near the growing edge, these coilings become less apparent in the mature regions of the colony. No autolytic excretions, distinct odours, diffusible pigments or exudates produced. Pustules are covered with numerous hyaline, straight, sterile hyphae that give them a hairy texture. Sporulation starts on Day Three. Small, light green pustules develop (0.5-1.5 mm), later becoming larger (2-3 mm) and darker shades of green as the pustules mature.

Conidiophores are *Pachybasium*-like, branching regularly in pairs along the stipe axis which can either be straight or curved. Sterile hyphal elongations can be seen extending from certain conidiophores. First and second level branching produced but no third level branching. Branches are perpendicular relative to the stipe axis or incline slightly towards the conidiophores apex. Phialides born directly from the stipe axis or on branches. Phialides solitary, or in whorls of 2-5 and diverge from one another. Phialides $(4.0-)5.0 \pm 7.0(-9.5) \times (2.5-)3.0 \pm 3.5(-4.0)$ µm (n=50), l/w ratio : $(1.0-)1.5 \pm 2.0(-3.5)$. Phialide base: $2 \pm 2.5(3.0)$ µm wide. Phialides ampuliform with symmetrical or slightly bent necks, and are thickest around the middle. Conidia $(3.0-)3.5 \pm 4.0(-4.5) \times 1.5 \pm 2.0$ µm, l/w ratio: $(1.0-)1.5 \pm 2.0(-3.5)$ born in wet heads, smooth, oval, containing one or two small guttules. Chlamydospores present in mature colonies, round or oval shaped, smooth-walled, forming mostly on hyphal termini. At 15°C: Colonies display restricted growth, no sporulation

* Please note that the descriptions provided here are preliminary and should not be cited as they will be published formally elsewhere

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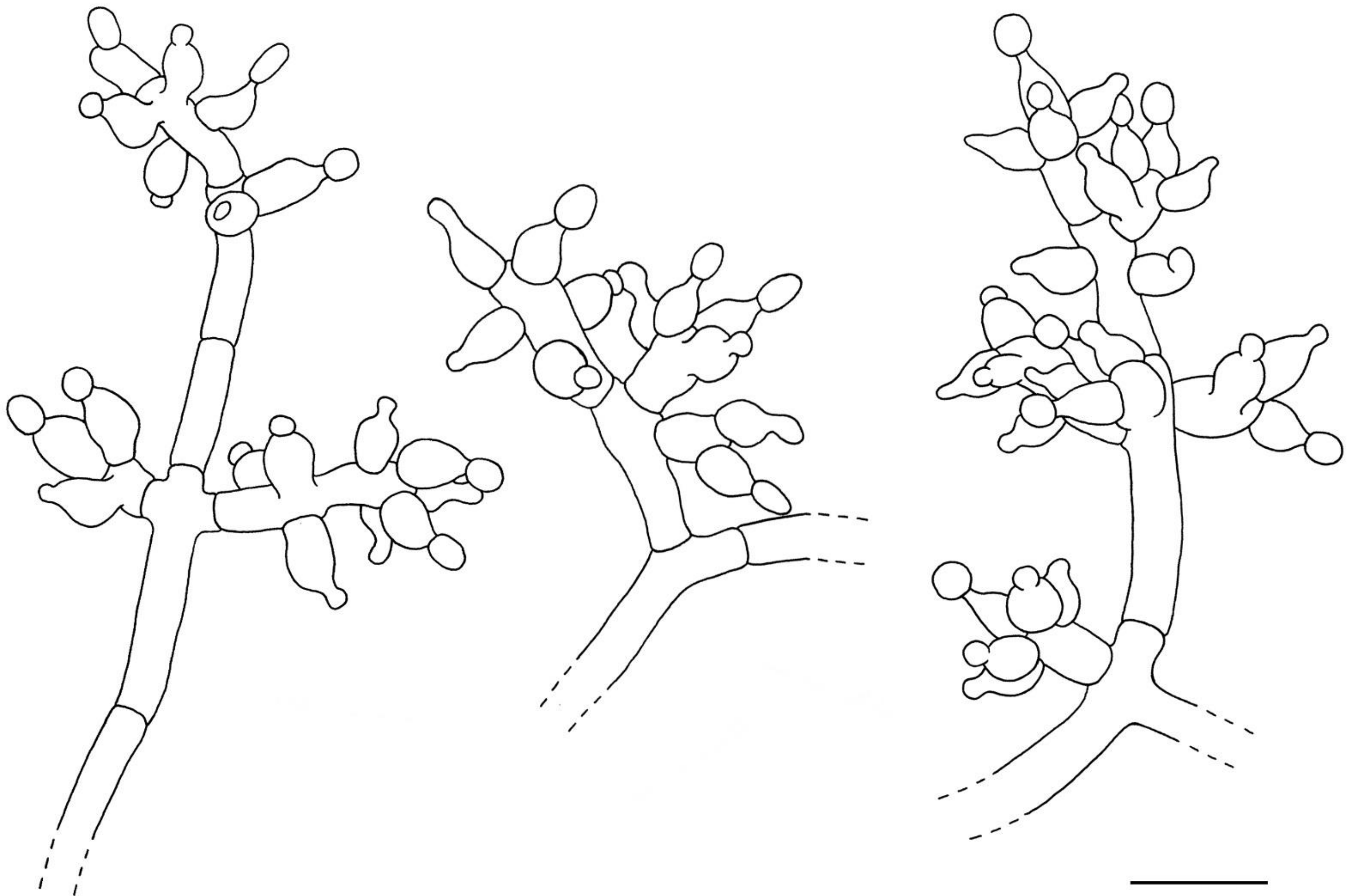

FIGURE 4. *Trichoderma restrictum*. Line drawings from strain Tri 144 (holotype) cultured on CMD. Scale bar = 10µm

occurs when incubated in darkness. Hyphae at the margins of the colony display signs of autolytic excretions. No hyphal coilings noted. At 30°C: colonies mostly resemble those grown at 25°C. However, growth occurs much slower and pustules are less well defined, fusing to form continuous fertile layers, no autolytic excretions noted.

PDA: optimum growth temperature at 25°C. Colony radius after 72h when grown at 25°C = 24 mm and 63 mm on Day Seven. At 15°C: colonies reach 4 mm radius after 72h and 13 mm on day 7. At 30°C colonies reach 11 mm radius after 72h and 31 mm on Day Seven. At 35°C no growth occurs. PDA at 25°C: colonies hyaline at non-sporulating zones, zonation is slightly less conspicuous than can be seen on CMA. Fertile zones bear small, downy pustules and hyaline zones in between sporulating zones show a few small, granular pustules. Fertile regions are light green in colour, becoming slightly darker as the conidiophores mature. No exudates, soluble pigments or hyphal coilings produced. Occasional hyphae along the growing margin

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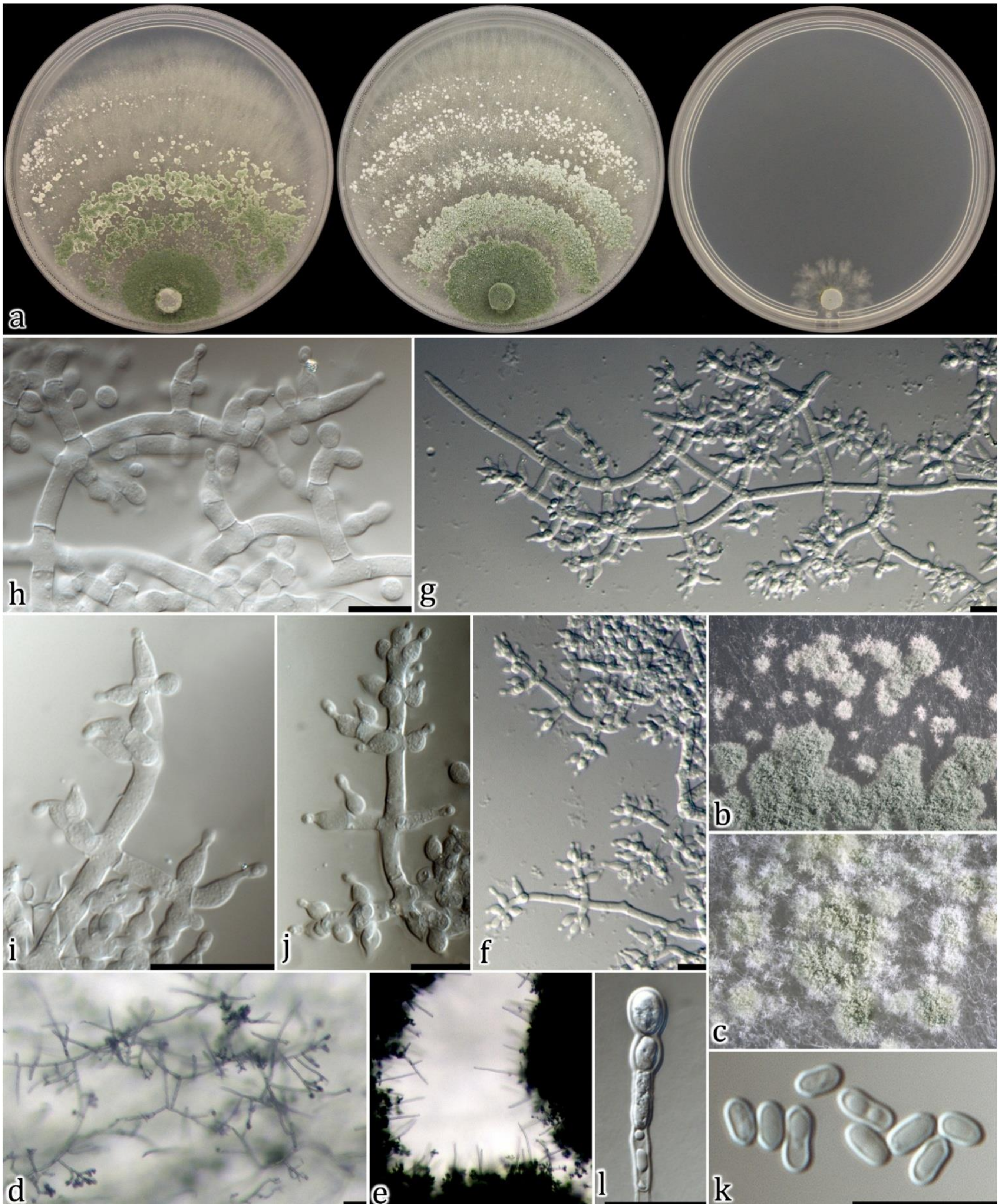

FIGURE 5. Morphological features characteristic of *Trichoderma restrictum*, Strain Tri 144. a. Colonies of *T. unknown* incubated on CMD for 7 days (left), PDA for 7 days (middle) and SNA for 7 days (right). b, c. Stereo microscope images, from CMD (top), PDA (bottom). d, e. Stereo microscope image from CMD. f, g. Conidiophores on CMA 400x magnification. h, i, j. Conidiophores formed on CMD 1000x. k. Conidia 1000x. l. Chlamydospores 1000x. (Scale bars = 10μm).

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show autolytic excretions, although this effect is not conspicuous. The colony starts to sporulate on Day Four. At 30°C; Colonies grow much slower than can be seen at 25°C. No sporulation occurs when grown in darkness. No autolytic excretions or hyphal coilings produced. At 15°C colonies also grow slowly, no sporulation occurs.

SNA: optimum growth temperature at 30°C. Colony radius after 7 days when grown at 30°C = 6 mm. At 15°C colonies reaching 3 mm radius on Day Seven. At 25°C colonies reach 6 mm radius on Day Seven. At 35°C no growth occurs. SNA at 30°C: Colonies grow poorly. Mycelium hyaline, growing subsurface. No sporulation occurs accept for area surrounding the inoculation plug. No distinct odors, soluble pigments, hyphal coilings or autolytic excretions produced. At 26 °C: colonies appear the same as at 30°C. At 15°C: colonies grow even slower than at 25°C, otherwise the colonies resemble those forming at 25°C.

Specimens examined: South Africa, Mpumalanga Province, Nelspruit (25°29'12.30"S 30°58'58.20"E). Isolated from bulk soils, Apr 2012, collected by I. L. du Plessis, ex-type culture Strain Tri 144 (HOLOTYPE).

Trichoderma undulatum I. L. du Plessis and K. Jacobs, prov. sp. nov.* **- Figs. 6, 7**

Etymology: Latin, *unda*: meaning wave, in reference to the curving hyphae that this fungus forms on the surface PDA.

CMD: optimum growth temperature at 25°C. Colony radius after 72h when grown at 25°C = 10 mm. Colonies reach 44 mm in radius on Day Seven. At 15°C colonies reach 6 mm radius after 72h and 21 mm on day 7. At 30°C colonies reach 10 mm radius after 62h and 47 mm on day seven. At 35°C no growth occurs. CMD at 25°C: Mycelium mostly subsurface, white aerial mycelium gives the colony a downy texture. Zonation inconspicuous and no hyphal coilings, soluble pigments or distinctive odours are produced. Conidiospore formation starts on day five, conidia produced scarcely, effuse conidiation can be seen in mature regions of the colony, fertile pustules also form and fuse to create continuous fertile regions. The growing edge of the colony forms hyphae which showed signs of autolytic excretion. Two different conidiophore types are produced: Conidiophores from pustules were *Trichoderma*-like, forming complex branching patterns that split from the conidiophore axis at unpaired, irregular levels.

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* Please note that the descriptions provided here are preliminary and should not be cited as they will be published formally elsewhere

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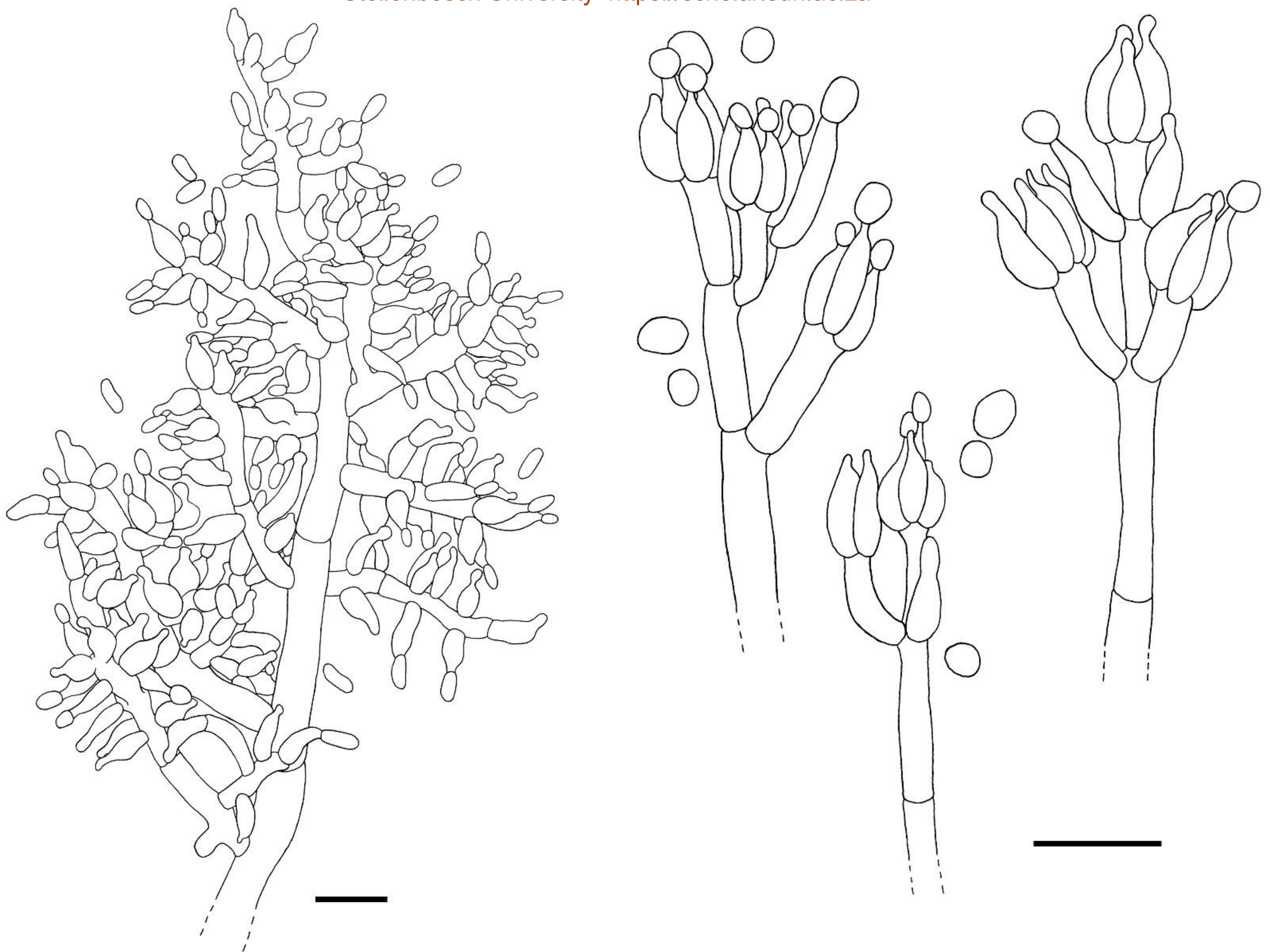

FIGURE 6. *Trichoderma undulatum*. Line drawing from Strain Tri 81 (holotype) cultured on CMD. Conidiophore morphology as can be seen forming in fertile pustules (left) and effusely sporulating regions of the colony (right). Scale bars = 10μm.

Branches tend to orientate towards the conidiophore apex and bear short, ampuliform, unpaired phialides at irregular levels that diverge from one another. Phialides do not develop along the conidiophore axis except at the terminus of the conidiophore. Conidiophores from effusely sporulating regions of the colony are *Gliocladium*-like and display relatively simple structures. Such conidiophores display a limited number of branchings that are orientated towards the conidiophore apex and bare ampuliform to lanceolate phialides. Phialides (from conidiophores in pustules) $(4.5-)5.5 \pm 6.5(-7.5) \times 2.0 \pm 2.5(-3.0)$ μm (n=50), l/w ratio: $(2.0-)2.0 \pm 3.0(-4.0)$. Phialide base: $1.5 \pm 2.0(-2.5)$ μm wide, phialides ampuliform with symmetrical or slightly bent necks, thickest most around the middle. Conidia $(2.0-)2.5 \pm 3.0 \times 1.5 \pm 2.0$ μm, l/w ratio: $1.2 \pm 1.5(-2.0)$ smooth, oval, less commonly subsphearoidal, containing one large guttule or few, small guttules. Round, smooth-walled chlamydospores are formed by mature colonies, mostly at hyphal termini.

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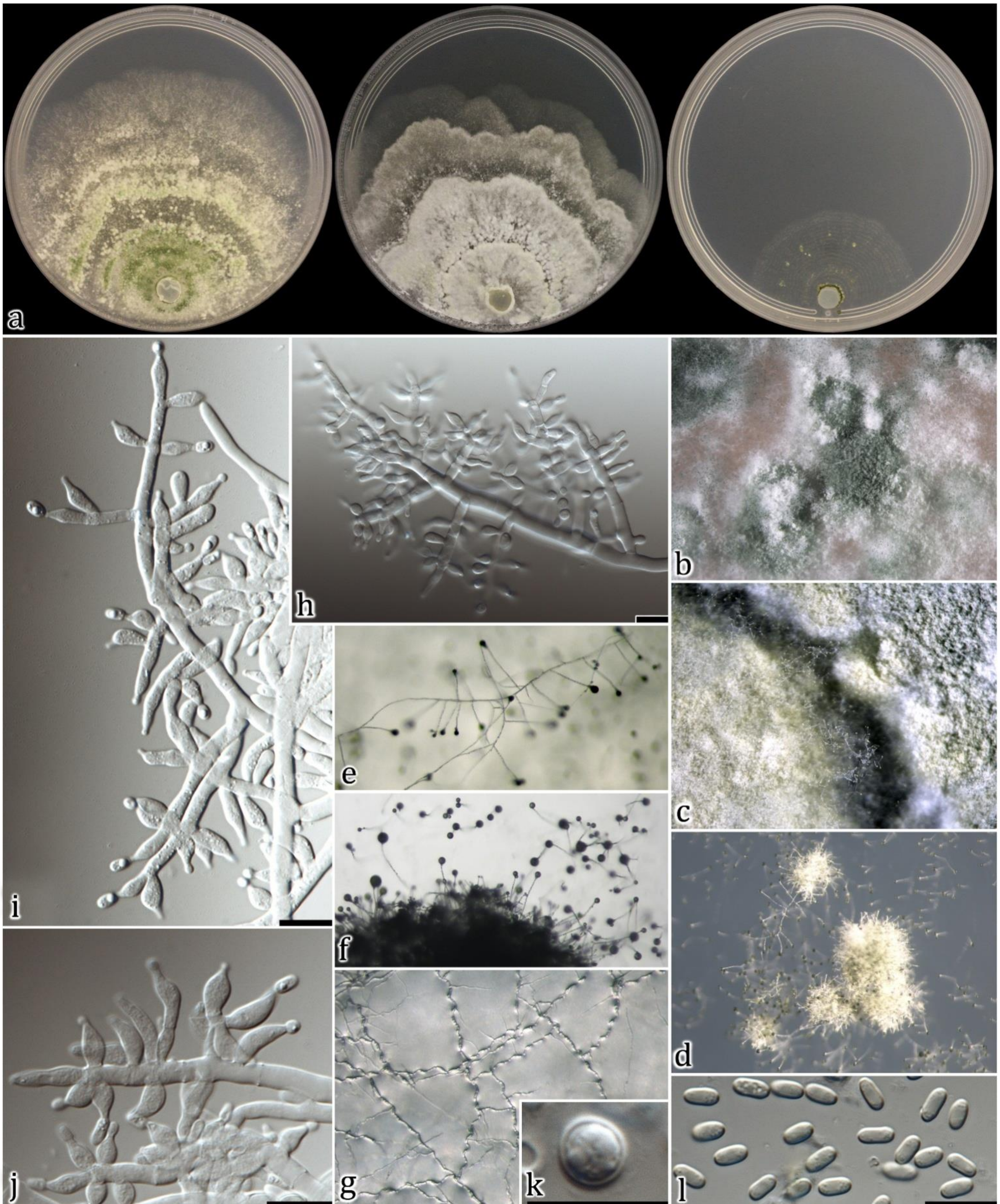

FIGURE 7. Morphological features characteristic of *Trichoderma undulatum*, Strain Tri 81. a. Colonies of *T. undulatum* incubated on CMD for 7 days (left), PDA for 7 days (middle) and SNA for 7 days (right). b, c, d. Stereo microscope images, from CMD (top), PDA (middle) and SNA (bottom). e, f. Stereo microscope images from SNA. g. Stereo microscope image on PDA. h. Conidiophore on CMA 400x magnification. i, j. Conidiophores formed on CMD 1000x. k. Chlamydospore 1000x. l. Conidia 1000x. (Scale bars = 10μm).

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PDA: optimum growth temperature at 30°C. Colony radius after 72h when grown at 30°C = 14 mm and reach 40 mm on Day Seven. At 15°C colonies reach 5 mm radius after 72h and 18 mm on day 7. At 25°C colonies reach 18 mm radius after 72h and 38 mm on Day Seven. At 35°C no growth occurs. PDA at 25°C: colony hyaline, mycelium mostly subsurface. Zonation conspicuous, the colony forms dense bundles of subsurface hyphae, this changes the appearance of the colony, hyphae that form during the dark phases are flat and more transparent whereas hyphae formed during the light phases are densely packed, opaque and slightly raised. Colony mostly sterile, white mycelia interweave to form a continuous mycelial lawn. No autolytic excretions, hyphal coilings, soluble pigments or distinctive odours noted. Hyphae that forms along the growing margin of the colony are undulated and form a serpentine pattern. At 15°C: colonies hyaline, restricted growth. No sporulation, autolytic excretions, hyphal coils or odors noted. At 30°C: Colonies resemble those formed at 25°C in all regards.

SNA: optimum growth temperature at 30°C. Colony radius after 72h when grown at 30°C = 10 mm and 30 mm on Day Seven. At 15°C colonies reach 7 mm radius on Day Seven. At 25°C colonies reach 9 mm radius after 72h of growth and 29 mm on Day Seven. At 35°C no growth occurs. SNA at 30°C; Colony thin, hyaline, zonation apparent but inconspicuous. Colony only lightly sporulating bearing conidia in wet heads. Mycelia mostly subsurface, small fertile, greyish green pustules formed. No autolytic excretions or hyphal coilings produced. At 15°C: colonies show poor growth. Hyphae subsurface, no sporulation. No autolytic excretions or hyphal coilings produced. At 25°C: colony hyaline, mycelia forming sub surface. Numerous hyphal ends break the surface of the agar and form large, wet, fertile heads, the stipes of such fertile hyphae appear roughened or warted under the dissecting microscope.

Specimens examined: South Africa, Western Cape Province, Swellendam (34°02'35.61"S 20°25'52.23"E). Isolated from bulk soils, Jul 2012, collected by I.L. du Plessis, ex-type culture Tri 81 (HOLOTYPE).

Discussion

This study isolated and described two novel *Trichoderma* species from soils collected in South Africa as part of a taxonomic inventory of the genus. Preliminary attempts to identify Strains Tri 144 and Tri 81, using the online barcoding based resource Trichokey, did not result in the identification of either strain.

Strain Tri 144 grouped with *Trichoderma* species from the *T. pachybasium* 'A' clade based on BLAST analyses using ITS and ef1α sequence data. Phylogenetic analyses showed that the *T. restrictum* is not closely related to other *Trichoderma* species currently recognized within the *T. pachybasium* 'A' clade (Fig. 2). The morphology of strain *T. undulatum* indicated its affiliation to the *T. pachybasium* clade by its conidiophore structures. *Trichoderma undulatum* forms intricately branched conidiophores with branches that bear numerous short, wide, ampuliform phialides with smooth, oval shaped conidia, which is consistent with the descriptions of *Pachybasium*-like conidiophores (Bissett 1991a) (Figs. 4 and 5).

It would be interesting to assess whether or not *T. restrictum* displays a similar potential for biocontrol application as some of its sister taxa in the *T. pachybasium* 'A' clade. A preliminary screening of Strain Tri 144 for its ability to produce volatile and non-volatile antibiotics that could inhibit the growth of a number of plant pathogens was recently done as part of a study which aimed to assess the biocontrol potential of native *Trichoderma* strains (See Chapter 5, Figures 1-4). These experiments revealed that *T. restrictum* showed only weak tendencies to inhibit the growth of selected plant pathogens through the production of antifungal metabolites *in vitro* relative to commercial biocontrol strains.

The sequence data from *T. undulatum* was divergent from the existing members of *Trichoderma*. For this reason the morphology of Strain Tri 81 was closely examined and it was found to display a number of morphological characteristics that would support its addition to the genus *Trichoderma* based on the descriptions outlined by Rifai (1969) and Bissett (1991a). In culture, Strain Tri 81 developed relatively fast growing colonies which formed white pustules that later turned green at the onset of conidiospore formation (Fig. 7). The strain also displayed micromorphological characteristics that are consistent with those of *Trichoderma.* Conidiophores, which developed within pustules, were complex and resembled the *Trichoderma*-like conidiophore type that was described by Jaklitsch (2009). These conidiophores branched extensively to form a treelike structure. Phialides from such conidiophores also tend to diverge from one another and bear single celled conidia (Fig. 7).

The phylogenetic placement of Strain Tri 81 within *Trichoderma* was made difficult by the fact that its DNA sequence data was highly divergent from other species in the genus. This made conventional phylogenetic analysis using ef1α sequence data impractical due to the fact that the sequence data from existing *Trichoderma* species and Strain Tri 81 were too divergent to align in a meaningful sense. For this reason a phylogenetic analysis was performed based on ITS sequence data, which is more conserved than ef1α sequence data, and could therefore be

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aligned without introducing a large number of ambiguous regions. This phylogenetic analysis revealed that Strain Tri 81 did not show a strong affinity towards any of the currently recognized clades within *Trichoderma* (Fig. 3). This species is, therefore, believed to constitute a novel lineage within *Trichoderma* that is distantly related to the other species.

Trichoderma restrictum and *T. undulatum* both show restricted distribution patterns in South Africa, each were isolated only once during this survey (Fig. 1). *Trichoderma restrictum* was isolated from garden soil in the North-east of the country and *T. undulatum* was isolated from the natural environment in the South-west. Therefore, little is known or can be inferred, regarding the ecology of these species or their function within the soil environment.

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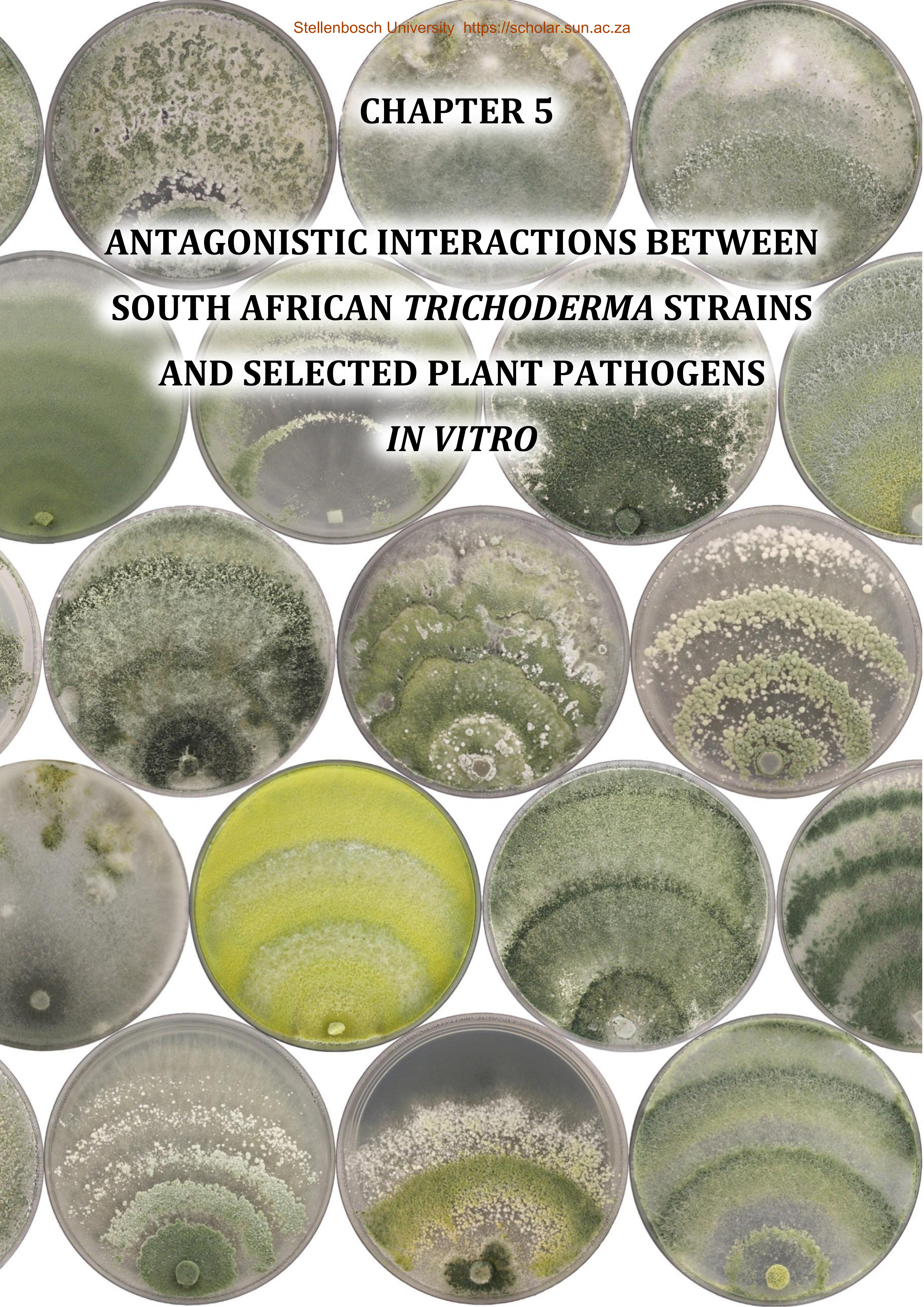

CHAPTER 5

ANTAGONISTIC INTERACTIONS BETWEEN SOUTH AFRICAN *TRICHODERMA* STRAINS AND SELECTED PLANT PATHOGENS *IN VITRO*

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Abstract

Trichoderma species are utilised as biological resources around the world and several species are routinely employed in agriculture as biological control agents or crop growth promoters. A recent survey of the *Trichoderma* population of South Africa has revealed that a number of such economically important *Trichoderma* species occur naturally in native soils. The South African agriculture sector makes use of *Trichoderma*-based biocontrol products, yet few studies have investigated the potential for native strains to antagonise plant pathogens. Such studies could lead to the possible discovery of native biocontrol agents and would facilitate the development of South Africa's biological resources. The aim of this study was, therefore, to gauge the ability of native *Trichoderma* strains to antagonise selected plant pathogens *in vitro* relative to commercial biocontrol strains. Perti dish assays were used to screen *Trichoderma* strains for their ability to produce volatile and non-volatile antifungal compounds active against several grapevine pathogens namely: *Neofusicoccum australe*, *Phompsis viticola*, *Botrytis cinerea* and *Eutypa lata*. In total, 20 native *Trichoderma* strains representing 14 different species were screened, several of which were found to outperform commercial biocontrol strains. The isolates of *B. cinerea* and *E. lata* were not inhibited by *Trichoderma* volatile and non-volatile metabolites to the same extent as *N. australe* and *P. viticola.* In some cases, total inhibition of the growth of the plant pathogens occurred, whereas in others, such metabolites had no noticeable affect. In addition, different *Trichoderma* species were found to vary significantly in terms of their antagonistic effects and this variation also occurred between different strains of the same species. The strong inhibitory effects that some of the native *Trichoderma* strains demonstrated against the selected plant pathogens indicate that their potential for future development as biocontrol agents.

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Introduction

The use of *Trichoderma* strains as biological control agents to protect crops from plant pathogens is well established (Whipps and Lumsden 2001, Harman *et al.* 2004, Anees *et al.* 2010, Segarra *et al.* 2010, Mbarga *et al.* 2012). Species such as *T. harzianum* and *T. asperellum* are examples of biocontrol agents that have proven to be effective at protecting a number of crops against a range of pathogens (Elad 1980, Inbar *et al.* 1994, Harman 2000, Verma *et al.* 2007). In addition, some *Trichoderma* species have also been shown to promote the growth of certain crops which can lead to greater yields (Ousley *et al.* 1993, 1994, Hoyos-Carvajal *et al.* 2009. Also see page 23). Lastly, *Trichoderma* biocontrol agents offer farmers a means to protect their crops whilst alleviating their dependency on chemical fungicides, some of which can have negative impacts on the environment and human health (Steenland *et al.* 1997, Brimner and Boland 2003, Farr *et al.* 2004, Komarek *et al.* 2010).

Trichoderma species are metabolically flexible and produce a diverse range of secondary metabolites which enable them to interact with other organisms in their environment in a number of different ways (Ghisalberti and Sivasithamparam 1991, Hoyos-Carvajal and Bissett 2011. Also see page 19). *Trichoderma* biocontrol agents antagonise plant pathogens through mechanisms such as mycoparasitism, direct competition, induction of the host plant immune system, as well as the production of antifungal metabolites (Benitez *et al.* 2004, Harman 2006, Harman and Shoresh 2007. Also see page 24).

In particular, the production of antifungal metabolites by *Trichoderma* strains have been reported as mechanisms of biocontrol in the past (Dennis and Webster 1971a and b, Ghisalberti and Sivasithamparam 1991, Ghisalberti and Rowland 1993, Kubicek *et al.* 2001, Bailey *et al.* 2008, Gal-Hemed *et al.* 2011). Antifungal compounds such as 6-pentyl-2H-pyran-2-one and tricholin have been identified as important antagonistic metabolites that are secreted by of a number of *Trichoderma* species (Lin *et al.* 1993, Vinale *et al.* 2008, Gal-Hemed *et al.* 2011).

Not all *Trichoderma* strains are suited for commercial application, so potential biocontrol strains are often initially screened *in vitro* based on their abilities to parasitize plant pathogenic fungi as well as to produce metabolites that prevent the growth of these plant pathogens (Askew and Laing 1994, Antal *et al.* 2000, Ortiz and Adriana 2001, Roco and Luz 2001, Hanson and Howell 2002, Kuckuk and Kicanic 2003, Samuels *et al.* 2006, Tondje *et al.* 2007). *Trichoderma* strains can exhibit different properties under field and lab conditions (Howell

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2003). For this reason, it is important to examine the effectiveness of candidate biocontrol strain *in vivo* through field trials or greenhouse experiments (Anees *et al.* 2010). These types of *in vivo* experiments are time consuming and expensive and it is, therefore, advantageous to limit the number of *Trichoderma* strains that need to be screened in this way by selecting only the most promising strains through *in vitro* screening experiments.

Candidate *Trichoderma* biocontrol strains can also be screened for traits other than those directly involved with antagonizing plant pathogens, such as for their capacity to promote the growth of certain crops or their ability to form mutualistic relationships with the roots of certain plant hosts (Yedidia *et al.* 2001, Ousley *et al.* 2003). *Trichoderma* strains that grow endophytically with the root system of a host plant are said to be rhizosphere competent (Harman 2000, 2006, Vinale *et al.* 2008, Hoyos-Carvajal *et al.* 2009). *Trichoderma* biocontrol agents that are rhizosphere competent are reportedly active within the soil for long periods of time, and are more effective at stimulating crop growth, than *Trichoderma* strains that do not possess this property (Harman *et al.* 2004).

The South African agriculture sector makes use of *Trichoderma* based biocontrol products and soil amendments on a commercial scale (Table 1). A number of these products are manufactured by companies based outside of South Africa (e. g. Adama from Israel, ABM from the USA and Koppert Biological Systems from the Netherlands). Some of these biocontrol products are also based on *Trichoderma* strains from outside of South Africa (e. g. Trianum®, which is based on *T. harzianum* Strain T-22 from the U.S.A). Relatively few studies have investigated the potential of South African *Trichoderma* strains to act as biocontrol agents. Askew and Laing (1994) were the first to report native *Trichoderma* strains that effectively antagonised strains of *Rhizoctonia solani in vitro*. In addition, Mutawila *et al.* (2011) and Kotze *et al.* (2011) demonstrated the biocontrol potential of a native *T. atroviride* strain in protecting grapevine pruning wounds from attack by pathogens *in vivo*. These studies demonstrate the capacity of native *Trichoderma* strains to antagonize plant pathogens more effectively than certain commercial strains and beg the question whether other native strains might also show similar potential for commercial development.

The grapevine industry of South Africa suffers fom the effects of certain stem pathogens. *Botrytis cinerea* is a causative agent of grey mould and *Phomopsis viticola* can cause cane and leafspot symptoms in grapevine (Holtz *et al.* 2003, O'Neill *et al.* 1996, Mostert *et al.* 2001). In addition, *Eutypa lata* and *Neofusicoccum australe* are responsible for causing dieback of grapevine (Molyneux *et al.* 2002, Amponasah *et al.* 2009). Past researchers have demonstrated

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that the use of *Trichoderma* based biocontrol approaches are viable strategies to counteract these plant pathogenic species: a study by O'Neill (1996) showed that a strain of *T. harzianum* was effective at preventing grey mould symptoms in grapevine and Mutawila *et al.* (2011) showed how *Eutypa* dieback on grapevine can be suppressed through the actions of *T. atroviride*.

The aim of this study was, therefore, to investigate South African *Trichoderma* strains for their capacity to antagonise selected grapevine pathogens *in vitro* relative to commercially used *Trichoderma* biocontrol strains.

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Materials and methods

Fungal strains used during this study

Trichoderma strains that were investigated during this study were isolated from soil samples that were collected from all across South Africa during a recent survey (See page 51). In total, 161 different *Trichoderma* strains were isolated. However, only a portion of these strains were screened. *Trichoderma* strains that belong to known biocontrol species were selected for screening, in addition to strains belonging to previously undescribed species. In total, 20 different *Trichoderma* strains representing 14 different species were screened.

A commercial strain of *T. harzianum* (Strain E77) was obtained from the Department of Plant Pathology at Stellenbosch University. A second *Trichoderma* biocontrol strain was isolated from a commercial product and was identified as *T. asperellum* Samuels, Lieckfeldt & Nirenberg (See page 93). These biocontrol *Trichoderma* strains served as reference strains during this study. Lastly, strains of *P. viticola* Saccardo, *N. australe* Slippers, Crous and Wingfield, *B. cinerea* Persoon and *E. lata* Tulasne and Tulasne were also obtained from the Department of Plant Pathology at Stellenbosch University.

Screening Trichoderma *strains for the production of non-volatile antifungal compounds*

Trichoderma strains were screened for the production of non-volatile antifungal compounds *in vitro* according to a Petri dish based assay (Dennis and Webster 1971a, Anees *et al.* 2010). MEA (Malt Extract Agar) (Merck, Germany) was prepared in 90 mm Petri dishes. MEA was selected for the experiment because it is a nutrient rich medium that supports the growth of a wide range of fungi. Cellophane membrane discs (Sigma, Germany) (90 mm in diameter) were autoclaved and placed flat on the surface of the medium. Care was taken to avoid air bubbles in between the membrane and the medium. Inoculation material for each of the *Trichoderma* strains were prepared by growing the strains on MEA and cutting agar plugs from the growing margins of the colonies. The inoculation plugs were then placed face down on top of the cellophane membranes in the center of the Petri dishes. These were then incubated for 48 hours at 25°C and were exposed to incidental light. Following incubation the cellophane membranes were removed and the Petri dishes were re-inoculated with the respective plant pathogenic strains using inoculation plugs that were prepared in the same way as with the *Trichoderma* strains described above. Petri dishes were incubated at 25°C for three to four days and the

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colony radius from each Petri dish was then recorded. The experiment was set up in triplicate and controls were prepared for the different plant pathogens by inoculating sets of Petri dishes with each respective plant pathogenic strain. The data from the test and negative control Petri dishes were then compared to one another through one-way ANOVA analyses in Excel.

It is possible that the *Trichoderma* strain might influence the growth of the plant pathogenic fungi during the assay by depleting the nutrient content of the medium as opposed to the production of antifungal compounds. Additional controls were set up to test this by incubating two different sets of cellophane Petri dishes for 72h. The first set was inoculated with the respective plant pathogens and the other set was left uninoculated. The cellophane membranes were then removed following incubation and the Petri dishes were inoculated with plant pathogenic strains. The colony radii from these two sets of Petri dishes were then compared to determine whether any inhibitory effects occurred as a result of nutrient depletion of the growth media (Dennis and Webster 1971a). Additional controls were prepared in order to determine whether or not the cellophane membranes influenced the growth of the plant pathogens.

Screening **Trichoderma** *strains for the production of volatile antifungal compounds*

Trichoderma strains were screened for the production of volatile antibiotics according to an *in vitro* assay (Dennis and Webster 1971b). Petri dishes were prepared with 20ml of MEA. *Trichoderma* strains were inoculated in the center of the Petri dishes using inoculation materials that were prepared in the same way as with the previous experiment. Petri dishes were incubated for 15 days at 25°C to allow the *Trichoderma* colonies time to develop to maturity and were wrapped in parafilm to prevent desiccation. Following incubation Petri dishes were removed from the incubator and their lids were replaced by the bottom sections of MEA plates that were newly inoculated in the center with plant pathogenic fungal strains in the same way as was described above. Parafilm was used to bind the two Petri dish bottoms together and they were incubated at 25°C for three to four days with the plant pathogen side facing down. Each strain was screened in triplicate. Control plates were set up in the same way except that the Petri dishes were not inoculated with *Trichoderma* but were instead inoculated with the plant pathogens themselves. The colony radii of the plant pathogens from each Petri dish were then recorded following incubation and the data was compared to the data from the negative controls through ANOVA analyses.

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Results and Discussion

This study showed that the growth of *N. australe* was strongly inhibited by a number of native *Trichoderma* strains (Fig. 1). Non-volatile metabolites from 11 of the 20 *Trichoderma* strains suppressed the growth of this plant pathogen by an average of more than 90% (Fig. 1). A similar result was seen when *P. viticola* was investigated, 9 of the 20 *Trichoderma* strains produced non-volatile metabolites which inhibited the growth of *P. viticola* by more than 90% (Fig. 2). However, this was not the case when *B. cinerea* and *E. lata* were investigated. Only a single *Trichoderma* strain was able to inhibit the growth of *B. cinerea* by more than 90% through the action of non-volatile metabolites (Fig. 3) and none of the *Trichoderma* strains inhibited *Eutypa lata* to the same extent (Fig 4). These results demonstrate that some of the native *Trichoderma* strains investigated here, such as Strain Tri 146 (*T. viride*) and Strain Tri 150 (*T. asperelloides*) are noteworthy producers of non-volatile metabolites that can inhibit the growth of *N. australe* and *P. viticola* effectively *in vitro*.

Results from experiments investigating the antagonistic effects that volatile *Trichoderma* antibiotics have on the growth of plant pathogens differed to a large extent from experiments investigating non-volatile metabolites. None of the volatile metabolites from native *Trichoderma* strains were able to completely suppress the growth of *N. australe,* but the greatest activity was shown by a strain of *T. atroviride* (65% growth inhibition) (Fig. 1). The same was true in the case of *B. cinerea* where no *Trichoderma* strains completely inhibited its growth (Fig. 3). The greatest activity in this case was demonstrated be a strain of *T. koningii* (80% growth inhibition) (Fig. 3). Similarly, the growth of *E. lata* was also only weakly inhibited by volatile *Trichoderma* metabolites and only *T. viride* was able to completely suppress the growth of this pathogen (Fig. 4). Lastly, *P. viticola* was also relatively weakly inhibited by the volatile metabolites of the *Trichoderma* strains and only *T. atroviride* and *T. koningiopsis* were able to inhibit the growth of this pathogen by more than 90% (Fig. 2).

Experimental controls revealed that none of the plant pathogenic species showed signs of growth inhibition when they were evaluated against themselves. Additional control petri dishes showed that cellophane membranes did not directly inhibit the growth of any of the plant pathogens. Finally, fungal growth which occurred on the cellophane membranes prior to their removal was not sufficient to deplete the medium of nutrients to the extent that growth of the plant pathogenic fungi were affected.

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The *Trichoderma* strains that were selected to serve as positive controls during this study varied in terms of their ability to inhibit the growth of the different plant pathogens through the production of antifungal metabolites. The first positive control strain, (*T. asperellum*) was not able to fully suppress the growth of any of the plant pathogens whereas the second strain, (*T. atroviride*) completely suppressed the growth of *N. australe* as well as *P. viticola* but not *E. lata* or *B. cinerea,* and only showed relatively moderate antagonistic action against these species.

Interestingly, the four new *Trichoderma* species that were isolated during this study all produced metabolites that were able to inhibit the growth of the plant pathogens to some degree. *Trichoderma vagum* prov. nom. completely inhibited the growth of *N. australe* through the production of non-volatile metabolites (Fig. 1) and also inhibited the growth of the remaining plant pathogens by more than 70% (Figs. 2, 3 and 4). Volatile metabolites from this species, however, did not affect the plant pathogens to the same extent, although a 75% reduction in the growth in *B. cinerea* was recorded relative to the negative control (Fig. 3). A previous study by Gal-Hemed *et al.* (2011) demonstrated the capacity for two strains of *T. vagum* to produce volatile and non-volatile metabolites that inhibited the growth of *Alternaria alternata* and *Rhizoctonia solani*. *Trichoderma terrigenum* prov. nom. almost totally inhibited the growth of *N. australe* through its non-volatile metabolites (99% growth reduction) (Fig. 1) and also showed notable antagonistic activity against *E. lata* and *B. cinerea* by inhibiting growth by more than 70% (Figs. 3 and 4). *Trichoderma restrictum* prov. nom. (Strain Tri 144) displayed a relatively moderate growth inhibitory effect of 85% on *P. viticola* through the production of volatile metabolites but did not yield noteworthy results in terms of its production of non-volatile metabolites. Lastly, *T. undulatum* prov. nom. (Strain Tri 81) caused a 92% reduction in the growth of *N. australe* through the production of non-volatile metabolites but performed relatively poorly against the remaining plant pathogens (Fig. 1).

A number of *Trichoderma* species that were included in the screening experiment seemed to display some variation between the different strains tested. For example, two strains of T. *asperellum* were included in this study. Strain Tri10 was isolated from soil whereas the remaining strain was a commercial biocontrol strain. These strains yielded different results in terms of their ability to produce non-volatile metabolites that were able to inhibit the growth of certain grapevine pathogens. The commercial strain inhibited the strain of *N. australe* by an average of 28% whereas Strain Tri10 inhibited this pathogen by 99% (Fig. 1). We can also see variation between the results from three *T. harzianum* strains that were included in the study.

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Some of these strains produced metabolites that inhibited the growth of *N. australe* by an average of as high as 90% whereas other strains only displayed an average of 43% inhibition (Fig. 1). These results were not unexpected as it has been reported in the past that biological traits related to the biocontrol properties of *Trichoderma* species are very strain specific and that variation is common among different strains of the same species (Harman 2000).

Overall, the plant pathogens, *N. australe* and *P. viticola,* were strongly inhibited by metabolites from a number of local *Trichoderma* strains. Results from several of the *Trichoderma* strains that were screened against these pathogens differed from the negative controls according to One-way ANOVA analyses (1DF, $P < 0.01$). On the other hand, the strains of *B. cinerea* and *E. lata* used during this study showed more resilience to the *Trichoderma* metabolites and were not inhibited to the same extent.

To conclude, this study reports a number of *Trichoderma* strains that effectively antagonized the growth of the selected plant pathogens and, therefore, show potential for possible future development as biocontrol agents. Previous authors have commented on the importance of confirming the biocontrol potential of *Trichoderma* strains through *in vivo* studies (Howell 2003, Anees *et al.* 2010). The *Trichoderma* strains reported here have only been subjected to preliminary *in vitro* screening trials and would, therefore, need to be evaluated further through *in vivo* trials before their potential for development as biocontrol agents can be fully assessed.

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TABLE 1 *Trichoderma* based biocontrol products commercially available in South Africa.

Product name	**Biocontrol agent**	**Target crop market**	**Target disease or pathogen**	**Company**	**Company websites**
Bio-Tricho	*T. harzianum* Unspecified strain	Various	Various	AgroOrganics	http://www.agro-organics.co.za/biotricho.php South African company
SabrEx	Unspecified *Trichoderma* sp.	Corn, wheat	Various	AMB	http://www.abm1st.com/crops-products/corn/sabrex/ Company from the USA
Trianum strain T-22	*T. harzianum* Unspecified strain	Various	Various	Koppert Biological Systems	http://www.trianum.com/en/products/trianum-g.html Company from the Netherlands
Tri-cure	*T. harzianum* Unspecified strain	Beans, potato	*Rhizoctonia*, *Fusarium*, and *Pythium* spp.	MBF International	http://www.mbfi.co.za/portfolio-view/tri-cure-wp/ South African company
Eco-77	*T. harzianum* Unspecified strain *T. viridescens* Strain E-77	Cucumber, grapevine, tomatoes, cucurbits	*Botrytis* spp. (tomatoes and cucumbers) *Eutypa* spp. (grapevine and cucurbits)	Plant Health Products	http://www.plant-health.co.za/index.html South African company
Trichodex-WP	*T. harzianum* Strain T39	Grapevine	Rot caused by *Botrytis* spp.	Adama	http://www.adama.com/en Company from Israel
Hygromix-T	*T. harzianum* Strain BD103	Unspecified	Unspecified	Hygrotech	http://www.hygrotech.co.za/images/SEEDLING%20PRODUCTS.pdf South African company
Trykocide	*T. harzianum* Unspecified strain	Various	Unspecified soil borne diseases	Axiom bio-product	South African company
Eco-T	*T. harzianum* Unspecified strain	Various	Unspecified root pathogens	Plant Health Products	http://www.plant-health.co.za/eco-t.html South African company
Trichoplus	*T. fertile* Unspecified strain	Various	Unspecified root pathogens	BASF	http://www.agro.basf.co.za Company from Germany supplying a product based on a South African *Trichoderma* strain
VineVax	*T. harzianum* Unspecified strain	Grapevine	Fungi causing trunk and root decay	Agrimm Technologies	http://www.agrimm.co.nz/contact.html Company from New Zealand

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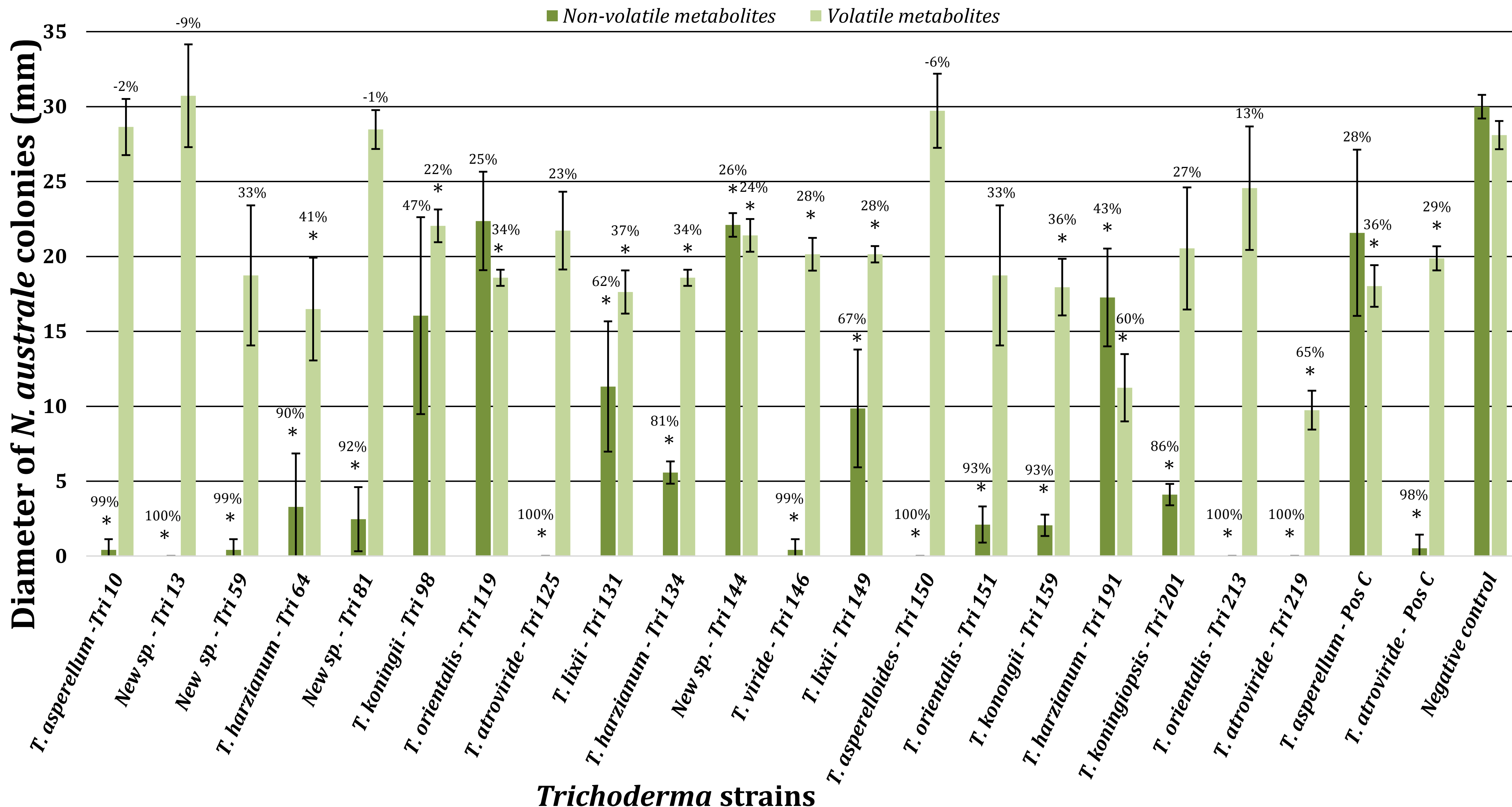

Figure 1. Bar graph illustrating the antagonistic effects of *Trichoderma* spp. on the growth of *N. australe*. Error bars represent standard deviation. Results from each *Trichoderma* strain were compared to the negative controls through One-way ANOVA analyses, asterix denotes results where P < 0.01 (1DF). The percentage inhibition relative to the negative control is indicated above each bar. Positive control strains are labelled:'Pos C'.

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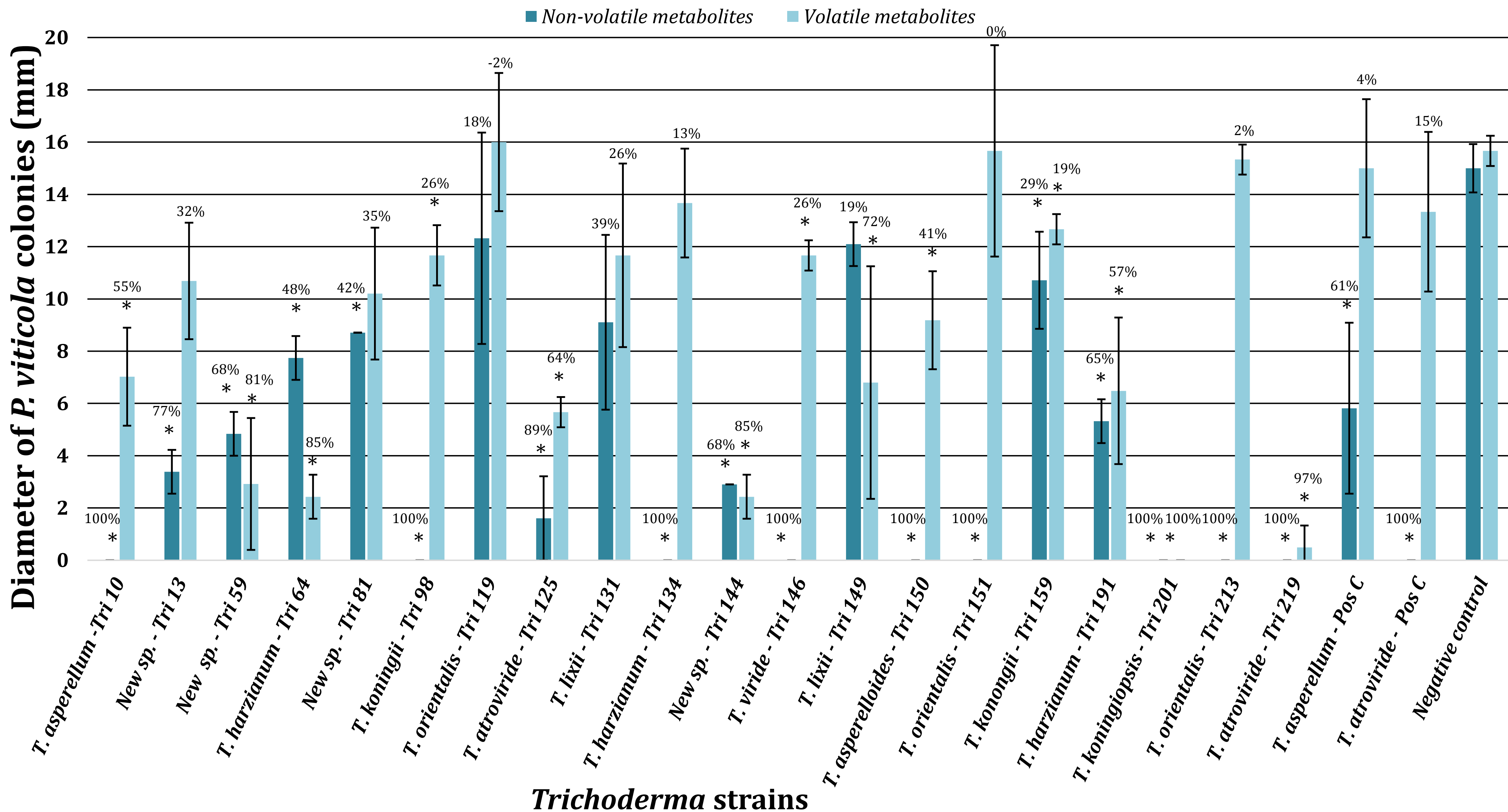

Figure 2. Bar graph illustrating the antagonistic effects of *Trichoderma* spp. on the growth of *P. viticola*. Error bars represent standard deviation. Results from each *Trichoderma* strain were compared to the negative controls through One-way ANOVA analyses, asterix denotes results where P < 0.01 (1DF). The percentage inhibition relative to the negative control is indicated above each bar. Positive control strains are labelled:'Pos C'.

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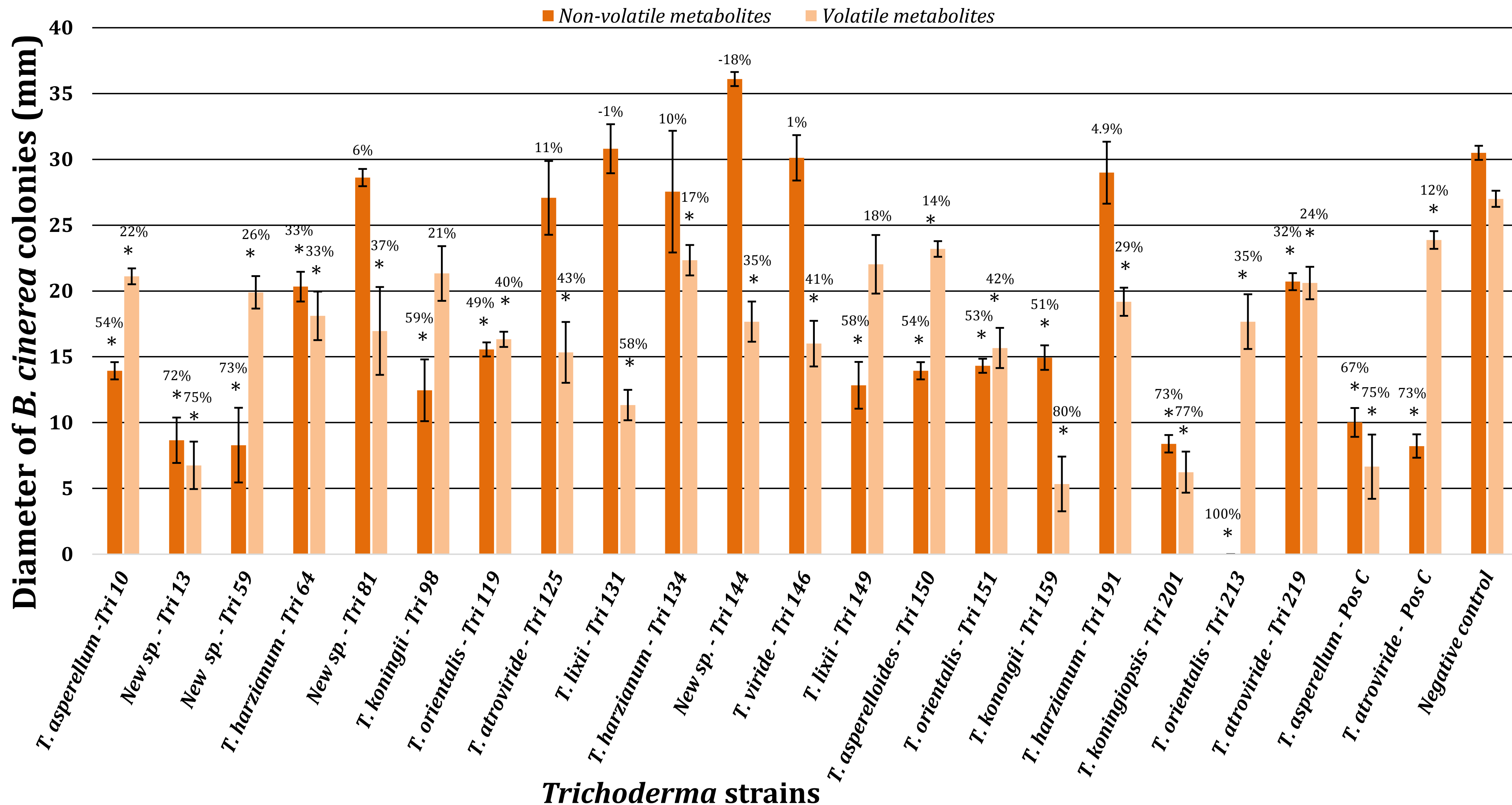

Figure 3. Bar graph illustrating the antagonistic effects of *Trichoderma* spp. on the growth of *B. cinerea*. Error bars represent standard deviation. Results from each *Trichoderma* strain were compared to the negative controls through One-way ANOVA analyses, asterix denotes results where $P < 0.01$ (1DF). The percentage inhibition relative to the negative control is indicated above each bar. Positive control strains are labelled:'Pos C'.

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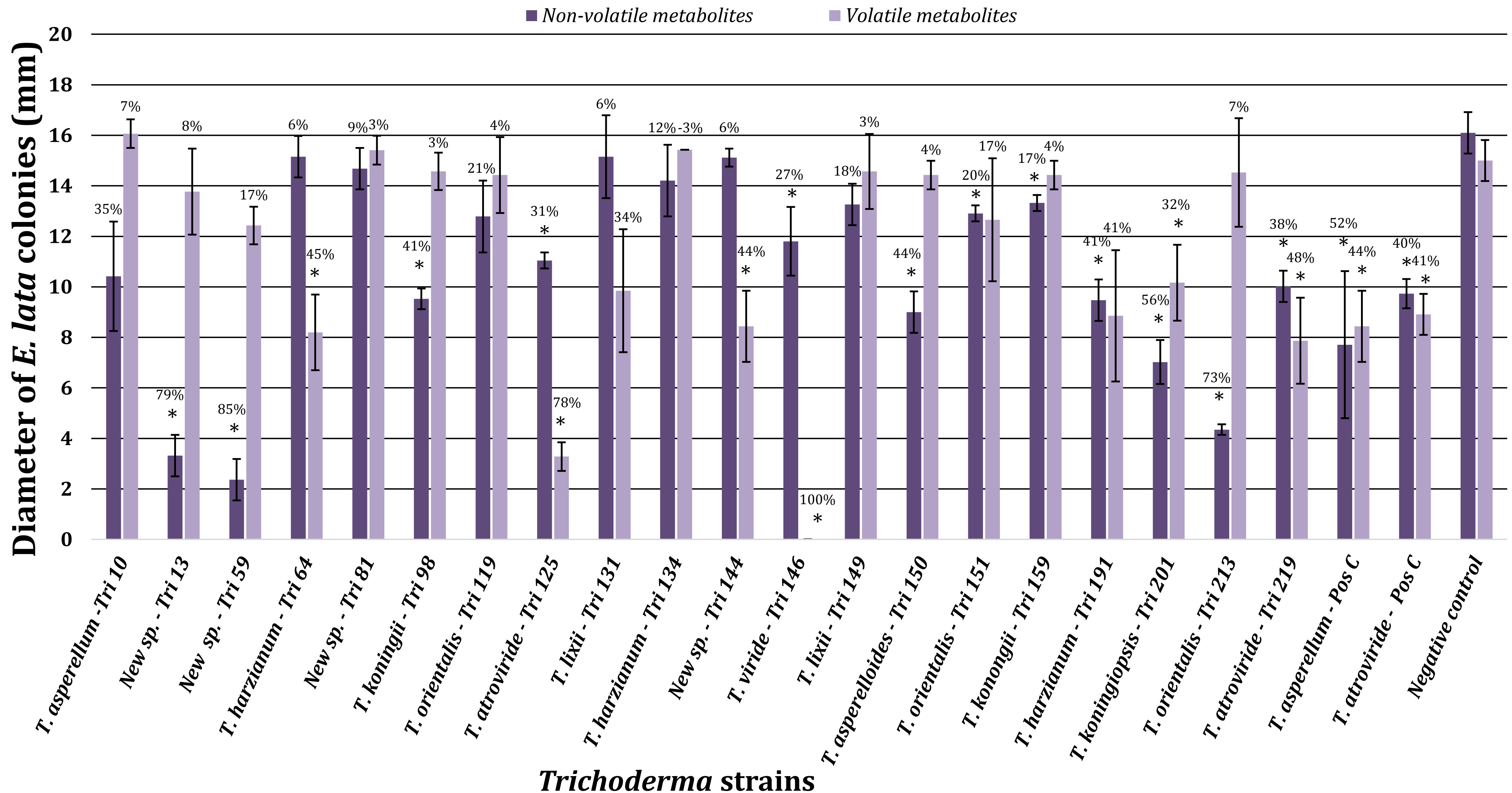

Figure 4. Bar graph illustrating the antagonistic effects of *Trichoderma* spp. on the growth of *E. lata*. Error bars represent standard deviation. Results from each *Trichoderma* strain were compared to the negative controls through One-way ANOVA analyses, asterix denotes results where P < 0.01 (1DF). The percentage inhibition relative to the negative control is indicated above each bar. Positive control strains are labelled:'Pos C'.

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Concluding remarks

The economic and agricultural importance of *Trichoderma* species are well known and numerous studies have investigated the diversity and application of these fungi worldwide. However, few such studies have been conducted in South Africa and only a handful of species have thus far been reported. Studies investigating the diversity and application of native *Trichoderma* strains would address certain research priorities expressed by government to document the biodiversity of South Africa and to promote the development of its biological resources. To this end, the primary objective of this study was to conduct a taxonomic inventory of the South African *Trichoderma* population. South Africa serves as an ideal location for such a study as the native plant and fungal communities are known to be diverse.

A total of 161 different *Trichoderma* strains were isolated from soil samples collected from across South Africa that were identified through phylogenetic and morphological analyses. *Trichoderma* isolates were found to represent 19 different species, four of which were discovered to be new to science. In addition, a number of *Trichoderma* species have not previously been reported from South Africa. These include *T. koningii, T. koningiopsis, T. asperelloides, T. lixii, T. virens* and *T. spirale*. Little overlap occurred between the *Trichoderma* species reported from South Africa by past investigators. This suggests that the known list of *Trichoderma* species in South Africa is not yet complete and that more species remain undiscovered and may come to light through continued exploration of the genus. The different *Trichoderma* species isolated during this study display different distribution patterns. Some species, such as *T. harzianum* and *T. saturnisporum*, seemed to display a widespread distribution whereas other species, such as *T. viride* and *T. asperellum*, were restricted to only certain parts of the country.

The two new species, *T. restrictum* prov. nom. and *T. undulatum* prov. nom. are each represented by a single strain and display morphological traits that are typical of *Trichoderma*. *Trichoderma restrictum* was found to belong to the *T. pachybasium* 'A' clade based on ITS and ef1α sequence data but did not group closely with any of the species currently recognized within this clade*. Trichoderma undulatum* displayed ef1*a* sequence data that was divergent from other *Trichoderma* species to the extent that meaningful multiple sequence alignments could not be made. Therefore, a genus wide phylogeny based on ITS sequence data was instead performed using representatives from each of the clades within the genus. This revealed that

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T. undulatum did not relate to any of the currently recognized clades within *Trichoderma* and, therefore, represents a novel lineage within the genus.

The remaining two new species, *T. vagum* prov. nom. and *T. terrigenum* prov. nom., belong to the *T. longibrachiatum* clade and were originally isolated from Hawaii and Israel during past studies. The novelty of these species were previously demonstrated through phylogenetic analyses using ef1α, cmd1 and chi18-5 sequence data but they were not formally described. These two new species were found to match two of the *Trichoderma* strains isolated during this study and were described.

A number of *Trichoderma* strains that were isolated during this study were identified as the human pathogen *T. orientalis*. Although this species was previously reported in South Africa, the extent of its distribution was not known. *T. orientalis* was found to be the most common species isolated during this study and also exhibited a wide distribution range. This is a concerning discovery, in light of the fact that *T. orientalis* is an opportunistic pathogen and a large portion of the South African population is immuneocompromised. The incidence of *Trichoderma* based disease in South Africa is currently not known. However, the fact that human deaths have been attributed to *T. orientalis* in the past in other parts of the world merits continued study to investigate the possible impact of this species in South Africa.

A number of important *Trichoderma* species, such as those that can be commercially employed as biocontrol agents and crop growth promoters, were isolated during this study. Such strains might have potential value as biological resources and would serve as ideal candidates for studies aiming to develop biocontrol agents or crop growth promoters in South Africa. Therefore, as a secondary objective, this study investigated South African *Trichoderma* strains for their ability to antagonize selected plant pathogens as a preliminary screening trial to assess their potential to serve as biocontrol agents. This was done by comparing native and commercial *Trichoderma* strains in terms of their ability to produce secondary metabolites that antagonize selected plant pathogens *in vitro*. Two different experiments were conducted to investigate the production of volatile and non-volatile metabolites. This showed that a number of native *Trichoderma* strains were capable of outperforming commercial strains in terms of their ability to produce non-volatile metabolites that could inhibit the growth of plant pathogens such as *N. australe* and *P. viticola*. These strains might, therefore, show potential for possible future development as biocontrol agents. Further *in vivo* screening experiments would be needed to gauge the potential of these strains for commercial development.

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To conclude, this study furthered our understanding of the diversity of *Trichoderma* in South Africa by identifying several species that were not previously known to occur in South Africa in addition to the discovery and description of several new species. Secondly this study identified a number of candidate *Trichoderma* strains that may show promise for possible future application as biocontrol agents and serves as a base from which future studies may further investigate *Trichoderma* species in South Africa.