Tulasnella spp. as saprotrophic and mycorrhizal fungi of tropical orchids: morphology, molecular taxonomy, and ecology

Dissertation

for the degree as a doctor of sciences

The Faculty of Biological Sciences

at the

Johann Wolfgang Goethe-University

submitted by

Darío Javier Cruz Sarmiento

from Loja-Ecuador

Frankfurt am Main, Germany 2016

vom Fachbereich Biowissenschaften der Johann Wolfgang Goethe-Universität als Dissertation angenommen

Dekanin

Prof. Dr. Meike Piepenbring

Gutachter

Prof. Dr. Meike Piepenbring

Prof. Dr. Franz Oberwinkler

Datum der Disputation

April 28, 2016

Form of the thesis

This dissertation is a cumulative dissertation based on the following three publications:

1.- Cruz DJ, Suarez JP, and Piepenbring M (2016) Morphological revision of Tulasnellaceae, with two new species of *Tulasnella* and new records of *Tulasnella* spp. for Ecuador. *Nova Hedwigia* 102(3–4): 279–338(60)

2.- Cruz DJ, Suarez JP, Kottke I, and Piepenbring M (2014) Cryptic species revealed by molecular phylogenetic analysis of sequences obtained from basidiomata of *Tulasnella*. *Mycologia* 106(4): 708–722

3.- Riofrio ML, Cruz DJ, Torres E, De La Cruz M, Iriondo JM, and Suarez JP (2013) Mycorrhizal preferences and fine spatial structure of the epiphytic orchid *Epidendrum rhopalostele*. *Am J Bot* 100(12): 1–10

Abstract

Tulasnella species (Tulasnellaceae, Cantharellales, Basidiomycota) form inconspicuous basidiomata on rotten branches or trunks of trees, difficult to find and recognize in nature. However, according to ultrastrucural and molecular data, species of Tulasnellaceae are the most frequent mycorrhriza forming fungi (mycobionts) of green, photosynthetic orchids worldwide. Species of Tulasnellaceae were also found as prominent mycobionts of the extraordinary diverse orchids in tropical montane rainforest of Southern Ecuador. Orchids obligately depend on mycobionts during the juvenile stage when the fungi have to deliver carbon to the non-photosynthetic protocorm and thus the fungi substantially influence the establishment of orchids in the wild. Species of Tulasnellaceae can acquire carbon from decaying bark or wood by specific saprotrophic capabilities as was recently proven through comparative genomics that included data on decay enzymes from *Tulasnella* cf. *calospora* isolated from orchid mycorrhizae (*Anacamptis laxiflora*, Italy). Thus, species of Tulasnellaceae can be saprotrophs and symbionts simultaneously.

It is currently under discussion, whether specific species of *Tulasnella* are required for seed germination and establishment of distinct terrestrial and epiphytic orchids in nature or if species of *Tulasnella* are generalists concerning their association with orchids. The inconsistences in species concepts and taxonomy of *Tulasnella* spp., however, strongly impede progress in this field of research. The aim of the present study was, therefore, to revise the species concepts by combining, for the first time, morphological and molecular data from basidiomata.

Specimens were collected in tropical Andean forest in Southern Ecuador and in temperate forests in Germany. Additional specimens were loaned from fungaria. In total, 205 specimens, corresponding to 16 own samples and 189 specimens from fungaria were analyzed. The mycobiont relationships of *Tulasnella* spp. with orchids from the sampling area in Ecuador were studied in populations of *Epidendrum rhopalostele.* The basis for molecular-phylogenetic analysis was completed by data obtained from own previous investigations on mycobionts from

the investigation area and *Tulasnella* isolates from Australia.

30 morphospecies are illustrated and delimited by a morphological key based on traditional species concepts. *Tulasnella andina* from Ecuador and *Tulasnella kirschneri* from China are presented as species new to science. *Tulasnella cruciata* is described from herbarium material for the first time. *Tulasnella* aff. *eichleriana* and *T. violea* are reported for the first time from Ecuador. Molecular sequences of two *Tulasnella* spp. isolated from mycobionts of *Epidendrum rhopalostele* cannot be related to any morphological species concept. Statistical analyses suggest that conventional diagnostic using morphological characteristics is ambiguous for delimiting morphologically similar species.

For the first time sequences of the ITS-5.8S rDNA region were obtained after cloning from fresh basidiomata. Extraction of DNA from herbarium specimens was, however, unsuccessful. Sequences from 16 fresh basidiomata, six pure cultures, and sequences of orchids mycorrhizae (e.g. from *Epidendrum rhopalostele*) available in the database GenBank were analyzed. Proportional variability of ITS-5.8S rDNA sequences within and among cultures and within and among specimens were used to designate morphospecies. Results suggest an intragenomic variation of less than 2 %, an intraspecific variation of up to 4 % and an interspecific divergence of more than 9 % for *Tulasnella* spp. Four percent of intraspecific divergence was defined as a minimum threshold for delimiting phylogenetic species. This threshold corroborates the so far used 3 % to 5 % divergence in delimitation of operational taxonomic units of *Tulasnella* mycobionts.

Quite a number of sequences of *Tulasnella* are available in GenBank, mostly obtained from direct PCR amplification from orchid mycorrhizae. By including closely related sequences in the phylogenetic analysis, several morphological cryptic species of *Tulasnella*, mostly from Ecuador, were found. Arguments are given for molecular support of the new species *Tulasnella andina* and the established species *Tulasnella albida*, *T. asymmetrica*, *T. eichleriana*, *T. tomaculum*, and *T. violea.* Thus, by combining molecular and morphological data species concepts in *Tulasnella* are improved. The definitions of *Tulasnella*

calospora and *T. deliquescens,* however, remain phylogenetically inconsistent. The present investigation is a first step to expand our knowledge on the intra- and interspecific morphological and molecular variability of *Tulasnella* spp. and to delimit species relevant for studies on ecology and communities of orchids and Tulasnellaceae.

Zusammenfassung

Die Familie der Tulasnellaceae gehört zur Ordnung der Cantharellales, welche eine basale Stellung innerhalb der Agaricomycetes (Basidiomycota) einnimmt. Die Tulasnellaceae bestehen aus drei Gattungen: *Pseudotulasnella*, *Stilbotulasnella* und *Tulasnella*. Die Gattung *Pseudotulasnella* mit der Art *Pseudotulasnella guatemalensis* sowie die Gattung *Stilbotulasnella* mit der Art *Stilbotulasnella conidiophora* sind monotypisch. Die Gattung *Tulasnella* ist die diverseste Gattung mit vielen Arten innerhalb dieser Familie. *Tulasnella* spp. sind hauptsächlich charakterisiert durch fortlaufend septierte Hyphen, die in manchen Arten Schnallen tragen. Die Basidien sind nicht septiert, generell kugelförmig oder subglobos oder keulenförmig bis obclavat. Cystiden, wenn vorhanden, sind Gloeocystiden. Die Sterigmen sind kugelförmig, ellipsoid oder spindelförmig. Manche Arten bilden auch Chlamydosporen. Die Basidiosporen sind glatt und können unterschiedliche Formen haben, wie z.B. kugelförmig, subglobos, länglich, elliptisch, bohnenförmig, allantoid, spiralig, sigmoid oder bauchig. Sie produzieren laufend sekundäre Basidiosporen. Weiterhin bilden *Tulasnella* spp. unscheinbare Basidiomata auf verrottenden Ästen oder Baumstämmen, welche in der Natur schwierig zu entdecken sind.

Asexuelle Stadien von *Tulasnella* spp. sind ebenfalls bekannt, welche hauptsächlich von Orchideenmykorrhizen isoliert wurden. In Kultur sind die monilioiden Zellen das wichtigste morphologisch-vegetative Merkmal der Anamorphen von *Tulasnella*. Diese anamorphen Arten wurden in die Gattung *Epulorhiza* eingeteilt, welche wiederum in der künstlichen Gruppe *Rhizoctonia* untergebracht wurden.

Die Tulasnellaceae beinhalten 54 offiziell beschriebene Arten. Nichtsdestotrotz lassen Studien von mykorrhizabildenden Pilzen (Mykobionten) in Orchideen eine größere Vielfalt der Arten von *Tulasnella* erahnen, basierend auf MOTU (molecular operational taxonomic units)-Daten. Basierend auf diesen molekularen Daten und ultrastrukturellen Beweisen (septierte Hyphen mit Doliporen und durchgehende Parenthosomen, die in den kortikalen Zellen der Orchideen enden) sind die Arten

der Tulasnellaceae die am weitesten verbreiteten Mykobionten von grünen, photosynthetisch aktiven Orchideen weltweit. Insofern sind *Tulasnella* spp. bedeutende Mykobionten der außerordentlich diversen Orchideen im tropischen Gebirgs-Regenwald von Süd Ecuador. Orchideen sind während ihrem juvenilen Stadium obligat von den Mykobionten abhängig, da die Pilze den Kohlenstoff zum nicht-photosynthetisch aktiven Protocorm liefern und die Pilze somit einen wesentlichen Einfluss auf die Etablierung der Orchideen in der Wildnis haben. Die Arten der Tulasnellaceae können durch spezielle saprotrophe Eigenschaften Kohlenstoff aus verfaulender Rinde oder Holz gewinnen, was kürzlich durch vergleichende Genomanalyse mit Daten von Abbau- Enzymen bewiesen werden konnte, welche aus *Tulasnella* cf. *calospora* stammten, isoliert aus Mykorrhizen der Orchidee *Anacamptis laxiflora* in Italien. Daher können die Arten der Tulasnellaceae offensichtlich gleichzeitig sowohl saprotrophe Organismen als auch Symbionten sein.

Zur Zeit wird diskutiert, ob spezielle Arten von *Tulasnella* für die Samenkeimung und zur Etablierung von unterschiedlichen terrestrischen und epiphytischen Orchideen in der Natur erforderlich sind, oder ob die Arten von *Tulasnella* in Bezug auf ihre Verbindung mit Orchideen eher Generalisten sind. Daher ist die Identifizierung von mit Orchideen assoziierten Mykorrhizapilzen auf der Populationsebene essentiell für die Planung von Schutzmaßnahmen für Orchideen. Rekrutierungsmuster könnten ebenfalls durch Faktoren wie Reichweite der Samenausbreitung und spezifische Umweltmerkmale bedingt sein. Die Inkonsistenz in Artkonzepten und der Taxonomie von *Tulasnella* spp. erschweren stark den Fortschritt innerhalb dieses Forschungsfeldes. Die Abgrenzung der Arten und die Suche nach einem geeigneten Schwellenwert, um die phylogenetischen Arten der Pilze zu definieren, sind in der Diskussion. Gerade die Arten der Tulasnellaceae sind aufgrund von unklaren morphologischen und molekularen Merkmalen innerhalb und zwischen den unterschiedlichen Arten schwer taxonomisch zu organisieren. Daher war das Ziel der vorliegenden Studie, die Artkonzepte innerhalb der Tulasnellaceae zu überarbeiten und zwar zum ersten

Mal durch die Kombination von morphologischen mit molekularen Daten der Basidiomata. Die traditionellen taxonomischen Methoden wie dichotome Bestimmungsschlüssel und computer-basierte numerische Algorithmen wurden ebenfalls genutzt. Die numerische Taxonomie dient als Hilfe zur Bestimmung der brauchbarsten Merkmale für die Erstellung des dichotomen Bestimmungsschlüssels.

Resupinate Basidiomata mit Merkmalen der Tulasnellaceae, wie wächserne bis fast schleimige Konsistenz und grau-violette oder hell-rosa Farbe, wurden von verrottendem Holz oder hinabgefallenen Ästen gesammelt. Die Sammelstellen befanden sich in immergrünen, tropischen Gebirgsregenwäldern der Anden in Süd Ecuador sowie in temperaten Wäldern in der Nähe von Frankfurt am Main in Deutschland. Die Sammelstellen in Ecuador befinden sich in der Reserva Biológica San Francisco (RBSF) und in einem sich erneuernden, annähernd 35 Jahre alten Wald. Das Sammeln in Deutschland wurde in sich regenerierenden Mischwäldern durchgeführt. Weitere Proben wurden aus Pilzsammlungen entliehen. Insgesamt wurden 205 Proben, davon 16 eigene Proben sowie 189 Proben aus Pilzsammlungen, analysiert. Sowohl die neuen Aufsammlungen als auch die Pilzherbar-Proben wurden durch mikroanatomische Untersuchungen verglichen, um bessere Einblicke in die unterschiedlichen Merkmale zu gewinnen. Kleine Stücke frischer Basidiomata von *Tulasnella* spp. wurden für die Sporulation mit anschließender Isolation auf festen PDA- und MEA-Agarmedien genutzt. Die mykobiontischen Beziehungen von *Tulasnella* spp. mit Orchideen (z.B. *Epidendrum rhopalostele*) aus dem Sammelgebiet in Ecuador wurden mit Hilfe von ITS-5.8S-Sequenzen aus GenBank phylogenetisch untersucht. Die Kolonisierung der Orchideenwurzeln durch die Mykobionten wurde zudem lichtmikroskopisch überprüft.

Die Basis der molekularphylogenetischen Untersuchung wurde durch Daten erweitert, die von den eigenen vorhergehenden Untersuchungen der Mykobionten aus den Forschungsarealen stammten sowie von acht *Tulasnella* Isolaten. Drei

Kulturen, die aus Australien stammten, wurden isoliert sowie identifiziert durch Warcup und Talbot als *Tulasnella asymmetrica*, drei Isolate von *Tulasnella* spp. aus Deutschland wurden von Franz Oberwinkler beigesteuert und zwei Kulturen wurden aus frischen Basidiomata gewonnen, welche in Deutschland gesammelt wurden. Die beiden zuletzt genannten Stämme wurden im Rahmen der vorliegenden Studie durch den Autor als *Tulasnella* cf. *pinicola* und *Tulasnella violea* identifiziert, gemäß ihrer teleomorphen Affinitäten.

Mikro-morphologische Strukturen von 30 Morphospezies wurden per Hand gezeichnet. Alle Morphospezies wurden durch morphologische Bestimmungsschlüssel, welche auf den traditionellen Artkonzepten beruhen, voneinander abgegrenzt. *Tulasnella andina* aus Ecuador und *Tulasnella kirschneri* aus China werden der Wissenschaft als neue Arten präsentiert. *Tulasnella cruciata* wurde zum ersten Mal detailliert beschrieben basierend auf Herbarmaterial. Zum ersten Mal wird von *Tulasnella* aff. *eichleriana* und *T. violea* in Ecuador berichtet. Die molekularen Sequenzen von zwei *Tulasnella* spp., isoliert als Mykobionten von *Epidendrum rhopalostele*, können keinem morphologischen Artkonzept zugeordnet werden. Bisher war es nämlich nicht möglich, diese beiden Arten als Fruchtkörper zu sammeln, was für eine formale Artbeschreibung notwendig ist. Studien an weiteren Populationen von *E. rhopalostele* sind notwendig, um die Bindung dieser Orchidee and bestimmte Arten von *Tulasnella* zu überprüfen.

Unterschiedliche Ansätze wurden durchgeführt, um bivariate Analysen, einfache lineare Regression und den Turkey-Test durchzuführen, um damit Arten und Gruppen aufgrund morphologischer Ähnlichkeiten zu definieren. Durch die Form der Basidiosporen können sieben Gruppen von *Tulasnella* spp. unterschieden werden. Andere morphologische Merkmale, wie das Vorhandensein von Schnallen oder Cystiden sind in allen sieben Gruppen verbreitet. Zudem wird die bekannte hohe Variabilität der Form und Größe der Basidiosporen innerhalb und zwischen den Arten von *Tulasnella* durch diese Analysen bestätigt. Statistische Analysen

lassen vermuten, dass konventionelle Diagnostik, welche morphologische Merkmale nutzt, zu uneindeutig für die Abgrenzung morphologisch ähnlicher Arten ist.

Zum ersten Mal wurden Sequenzen der ITS-5.8S rDNA-Region von *Tulasnella* spp. gewonnen, nachdem diese aus frischen Basidiomata kloniert wurden. Die Extraktion der DNA aus Herbarbelegen war hingegen nicht erfolgreich. Die Pilzherbar Proben waren 20-40 Jahre alt und oftmals in schlechtem Zustand, da sie kollabierte und degradierte Strukturen aufwiesen. Sequenzen aus 16 frischen Basidiomata, sechs Reinkulturen und Sequenzen von Orchideen-Mykorrhizen (zum Beispiel von *Epidendrum rhopalostele*), vorliegend in GenBank, wurden analysiert. Die proportionale Variabilität der ITS-5.8S rDNA-Sequenzen innerhalb sowie zwischen den Proben wurde genutzt, um die Morphospezies zu kennzeichnen. Weiterhin wurde die intragenomische Variabilität der ITS-5.8S rDNA-Region aus klonierten Sequenzen von Reinkulturen bestimmt. Die sogenannte "Barcode-Lücke" (barcode–gap) wurde definiert als der Abstand zwischen der maximalen intraspezifischen Variabilität und der minimalen interspezifischen Divergenz, um so einen zuverlässigen molekularen Schwellenwert für die Abgrenzung der Arten von *Tulasnella* zu gewinnen.

Die Ergebnisse deuten auf eine intragenomische Variabilität von weniger als 2%, eine intraspezifische Variabilität von bis zu 4% und eine interspezifische Divergenz von mehr als 9% für *Tulasnella* spp. hin. Vier Prozent Divergenz wurde als Minimum-Schwellenwert für die Abgrenzung der phylogenetischen Arten definiert. Dieser Schwellenwert bestätigt die bis jetzt genutzte 3% bis 5% Divergenz bei der Abgrenzung von praktikablen taxonomischen Einheiten von *Tulasnella*-Mykobionten oder für das Abschätzen der Diversität in anderen Pilzgruppen.

Viele Sequenzen von *Tulasnella* sind über GenBank erhältlich, hauptsächlich gewonnen durch direkte PCR-Amplifikation aus Orchideen-Mykorrhiza oder aus Lebermoosen der Familie Aneuraceae. Manche Sequenzen stammen von keimfreien, anamorphen Kulturen, welche aus Mykorrhizen gewonnen wurden. Durch das Einbeziehen von eng verwandten Sequenzen und 4% der molekularen Divergenz als Minimum-Schwellenwert in der phylogenetischen Analyse, konnten viele morphologisch kryptische Arten von *Tulasnella*, hauptsächlich aus Ecuador, gefunden werden. Viele andere Sequenzen, welche aus Basidiomata von *Tulasnella* spp. aus verschiedenen Abstammungsgemeinschaften gewonnen wurden, gruppierten sich zusammen mit Sequenzen, die von Orchideen-Mykorrhizen stammten, welche an der gleichen Stelle in Ecuador gesammelt wurden. Bedenkt man die Abhängigkeit der Orchideen von Mykobionten und *Tulasnella* im Speziellen, können viele weitere *Tulasnella*-Arten in Ecuador erwartet werden, sowie in anderen Regionen, die artenreich bezüglich der Orchidaceae sind.

Die molekularen Informationen zeigen klare Grenzen für die neue Art *Tulasnella andina*. Nach bestem Wissen ist dies die erste Art, die in dieser Gattung beschrieben wurde, basierend auf morphologischen und molekularen Daten. Vieles spricht dafür, dass die molekularen Daten die bisher aufgrund morphologischer Merkmale vorgenommenen Artzuordnungen von *Tulasnella albida*, *Tulasnella asymmetrica*, *Tulasnella eichleriana*, *Tulasnella tomaculum* und *T. violea* unterstützen. Somit sind durch die Kombination von molekularen und morphologischen Daten diese Artkonzepte von *Tulasnella* bestätigt worden. Die Definitionen von *Tulasnella calospora* und *Tulasnella deliquescens* bleiben jedoch phylogenetisch inkonsistent. Die vorliegenden Untersuchungen sind ein erster Schritt, um das Wissen über die intra- und interspezifische morphologische und molekulare Variabilität zu erweitern sowie um Arten abzugrenzen, die für Studien der Ökologie und der Lebensgemeinschaften von Orchideen und Arten der Tulasnellaceae relevant sind.

1 Introduction

The family Tulasnellaceae Juel belongs to the order Cantharellales placed in a basal position within the Agaricomycetes, Basidiomycota (Moncalvo 2006; Hibbett et al. 2007; Veldre et al. 2013; Kohler 2015). Tulasnellaceae consist of three genera, *Pseudotulasnella* Lowy*, Stilbotulasnella* Oberw. & Bandoni, and *Tulasnella* J. Schröt., and include 54 formally described species (Kirk et al. 2008). Nonetheless studies of orchid mycobionts suggest more diversity of species of *Tulasnella* based on MOTUs (Molecular operational taxonomic units) (Jacquemyn et al. 2011; 2015; Martos et al. 2012; Girlanda et al. 2011; Kottke et al. 2013). The genus *Pseudotulasnella* with the species *P. guatemalensis* Lowy is monotypic and characterized by apically partly septate basidia and lack of clamps at the septa (Lowy 1964). The genus *Stilbotulasnella* is monotypic as well. *Stilbotulasnella conidiophora* Bandoni & Oberw. is characterized by prominent synnemata with blastic conidia. This species lacks an organized hymenium and the hyphae are septate with dolipores without parenthesomes (Bandoni and Oberwinkler 1982).

The genus *Tulasnella* was established in 1888 by Schröter, with *Gloeotulasnella* Höhn. & Litsch. (1908) being a synonym according to Olive (1957) and Roberts (1994b). *Tulasnella* spp. are characterized by hyphae that are continuously septate, in some cases with clamps. Basidia are not septate, generally globose to subglobose or clavate to obclavate. Cystidia, when present, are gloeocystidia. Sterigmata are globose, ellipsoidal or fusiform. Chlamydospores are generated by some species. Basidiospores are smooth and present different shapes, i.e., globose, subglobose, oblong, elliptic, phaseoliform, allantoid, spiral, sigmoid, or ventricose. They produce secondary basidiospores by repetition (based on Cruz et al. 2016).

Asexual states are known from cultures of *Tulasnella* spp. isolated mainly from orchid mycorrhizae (Warcup and Talbot 1967; 1971; 1980), few were obtained from basidiomata (Oberwinkler, pers. com.). These anamorphs have been classified in the genus *Epulorhiza* and accommodated in the artificial group *Rhizoctonia* (Moore 1987). *Epulorhiza repens* (Bernard) Moore [teleomorph:

Tulasnella deliquescens (Juel) Juel] is the type species. In culture, the monilioid cells are the main morphological vegetative characteristic of *Epulorhiza*. Petersen (1968) showed asexual spore production in a culture of *Tulasnella pinicola* Bresadola [= *Gloeotulasnella pinicola* (Bresadola) Rogers]. Ingold (1984) reported short conidiophores from which stellate conidia are formed in succession for *Tulasnella cystidiophora* Höhn. & Litsch., on the surface of the culture medium. Teleomorphic states of *Tulasnella* spp. were generated from isolates of Australian orchid mycorrhiza by Warcup and Talbot (1967; 1971; 1980).

Pseudotulasnella guatemalensis is only reported from Guatemala (Lowy 1964), and *Stilbotulasnella conidiophora* only from Hawaii (Bandoni and Oberwinkler 1982). *Tulasnella* spp. are known to the northern hemisphere, i.e., for Belgium, England, France, Germany, Norway, Spain, Turkey, Wales (Bourdot and Galzin 1909; 1928; Dueñas 2005; Jülich and Jülich 1976; Olive 1957; Ordynets 2012; Roberts 1992; 1993a and b; 1994 and b; 1999; Rogers 1933; Van de Put and Antonissen 1996) but also from Australia (Warcup and Talbot 1967; 1971; 1980), Tahiti (Olive 1957), and South America (Cruz et al. 2011; 2014; Greslebin and Rajchenberg 2001). Almost all described species in Tulasnellaceae appear to be saprotrophic (Bandoni and Oberwinkler 1982; Cruz et al. 2011; Greslebin and Rajchenberg 2001; Lowy 1964; Roberts 1992; 1993a and b; 1994a and b; 1999). In addition, potentially almost all species are important mycobionts associated with roots of epiphytic and terrestrial orchids worldwide, as shown by molecular data (Bougoure et al. 2005; Girlanda et al. 2011; Jacquemyn et al. 2011; Ma et al. 2003; Martos et al. 2012; McCormick et al. 2004; Pereira et al. 2003; 2005; 2014; Riofrio et al. 2013; Suarez et al. 2006; Kottke et al. 2010; Warcup and Talbot 1967; 1971; 1980; Kohler et al. 2015). *Tulasnella* spp. were reported as the most common mycobionts associated with roots of epiphytic and terrestrial orchids in a tropical mountain rainforest of the Northern Andes of Ecuador (Kottke et al. 2010; Riofrio et al. 2013). *Tulasnella* spp. were also reported as symbionts of liverworts (Bidartondo et al. 2003; Kottke et al. 2003). *Tulasnella zooctonia* is parasitic on *Amoeba terricola* (Drechsler 1969).

Species concepts in Tulasnellaceae were based on macroscopic characteristics of basidiomata first (Fig. 1A) (Bourdot and Galzin 1909), later on micro-morphological characteristics of the teleomorph (Fig. 1B-D) (Bandoni and Oberwinkler 1982; Lowy 1964; Roberts 1999; Rogers 1933) or on structures of the anamorph (Pereira et al. 2003; 2005; 2014). The morphology of basidiospores is probably the most commonly used characteristic of taxonomical significance to distinguish species of fungi (Parmasto and Parmasto 1987; 1992) and probably important in evolutionary trends in Basidiomycota (Oberwinkler 2012).

Traditional taxonomy methods like dichotomous keys were revolutionized by computer-based numerical algorithms (Sokal and Sneath 1963; Sokal 1966). The algorithms are an essential tool in taxonomy based on quantitative morphological characteristics of organisms such as fungi that are otherwise hard to distinguish (Erdem et al. 2011; Morgan 1971; Stielow et al. 2011). Numerical taxonomy may help to select the most useful characteristics for dichotomous keys. Dichotomous or sequential keys for species of the genus *Tulasnella* are available from Roberts (1994b; 1999), but actually improvement and incorporation of new taxa is needed.

Meanwhile nuclear ribosomal DNA (rDNA) sequences and molecular-phylogenetic analyses revealed numerous “cryptic” species with unresolved species concepts (Taylor and McCormick 2008). Most sequences available for *Tulasnella* spp. were so far obtained by direct PCR amplification from orchid mycorrhizae or Aneuraceae liverworts (Girlanda et al. 2011; Jacquemyn et al. 2010; 2011; Kottke et al. 2003; Martos et al. 2012; McCormick et al. 2004; Suarez et al. 2006; Taylor and McCormick 2008; Preußing et al. 2010). Some sequences stem from axenic, anamorphic cultures obtained from mycorrhizae (McCormick et al. 2004; Suarez et al. 2006). No sequences from basidiomata were available at the beginning of the present study, impeding correlation of morphological species and molecular genotypes.

The internal transcribed spacer (ITS) was suggested as general marker for species delimitation in Fungi (Schoch et al. 2012). Beside other regions (5.8S rDNA) and mitochondrial sequences, the ITS region was widely used to define genotypes in

Tulasnella spp. (Shefferson et al. 2005; 2007; Chutima et al. 2011; Cruz et al. 2011; 2014; Chen et al. 2012; Martos et al. 2012; Okayama et al. 2012; Valadares et al. 2012; Oliveira et al. 2013). However, *Tulasnella* spp. exhibit exceptionally high molecular variability in rDNA regions, similar to other cantharelloid fungi (Moncalvo et al. 2006). Sequence alignments are, therefore, difficult and need improvement. The threshold for species delimitation, currently proposed with a value of 3 % divergence in the ITS sequences of fungi, varies in different investigations on Tulasnellaceae (3 % to 5 %).

The idea of the presented study was, therefore, to isolate and sequence DNA from basidiomata and cultures of *Tulasnella* spp. to clarify intra- and interspecific variation of the ITS-5.8S rDNA region. Further, the intra-genomic variation of the ITS region was determined from clone sequences of pure cultures. The so-called "barcode gap" should be defined, as distance between the maximal intra-specific variation and the minimal inter-specific divergence (Meyer and Paulay 2005) to obtain a reliable molecular threshold for species delimitation in *Tulasnella*. In a further step, integration of morphological and molecular-phylogenetic characteristics should be achieved for *Tulasnella* species.

In the present dissertation I present new data for *Tulasnella* species, based on new morphological observations and molecular sequences from own samples from tropical and temperate forests and fungaria material. The first part of the investigation (publication) yielded descriptions of species and revisions of quantitative morphological characteristics, including a new dichotomous key for 30 species. In the second publication for the first time ITS sequences obtained from basidiomata were correlated with morphological data. An improved concept for *Tulasnella* spp. delimitation is presented and two new species are established. In the third publication, mycorrhizal partners of *Epidendrum rhopalostele* Hágsater & Dodson were detected by analyses of DNA sequence data that showed the dominance of two *Tulasnella* spp. Furthermore, evidence about the life-style of *Tulasnella* spp. as saprotrophs and symbionts is presented.

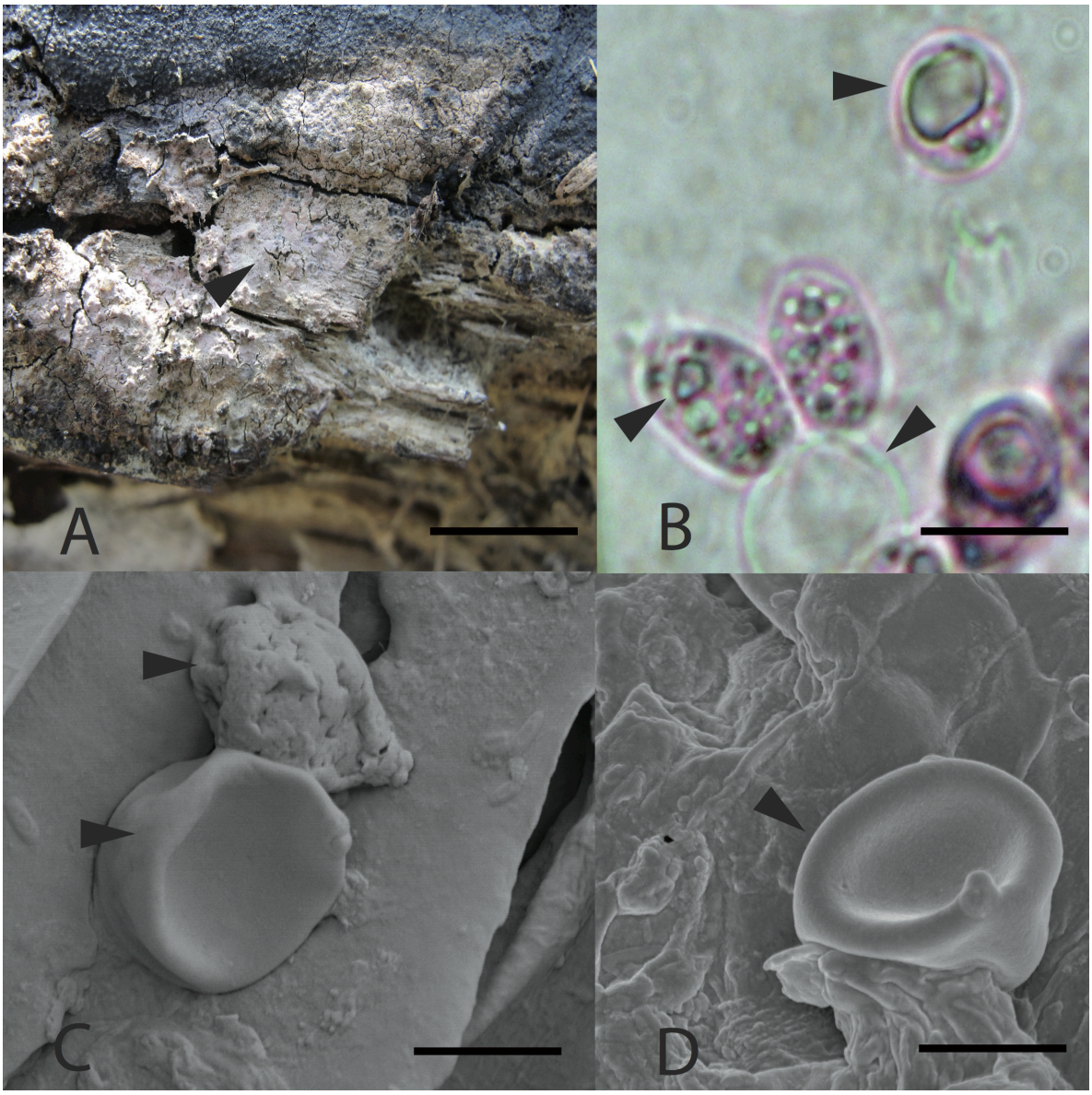

Figure 1. (A) Resupinate pinkish basidioma on decaying wood (black arrowhead) formed by *Tulasnella violea*. (B) Typical tullasnelloid basidium, sterigmata and globose basidiospore (black arrowheads) of *Tulasnella* sp. The microstructures are stained with phloxine 1 % and observed by light microscopy. (C-D) Scanning electron microscopy (SEM) photographs of globose basidiospores of *Tulasnella violea*. The preparation to SEM was from air-dried material of the specimen DC293, directly mounted and sputtered with gold for 60 sec. Bars: (A) = 4 cm. (B) = 8 μm. (C-D) = 4 μm.

2 Material and Methods

2.1 Sampling of basidiomata and mycorrhizae

Sampling of resupinate fungi with tulasnelloid characteristics (Fig. 1A) (Roberts 1999; Cruz et al. 2011) was carried out in evergreen upper montane tropical rainforest in Ecuador (Beck et al. 2008; Bendix et al. 2013) and temperate forests near Frankfurt am Main, Germany, 2007–2010. Sampling sites in Ecuador included the Reserva Biológica San Francisco (RBSF), on the eastern slope of the Cordillera El Consuelo in the northern Andes, Zamora Chinchipe province (3°58′ S, 79°04′ W, 1900–2500 m a.s.l) and a fragment of regenerating, approximately 35 years old forest on the eastern slope of the Cordillera Real in the northern Andes, Zamora Chinchipe province (3°59′ 17″ S, 79°6′ 4″ W, 2280 m a.s.l). Sampling in Germany was carried out in regenerating, mixed forests in or close to Frankfurt am Main, such as the Ginnheim forest (8°38′ E, 50°08′ N, 112 m a.s.l), the Stadtwald, the Louisa forest (8°40' E, 50°5' N, 149 m a.s.l), and the forest of Apfelbach, Groß Gerau (8°29′ E, 49°55′ N, 140 m a.s.l).

Basidiomata were collected from decayed wood or fallen branches. DNA was extracted from the fresh basidiomata. All samples of *Tulasnella* spp. from Ecuador and Germany were dried and deposited in the fungarium section of the Herbarium Universidad Tecnica Particular de Loja (HUTPL), Loja, Ecuador.

Sequences of two *Tulasnella* spp. that form orchid mycorrhiza with *Epidendrum rhopalostele* in the tropical forest Cordillera El Consuelo southern Ecuador (Riofrio et al. 2013) were included in our analysis.

2.2 Specimens loaned from fungaria

The morphological revision is based on 205 specimens, including 189 specimens from fungarium material and 16 specimens from own collections. *Tulasnella* specimens were loaned from the following fungaria: National Botanic Garden of Belgium (BR), Kew Royal Botanic Gardens (K), Eberhard-Karls-Universität Tübingen (TUB). The specimens collected by Roland Kirschner (RoKi) are

deposited in the National Museum of Natural Science of Taiwan (TNM) and fungal herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN-HKAS).

2.2.1 *Axenic cultures of Tulasnella*

Eight axenic cultures of *Tulasnella* spp. were included in the molecular analyses. Three cultures originated from Australia and were isolated and identified by Warcup and Talbot (1967) and Warcup (1973) as *Tulasnella asymmetrica* Warcup & P.H.B. Talbot (culture ID: MAFF 305806, MAFF 305808, MAFF 305809). Three isolates from Germany, *Tulasnella* sp. FO 35532, *Tulasnella* sp. FO 24380a, and *Tulasnella* sp. FO 24462a were contributed by Franz Oberwinkler, culture collection of the Institute of Ecology, Evolutionary Ecology of Plants, Eberhard-Karls-University Tübingen, Germany. Two cultures were obtained from fresh basidiomata sampled in Germany by the author. The strains (DC309 and DC292) were identified as *Tulasnella* cf. *pinicola* Bres. and *T. violea* (Quél.) Bourdot & Galzin, respectively, according to their teleomorph affinities.

2.3 Morphological diagnosis of basidiomata

Free-hand sections of basidiomata of the fresh samples and the fungarium material were stained with 1 % phloxine. Amyloid reaction was evaluated by use of Melzer's reagent. Sections were examined at 100 to 1000-fold magnification by use of a Leitz SM-LUX or Zeiss Axioskop 2 microscope. Illustrations of micro-morphological structures were done by hand using a scale of 1 × 1 cm^2 corresponding to 5 × 5 μm^2. The different shapes of the basidiospores (Fig. 2 A-P) were classified mostly according to Largent et al. (1973). Hyphal diameter, length/width of cystidia, basidia, sterigmata, and basidiospores were measured based on 30 basidiospores and at least 25 further structures per specimen. Q-values as length/width were calculated for basidiospores (Tulloss and Lindgren 2005). Morphospecies identification of the new specimens was obtained by using the key of Roberts (1999). Identifications were corroborated by comparison with the fungaria collections.

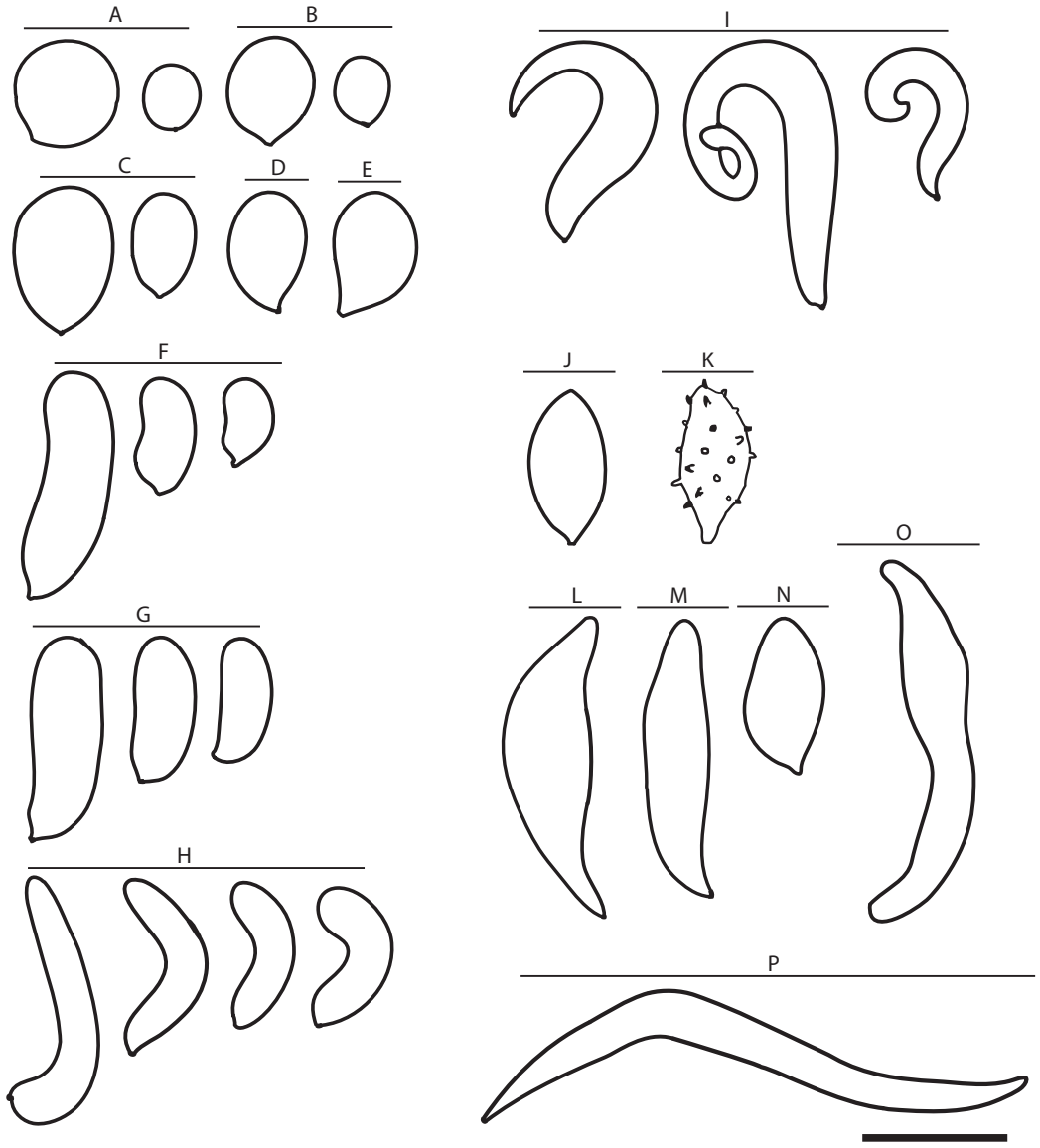

Figure 2. Shapes of basidiospores in species of Tulasnellaceae. (A) globose. (B) subglobose. (C) oblong. (D) elliptical. (E) pip-shaped. (F) phaseoliform. (G) subcylindrical. (H) allantoid. (I) spiral. (J) fusiform. (K) subfusiform and spiny. (L) subfusiform, tapering and curved to both apices. (M) subfusiform, ventral concavity next to the apiculus and tapering to apex. (N) subfusiform. (O) ventricose. (P) sigmoid. Scale bar = 10 µm. The shapes K and O are redrawn from Roberts´ illustrations (Roberts 1994a; 2003). Taken from Cruz et al. (2016).

2.4 Statistical analysis of morphological characteristics

2.4.1 Bivariate analysis

Two different approaches were used to conduct bivariate analyses to evaluate groups of morphological similarities. The first one was based on Jaccard analysis (Jaccard 1908) as detailed in Cruz et al. (2016). The second was based on Euclidean distances (Sokal and Sneath 1963) including between-linkage clustering detailed in Cruz et al. (2014). Both analyses were visualized as dendrograms performed in SPSS 11.0 (IBM Corporation, Somers, NY, USA). The shapes of basidiospores (Fig. 2 A-P), and presence or absence of clamp connections, cystidia, chlamydospores, and conidia (blastoconidia or other kinds of conidia) were used (Cruz et al. 2016). Data from 50 morphospecies of Tulasnellaceae, including those represented by own samples and others with information available from literature (Bandoni and Oberwinkler 1982; Bourdot and Galzin 1909; 1928; Lowy 1964; Roberts 1994b; 1999; Rogers 1933; Van de Put and Antonissen 1996) were compiled to evaluate groups formed by morphological similarities. Details are given in Cruz et al. (2016).

Euclidean distances of shape and size of basidiospores of 91 specimens were calculated to further evaluate seven morphologically close species (*Tulasnella albida*, *T. andina*, *T.* aff. *andina*, *T. eichleriana*, *T. pinicola*, *T. pruinosa, T. violea*). Details are given in Cruz et al. (2014).

2.4.2 Simple linear regression and Tukey test

The mean values of basidiospore length, width, and Q-values were analyzed by simple linear regression or Tukey test in order to compare or correlate different species. The number of specimens and morphospecies included in these two analyses are given in Cruz et al. (2014; 2016).

2.5 Molecular analysis of Tulasnella *basidiomata, mycorrhizae, and cultures*

2.5.1 DNA isolation, PCR, cloning, and sequencing

DNA was extracted from fresh basidiomata, dried fungarium material, mycorrhizae, and axenic cultures using a DNeasy Plant Mini Kit (Qiagen). The DNA amplification, PCR, and cloning conditions are given in detail in Cruz et al. (2011; 2014; 2016). Sequences were obtained from fresh samples of basidiomata and mycorrhizae and from the cultures. Attempts to obtain sequences from fungarium specimens were unsuccessful. These specimens were frequently in poor condition showing collapsed and degraded structures.

2.5.2 Alignment of DNA sequence data

The sequence chromatograms were verified using the software Sequencher 4.6 (Gene Codes, Ann Arbor, MI). Basidiomata, cultures (121 new sequences), and mycorrhizae i.e four sequences from *Epidendrum rhopalostele* (Riofrio et al. 2013) and six sequences from basidiomata were provided by M. Bidartondo, Kew Garden yielded 131 sequences for our analysis. These sequences were aligned separately or together with the most similar *Tulasnella* sequences available from GenBank (http://www.ncbi.nlm.nih.gov/) progressing in two steps. First, a phylogenetic tree was created with data from the 5.8S ITS rDNA region (about 165 bp). This tree yielded four well-supported groups within which the entire ITS-5.8S rDNA region (approximately 536 to 878bp) was realigned. Details are given in Riofrio et al. (2013) and Cruz et al. (2014).

2.5.3 Phylogeny, intra-genomic, intra-specific, and inter-specific analysis

Phylogenetic trees were calculated from the four subgroups including related sequences from GenBank. Phylogenetic calculations were performed with the programs RAxML v7.0.4 (Stamatakis 2006) and MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001).

Pairwise differences of the ITS-5.8S rDNA sequences were calculated in MEGA 5.2.2 software (Tamura et al. 2011) in order to evaluate intra-genomic, intra-specific, and inter-specific variation as detailed in Cruz et al. (2014).

An additional phylogenetic analysis was carried out to corroborate the placement of

the new species *Tulasnella andina* D. Cruz, J.P. Suárez & M. Piepenbr. and the new records of *T.* aff. *eichleriana* and *T. violea* from Ecuador against closely related sequences from GenBank. Divergent portions of the alignment were eliminated using the Gblocks program v. 0.91b (Castresana 2000). The tree was generated using MEGA 5.2.2 (Tamura et al. 2011). Details are given in Cruz et al. (2016).

3 Results

3.1 Morphological diagnosis and taxonomy

The revised fresh and fungarium material, 180 specimens on total, represent 30 morphospecies (Table 1), described by Cruz et al. (2016). *Tulasnella andina* from Ecuador and *Tulasnella kirschneri* from China are considered as species new to science (Figs. 3, 4) and are published in Cruz et al. (2016). *Tulasnella* aff. *cruciata* is reported from basidiomata of fungarium material for the first time (Fig. 5), the description is available in Cruz et al. (2016).

Table 1: Morphospecies analyzed and defined in this study (Cruz et al. 2016).

	Morphospecies name	Type specimen	Number of specimens
1	*Tulasnella albida* Bourdot & Galzin		10
2	*Tulasnella allantospora* Wakef. & A. Pearson		9
3	*Tulasnella andina* D. Cruz, J.P. Suárez & M. Piepenbr.	DC225 (holotype)	10
4	*Tulasnella anguifera* P. Roberts	K 18541 (holotype)	1
5	*Tulasnella brinkmannii* Bres.		2
6	*Tulasnella calospora* (Bourd.) Juel		6
7	*Tulasnella* aff. *cruciata* Warcup & P.H.B. Talbot		1
8	*Tulasnella curvispora* Donk		2
9	*Tulasnella cystidiophora* Honh. & Litsch.		2
10	*Tulasnella danica* Hauerslev		1
11	*Tulasnella deliquescens* (Juel) Juel		4
12	*Tulasnella eichleriana* Bres.		29
13	*Tulasnella falcifera* P. Roberts	K 18539 (holotype)	1
14	*Tulasnella fuscoviolacea* Bres		2
15	*Tulasnella griseorubella* Litsch.		1
16	*Tulasnella helicospora* Raunk.		2
17	*Tulasnella hyalina* Höhn. & Litsch.		12
18	*Tulasnella interrogans* P. Roberts		1
19	*Tulasnella kirschneri* D. Cruz, J.P. Suárez & M. Piepenbr.	R. Kirschner 923 (holotype)	1
20	*Tulasnella pallida* Bres.		11
21	*Tulasnella pinicola* Bres.		6
22	*Tulasnella pruinosa* Bourdot & Galzin		7
23	*Tulasnella rubropallens* Bourdot & Galzin		8
24	*Tulasnella saveloides* P. Roberts	Paratype P. Roberts 114 (K 21326)	1
25	*Tulasnella subglobospora* Hjortstam		4
26	*Tulasnella thelephorea* (Juel) Juel		7
27	*Tulasnella tomaculum* P. Roberts	Paratype P. Roberts 366 (K 23190)	7
28	*Tulasnella valentini* Van de Put	BR 6616006 (lectotype)	1
29	*Tulasnella violea* (Quél.) Bourdot & Galzin		31
Doubtful species			
30	*Tulasnella quasiflorens* P. Roberts	Paratype P. Roberts 683 (K 26234)	1
Total of specimens investigated:			**180**

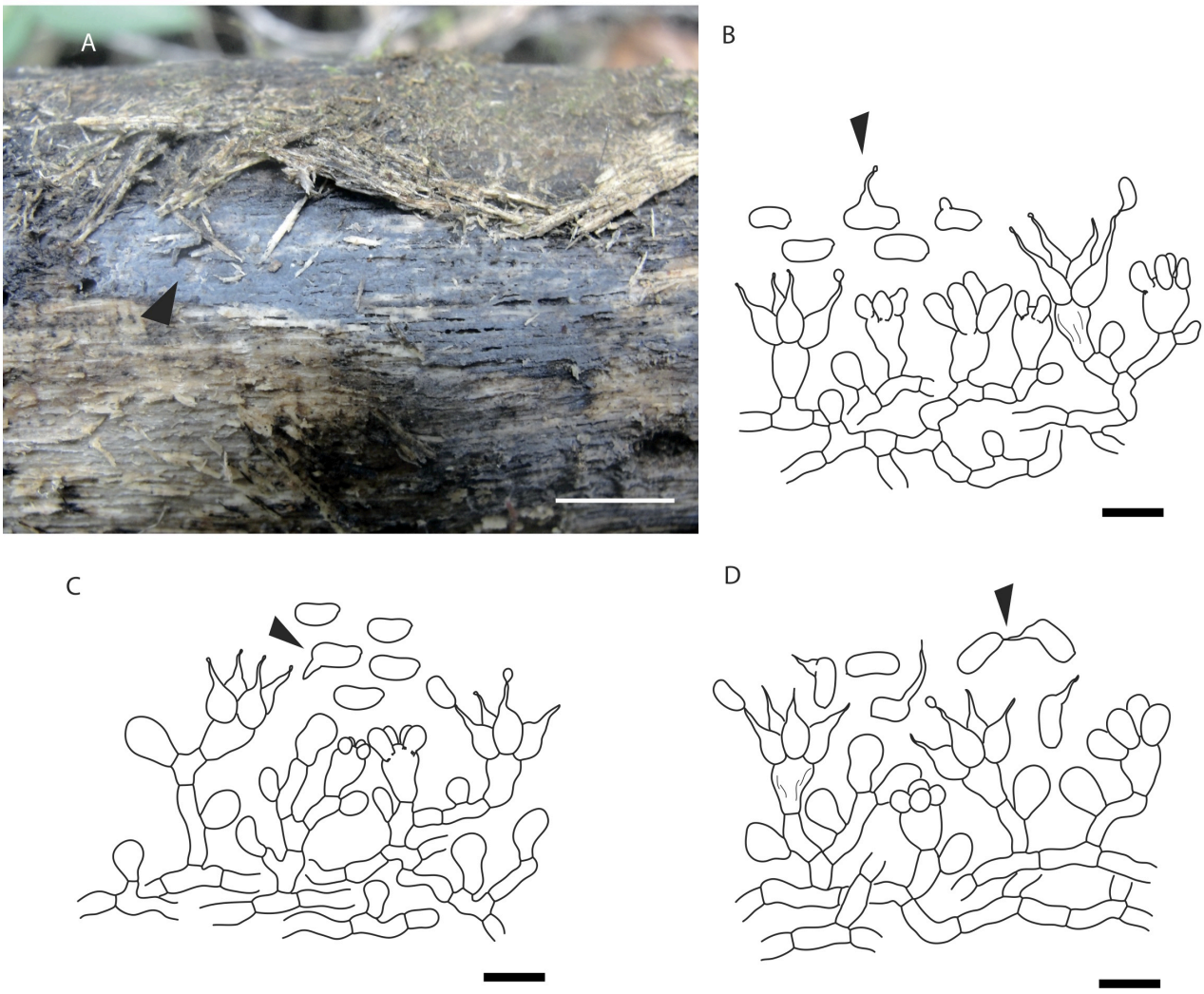

Figure 3. *Tulasnella andina.* (A) Resupinate basidioma (indicated by black arrowhead) on decaying fallen branch (holotype DC225). Scale bar = 2 cm. (B-D) Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowhead). (B) Holotype DC225. (C) DC157. (D) DC245. Scale bars = 10 μm. Taken from Cruz et al. (2016).

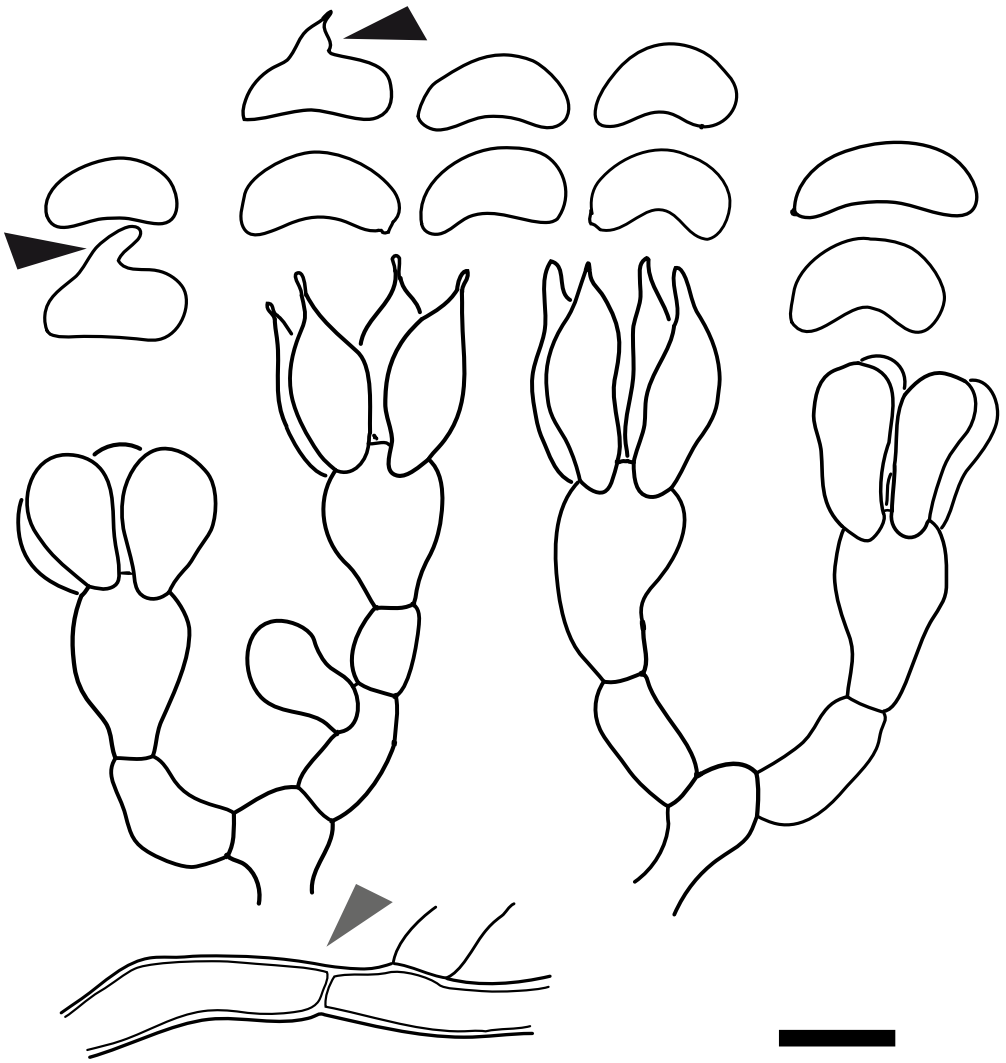

Figure 4. *Tulasnella kirschneri*. Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). The gray arrowheads indicate thick-walled hyphae. RoKi 923. Scale bar = 10 μm. Taken from Cruz et al. (2016).

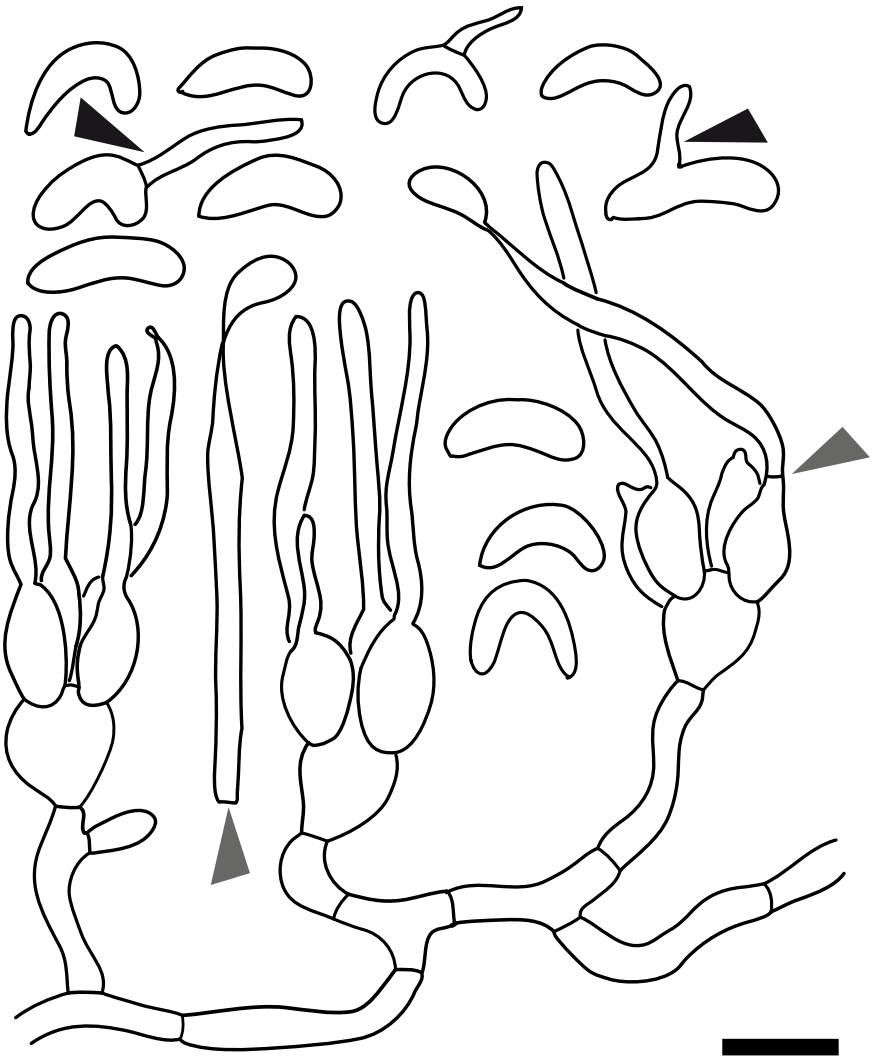

Figure 5. *Tulasnella* aff. *cruciata.* Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowhead). The gray arrowheads indicate adventitious septa in the sterigmata. BR 6612669. Scale bar = 10 μm. Taken from Cruz et al. (2016).

3.2 Statistical analysis

Based on bivariate analyses, seven groups were separated by the shape of basidiospores (Fig. 6). Clamp connections, cystidia, and other morphological characteristics are spread among the seven groups as shown in the dendrogram. The groups are also heterogeneous concerning the shape and size of basidiospores of rather similar morphospecies like *Tulasnella albida*, *T. andina*, *T.* aff. *andina, T. eichleriana*, *T. pinicola*, *T. pruinosa*, and *T. violea* (Fig. 7). Thus, we deal with morphological species concepts that are often difficult to apply (Cruz et al. 2014; 2016). Morphological variability and overlapping of shape and size of the basidiospores of several morphospecies is also indicated by simple linear regression (Cruz et al. 2014) and Tukey test analysis represented in a histogram (Fig. 8) (Cruz et al. 2014; 2016).

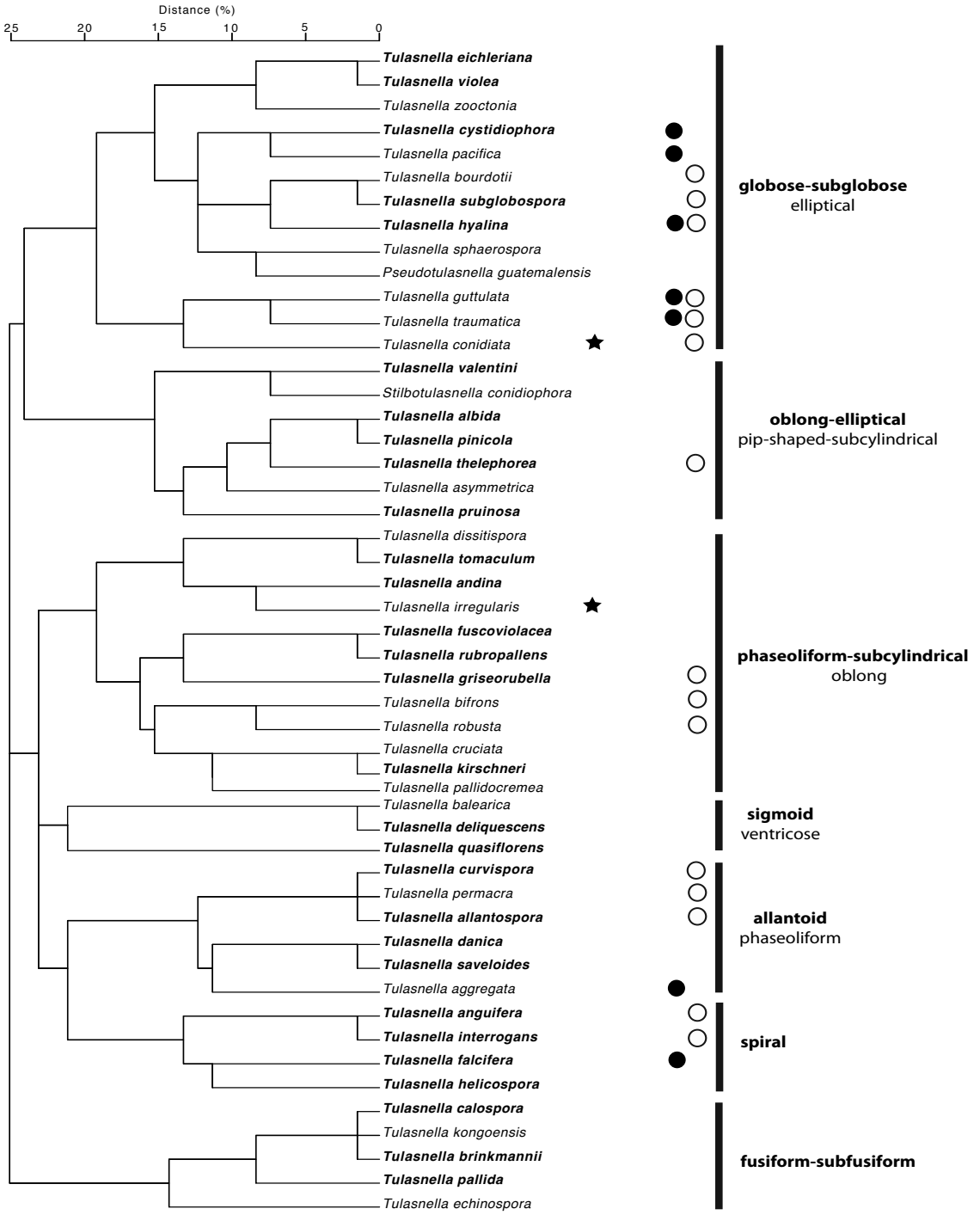

Figure 6. Dendrogram of species of Tulasnellaceae inferred by Jaccard analysis of all available structures (see text) from 48 taxa (Index Fungorum, http://www.IndexFungorum.org) including the new species *Tulasnella andina* and *T. kirschneri*. Names of species presented in detail by Cruz et al. (2016) are written in bold front. Seven groups are defined based on the main shapes of the basidiospores. Less common structures are indicated by symbols: clamp connections (o), cystidia (●), chlamydospores (⋆). Taken from Cruz et al. (2016).

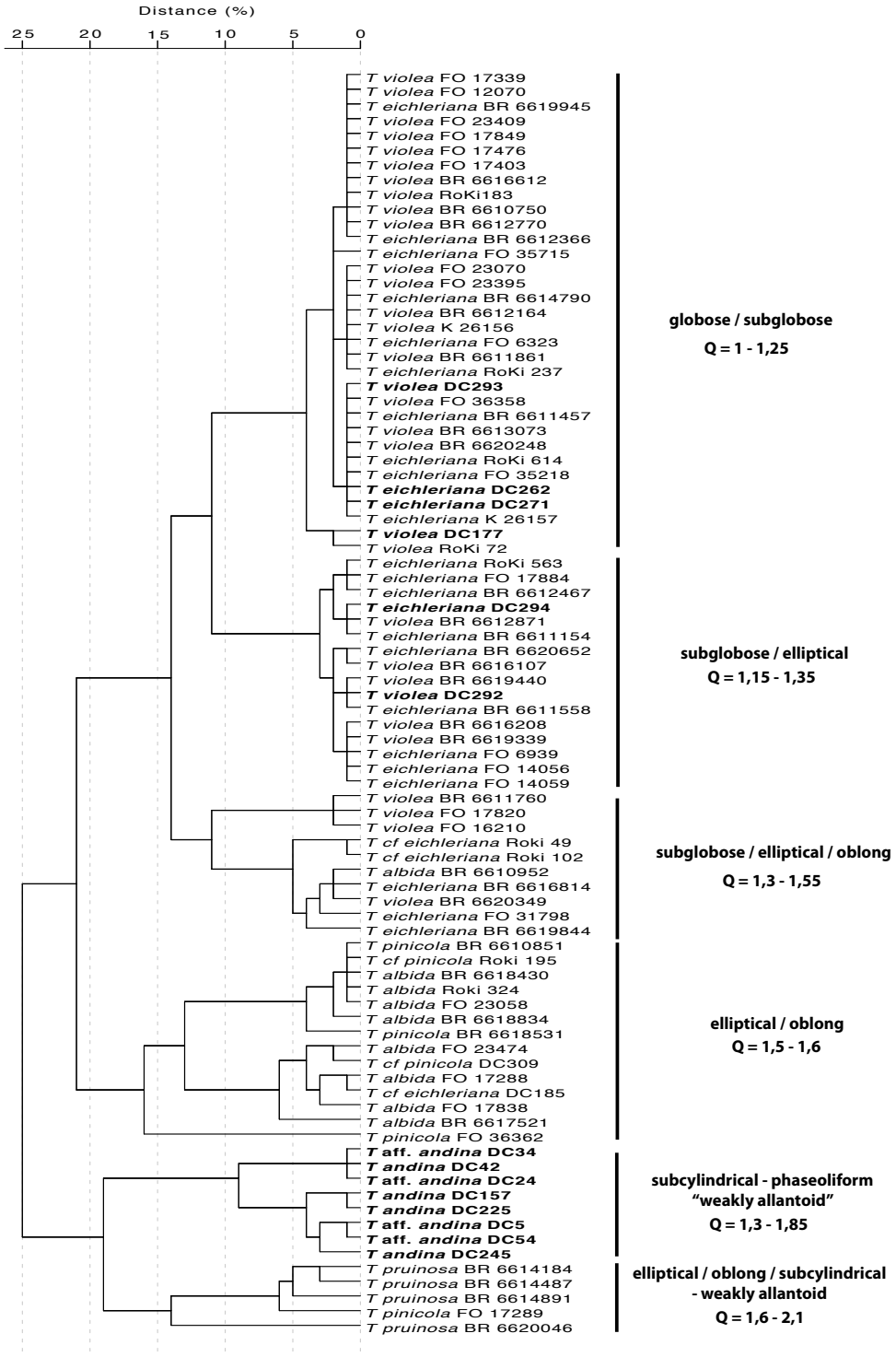

Figure 7. Dendrogram inferred by Euclidean distance analysis of Q values of basidiospores. Preliminary assignations of names are based on the taxonomic key in Roberts (1999). The scale indicates percentages of distances between the specimens. Modified after Cruz et al. (2014).

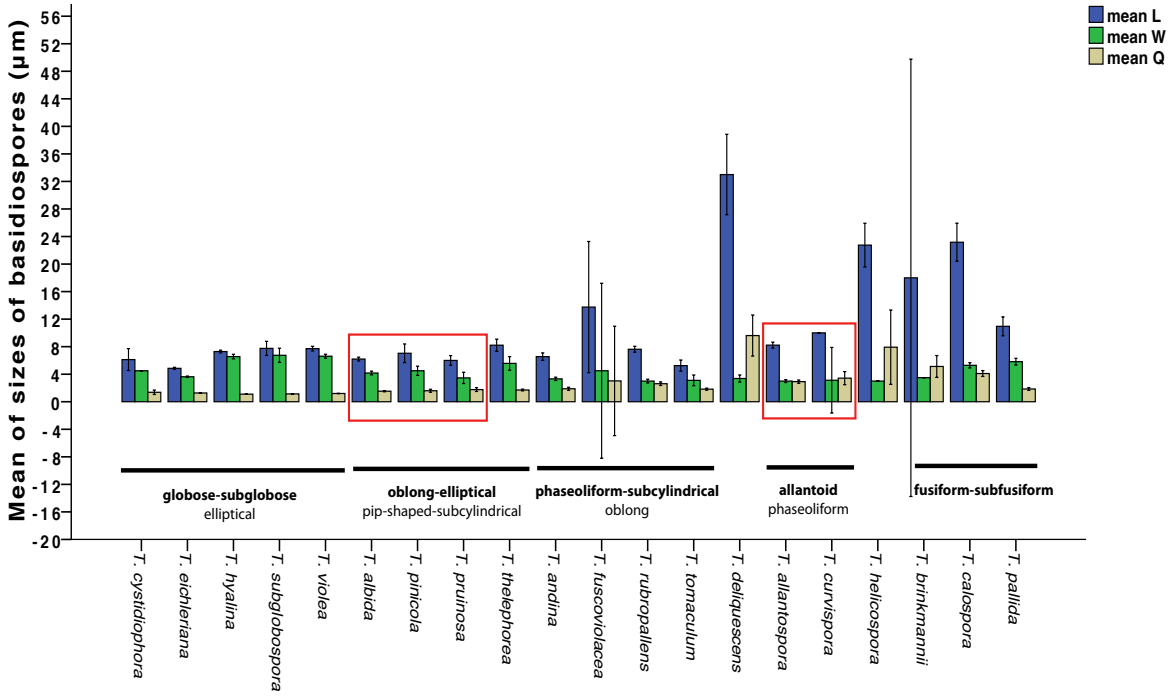

Figure 8. Histogram showing the means of basidiospore length, width (both in µm), and Q-values (length/width) of 20 morphospecies of *Tulasnella* available by more than one specimen. Error bars on the histograms indicate 95 % of confidence intervals. Groups of morphospecies that are conflictive by high similarity and without significant differences in length, width, or Q-values of basidiospores are highlighted in red. The shape of the basidiospores is indicated below the histograms. Taken from Cruz et al. (2016).

3.3 Dichotomous key for species of Tulasnellaceae

A dichotomous key to genera of Tulasnellaceae and species of *Tulasnella* was constructed and is available in Cruz et al. (2016).

3.4 Species delimitations of Tulasnella *by molecular analysis*

The phylogenetic tree based on 127 sequences of the conserved 5.8S rDNA region is composed of four groups (I– IV), well supported by maximum likelihood bootstrap values and Bayesian posterior probabilities, respectively (73–100 % BS

and PP) (Fig. 9). The four groups (Cruz et al. 2014) are consistent with the phylogenetic tree treated with Gbloks software (Fig. 10) (Cruz et al. 2016). The four groups were further analyzed using the complete ITS1-5.8S rDNA regions, results are shown in Fig. 10 (Cruz et al. 2014).

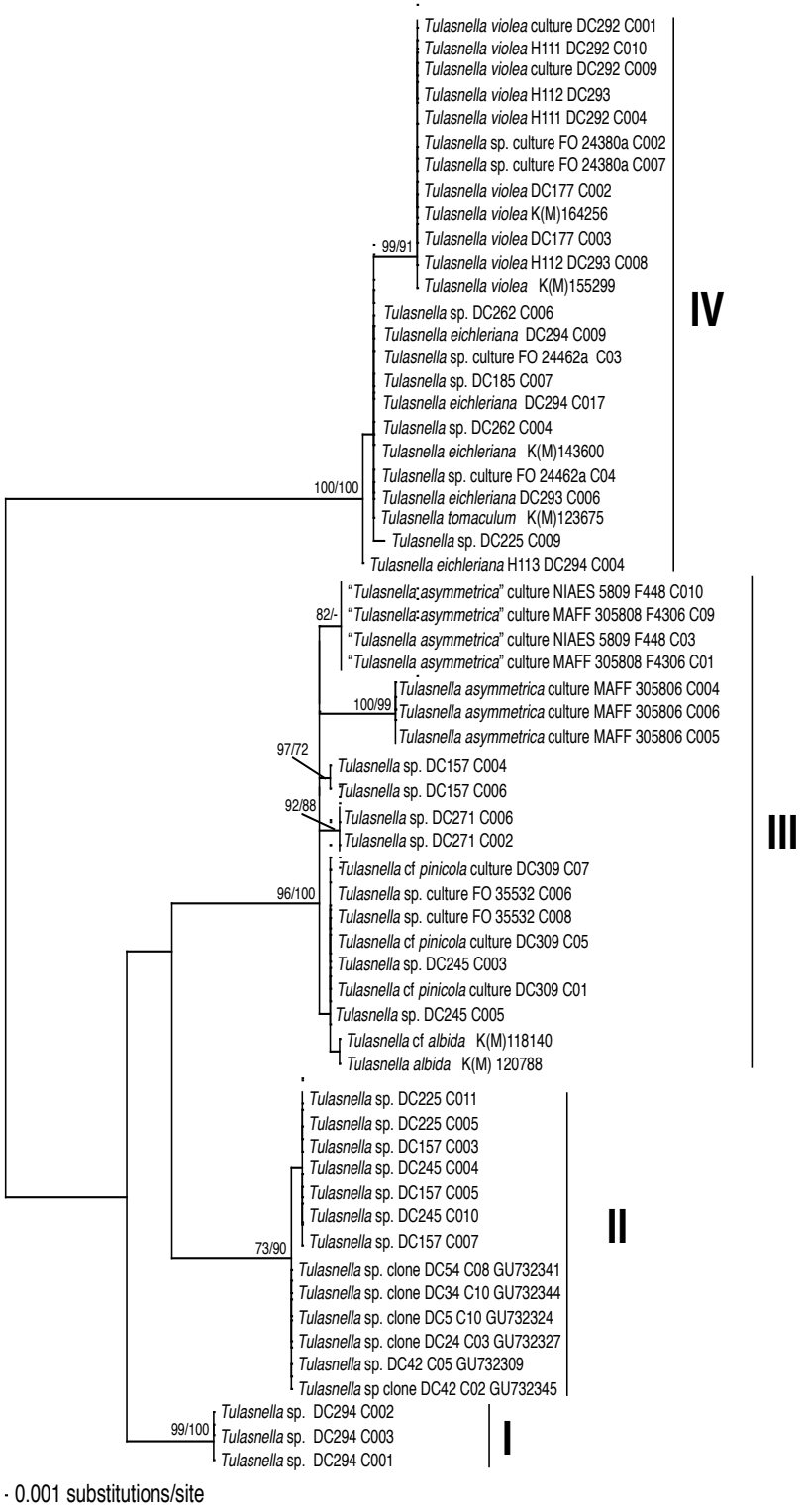

Figure 9. Phylogenetic hypothesis based on 5.8S rDNA region including most of the new sequences of *Tulasnella* spp. Values on the nodes correspond to bootstrap values obtained by Maximum Likelihood (left) and Bayesian posterior probabilities (right; converted to %). Only values larger than 70 % are shown on nodes. Dubious species names are written between inverted commas. The numbers I-IV represents the different groups further aligned and analyzed phylogenetically based on the ITS-5.8S rDNA regions (Figs. 2 to 5 in Cruz et al. 2014). The tree is rooted at its midpoint. Modified after Cruz et al. (2014).

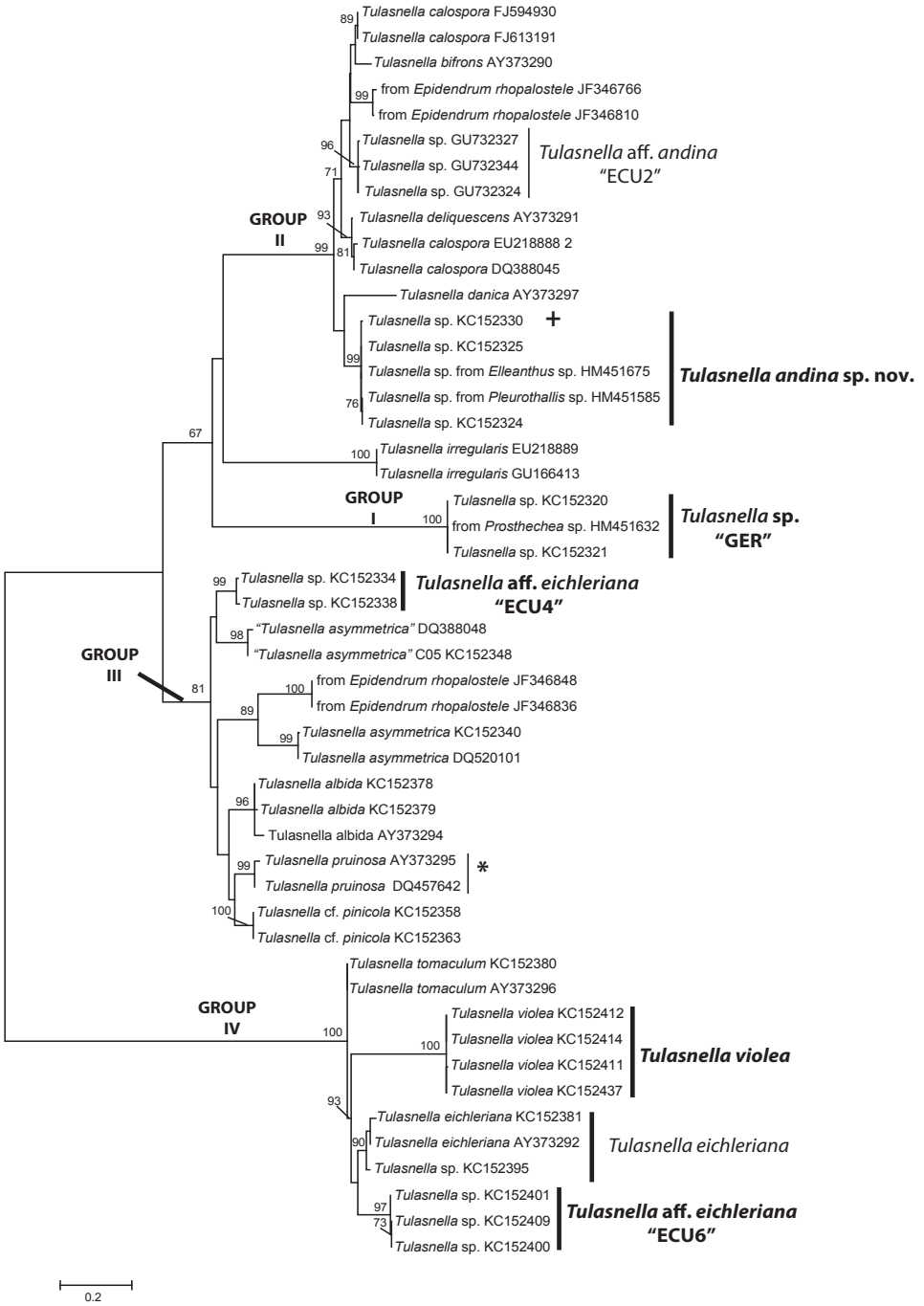

Figure 10. Phylogenetic hypothesis based on ITS-5.8S rDNA sequences obtained from own specimens and from NCBI (http://www.ncbi.nlm.nih.gov/). Values close to the nodes correspond to maximum likelihood bootstrap values. Only values larger than 60 % are shown. In bold: *Tulasnella andina* as new species and *T*. aff. *eichleriana* (ECU4; ECU6) and *T. violea* as new records for Ecuador. The type sequence of *T. andina* is marked by (**+**). *Tulasnella pruinosa* sequences are indicated by *. The designation of Groups I, II, III, and IV correspond to the groups presented by Cruz et al. (2014). Modified after Cruz et al. (2016)

Group I, named *Tulasnella* sp. GER (Fig. 10) contains new sequences from one specimen (DC294) sampled by the author in RBSF and sequences of mycorrhizae of *Prostechea* sp. (Orchidaceae) sampled in RBSF, Ecuador (Kottke et al. 2013). Group II contains two separated clades of specimens sampled in Ecuador (Fig. 10). The first clade, named *Tulasnella andina* (Cruz et al. 2016), corresponds to *T*. sp. ECU 1 in Cruz et al. (2014). The sequences from *Tulasnella andina* are nested with sequences obtained from terrestrial and epiphytic orchids sampled in the RBSF. The second clade named *Tulasnella* aff. *andina* (Cruz et al. 2016) correspond to *T.* sp. ECU 2 in Cruz et al. (2014). Both clades are related to sequences of *Tulasnella* spp. named *T. bifrons* Bourdot & Galzin, *T. calospora* (Boud.) Juel, and *T. deliquescens* (Juel) Juel obtained from GenBank (Fig. 10).

Group III contains several clades from cultures attributed to the species *T. asymmetrica,* "*T. asymmetrica*", *T.* cf. *pinicola* (Fig. 10) (Cruz et al. 2014). Other clades are assigned to *T. albida*, *T. pruinosa*, *T.* aff. *eichleriana* based on their teleomorph information (Cruz et al. 2014). Group III also contains sequences from mycorrhizae of *Epidendrum rhopalostele* (Riofrio et al. 2013) without known morphological affinities (Fig. 10).

Group IV contains one clade (Fig. 10) formed by sequences considered to represent *Tulasnella violea*, obtained from five basidiomata sampled in Ecuador, England, Germany, and Wales (see Fig. 5 in Cruz et al. 2014). One clade in this group is attributed to *T. eichleriana* consisting of sequences from basidioma DC294 and the culture FO 24462a obtained from a basidioma of *Tulasnella* sp. (non vid.) collected in Germany. The clade *T.* aff. *eichleriana* ECU6 (Cruz et al. 2016) corresponds to *T.* sp. ECU6 in Cruz et al. (2014). This clade is formed by sequences obtained from one basidioma collected in Ecuador. An additional clade is designate to the correct *T. tomaculum* based on sequences obtained from basidiomata (see Fig. 5 in Cruz et al. 2014).

Intragenomic variability among sequences from individual cultures is 0–2 %. The pairwise differences between sequences from specimens considered as a certain species have a sequence variability of 0–1 % in *Tulasnella albida*, *T. asymmetrica*

and *T.* cf. *pinicola*, 0–3 % in *Tulasnella* spp. belonging to the groups II and IV (Table 2). The highest variation (4 %) occurs in *T. eichleriana*. The interspecific

Table 2. Intragenomic variation in cultures, as well as intra- and interspecific sequence variability among specimens defined as morphospecies of *Tulasnella* within the respective groups. Pairwise distances were calculated in MEGA 5 software (Tamura et al. 2011) with Kimura-2-parameter distances (Kimura 1980) with gap deletion (Modified after Cruz et al. 2014).

Group	Name of the clade	Number of specimens in each clade	Length of ITS-5.8S in base pairs	Intragenomic difference	Intraspecific difference	Interspecific difference
I	*Tulasnella* sp. GER	1	536	—	—	—
II	*Tulasnella andina* ECU 1	4	564	—	0–3 %	9–13 %
	Tulasnella aff. *andina* ECU 2	5		—	0–3 %	
III	*Tulasnella albida*	2	582	—	0–1 %	12–44 %
	Tulasnella sp. ECU 3	1		—	—	
	Tulasnella asymmetrica	1		0–1 %[*1]	0–1 %	
	"Tulasnella asymmetrica" DQ388048, KC152348	2		0–2 %[*2] 0–2 %[*3]	0–3 %	
	Tulasnella aff. *eichleriana* ECU 4	1		—	—	
	Tulasnella cf. *pinicola*	3		0 %[*4] 0–1 %[*5]	0–1%	
IV	*Tulasnella* sp. ECU 5	1	878	—	—	11–43 %
	Tulasnella eichleriana	3		0–1%[*6]	0–4 %	
	Tulasnella aff. *eichleriana* ECU 6	2		—	0–2 %	
	Tulasnella tomaculum	1		—	—	
	Tulasnella violea	6		0 %[*7] 0–1 %[*8]	0–3 %	

* Pure cultures: [1] MAFF 305806, [2] MAFF 305808, [3] MAFF 305809, [4] DC309, [5] FO 35532, [6] FO 24462a, [7] FO 24380a, [8] DC292.

divergences are at least 9 % and can reach 44 %. The lowest variation, 9–13 %, occurs between the closely related *Tulasnella andina* ECU1 and *T.* aff. *andina* ECU2 from Ecuador (Group II). The supported clades in each group containing

sequences with up to 4 % of proportional differences are considered as one phylogenetic species (Cruz et al. 2014).

4 Discussion

4.1 Morphological delineation of Tulasnella *species*

The genus *Tulasnella* has been studied morphologically and illustrated by many authors (Bandoni and Oberwinkler 1982; Bourdot and Galzin 1909; 1928; Dueñas 1988; 1989; Greslebin and Rajchenberg 2001; Jülich and Jülich 1976; Lowy 1964; Martin 1939; Olive 1957; Ordynets 2012; Roberts 1992; 1993a; 1993b; 1994a 1994b; 1999; Rogers 1933; Van de Put and Antonissen 1996; Warcup and Talbot 1967; 1971; 1980). Different species concepts were suggested (Bourdot and Galzin 1909; 1928; Rogers 1933). The author reinvestigated available material from fungaria and studied specimens from personal collections to define 30 morphospecies (Table 1). Comparison of microstructures let us conclude that *Tulasnella andina* from Ecuador and *T. kirschneri* from China are new to science (Figs. 3, 4).

Tulasnella andina was found as saprotrophic fungus mostly growing on decaying trunks or fallen branches that previously might have carried epiphytic orchids. However, *T. andina* is a potential mycorrhizal fungus of orchids according to molecular sequences obtained from orchid mycorrhizae (Cruz et al. 2014). It is morphologically similar to *Tulasnella pruinosa* Bourdot & Galzin because *T. pruinosa* can present phaseoliform basidiospores. However, the basidiospores in *T. andina* are less variable in length [(5-)6-8 × (2-)3-4(-5) μm] than the basidiospores present in *T. pruinosa* [(3.5-)5-7(-7.5) × (2.5-)3-4(-5) μm]. Because the shape of the basidiospores is extremely variable in specimens of *T. pruinosa* (own observations, see the description of *T. pruinosa* in Cruz et al. 2016), it is considered to be an uncertain and ill-defined species (Roberts 1994b).

Tulasnella kirschneri collected in China by R. Kirschner is proposed as a new species to science because it morphologically differs from all other known species as discussed in Cruz et al. (2016).

Further specimens recently collected in Ecuador were identified as *Tulasnella* aff. *eichleriana* and *T. violea*, and correspond to new records for Ecuador (Cruz et al. 2016). In this country, 13 undetermined species of *Tulasnella* are listed according to molecular data (Liede-Schumann and Breckle 2008) and one *Tulasnella* aff. *andina* similar to *T. andina* is known with morphological and molecular data (Cruz et al. 2011). According to literature on the distribution of *Tulasnella* spp. in the neotropics (Cruz et al. 2011; Greslebin and Rajchenberg 2001; Lowy 1964; Martin 1939; Roberts 2006), these records are new for Ecuador.

The two clades formed by sequences of *Tulasnella* spp. obtained from orchid mycorrhizae of *Epidendrum rhopalostele* are distinct from the clades formed by sequences from basidiomata found in the same forest (Fig. 10) (see Figs. 3 and 4 in Riofrio et al. 2013). As the author spent many hours in this forest looking for basidiomata of *Tulasnella* spp. and closely investigated branches carrying this orchid, these two species apparently predominantly develop asexually.

Basidiospore shapes, Q-values, and measurements were previously considered as the main characteristics to separate species in Tulanellaceae (Jülich and Jülich 1976; Lowy 1964; Martin 1939; Olive 1957; Roberts 1992; 1993a; 1993b; 1994a 1994b; 1999; Rogers 1933; Van de Put and Antonissen 1996; Warcup and Talbot 1967; 1971; 1980). In the presented study, the shape of basidiospores separated seven groups of *Tulasnella* spp. (Fig. 6). However, only three of these groups (allantoid, globose-subglobose, spiral) are in concordance with Roberts´ species concept. Other morphological characteristics, like the presence of clamp connections or cystidia are spread among the seven groups (Fig. 6). Further, the known, high variability of shape and size of basidiospores within and among species of *Tulasnella* is corroborated by bivariate analysis (Fig. 7), simple linear regression (Fig. 1 in Cruz et al. 2014), and Tukey-test in our investigation. Distinct species like *Tulasnella allantospora* and *T. curvispora* or *T. albida* and *T. pinicola* do not show significant difference by basidiospores analysis (Fig. 8) (see Table 3 in Cruz et al. 2016). Our analysis suggests that morphological characteristics alone are insufficient to delimit several species of *Tulasnella* (Figs. 7 and 8). The high variability of morphological characteristics appears to be common in

Tulasnellaceae (Cruz et al. 2014; Moncalvo et al. 2006; Hibbett et al. 2007). This problem appears to be ubiquitous in fungi, impeding morphological species recognition (Gazis et al. 2011).

4.2 Molecular information and correlation with morpho-species

Molecular information clearly delimits and supports the new species *Tulasnella andina.* To our best knowledge it is the first species described in this genus based on morphological and molecular data. In case of the new species *Tulasnella kirschneri* no sequences were obtained from the type material and the phylogenetic position remains unclear.

Two of the seven morphological groups, oblong-elliptical and globose-subglobose, can be only partially correlated to the phylogenetic groups III and IV, respectively (Cruz et al. 2014; 2016). Species like *Tulasnella* aff. *eichleriana* with mostly globose-subglobose basidiospores clustered into the phylogenetic group III and *Tulasnella tomaculum* with mostly phaseoliform or “weakly allantoid” basidiospores clustered into the phylogenetic group IV. Thus, the morphological groups in Tulasnellaceae are not supported by phylogenetic hypotheses. On contrast morphologically defined higher taxa of fungi are often supported by molecular phylogenetic hypotheses (Hibbett et al. 2004; 2007).

Phylogenetic species can be defined when the genetic sequence distances within and between species is clarified (Cruz et al. 2014; 2016). The 4 % of intraspecific variation (Table 2) found in this study indicates that variation among *Tulasnella* species can be higher than 3 %, the widely accepted divergence threshold for defining phylogenetic species in other groups of fungi (Nilsson et al. 2008). The found variation is however within the range of 3–5 % threshold used for defining operational taxonomic units (OTUs) of *Tulasnella* mycobionts of orchids by many authors (Girlanda et al. 2011; Jacquemyn et al. 2011; Martos et al. 2012; Linde et al. 2013; Kottke et al. 2013). Consequently we can define the barcode gap used to delimit species in other organisms between the maximum intraspecific variation of 4 % and the minimum interspecific divergence of 9 % (Meyer and Paulay 2005; Meier et al. 2008; Ni et al. 2012; Puillandre et al. 2012; Cruz et al. 2014).

Intragenomic variation obtained from clones of pure *Tulasnella* spp. cultures in this investigation is up to 2 %. This variation corresponds to the 1.8 % of intragenomic variation found for isolates of *Tulasnella* spp. by Linde et al. (2013).

Phylogenetic and morphological information (Cruz et al. 2016) was found congruent for many of the treated species. In addition, the study reveals several cryptic species of *Tulasnella,* phylogenetically but not morphologically distinct taxa. *Tulasnella andina* in Group II is indistinguishable morphologically from *T.* aff. *andina* ECU 2, but clearly separated by sequence data. *Tulasnella* aff. *eichleriana* ECU 4 and *Tulasnella* aff. *eichleriana* ECU 6 in groups III and IV were both identical morphologically to *T. eichleriana* (see descriptions in Cruz et al. 2016), but distinct phylogenetic species. A further species is revealed molecularly from clones of a specimen sampled in Germany *T.* sp. GER (Fig. 10). Different clades formed by sequences named as distinct epithets (*Tulasnella albida*, *T. asymmetrica*, *T. eichleriana, T. irregularis, T.* cf. *pinicola*, *T. tomaculum*, and *T. violea*) are suggested to be correlated to these species and discussed concerning their molecular delimitation by Cruz et al. (2014). Other clades (Fig. 10), generated from non-type sequences, obtained from cultures can be related to *Tulasnella bifrons, T. danica*, and *T. pruinosa* (Cruz et al. 2014). The definitions of *T. calospora* and *T. deliquescens* are phylogenetically inconsistent (Fig. 10). This point was already discussed by Suarez et al. (2006) and Taylor and McCormick (2008). Identity of these taxa could not be resolved here.

4.3 Ecology

Tulasnella spp. are known to be saprotrophs and important orchid mycobionts simultaneously, in the tropics and worldwide (Cruz et al. 2011; 2014; 2016; Girlanda et al. 2011; Jacquemyn et al. 2010; 2011; Martos et al. 2012; McCormick et al. 2004; Roberts 1999; Suarez et al. 2006; Taylor and McCormick 2008). For example, our phylogenetic data on ITS-5.8S rDNA regions obtained from basidiomata of *Tulasnella andina* suggest that this species might be a mycobiont of terrestrial and epiphytic orchids, as basidiomata were found on rotten tree branches often close to orchid individuals and sequences cluster together in one

clade (Fig. 10) (Cruz et al. 2014; 2016). Other species of *Tulasnella* (e.g. *T. eichleriana*, *T. calospora*, *T. irregularis*, *T. violea*) were found on decomposing organic matter and form mycorrhizae with orchids in nature and artificially, promoting orchid seed germination and development of the protocorm (Roberts 1992; 1993a and b; 1994a and b; 1999; Warcup and Talbot 1967; 1971; 1980; Zettler et al. 2013). *Tulasnella* spp. form mycorrhizae with a very broad spectrum of orchid species belonging to taxa from the basal Apostasioidae to higher Epidendroideae, mostly by broad sharing among partners (Kottke and Suarez 2009; Kottke et al. 2013; Jacquemyn et al. 2011). Under certain, still unclear circumstances distinct *Tulasnella* spp. may, however, preferentially associate with certain orchid species or life forms like epiphytes or terrestrial plants (Martos et al. 2012; Jacquemyn et al. 2015). In the presented study, one population of epiphytic orchids was found associated with only two *Tulasnella* spp., one of Group II, the other in Group III (Riofrio et al. 2013). The orchid individuals were found to preferentially grow on trunks of dead trees, i.e. on more or less rotten organic material. We may speculate that the associated two *Tulasnella* mycobionts grow well on the rotten trunks and promote orchid establishment by supplying carbon and other nutrients to the orchid protocorm, the juvenile, non-photosynthetic state of the orchids. Recent comparative genomic approaches documented that a *Tulasnella* cf. *calospora* isolate preserved genes for some decay enzymes that allow carbon acquisition from rotten organic material (Kohler et al. 2015). Similar gene equipment was found in Sebacinaceae, the second wide spread mycobionts of green orchids, but is lost continuously in ectomycorrhizal fungi of Agaricomycetes. Tulasnellaceae and Sebacinaceae take a basal position in the Agaricomycetes consisting mainly of saprotrophs and mycobionts (Kohler et al. 2015). The work by Kohler et al. (2015) explains why just Tulasnellaceae, the sister group Ceratobasidiaceae and part of Sebacinaceae are the important and the only mycobionts of green orchids in Agaricomycetes: only these fungal groups have, according to current knowledge, saprotrophic and symbiotic capabilities simultaneously. In order to understand the importance of species of Tulasnellaceae for individual orchid establishment and, thus, orchid communities further studies on their ecological requirements are necessary. Further studies are needed to clarify if

Tulasnella species differ in their ecological demands and/or preferences for orchid species. As basis for such ecological studies, a clear concept of phylogenetically supported species delimitation in Tulasnellaceae is indispensable. The presented work is a first step in this direction.

5 References

Bandoni RJ, and Oberwinkler F (1982) *Stilbotulasnella*: a new genus in the Tulasnellaceae. *Canad J Bot* 60: 1875–1879

Beck E, Makeschin F, Haubrich F, Richter M, Bendix J, and Valarezo C (2008) The ecosystem (Reserva Biológica San Francisco). In: Beck E, Bendix J, Kottke I, Makeschin F, Mosandl R (eds) Gradients in a tropical mountain ecosystem of Ecuador. *Ecol Studies*, no.198. Springer, Berlin Heidelberg New York, pp 1–14

Bendix J, Beck E, Braüning A, Makeschin F, Mosandl R, Scheu S, et al. (eds.) (2013) Ecosystem services, biodiversity and environmental change in a tropical mountain ecosystem of South Ecuador, *Ecol Stud* 221, doi 10.1007/978-3-642-38137-9_18, © Springer-Verlag Berlin Heidelberg

Bidartondo MI, Bruns TD, Weiss M, Sergio C, and Read DJ (2003) Specialized cheating of the ectomycorrhizal symbiosis by an epiparasitic liverwort. *Proc R Soc Lond [Biol]* 270: 835–842

Bougoure JJ, Bougoure DS, Cairney WG, and Dearnaley DW (2005) ITS-RFLP and sequence analysis of endophytes from *Acianthus, Caladenia* and *Pterostylis* (Orchidaceae) in southeastern Queensland. *Mycol Res* 109: 452–460

Bourdot H, and Galzin A (1909) Hymenomycetes de France. I Heterobasidiés. Bull Soc *Mycol Fr* 25: 15–36

Bourdot H, and Galzin A (1928) Hymenomycetes de France. Heterobasidiés. III Tulasnellaceae. *Biblioth Mycol,* Edited by J. Cramer, vol 23 (reprint, 1969)

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* 17:540–552

Cruz DJ, Suarez JP, Kottke I, Piepenbring M, and Oberwinker F (2011) Defining species in *Tulasnella* by correlating morphology and nrDNA ITS-5.8s sequence data of basidiomata from a tropical Andean forest. *Mycol Prog* 10: 229–238

Cruz DJ, Suarez JP, Kottke I, and Piepenbring M (2014) Cryptic species revealed by molecular phylogenetic analysis of sequences obtained from basidiomata of *Tulasnella*. *Mycologia* 106(4): 708–722

Cruz DJ, Suarez JP, and Piepenbring M (2016) Morphological revision of Tulasnellaceae, with two new species of *Tulasnella* and new records of *Tulasnella* spp. for Ecuador. *Nova Hedwigia* 102(3–4): 279–338(60)

Chen J, Wang H, and Guo SX (2012) Isolation and identification of endophytic and mycorrhizal fungi from seeds and roots of *Dendrobium* (Orchidaceae). *Mycorrhiza* 22:297–307

Chutima R, Dell B, and Lumyong S (2011) Effects of mycorrhizal fungi on symbiotic seed germination of *Pecteilis susannae* (L.) Rafin (Orchidaceae), a terrestrial orchid in Thailand. *Symbiosis* 53:149–156

Drechsler C (1969) A *Tulasnella* parasitic on *Amoeba terricola*. *Am J Bot* 56: 1217–1220

Dueñas M (1988) Fragmenta Chorologica Occidentalia, Fungi, 1282–1296 *Anales Jard Bot Madrid* 45: 287–289 (Corol. Basidiomycotina, Dacrymicetales. Tremellales, Tulasnellales, LE, O, P. S)

Dueñas M (1989) Phragmobasidiomycetes del norte de España. *Anales Jard Bot Madrid* 46(1): 229–232

Dueñas M (2005) New and interesting Iberian heterobasidiomycetous fungi. 1. *Nova Hedwigia* 81: 177–198

Erdem B, Kariptas E, Çil E, and Isik K (2011) Biochemical identification and numerical taxonomy of *Aeromonas* spp. isolated from food samples in Turkey. *Turk J Biol* 35: 463–472

Gazis R, Rehner S, Chaverri P (2011) Species delimitation in fungal endophyte diversity studies and its implications in ecological and biogeographic inferences. *Mol Ecol* 20: 3001–3013

Girlanda M, Segreto R, Cafasso D, Liebel HB, Rodda M, Ercole E, et al. (2011) Photosynthetic mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations. *Am J Bot* 98: 1148–1163

Greslebin GA, and Rajchenberg M (2001) The genus *Tulasnella* with a new species in the Patagonian Andes forests of Argentina. *Mycol Res* 105(9): 1149–1151

Hibbett D (2004) Trends in morphological evolution in Homobasidiomycetes inferred using maximum likelihood: a comparison of binary and multistate approaches. *Syst Biol* 53: 889–903.

Hibbett DS, Binder M, JF, Bischoff M, Blackwell PF, Cannon OE, Eriksson S, et al. (2007) A higher-level phylogenetic classification of the Fungi. *Mycol Res* 111: 509–547

Huelsenbeck JP, and Ronquist FR (2001) MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755

Ingold TC (1984) Patterns of ballistospore germination in *Tilletiopsis, Auricularia* and *Tulasnella. Trans Br Mycol Soc* 83(4): 583–591

Jaccard P (1908) Nouvelles recherches sur la distribution florale. *Bull Soc Vaudoise Sci Nat* 44: 223–270.

Jacquemyn H, Honnay O, Cammue BPA, Brys R, and Lievens B (2010) Low specificity and nested subset structure characterize mycorrhizal associations in five closely related species of the genus *Orchis*. *Mol Ecol* 19: 4086–4095

Jacquemyn H, Merckx V, Brys R, Tyteca D, Cammue BPA, Honnay O, et al. (2011) Analysis of network architecture reveals phylogenetic constraints on mycorrhizal specificity in the genus *Orchis* (Orchidaceae). *New Phytol* 192: 518–528

Jacquemyn H, Brys R, Waud M, Busschaert P, and Lievens B (2015) Mycorrhizal networks and coexistence in species-rich orchid communities. *New Phytol* 206(3): 1127-34. doi: 10.1111/nph.13281

Jülich W, and Jülich U (1976) A contribution towards a revision of the genus *Tulasnella*. *Persoonia* 2: 49–64

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120

Kirk PM, Cannon PM, David JC, and Stalpers JA (2008) Ainsworth and Bisby's Dictionary of the Fungi. 10th edn. CAB International, Wallingford. Oxon

Kohler A, Kuo A, Nagy LG, Morin E, Barry KW, Buscot F, et al. (2015) Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists**.** *Nature Genetics* 47: 410–415 doi: 10.1038/ng.3223

Kottke I, Beiter A, Weiß M, Haug I, Oberwinkler F, and Nebel M (2003) Heterobasidiomycetes form symbiotic associations with hepatics: Jungermanniales have sebacinoid mycobionts while *Aneura pinguis* (Metzgeriales) is associated with a *Tulasnella* species. *Mycol Res* 107: 957–968

Kottke I, and Suárez JP (2009) Mutualistic, root-inhabiting fungi of orchids identification and functional types. In Proc. Second Scientific Conf. on Andean Orchids (eds Pridgeon AM, and Suárez JP), pp. 84–99. Loja, Ecuador: Universidad Técnica Particular de Loja (ISBN 978-9942-00-502-1)

Kottke I, Suarez JP, Herrera P, Cruz D, Bauer R, Haug I, et al. (2010) Atractiellomycetes belonging to the 'rust' lineage (Pucciniomycotina) form mycorrhizae with terrestrial and epiphytic neotropical orchids. *Proc R Soc Lond [Biol]* 277: 1289–1296

Kottke I, Setaro S, Haug I, Herrera P, Cruz DJ, et al. (2013) Mycorrhiza networks promote biodiversity and stabilize the tropical mountain rain forest ecosystem – Perspectives for understanding complex communities. In: Ecosystem Services, Biodiversity and Environmental Change in a Tropical

Mountain Ecosystem of South Ecuador, *Ecological Studies*, Springer Verlag, Heidelberg

Largent DL, Johnson D, and Watling R (1973) How to identify mushrooms to genus III: Microscopic features. Eureka, CA: *Mad River Press*. 148 pp

Liede-Schumann S, and Breckle S-W (2008) Provisional Checklist of Flora and Fauna of the San Francisco valley and its surroundings (Reserva Biológica San Francisco, Province Zamora-Chinchipe, southern Ecuador). Ecotropical Monographs 4

Linde CC, Phillips RD, Crisp MD, and Peakall R (2013) Congruent species delineation of *Tulasnella* using multiple loci and methods. *New Phytol* doi: 10.1111/nph.12492

Lowy B (1964) A new genus of the Tulasnellaceae. *Mycol* 56: 696–700

Ma M, Tan TK, and Wong SM (2003) Identification and molecular phylogeny of *Epulorhiza* isolates from tropical orchids. *Mycol Res* 107: 1041–1049

Martin GW (1939) New or noteworthy fungi from Panama and Colombia. III. *Mycol* 31: 239–249

Martos F, Munoz F, Pailler T, Kottke I, Gonneau C, and Selosse MA (2012) The role of epiphytism in architecture and evolutionary constraint within mycorrhizal networks of tropical orchids. *Mol Ecol* 21: 5098–5109

McCormick MK, Whigham DF, and O'Neill J (2004) Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytol* 163: 425–438

Meier R, Zhang G, and Ali F (2008) The use of mean instead of smallest interspecific distances exaggerates the size of the "barcoding gap" and leads to misidentification. *Syst Biol* 57: 809–813

Meyer CP, and Paulay G (2005) DNA barcoding: Error rates based on comprehensive sampling. *PLoS Biol* 3: 2229–2238

Moncalvo JM, Nilsson RH, Koster B, Dunham SM, Bernauer T, Matheny PB, et al. (2006) The cantharelloid clade: Dealing with incongruent gene trees and phylogenetic reconstruction methods. *Mycol* 98: 937–948

Moore RT (1987) The genera of *Rhizoctonia-like* fungi: *Ascorhizoctonia, Ceratorhiza* gen. nov., *Epulorhiza* gen. nov., *Moniliopsis,* and *Rhizoctonia*. *Mycotaxon* 29:91–99

Morgan DJ (1971) Numerical taxonomic studies of the genus *Botrytis* II. Other *Botrytis* taxa. *Trans Br Mycol Soc* 56: 327–335

Ni L, Li Q, Kong L, Huang S, and Li L (2012) DNA barcoding and phylogeny in the family Mactridae (Bivalvia: Heterodonta): Evidence for cryptic species. *Biochem Sys Ecol* 44: 164–172

Nilsson RH, Kristiansson E, Ryberg M, Hallenberg N, and Larsson K-H (2008) Intraspecific ITS variability in the Kingdom Fungi as expressed in the internal sequence databases and its implications for molecular species identification. *Evol Bioinformatics* 4: 193–201

Oberwinkler F (2012) Evolutionary trends in Basidiomycota, *STAPFIA* 96: 45–104

Okayama M, Yamato M, Yagame T, and Iwase K (2012) Mycorrhizal diversity and specificity in Lecanorchis (Orchidaceae). *Mycorrhiza* (7): 545–53. doi: 10.1007/s00572-012-0429-z

Olive LS (1957) Tulasnellaceae of Tahiti. A revision of the family. *Mycologia* 49: 663–679

Oliveira SF, Bocayuva MF, Veloso TGR, Bazzolli DMS, Silva CC, Pereira OL, et al. (2013) Endophytic and mycorrhizal fungi associated with roots of endangered native orchids from the Atlantic Forest, Brazil. *Mycorrhiza* (1): 55–64. doi: 10.1007/s00572-013-0512-0

Ordynets O (2012) New records of corticioid fungi with heterobasidia from Ukraine. *Turk J Botany* 36: 590–602 doi:10.3906/bot-1109-1

Parmasto E, and Parmasto I (1987) Variation of Basidiospores in the Hymenomycetes and its significance to their taxonomy. *Bibl Mycol*, Band 115. J. Cramer, Berlin, Germany

Parmasto E, and Parmasto I (1992) Size and shape of basidiospores in the Hymenomycetes. *Mycol Helv* 5: 47–78

Pereira M, Coelho I, Valadares R, Oliveira F, Bocayuva M, Pereira O, et al. (2014) Morphological and molecular characterization of *Tulasnella* spp. fungi isolated from the roots of *Epidendrum secundum*, a widespread Brazilian orchid. *Symbiosis* 62(2): 111–121. doi:10.1007/s13199-014-0276-0.

Pereira OL, Kasuya MCM, Borges AC, and Fernandes de Araújo E (2005) Morphological and molecular characterization of mycorrhizal fungi isolated from neotropical orchids in Brazil. *Can J Bot* 83: 54–65

Pereira OL, Rollemberg CL, Borges AC, Matsuoka K, and Kasuya MCM (2003) *Epulorhiza epiphytica* sp. nov. isolated from mycorrhizal roots of epiphytic orchids in Brazil. *Mycoscience* 44: 153–155

Petersen RH (1968) Hyphal spore germination and asexual spore production in *Gloeotulasnella pinicola***.** *Mycopathol Mycol Appl* 35: 145–149

Preußing M, Nebel M, Oberwinkler F, and Weiß M (2010) Diverging diversity patterns in the *Tulasnella* (Basidiomycota, Tulasnellales) mycobionts of *Aneura pinguis* (Marchantiophyta, Metzgeriales) from Europe and Ecuador. *Mycorrhiza* 20(3): 147–59

Puillandre N, Modica MV, Zhang Y, Sirovich L, Boisselier MC, Cruaud C, et al. (2012) Large-scale species delimitation method for hyperdiverse groups. *Mol Ecol* 21:2671–2691

Riofrio Ml, Cruz DJ, Torres E, De La Cruz M, Iriondo J-M, and Suarez JP (2013) Mycorrhizal preferences and fine spatial structure of the epiphytic orchid *Epidendrum rhopalostele. Am J Bot* 100(12): 1–10

Roberts P (1992) Spiral-spored *Tulasnella* species from Devon and the New Forest. *Mycol Res* 96 (3): 233–236

Roberts P (1993a) The genus *Tulasnella* in Norway. *Windahlia* 20: 67–74. Göteborg

Roberts P (1993b) Allantoid-spored *Tulasnella* species from Devon. *Mycol Res* 97: 213–220

Roberts P (1994a) Long-spored *Tulasnella* species from Devon, with additional notes on allantoid-spored species. *Mycol Res* 98: 1235–1244

Roberts P (1994b) Globose and ellipsoid-spored *Tulasnella* species from Devon and Surrey, with a key to the genus in Europe. *Mycol Res* 98: 1431–1452

Roberts P (1999) Rhizoctonia-forming Fungi: a taxonomic guide. *Royal Botanic Gardens*, Kew

Roberts P. (2006) Caribbean heterobasidiomycetes: 2. Jamaica. *Mycotaxon* 96: 83–107

Rogers DP (1933) A taxonomic review of the Tulasnellaceae. *Ann Mycol* 31, 181–203

Schoch C, Seifert K, Huhndorf S, Robert V, Spouge JL, Levesque CA, et al. (2012) The nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc Natl Acad Sci* USA 109: 6241–6246

Shefferson RP, Weiß M, Kull T, and Taylor DL (2005) High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. *Mol Ecol* 14: 613–626

Shefferson RP, Taylor DL, Weiß M, Garnica S, McCormick MK, Adams S, et al. (2007) The evolutionary history of mycorrhizal specificity among lady's slipper orchids. *Evol* 61: 1380–1390

Sokal RR, and Sneath PHA (1963) Principles of numerical taxonomy. San Francisco: *Freeman WH and Company*

Sokal RR (1966) Numerical taxonomy. *Sci Amer* 215: 106–116.

Stamatakis A (2006) RAxML-VI-HPC: Maximum Likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22(21): 2688–2690

Stielow B, Bratek Z, Orczán AKI, Rudnoy S, Hensel G, Hoffmann P, et al. (2011) Species delimitation in taxonomically difficult fungi: The case of *Hymenogaster*. *PLoS ONE* 6(1): e15614. doi:10.1371/journal.pone.0015614

Suarez JP, Weiss M, Abele A, Garnica S, Oberwinkler F, and Kottke I (2006) Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. *Mycol Res* 110: 1257–1270

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol Biol Evol* 28: 2731–2739

Taylor DL, and McCormick MK (2008) Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. *New Phytol* 177: 1020–1033

Tulloss RE, and Lindgren JE (2005) *Amanita aprica* a new toxic species from western North America. *Mycotaxon* 91: 193–205

Valadares RB, Pereira MC, Otero JT, and Cardoso EJ (2012) Narrow fungal mycorrhizal diversity in a population of the orchid *Coppensia doniana. Biotropica*. 44(1): 114–122 doi: 10.1111/j.1744- 7429.2011.00769.x

Van de Put K, and Antonissen I (1996) *Tulasnella's* uit Vlaanderen. *Sterbeeckia* 17: 44–69

Veldre V, Abarenkov K, Bahram M, Florent Martos F, Selosse M-A, Tamm H, et al. (2013) Evolution of nutritional modes of Ceratobasidiaceae (Cantharellales, Basidiomycota) as revealed from publicly available ITS sequences. *Fungal Ecol* 6: 256–268

Warcup JH, and Talbot PHB (1967) Perfect states of Rhizoctonias associated with orchids I. *New Phytol* 66: 631–641

Warcup JH, and Talbot PHB (1971) Perfect states of Rhizoctonias associated with orchids II. *New Phytol* 76: 35–40

Warcup JH (1973) Symbiotic germination of some Australian terrestrial orchids. *New Phytol* 72: 387–392

Warcup JH, and Talbot PHB (1980) Perfect states of Rhizoctonias associated with orchids III. *New Phytol* 86: 267–272

Zettler WW, Corey AL, Jacks AL, Gruender LT, and Lopez AM (2013) *Tulasnella Irregularis* (Basidiomycota: Tulasnellaceae) from roots of *Encyclia tampensis* in South Florida, and confirmation of its mycorrhizal significance through symbiotic seed germination. *Lankesteriana* 13(1–2): 119–128

6 Acknowledgments

Foremost, I would like to express my sincere gratitude to my tutor Meike Piepenbring for her encouragement, guidance, support, patience, motivation, and understanding from the initial stages of my study to the final level of developing my doctoral thesis. My gratitude to her for kindly supporting this study at the Department of Mycology at the J.W. Goethe-University in Frankfurt am Main and for her advice during these years. Pipenbring´s guidance helped me in all areas of scientific research and the writing of this thesis.

I am heartily thankful to my supervisors Juan Pablo Suarez in Ecuador, and Ingrid Kottke in Germany, whose encouragement, guidance, academic experience, constructive criticism, and invaluable discussions allowed me to achieve many objectives within my doctoral studies.

Furthermore, I am grateful to Roland Kirschner for sharing his academic experience at the beginning of my doctoral studies. I also thank Franz Oberwinkler and Ralph Mangelsdorff for their constructive criticism, as well as the many inspiring and invaluable discussions. I thank them for their critical review of some of my manuscripts.

In particular, I would like to acknowledge my colleagues and Jascha Weisenborn in Germany and Paulo Herrera in Ecuador for many of their professional and personal experience in molecular phylogenetic analysis and fungi isolation and preservation. I am grateful to different students such as Juan Sebastian Eguiguren for joining our fieldwork and for his help and assistance in the laboratory in Ecuador.

A special thank goes to Carola Glatthorn for her secretarial assistance and her

friendly support in different University processes. Additionally, I thank my other colleagues at the Department of Mycology, Melissa Mardones, Miguel Rosas Medina, and Caroline Judith for creating a friendly and comfortable working atmosphere.

I offer my acknowledgments to all authorities belonging to the different institutions: Universidad Técnica Particular de Loja, Deutsche Forschungsgemeinschaft (DFG - RU 816), and the German Academic Exchange Service (DAAD) who economically supported my doctoral studies. I thank the curators of the fungaria of the Eberhard-Karls University of Tübingen (TUB), National Botanic Garden of Belgium (BR), and Kew Royal Botanic Gardens (K) for answering requests and for the loan of specimens.

Above all, I would like to thank my family for their support. I thank my mum Blanca and my dad Arturo for giving me strength in my studies. My brothers (Fabián, Fernando) and sisters (Jenny, Karina and Mónica) for their unconditional love, care and understanding.

Special thanks to my beloved wife Karla, and my children Paula, Matías and Nayeli who make me feel at home and provide me with confidence, motivation, and inspiration in all my actions and throughout my study.

7 Publications

The results of this study were compiled in the following three publications:

The following page numbers of 1–60 are the page numbers of the following publication.

1.- Cruz DJ, Suarez JP, and Piepenbring M (2016) Morphological revision of Tulasnellaceae, with two new species of *Tulasnella* and new records of *Tulasnella* spp. for Ecuador. *Nova Hedwigia* 102(3–4): 279–338(60)

Status: 2016

Title of the magazine: Nova Hedwigia

Participating authors:

1.- DJC: Darío Javier Cruz

2.- JPS: Juan Pablo Suárez

3.- MP: Meike Piepenbring

To which extend did the doctoral candidate or the co-authors contribute individually to the dissertation?

(1) Development and planning

1.- DJC: 60%

2.- JPS: 15%

3.- MP: 25%

(2) Performance of individual research and experiments

1.- DJC: 90%

2.- JPS: 5%

3.- MP: 5%

DJC did all the sampling of basidiomata of *Tulasnella* spp. in a tropical rain forest in Ecuador and a regenerated forest in Germany. DJC did all the molecular work and phylogenetic analysis.

(3) Composition of data gathering and images

1.- DJC: 90%

2.- JPS: 5%

3.- MP: 5%

All the specimens were revised, illustrated, and described microscopically by DJC. The illustrations published here were done by DJC and improved after comments by MP. The specimens that correspond to the new species *Tulasnella andina* and the new records *Tulasnella* aff. *eichleriana* and *T. violea* from Ecuador were collected by DJC. The specimen corresponding to the new species *Tulasnella kirschneri* was collected by R. Kirschner. Two pure cultures (*Tulasnella* cf. *pinicola*, DC309 and *T. violea*, DC292) from fresh basidiomata were isolated by DJC.

DJC carried out all the DNA isolation from basidiomata (fresh and herbarium material) and cultures followed by PCR. The standardized protocol of PCR including a nested PCRs was improved by DJC in collaboration with JPS.

(4) Analysis and interpretation of the data

1.- DJC: 90%

2.- JPS: 5%

3.- MP: 5%

All the statistical analyses were done by DJC and revised by mathematicians of the UTPL. The phylogenetic analyses were performed by DJC and improved according to recommendations by JPS.

The morphological descriptions were written by DJC and improved after comments by MP.

(5) Preparation of the manuscript

1.- DJC: 80%

2.- JPS: 10%

3.- MP: 10%

Nova Hedwigia
published online October 2015

PrePub Article

Morphological revision of Tulasnellaceae, with two new species of *Tulasnella* and new records of *Tulasnella* spp. for Ecuador

Darío Cruz[1]*, Juan Pablo Suárez[1] and Meike Piepenbring[2]

[1] Museum of Biological Collections, Section of Basic and Applied Biology, Department of Natural Sciences, Universidad Técnica Particular de Loja, San Cayetano Alto s/n C.P. 11 01 608, Loja, Ecuador.

[2] Cluster for Integrative Fungal Research (IPF), Institute of Ecology, Evolution, and Diversity, Goethe-University Frankfurt am Main, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany.

With 33 figures and 3 tables

Abstract: Due to ambiguous morphological and molecular characteristics, species of Tulasnellaceae are difficult to organize taxonomically. In this study a morphological revision of the family and 30 reinvestigated morphospecies of *Tulasnella* are presented. Illustrations, descriptions, statistical analysis, and discussions of the species are based on exsiccata from fungaria and own specimens collected in Ecuador and Germany. A dichotomous key to genera of Tulasnellaceae and the species of *Tulasnella* described in the present publication is compiled. *Tulasnella andina* from Ecuador and *Tulasnella kirschneri* from China are presented as species new to science. *Tulasnella cruciata* was described from Australia from a basidioma grown in culture and a species similar but not identical to this species was obtained from a basidioma found in herbarium material for the first time. *Tulasnella* aff. *eichleriana* and *T. violea* are reported for the first time from Ecuador. Phylogenetic placements of the new species *T. andina* and new records for Ecuador are presented based on sequences previously published by us. The morphological reinvestigations exemplify a vast intra-specific structural variability in species of *Tulasnella* and call for further specimens for morphological analysis and molecular data to define species.

Key words: basidiospores, morphospecies, morphological variability, phylogenetic analyses.

Introduction

The members of Tulasnellaceae Juel belong to the order Cantharellales within the Agaricomycetes (Moncalvo 2006, Hibbet et al. 2007, Veldre et al. 2013). Tulasnellaceae consist of three genera, *Pseudotulasnella* Lowy, *Stilbotulasnella* Oberw. & Bandoni,

* Corresponding author: djcruz@utpl.edu.ec

 DOI: 10.1127/nova_hedwigia/2015/0304

www.borntraeger-cramer.de
0029-5035/2015/0304 $ 15.00

and *Tulasnella* J. Schröt., and include 54 formally described species (Kirk et al. 2008). Species of Tulasnellaceae are characterized by resupinate or effused basidiomata that are ceraceous to subgelatinous refracting light, hyaline or frequently violaceous grey when fresh, and almost invisible macroscopically when dry. Asexual sporulation is unknown except for chlamydospores in some species, simple conidiophors in *Tulasnella valentine* Van de Put, and synnemata in *Stilbotulasnella conidiophora* Bandoni & Oberw. (Bandoni & Oberwinkler 1982, Van de Put & Antonissen 1996). Hyphae are bi- or multinucleate (Andersen 1996), hyaline, monomitic, and with or without clamp connections. Septal pores are dolipores with continuous or pauciperforate parenthesomes (Andersen 1996, Moore 1996, Van Driel et al. 2009) or without parenthesomes (Bandoni & Oberwinkler 1982). Cystidia correspond to gloeocystidia and are present only in some species. Basidia are usually globose, sphaeropedunculate to clavate, without septa (holobasidia) or apically incompletely cruciate-septate (Lowy 1964). Sterigmata are swollen with an adventitious septum at the base. They are globose to ellipsoidal when young, and later become cylindrical to clavate with an apical extension in many species. Basidiospores are hyaline, thin-walled, smooth or rarely spiny, e.g., in *Tulasnella echinospora* P. Roberts (Roberts 2003). They show a great variety of shapes, being globose, spiral, allantoid, fusiform, or sigmoid-bacilliform with many intermediate forms. They produce secondary spores (ballistospores) by repetition.

The genus *Pseudotulasnella* with the species *P. guatemalensis* Lowy is monotypic and characterized by apically partly septate basidia and lack of clamps on the septa (Lowy 1964). The genus *Stilbotulasnella* is monotypic as well. *Stilbotulasnella conidiophora* is characterized by prominent synnemata with blastic conidia. This species lacks an organized hymenium and the hyphae are septate with dolipores without parenthesomes (Bandoni & Oberwinkler 1982).

The genus *Tulasnella* was established in 1888 by Schröter, with *Gloeotulasnella* Höhn. & Litsch. (1908) being a synonym according to Olive (1957) and Roberts (1994b). The type species of *Tulasnella* is *Tulasnella lilacina* Schröt. The concept of this species is not well known, because no type collection is available. This species is probably a synonym of *T. violea* (Quél.) Bourdot & Galzin (Bourdot & Galzin 1923, Roberts 1994b, 1999, Roberts & Piątek 2004). *Tulasnella* spp. are characterized by hyphae that are continuously septate, in some cases with clamps. Basidia are not septate, generally globose to subglobose or clavate to obclavate. Cystidia, when present, are gloeocystidia. Sterigmata are globose, ellipsoidal or fusiform. Chlamydospores are generated by some species. Basidiospores are smooth and present different shapes, i.e., globose, subglobose, oblong, elliptic, phaseoliform, allantoid, spiral, sigmoid, or ventricose. They produce secondary basidiospores by repetition.

Ecologically, *Tulasnella* spp. have been mostly reported as saprotrophs on decayed wood (Roberts 1999, Greslebin & Rajchenberg 2001, Cruz et al. 2011). Many species are important mycobionts associated with roots of epiphytic and terrestrial orchids worldwide (Bougoure et al. 2005, Girlanda et al. 2011, Jacquemyn et al. 2011, Ma et al. 2003, Martos et al. 2012, McCormick et al. 2004, Pereira et al. 2003, 2005, 2014, Riofrio et al. 2013, Suárez et al. 2006, Kottke et al. 2010, Warcup & Talbot 1967, 1971, 1980).

In a previous investigation we presented a species concept based on molecular data of own collected specimens and data from GenBank, NCBI (Cruz et al. 2014). However, morphological and molecular species concepts in *Tulasnella* still leave many taxonomic inconsistencies unsolved (McCormick et al. 2004, Suárez et al. 2006, Taylor & McCormick 2008, Pereira et al. 2014). Here we compile morphological information on 30 morphospecies of *Tulasnella* based on investigation of 205 specimens from own sampling activity and fungarium material. Data for the monotypic genera *Pseudotulasnella* and *Stilbotulasnella* are included based on literature. The morphospecies of *Tulasnella* are described, illustrated, and discussed comparing morphologically similar species. Molecular data are included in the discussion of species concepts, but they are only available for few of the described species and very rarely from type material. Distinctive morphological characteristics are summarized in a dichotomous key for genera of Tulasnellaceae and for the 30 analysed morphospecies of *Tulasnella*.

Material and methods

Specimens: The morphological revision is based on 205 specimens, including 189 specimens from fungarium material and 16 specimens from recent collections. However, 25 specimens from fungarium material were excluded from the analysis due to partial degradation, lacking tulasnelloid structures or were mixed with other fungi. *Tulasnella* specimens were loaned from the following fungaria: National Botanic Garden of Belgium (BR); Kew Royal Botanic Gardens (K); Eberhard-Karls-Universität Tübingen (TUB). The specimens collected by Roland Kirschner (RoKi) are deposited in the National Museum of Natural Science of Taiwan (TNM) and fungal herbarium of Kunming Institute of Botany, Chinese Academy of Science (KUN-HKAS).

The first author collected specimens in the evergreen upper montane tropical rain forest in Ecuador and in temperate forests near Frankfurt, Germany. Detailed information on the sampling sites is given by Cruz et al. (2011 and 2014). Vouchers of these collections are stored in the fungarium of the Universidad Técnica Particular de Loja (HUTPL) at Loja, Ecuador.

Small pieces of basidiomata of *Tulasnella* spp. were used for sporulation and isolation on solid PDA and MEA agar media. However, isolation of *Tulasnella* spp. failed except for two specimens (*T. violea* and *T.* cf. *pinicola*) collected in Germany.

Morphological diagnosis: Free-hand sections of basidiomata of the fresh samples and the fungarium material were stained with 1% phloxine. Amyloid reaction was evaluated with Melzer's reagent. Sections were examined at 100 to 1000-fold magnification by use of a Leitz SM-LUX or Zeiss Axioskop 2 microscope. Hyphal diameter, length/width of cystidia, basidia, sterigmata, and spores were measured based on 30 basidiospores and at least 25 other structures per specimen. Q-values as length/width were calculated for basidiospores (Tulloss & Lindgren 2005). The different shapes of the basidiospores (Fig. 1) were classified mostly according to Largent et al. (1973).

Illustrations were done by hand using a scale of 1×1 cm^2 corresponding to 5×5 µm^2. Morphospecies identifications of the new specimens were obtained by using the key provided by Roberts (1999). The identifications were confirmed by comparison with the fungaria collections.

Numerical analysis of morphological characteristics

Bivariate analysis: Basidiospore shapes (globose, subglobose, elliptical, oblong, phaseoliform or weakly allantoid, pip-shaped, subcylindrical, sigmoid, ventricose, allantoid, spiral, fusiform, subfusiform) and presence or absence of clamp connections, cystidia, chlamydospores, and conidia (blastoconidia or other kinds of conidia) were used in the statistical analysis. In total, 49 morphospecies of Tulasnellaceae, including those represented by own samples and others with information available from literature (Bandoni & Oberwinkler 1982, Bourdot & Galzin 1909, 1928, Lowy 1964, Roberts

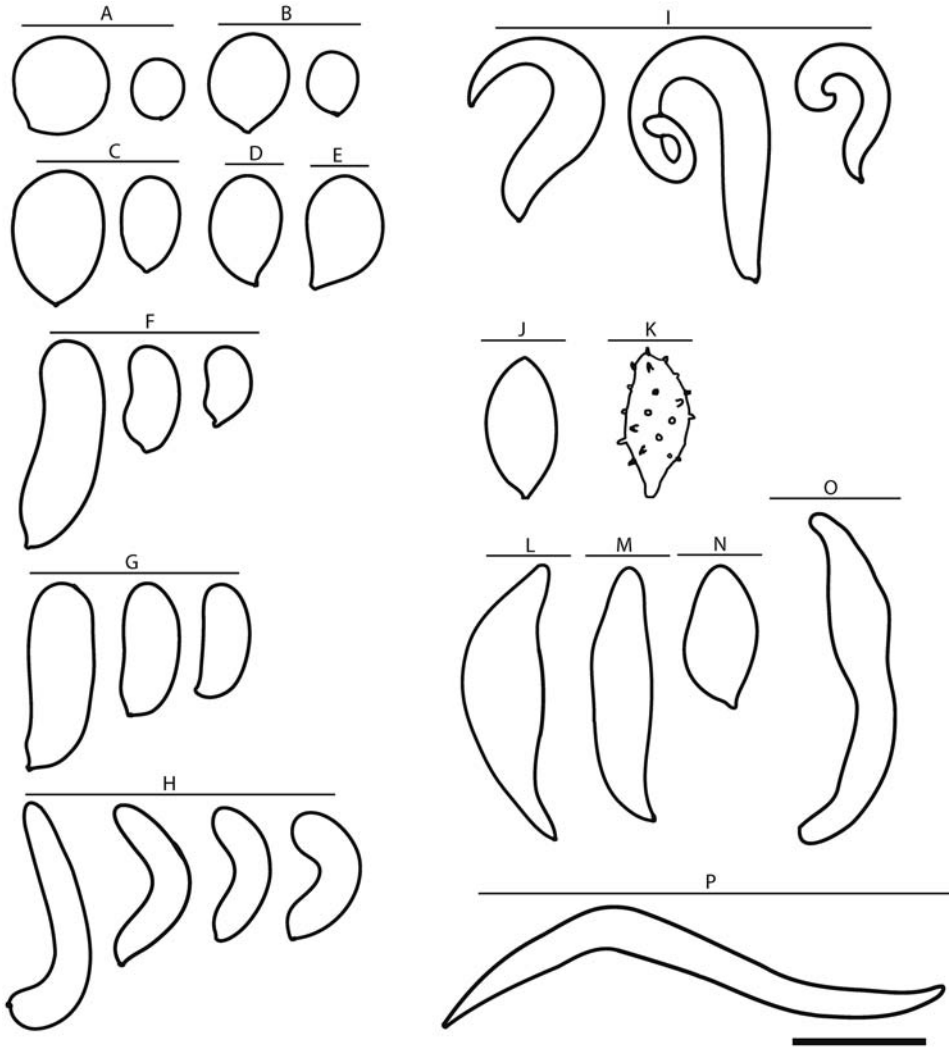

Fig. 1. Shapes of basidiospores in species of Tulasnellaceae. (A) globose. (B) subglobose. (C) oblong. (D) elliptical. (E) pip-shaped. (F) phaseoliform. (G) subcylindrical. (H) allantoid. (I) spiral. (J) fusiform. (K) subfusiform and spiny. (L) subfusiform, tapering and curved to both apices. (M) subfusiform, ventral concavity next to the apiculus and tapering to apex. (N) subfusiform. (O) ventricose. (P) sigmoid. Scale bar = 10 µm. The shapes 23 and 27 are redrawn from Roberts' illustrations (Roberts 1994a, 2003).

1994b, 1999, Rogers 1933, Van de Put & Antonissen 1996) were compiled to evaluate groups formed by morphological similarities. These characteristics were clustered by Jaccard analysis (Jaccard 1908) and visualized as a dendrogram performed in SPSS 11.0 (IBM Corporation, Somers, NY, USA).

Some species names were excluded due to ambiguous references or synonyms as presented in revisions by Roberts (1992, 1993b, 1994a, 1994b and 1999).

Tukey-test: The mean values of spore length, width, and Q-values of 170 specimens corresponding to 20 morphospecies were used to perform the Tukey-test to compare the species. Ten morphospecies with only one specimen were excluded from this statistical analysis.

Dichotomous key for Tulasnellaceae: The morphological characteristics considered in the bivariate analysis and Tukey-test were used to generate a dichotomous key to genera of Tulasnellaceae and species of *Tulasnella*. All basidiospore measurements were obtained in this study except for *Tulasnella quasiflorens* P.Roberts, for which data published by Roberts (1994a) were used.

Phylogenetic analysis: A phylogenetic analysis (Fig. 2) was carried out based on sequences of the internal transcribed spacer (ITS) obtained in previous studies from basidiomata (Cruz et al. 2011 and 2014) and complemented by sequences from GenBank, NCBI (http://www.ncbi.nlm.nih.gov/). The sequences mostly stem from strains obtained from basidiomata or from DNA isolated from orchid mycorrhizae (Table 1). The sequences were aligned using MAFFT v. 5.667 (Katoh et al. 2005) under the G-INS-i option. Because of the heterogeneity in the alignment, the highly divergent portions were eliminated using the Gblocks program v. 0.91b (Castresana 2000). The options were as in the following: Minimum Number of Sequences for a Conserved Position to (47), Minimum Number of Sequences for a Flank Position to (78), Maximum Number of Contiguous Nonconserved Positions to (8), Minimum Length of a Block to (10), and Allowed Gap Positions to (With half). A phylogentic Maximum-likelihood tree was inferred using the genetic GTRMIX model of DNA substitution with 1000 rapid bootstrap replicates (Felsenstein 1985). The tree was generated using MEGA 5.2.2 (Tamura et al. 2011).

Results

Morphological diagnoses, taxonomy, and illustrations

Species descriptions

Tulasnella albida Bourdot & Galzin, Hymen. de Fr.: 59 (1928). *Tulasnella intrusa* Hauerslev, Opera Bot. 100: 114 (1989). (Synonym according to Roberts 1994b) Fig. 3

Basidiomata resupinate, almost imperceptible, but refracting the light in fungarium material. Hyphae sinuous, continuously septate, producing ramifications in angles of up to 90°, unclamped, 3-4 µm diam. Cystidia absent. Basidia clavate to obpyriform, commonly found growing in clusters together to young basidia, (8–)10–13(–15) × (5–)6–7(–8) µm. Sterigmata four per basidium, mainly fusiform, (10–)12–17 × 4–5(–6) µm. Basidiospores oblong to elliptical, rarely pip-shaped or subglobose, (5–)6–7(–8) × (3.5–)4–5 µm, Q-values (1.1–)1.4–1.8(–2), hyaline, smooth, producing secondary basidiospores by repetition.

Specimens examined: EUROPE: **Belgium**, Zoersel, 06 May 1989, Van de Put K. 89050601 (6610952); Antwerpen, on *Pinus sylvestris*, 27 May 1995, Van de Put K. 95052703 (BR 6617521); Stekene, on hardwood, 11 Nov. 1995, Van de Put K. 95111109 (BR 6618430); Walem, on *Pinus sylvestris*, 11 Jan. 1996, Van de Put K. 96011101 (BR 6618834). **Germany**, Bayer-Schwaben, forest north of Reinhartshausen, south-west of Augsburg, ± 570-580 m, 25 Oct. 1970, F.Oberwinkler 17288; Oberbayern, Bernrieder forest between Holzhausen and Rottenried, south of Fürstenfeldbruck, ± 570 m, 03 Apr. 1972, F.Oberwinkler 17838; Baden-Württemberg, Schwarzwald, upland moor at Hinterzarten, 03 Sept. 1975, F.Oberwinkler 23058; Baden-Württemberg, Tübingen Hagelloch, Bogentor, ± 460 m, 4 Nov. 1975, F.Oberwinkler 23353; Baden-Württemberg, Schönbuch, between Hagelloch and Hohenentringen, "Bruderhaus", ± 500 m, from bark of *Picea abies*, 17 Feb. 1976, F.Oberwinkler 23474; Baden-Württemberg, Tübingen, Heuberger Tor, on bark of *Picea abies*, 15 Mar. 1998, R.Kirschner 324. **United Kingdom,** England, Surrey, Kew Royal Botanic Gardens at conservation area, on bark of *Salix* sp., mixed with *Sistotrema brinkmannii*, 12 Dec. 2003, A.Henrici, without number (K 120788).

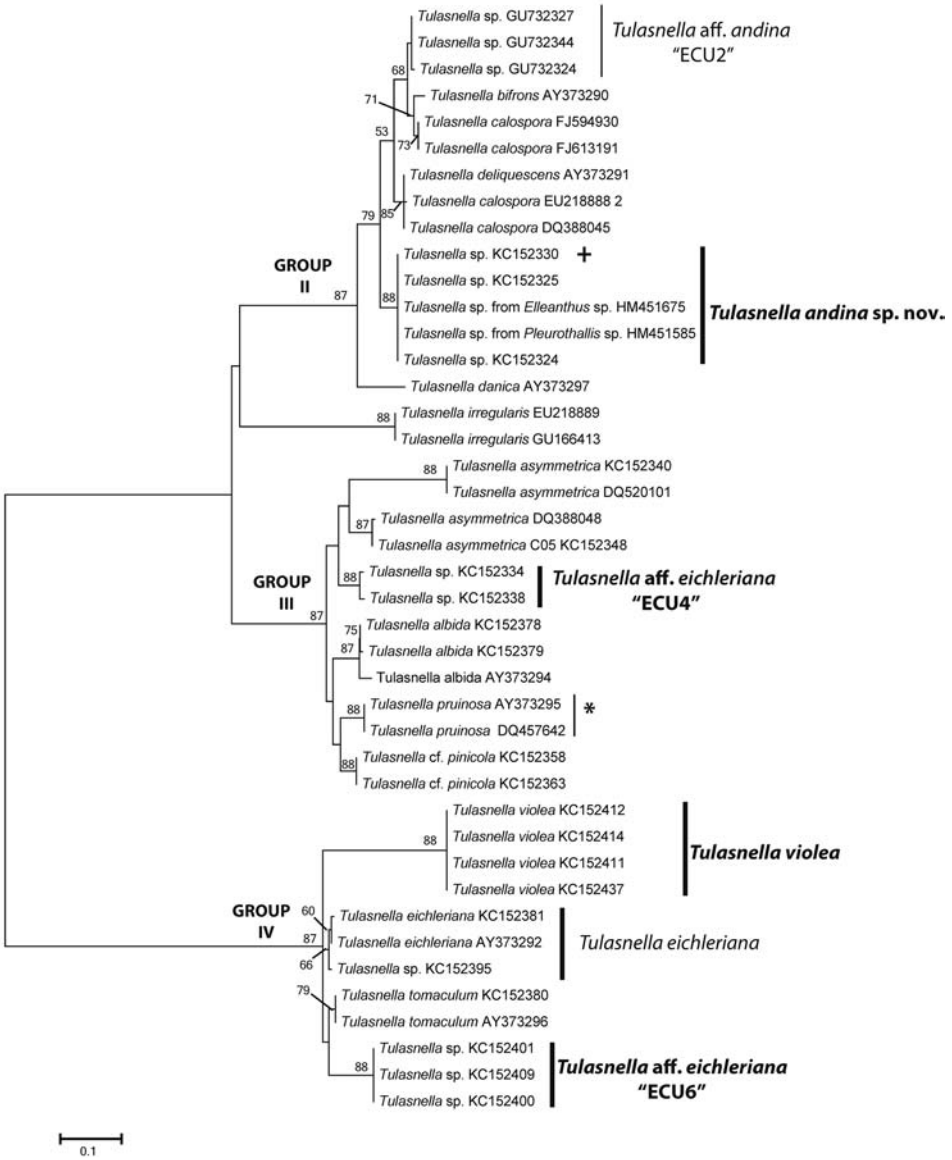

Fig. 2. Phylogenetic hypothesis based on ITS sequences obtained from own specimens and from NCBI (http://www.ncbi.nlm.nih.gov/). Values close to the nodes correspond to maximum likelihood bootstrap values. Only values larger than 60% are shown. Written in bold: *Tulasnella andina* as new species and *T*. aff. *eichleriana* (ECU4; ECU6) and *T. violea* as new records for Ecuador. The type sequence of *T. andina* is marked with (+). *Tulasnella pruinosa* sequences are indicated by *. The designation of Groups II, III, and IV correspond to the groups presented by Cruz et al. (2014).

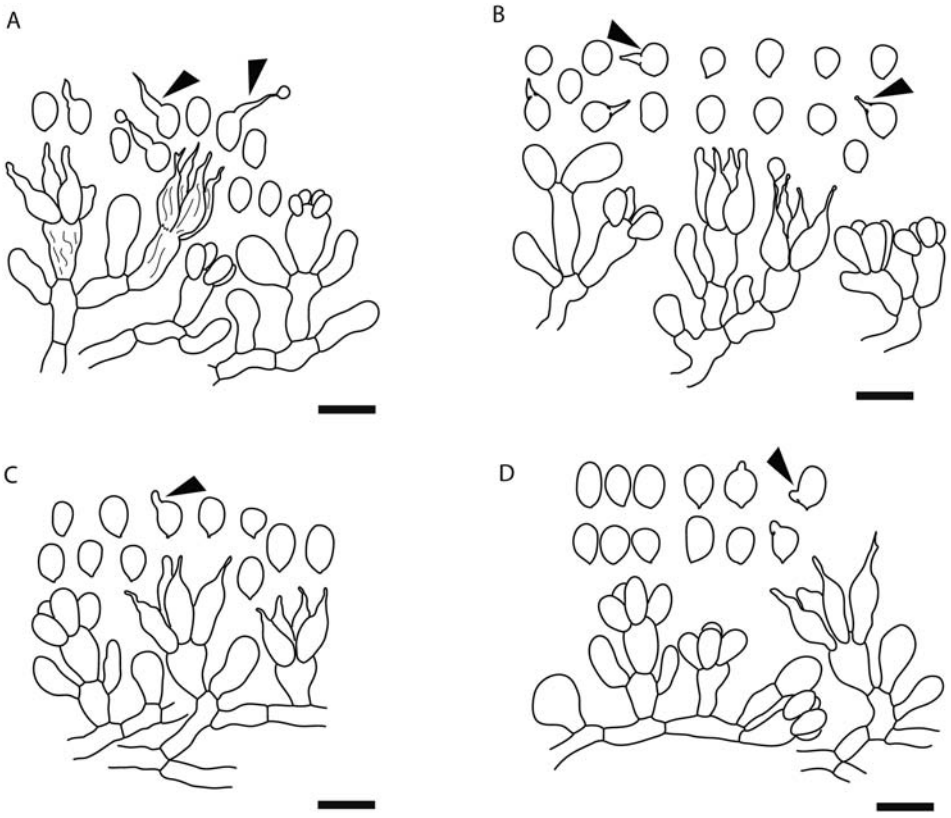

Fig. 3. *Tulasnella albida*. (A-D) Hyphae, basidia, sterigmata in different developmental stages, and basidiospores germinating by repetition. (A) K 120788. (B) BR 6618834. (C) BR 6610952. (D) BR 6618430. Scale bars = 10 μm.

Tulasnella albida is closely related to *T. pinicola* (Roberts 1994b). The basidiospores of both species are mainly oblong to elliptical and rarely pip-shaped or subglobose. The basidiospores of *T. albida*, however, measure (5–)6–7(–8) × (3.5)4–5 μm and those of *T. pinicola* (5–)7–9 × 4–6(–6.5) μm, so the basidiospores of *T. albida* are shorter than those of *T. pinicola* (Roberts 1994b).

The hymenium of *Tulasnella albida* forms basidia frequently in clusters growing from short cells. This is not the case in *T. pinicola*. The basida of *T. albida* measure (8–)10–13(–15) × (5–)6–7(–8) μm and the sterigmata (10–)12–17 × 4–5(–6) μm, so they are shorter than those of *T. pinicola* [16–23(–27) × 7–9 μm and (13–)15–33(–37) × (3–)5(–6) μm]. However, the measurements of the basidiospores, basidia, and sterigmata can be very variable and can be overlapping in these species impeding a correct identification. These characteristics cannot easily be used to separate both species.

Table 1. GenBank accession numbers of species used in the molecular-phylogenetic analysis.

Species	Collection number	GenBank accession no.	Country	Reference
Tulasnella albida	KC 110 (strain)	AY373294	England	McCormick et al. 2014
Tulasnella albida	K120788 (basidioma)	KC152379	England	Cruz et al. 2014
Tulasnella albida	K118140 (basidioma)	KC152378	Wales	Cruz et al. 2014
Tulasnella asymmetrica	AFTOL ID 1678 = MAFF 305807 (strain)	DQ520101	Australia	Garnica, S. and M.Weiss Direct submission
Tulasnella asymmetrica	MAFF 305806 (strain)	KC152340	Australia	Cruz et al. 2014
Tulasnella asymmetrica	MAFF 305808 (strain)	KC152348	Australia	Cruz et al. 2014
Tulasnella asymmetrica	MAFF P305809 (strain)	DQ388048	Australia	Suárez et al. 2006
Tulasnella bifrons	BPI 724849 (strain)	AY373290	Canada	McCormick et al. 2004
Tulasnella calospora	CBS 573 83 (strain)	EU218888 2	Australia	Taylor and McCormick 2008
Tulasnella calospora	H0402 37 (strain)	FJ613191	China	Yang,K., J.Wuand L.Li Direct submission
Tulasnella calospora	MAFF P305805 (strain)	DQ388045	Australia	Suárez et al. 2006
Tulasnella calospora	H1401 23 (strain)	FJ594930	China	Yang,K., J.Wu and L.Li Direct submission
Tulasnella* cf. *pinicola	DC309 (strain)	KC152358	Germany	Cruz et al. 2014
Tulasnella* cf. *pinicola	DC309 (strain)	KC152363	Germany	Cruz et al. 2014
Tulasnella danica	KC 388 (strain)	AY373297	England	McCormick et al. 2004
Tulasnella deliquescens	DAOM 478 (strain)	AY373291	–	McCormick et al. 2004
Tulasnella eichleriana	KC 852 (strain)	AY373292	England	McCormick et al. 2004
Tulasnella eichleriana	K143600 (basidioma)	KC152381	England	Cruz et al. 2014
Tulasnella irregularis	CBS 57483 (strain)	EU218889	Australia	Taylor and McCormick 2008
Tulasnella irregularis	D1 KT TC 1 (strain)	GU166413	Thailand	Nontachaiyapoom et al. 2010
Tulasnella pruinosa	AFTOL 610 = DAOM 17641 (strain)	DQ457642	Canada	Matheny et al 2006
Tulasnella pruinosa	DAOM 17641 (strain)	AY373295	Canada	McCormick et al. 2004
Tulasnella tomaculum	KC 429 (strain)	AY373296	Wales	McCormick et al. 2004
Tulasnella tomaculum	K123675 (basidioma)	KC152380	England	Cruz et al. 2014
Tulasnella violea	DC292 (strain)	KC152412	Germany	Cruz et al. 2014
Tulasnella violea	DC177 (basidioma)	KC152414	Ecuador	Cruz et al. 2014
Tulasnella violea	DC293 (basidioma)	KC152437	Germany	Cruz et al. 2014
Tulasnella violea	K164256 (basidioma)	KC152411	England	Cruz et al. 2014
***Tulasnella* sp.**	DC294 (basidioma)	KC152395	Germany	Cruz et al. 2014
***Tulasnella* sp.**	DC157 (basidioma)	KC152325	Ecuador	Cruz et al. 2014
***Tulasnella* sp.**	DC185 (basidioma)	KC152401	Ecuador	Cruz et al. 2014
***Tulasnella* sp.**	DC225 (basidioma)	KC152330	Ecuador	Cruz et al. 2014

***Tulasnella* sp.**	DC245 (basidioma)	KC152324	Ecuador	Cruz et al. 2014
***Tulasnella* sp.**	DC262 (basidioma)	KC152409	Ecuador	Cruz et al. 2014
***Tulasnella* sp.**	DC262 (basidioma)	KC152400	Ecuador	Cruz et al. 2014
***Tulasnella* sp.**	DC271 (basidioma)	KC152334	Ecuador	Cruz et al. 2014
***Tulasnella* sp.**	DC271 (basidioma)	KC152338	Ecuador	Cruz et al. 2014
***Tulasnella* sp.**	DC5 (basidioma)	GU732324	Ecuador	Cruz et al. 2011
***Tulasnella* sp.**	DC24 (basidioma)	GU732327	Ecuador	Cruz et al. 2011
***Tulasnella* sp.**	DC34 (basidioma)	GU732344	Ecuador	Cruz et al. 2011
***Tulasnella* sp.**	from *Elleanthus* sp. 3TB2 1	HM451675	Ecuador	Herrera et al. Direct submission
***Tulasnella* sp.**	from *Pleurothallis* sp. 1TC2 8	HM451585	Ecuador	Herrera et al. Direct submission

Tulasnella albida can produce subglobose basidiospores similar to those of *T. eichleriana* [4–5(–6) × (3–)3.5–4 µm] or *T. violea* [(5–)6–9(–12) × (5–)6–8 µm]. This also can generate confusion. However, basidiospores of the latter species are generally globose to subglobose with Q-values between 1 to 1.5, and in *T. albida* the basidiospores are mainly oblong to elliptical with Q-values between 1.4 to 1.8.

Due to the lack of molecular data from the type specimen, this species concept has not yet been confirmed by molecular phylogenetic analyses. Nonetheless, Cruz et al. (2014) proposed one clade for *Tulasnella albida* according to sequences obtained from morphologically similar basidiomata in sexual state (Table 1).

Tulasnella allantospora Wakef. & A.Pearson, Trans. Br. mycol. Soc. 8(4): 220 (1923); *Gloeotulasnella caroliniana* L.S.Olive, Bull. Torrey Bot. Club 80 (1): 41 (1953). (Synonym according to Roberts 1993b). Fig. 4

Basidiomata resupinate, effused, waxy, slightly blackish in fungarium material. Hyphae branching, continuously septate, all septa with clamps, (2–)2.5–3.5(–4) µm diam. Cystidia absent. Basidia mostly inverted pyriform, subglobose clavate, and solitary, (7–)9–15(–21) × 5–7(–8) µm. Sterigmata four per basidium, obclavate and basally globose, (8–)10–16(–20) × 4–5(–6) µm. Basidiospores mainly allantoid, (6–)7–10(–11) × 2.5–3.5(–4) µm, Q-values (2.3–)2.4–3.3(–4), hyaline, germinating by repetition.

Specimens examined: EUROPE: **Belgium**, Zoersel, on *Quercus robur*, 14 Jul. 1990, Van de Put K. 90071402 (BR 6611659); same site, on *Pinus* sp., 28 Dec. 1991, Van de Put K. 91122805 (BR 6612265); same site, 20 Feb. 1993, Van de Put K. 93022013 (BR 6613275); Walem, on *Pinus sylvestris*, 06 Jan. 1994, Van de Put K. 94010601 (BR 6614285); Zoersel, 08 Oct. 1994, Van de Put K. 94100808 (BR 6615295); Walem, on *Pinus sylvestris*, 20 Dec. 1994, Van de Put K. 94122004 (BR 6615703); Zoersel, on *Pinus sylvestris*, 04 Mar. 1995, Van de Put K. 95030403 (BR 6616511); same site, on *Quercus robur*, Van de Put K. 96030220 (BR 6619541). **Germany**, Bayer-Schwaben, Reinhartshausen, southwest of Augsburg, 570-580 m, 01 Nov. 1970, F.Oberwinkler 17315. **United Kingdom,** England, Devon, Dartmoor Nat. Park, on fallen branch of *Picea* sp., 1 Dec. 1991, P.Roberts 340 (K 23181).

Tulasnella allantospora is characterized by completely clamped hyphae and allantoid basidiospores. Van de Put (1996) reported that the size of the basidiospores is in average (6.5–)7–9 × 2.5–3(–3.5) µm. Substantial variability in size of basidiospores

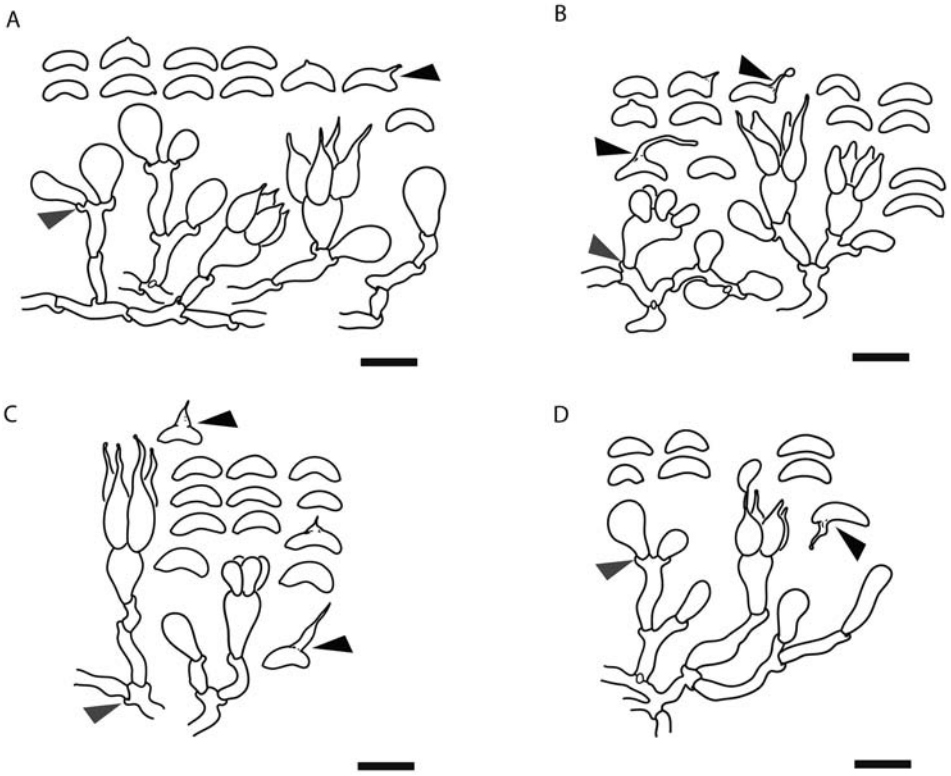

Fig. 4. *Tulasnella allantospora.* (A-D) Hyphae with clamps (indicated by gray arrowhead), basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowhead). (A) BR 6611659. (B) BR 6613275. (C) BR 664285. (D) BR 6615295. Scale bars = 10 μm.

was found in our revision of the same material with (6–)7–10(–11) × 2.5–3.5(–4) μm. The two averages are close to 6.5–9(–10) × 2.5–3 μm, which is the size range observed for the holotype of *T. allantospora*, revised by Roberts (1993b). Similar descriptions corresponding to this species concept have been presented by Roberts (1993a, 1994a). This morphological information suggests that all these specimens correspond to the same morphospecies. Due to the lack of molecular data, this species concept has not yet been confirmed by molecular phylogenetic analyses.

Tulasnella andina D.Cruz, J.P.Suárez & M.Piepenbr., **sp. nov.** Fig. 5

MycoBank: MB 814018

Etymology: The epithet of this new species refers to the montane tropical rainforest (Reserve Biológica San Francisco – RBSF) located at northern Andes in southern Ecuador.

Basidiomata resupinate, waxy pruinose, in fresh sample grey, violaceous grey, or slightly pinkish, extending on the substrate to about 10 cm^2, inconspicuous to the

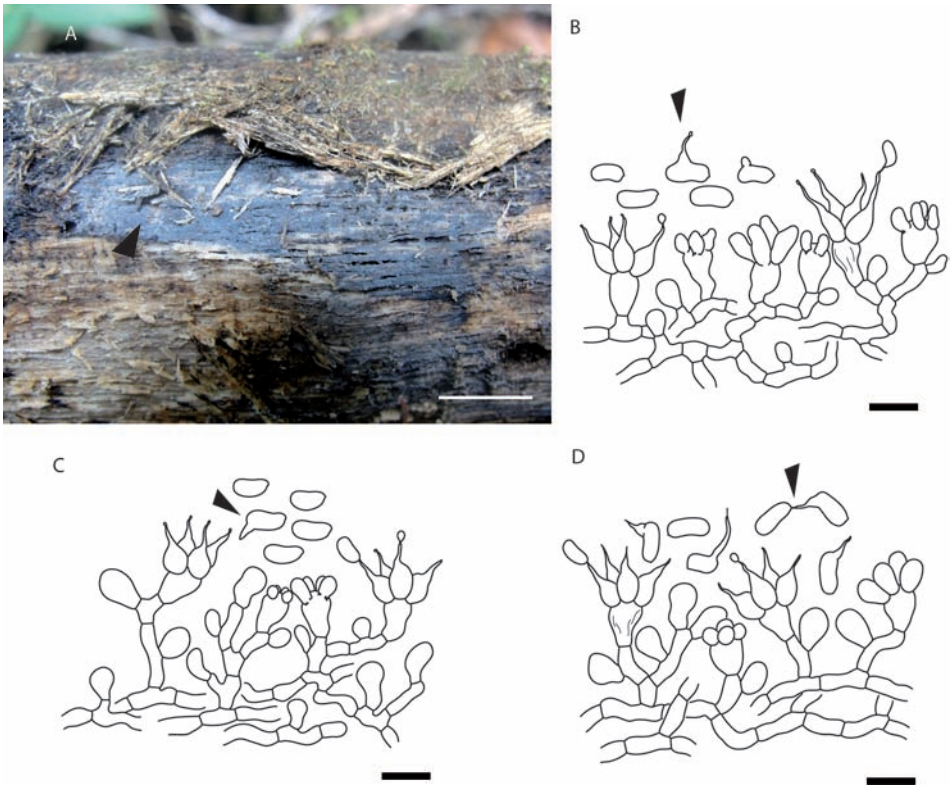

Fig. 5. *Tulasnella andina.* (A) Resupinate basidioma (indicated by black arrowhead) on decaying fallen branch (holotype D.Cruz 225). Scale bar = 2 cm. (B–D) Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowhead). (B) Holotype D.Cruz 225. (C) D.Cruz 157. (D) D.Cruz 245. Scale bars = 10 μm.

unaided eye in dried material. Hyphae loosely connected, forming irregular and rarely compact layers, hyaline, hyphae with a diameter of 2–3(–4) μm, without clamps, branched with "T" shape, sometimes constricted at the base of ramifications. Cystidia absent. Basidia mostly clavate, somewhat variable and sometimes subglobose to oblong in different specimens, containing several oil droplets (guttules) inside each cell, (7–)8–11(–12) × 5–6(–7) μm. Sterigmata four per basidium, pyriform to fusiform, in mature state, (6–)8–12(–15) × 4–5(–6) μm, often containing guttules. The length of the sterigmata is variable within and among specimens. Basidiospores phaseoliform or weakly allantoid to rarely subcylindrical, (5–)6–8 × (2–)3–4(–5) μm, Q-values (1.5–)1.7–2.3(–2.7), hyaline, smooth, sometimes with one or two guttules in each cell. Basidiospores produce secondary basidiospores by repetition.

Holotype: SOUTH AMERICA: **Ecuador**, southern Andes in southern Ecuador, Zamora Chinchipe Province, Cordillera El Consuelo, Reserva Biológica San Francisco, 3°58'S, 79°04'W, aprox. 1900 m, on decaying fallen branch, 27 Oct. 2009, D.Cruz 225 (HUTPL).

Further specimens examined: SOUTH AMERICA: Ecuador, Zamora Chinchipe, Cordillera El Consuelo, forest on the border of the Podocarpus National Park, km 24 between Loja and Zamora road, on a decaying fallen branch, 16 Jul. 2009, D.Cruz 157; Reserva Biológica San Francisco, on decaying fallen branch, 27 Oct. 2009, D.Cruz 225; same site, on decayed wood, 11 Nov. 2009, D.Cruz 245.

Tulasnella andina is a saprotrophic fungus mostly growing on decaying trunks or fallen branches that previously might have carried epiphytic orchids. The morphologically characterized species is relatively uniform with respect to the phaseoliform to subcylindrical shape of basidiospores. It is morphologically similar to *Tulasnella pruinosa* Bourdot & Galzin because *T. pruinosa* can present phaseoliform basidiospores. However, the basidiospores in *T. andina* are less variable in length [(5–)6–8 × (2–)3–4(–5) µm] than the basidiospores present in *T. pruinosa* [(3.5–)5–7(–7.5) × (2.5–)3–4(–5) µm]. *T. pruinosa* shows a high variability in the shape of the basidiospores, which are ellipsoid to oblong, often tapering towards the apiculus and occasionally ventrally depressed (own observation), distinct to the phaseoliform or weakly allantoid to subcylindrical basidiospores in *T. andina*. Different researchers remark this variability in shape of basidiospores for *T. pruinosa* (Bourdot & Galzin 1909, Dueñas 2001, Jülich & Jülich 1976, Roberts 1994b, Van de Put & Antonissen 1996).

The type material of *T. pruinosa* was not revised, however, the specimen P.Roberts 121, K 26130 mentioned by Roberts (1994b) was analysed. This specimen appears to be identical in all the sizes and morphological structures to the lectotype specimen A.Galzin 11012 (Bourdot 8745) illustrated by Jülich & Jülich (1976) and Roberts (1994b). In addition, the sterigmata of *T. pruinosa* are globose to ellipsoidal when young, and clavate to fusiform or mitriform when mature, with approximately 4 µm diameter at the widest part (Roberts 1994b and own observations). The sterigmata of *T. andina* are pyriform and 4–6 µm wide when mature. Because the shape of the basidiospores is extremely variable in specimens of *T. pruinosa* (own observation, see the description of *T. pruinosa*), it is considered to be an uncertain and ill-defined species (Roberts 1994b).

Tulasnella andina and *T. pruinosa* are separate species according to the phylogenetic analysis presented by Cruz et al. (2014) and in Fig. 2. The sequences corresponding to specimens morphologically assigned as *T. andina* cluster in the clade *T.* sp. ECU 1 in Group II while the *T. pruinosa* sequence (Accession in GenBank AY373295, culture DAOM 17641 isolated from basidioma of *T. pruinosa*) clusters in Group III (Cruz et al. 2014). The clade assigned to *T. andina* also includes sequences isolated from orchid roots sampled on the same study site or close by (Suárez et al. 2006, Herrera pers. com.). This indicates that *T. andina* probably is an orchid mycobiont.

Tulasnella andina appears morphologically identical to specimens (DC5; DC24; DC34; DC42; DC54) described as *T.* sp. by Cruz et al. (2011). Cruz et al. (2014) presented these mosphospecies as cryptic species phylogenetically clustered in two different clades (*T.* sp. ECU 1 and *T.* sp. ECU 2). The interspecific distance found between *T.* sp. ECU 1 and *T.* sp. ECU 2 species was more than 9% in the ITS region. *T.* sp. ECU 2 (Cruz et al. 2011, 2014) thus, still remains without a scientific name and is provisionally mentioned here as *T.* aff. *andina*.

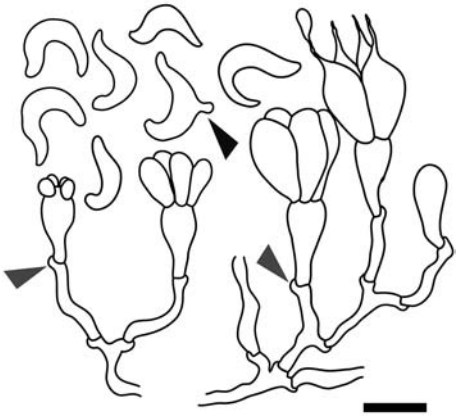

Fig. 6. *Tulasnella anguifera*. Hyphae with clamps (indicated by gray arrowhead), basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowhead). K 18541. Scale bar = 10 μm.

Tulasnella anguifera P.Roberts, Mycol. Res. 96(3): 235 (1992). Fig. 6

Basidiomata resupinate, effused, crusty, and blackish in fungarium material. Hyphae rarely branching, forming long septate cells at regular intervals, all septa with clamps, hyphae (1.5–)2–2.5 μm diam. Cystidia absent. Basidia clavate, usually solitary, (8–)10–14 × 5–5.5 μm. Sterigmata four per basidium, clavate or globose when immature, and subfusiform to fusiform when mature, (11–)16–18 × 6–7 μm. Basidiospores anguiform resembling worms or snakes, (12–)16–20 × 3–4 μm, Q-values (4–)4.8–5.3. Basidiospores germinate by repetition.

Specimen examined: EUROPE: **United Kingdom**, England, Devon, Newton Abbot, Stover Park, on wood of *Picea* sp., 29 Dec. 1990, P.Roberts 109 (Holotype, K 18541).

Tulasnella anguifera forms part of the group of species with spiral basidiospores (Roberts 1992). *T. anguifera* and *T. interrogans* are rather similar because both species have completely clamped hyphae and spiral basidiospores. *T. anguifera* differs from *T. interrogans* by basidiospores that are anguiform and measure (12–)16–20 × 3–4 μm, while the basidiospores of *T. interrogans* mostly have the shape of question marks and measure (13–)15–17(–19) × 1.5–2 μm. This species concept has not yet been confirmed by molecular phylogenetic analyses because sequences data from the type specimen are still lacking.

Tulasnella brinkmannii Bres., Annls mycol. 18(1/3): 50 (1920). Fig. 7

Basidiomata resupinate, effused, slightly waxy, violaceous grey in dry material. Hyphae formed by long septate cells, unclamped, 2–3 μm diam. (specimen from Belgium) to 4–5 μm diam. (specimen from Germany). Cystidia absent. Basidia sphaeropedunculate to clavate, usually solitary and sometimes two basidia clustered, (9–)10–15 × 6–8 μm (specimen from Belgium) to (17–)22–25 × (8–)9–10 μm (specimen from Germany).

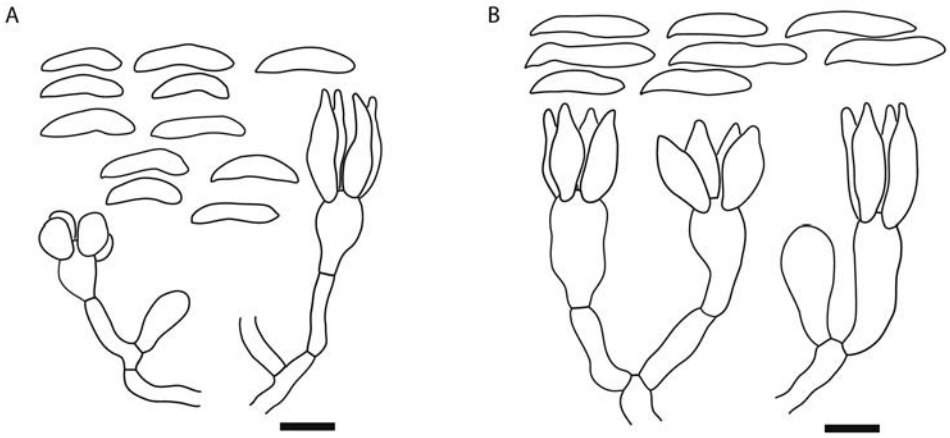

Fig. 7. *Tulasnella brinkmannii*. (A–B) Hyphae lacking clamps, basidia, sterigmata in different developmental stages, and basidiospores. (A) BR 6615093. (B) R. Kirschner 91. Scale bars = 10 μm.

Sterigmata four per basidium, fusiform to subfusiform, tapering to the apex and cylindrical and slightly depressed to the ventral part when mature, (16–)18–23 × 7–6 μm. Basidiospores mainly subfusiform, sometimes fusiform, with ventral concavity next to the apiculus and a tapering apex, rarely subcylindrical, (13–)17–23(–25) × 3–4(–5) μm, Q-values 4.2–6.3(–7.3), hyaline, smooth, germinating by repetition.

Specimens examined: EUROPE: **Belgium**, Zoersel, 16 Apr. 1994, Van de Put K. 94041604 (BR 66150933). **Germany**, Bayern, Allgäu, between Wertach and Oberjoch, 08 Oct. 1998, R.Kirschner 91.

Tulasnella brinkmannii can be confused with *T. calospora*, because both species can develop fusiform basidiospores tapering and curving to the apex. However, *T. brinkmannii* has subfusiform basidiospores with a ventral concavity next to the apiculus, tapering to the apex, and measuring (13–)17–23(–25) × 3–4(–5) μm. Basidiospores of *T. calospora* are mainly fusiform, measure (15–)18–28(–35) × 4.5–6(–7) μm, and are usually tapering and curved at both apices. In addition to this, sterigmata of *T. brinkmannii* measure (16–)18–23 × 7–6 μm and are mostly subfusiform, tapering to the apex. Mature sterigmata are cylindrical to slightly compressed at the ventral part. In *T. calospora*, mature sterigmata are obclavate, completely globose at the base and very tiny at the apical part, measuring (15–)18–23 × 7–9(–10) μm.

The two revised specimens from Germany or Belgium may correspond to distinct species, because of differences found in measurements of the hyphae and the basidia. The morphological characteristics of the type material [(*non vidi*) Germany, W.Brinkmann, Bourdot 77011 *sensu* Jülich & Jülich (1976) and Roberts (1994a)], mostly correspond to the characteristics of the specimen collected in Germany. However, high variability in sizes of hyphae and basidia are present and thus, the specimens are retained together as *T. brinkmannii*. So far no molecular data are available from type material or any other specimen of this species. This impedes to confirm this species concept by molecular phylogenetic analyses.

Tulasnella calospora (Boud.) Juel, Bih. Svensk. Vet. Akad. Handl. 23(3:12): 23 (1897); *Prototremella calospora* Boud., Journ. de Bot. 10: 85 (1896); *Gloeotulasnella calospora* (Boud.) D. P. Rogers, Annls Mycol. 31: 201 (1933). (Synonyms according to Roberts 1994a). Fig. 8

Basidiomata resupinate, inconspicuous, violaceous grey in dry material. Hyphae unclamped, 4–6 µm diam. Cystidia absent. Basidia clavate, with the upper part completely globose and thin at the basal part, rarely sphaeropedunculate, (13–)16–25 × (9–)12–18 µm. Sterigmata globose at the base and apically extended, sometimes fusiform, (14–)16–23 × (7–)8–10 µm. Basidiospores elongate, mainly fusiform to subfusiform, tapering and usually curved to both apices or sometimes presenting one side more curved than the other, (15–)18–28(–35) × 4.5–6(–7) µm, Q-values (2–)2.8–4.8(–6), hyaline, smooth, germinating by repetition.

Specimens examined: EUROPE: **Belgium**, Walem, on *Pinus sylvestris*, 10 Feb 1994, Van de Put K. 94021001 (BR 6614689); same site, on *Pinus sylvestris*, 31 Jan. 1995, 95013101 (BR 6616309, as *T. pallida* det. Van de Put K. & Antonissen); 25 Mar. 1995, Van de Put K. 95032506 (BR 6616915); same site, on *Pinus sylvestris*, 26 Mar. 1995, Van de Put K. 95032601 (BR 6617016). **Germany**, Bayern-Schwaben, Gut Bannacker at Bergheim, southwest of Augsburg, ± 520 m, 30 Mar. 1970, C.Mayr and F.Oberwinkler 16221. **United Kingdom**, England, Devon, on fallen branch with *Botryobasidium aureum*, 17 Oct. 1992, P.Roberts 478 (K 26126).

Tulasnella calospora is closely related to *T. brinkmannii* and *T. pallida* because of fusiform basidiospores. However, the basidiospores of *T. pallida* are shorter, measure (7–)8–12(–15) × (4–)5–6 µm, and are mainly fusiform to subfusiform without curved apex. The basidiospores of *T. calospora* are elongate, measure (15–)18–28(–35) × 4.5–6(–7) µm, are mainly fusiform to subfusiform, and have tapering and usually curved apices. Sometimes an apex can be more curved on one side than on the other.

Regarding calosporoid basidia and sterigmata, *Tulasnella calospora* is related to *T. deliquescens* and *T. sphaerospora*. *T. calospora* is easy to distinguish from *T. deliquescens* by the basidiospores. In the latter species, the basidiospores are longer and the shape is constantly sinuous (Roberts 1994a, 1999). In *T. sphaerospora*, basidiospores are spherical (Martin 1939), thus different from the fusiform or subfusiform basidiospores of *T. calospora.*

Many sequences in the databases (i.e., GenBank; http://www.ncbi.nlm.nih.gov/) are named as *T. calospsora* (e.g. accession no. DQ388045, strain JHW 0689; accession no. EU218888 2, isolate CBS 573 83, strain from *Caladenia reticulata* JHW 062, McCormick et al. 2004) isolated from terrestrial Australian orchids by Warcup and Talbot (1967). Roberts (1999), however, renamed the strains assigned to *T. calospora* by Warcup & Talbot (1967) as *T. deliquescens* corresponding to the teleomorph of *Epulorhiza repens* (Bernard) Moore, because the morphological descriptions by Warcup do not correspond to the *T. calospora* species concept. Other different sequences named as *Tulasnella calospora* (e.g. accession no. FJ613191, FJ594930) lack references concerning their teleomorphic states. Suárez et al. (2006) presented taxonomic problems or misidentifications concerning the species concept of *T. calospora*. Molecular data from the type specimen and basidiomata are required to confirm this species concept by molecular phylogenetic analyses.

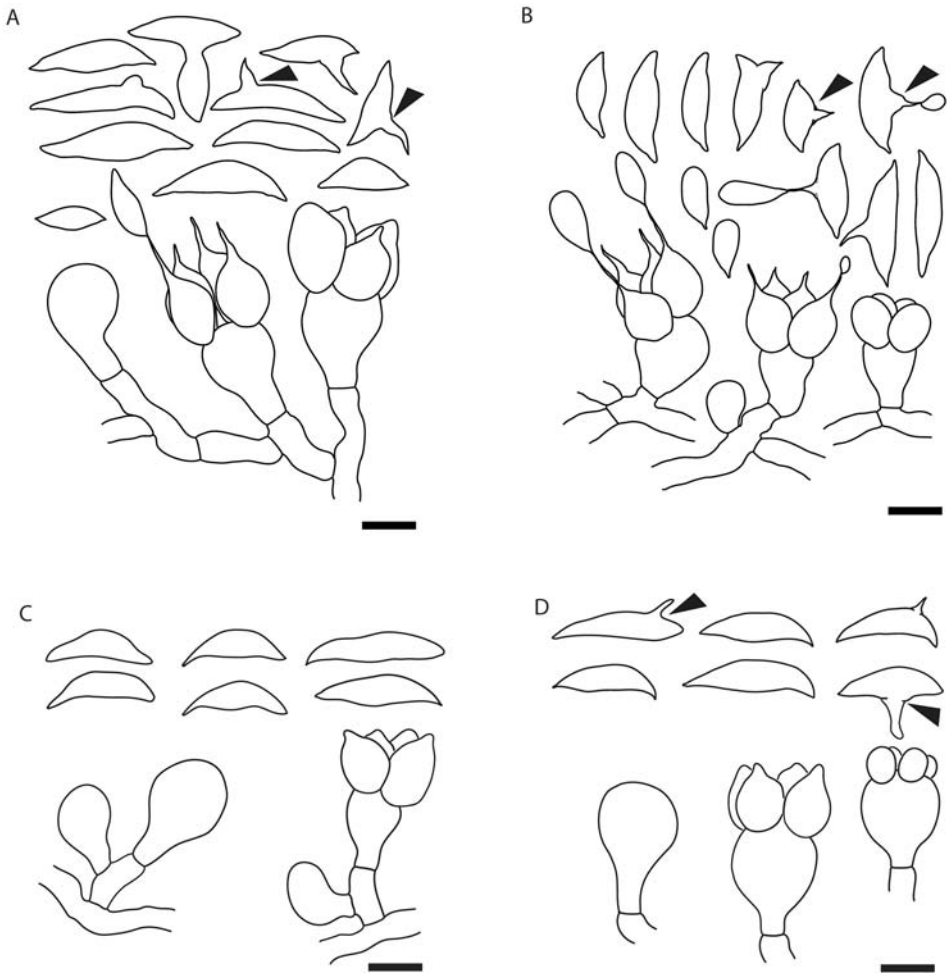

Fig. 8. *Tulasnella calospora.* (A–D) Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition. (A) BR 6614689. (B) BR 6616309. (C) BR 6616915. (D) K 26126. Scale bars = 10 μm.

Tulasnella* aff. *cruciata Warcup & P.H.B.Talbot, New Phytol. 70: 37 (1971). Fig. 9

Basidiomata resupinate, inconspicuous, slightly pruinose, grayish to black in dry fungarium material. Hyphae straight, forming lateral branches with angles of ca. 90°, septate, unclamped, 2–2.5 μm diam. Cystidia absent. Basidia clavate to subglobose, thin at the base, 10–15 × 8–9 μm. The basidia are mostly solitarily generated from long basal hyphae. Sterigmata four per basidium, basally obclavate to globose, with bacilliform or cylindrical spicula elongated at the apex, 36–45(–50) × (5–)6–7 μm. The basal part and the elongated spicula of the sterigmata often are loosely connected by an

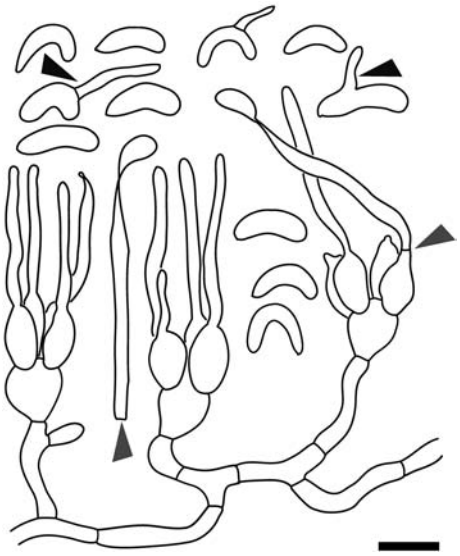

Fig. 9. *Tulasnella* aff. *cruciata.* Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowhead). The gray arrowheads indicate adventitious septa in the sterigmata. BR 6612669. Scale bar = 10 μm.

adventitious septum. Basidiospores mainly subcylindrical to allantoid, (10–)12–15(–20) × (2.5)3–4 μm, Q-values 2.4–5(–6.6), hyaline, smooth, germinating by repetition.

Specimen examined: EUROPE: **Belgium**, Antwerpen, Linker-Oever, St. Annabos, 25 Apr. 1992, Van de Put K. 92042501 as *Tulasnella calospora* (BR 6612669).

Tulasnella cruciata is a species isolated from Australian orchid roots (Warcup & Talbot 1971). The specimen from Belgium revised here shows characteristics similar to the species described by Warcup & Talbot (1971). However, the sterigmata in the Belgian specimen were found consistently elongated to 36–45(–50) μm, while the sterigmata in the type specimen were mostly 32(–72) μm long. Only one adventitious septum was observed in the Belgian material, different to the two adventitious septa described for the type specimen. The basidiospores are subcylindrical to allantoid in both cases with measurements of (10–)12–15(–20) × (2.5)3–4 μm in the Belgian specimen and 12–18 × 3.5–4.5 μm in the type specimen. Nonetheless, the allantoid basidiospores appear to be more strongly curved in the Belgian specimen than the allantoid basidiospores illustrated from the type specimen (Warcup & Talbot 1971). Based on these characteristics, the basidioma described here is named *Tulasnella* aff. *cruciata.*

Tulasnella aff. *cruciata* is related to *T. danica* and *T. irregularis* by the allantoid basidiospores and similar shapes of basidia and sterigmata (Roberts 1993a, 1994a, Warcup & Talbot 1971, 1980). The specimen named and revised here as *T. danica* does not have adventitious septa in sterigmata between the basal part and the spicula.

In addition, the basidiospores in *T. danica* were observed constantly as allantoid [(10–)12–16 × 3–4 µm]. *T.* aff. *cruciata* differs from *T. irregularis* which has shorter and wider basidiospores [11–14 × 4.5–5 µm]. Due to a lack of molecular data, the species concept has not yet been confirmed by phylogenetic analyses.

Tulasnella curvispora Donk, Persoonia 4(3): 263 (1966). Fig. 10

BASIDIOMATA resupinate, waxy, inconspicuous, blackish to bright in dry material. HYPHAE tortuous and branched, septate, all septa clamped, 2–3(–3.5) µm diam. CYSTIDIA absent. BASIDIA inverted pyriform to clavate, usually solitary, (8–)9–12 × 5–7 µm. STERIGMATA four per basidium, obclavate and basally globose, 12–16 × 5–6 µm. BASIDIOSPORES allantoid, 9–11(–12) × (2.5–)3–4 µm, quotient Q-values (2.6–)3–4(–4.8), smooth, germinating by repetition.

SPECIMENS EXAMINED: EUROPE: **Belgium**, Zoersel, 20 Feb. 1993, Van de Put K. 93022015 (BR 6613376); same site, on *Salix* sp., 2 Mar. 1996, Van de Put K. 96030208 (BR 6619238).

The specimens called *Tulasnella curvispora* in this study are morphologically identical to specimens called *T. allantospora*, especially the material collected and studied by Van de Put (1996). Van de Put (1996) assigned the specimens to the two species based on the size of the basidiospores: those of *T. curvispora* with 8–14 × 3–4 µm or (7–)9–15(–15.5) × (2–)2.2–3(–3.5) µm and those of *T. allantospora* with (6.5–)7–9 × 2.5–3(–3.5) µm. In our study we found a range in size of the basidiospores of 9–11(–12) × (2.5–)3–4 µm similar to the size of basidiospores of specimens named *T. curvispora*. Nevertheless, more variability in size was found to *T. allantospora* [(6–)7–10(–11) × 2.5–3.5(–4) µm]. Clearly, basidiospore measurements overlap in both species, making it difficult to separate them.

This species concept has not yet been confirmed by molecular phylogenetic analyses. Molecular data from the type specimen or other specimens are not available in the GenBank databases.

Tulasnella cystidiophora Höhn. & Litsch., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 115: 1557 (1906); *Gloeotulasnella cystidiophora* (Höhn. & Litsch.) Juel, Ark. Bot. 14(no. 6): 8 (1914); *Prototremella tulasnei* P.Karst., Hedwigia 35(1): 45 (1896). (Synonyms according to Roberts 1993). Fig. 11

BASIDIOMATA inconspicuous, waxy effused, slightly grayish to bright in dry material. HYPHAE slightly tortuous, with lateral branches presenting "T" shapes due to angles close to 90°, continuously septate, without clamps, 2–3.5(–4) µm diam. GLOEOCYSTIDIA abundant, cylindrical to irregularly swollen, projecting up, (21–)30–42 × 4–6 µm. BASIDIA usually clavate, sometimes sphaeropedunculate, growing in clusters or solitary, (9–)10–14 × (6–)8–11(–12) µm. STERIGMATA four per basidium, obclavate basally globose, tapered and extended to the apex, when young resembling to "pines of bowling", 15–20 × 5–7 µm. BASIDIOSPORES mainly globose to subglobose, less common elliptical, 5–7 × 4–5(–6.5) µm, Q-values (1–)1.2–1.6(–1.8), smooth, germinating by repetition.

SPECIMENS EXAMINED: EUROPE: **Belgium**, Zoersel, 15 Oct. 1994, Van de Put K. 94101502 (BR 6615396). **United Kingdom**, Wales, Monmouthshire, Wentwood forest, on fallen branch, 28 May 1992, P.Roberts 396 (K 24627).

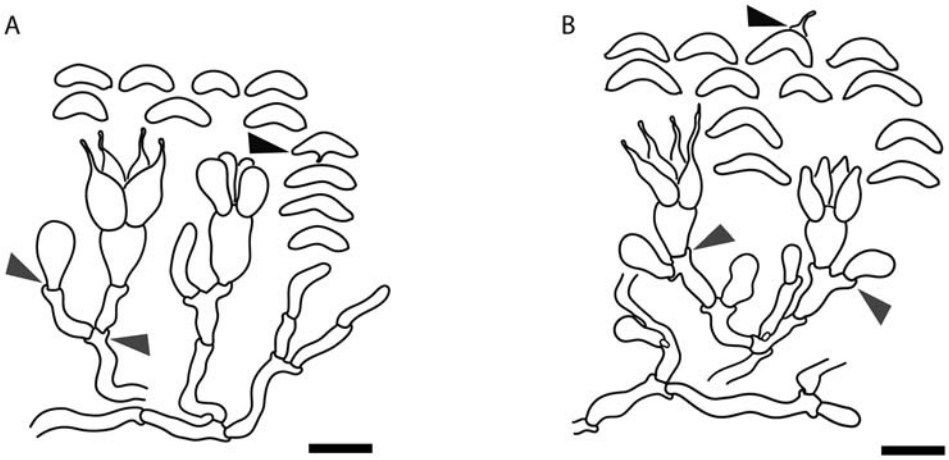

Fig. 10. *Tulasnella curvispora.* (A–B) Hyphae with clamps (indicated by gray arrowhead), basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowhead). (A) BR 6615316. (B) BR 6619258. Scale bars = 10 μm.

Basidiospores in *Tulasnella cystidiophora* are globose to subglobose as in *T. eichleriana*, *T. violea*, and *T. subglobospora*. *T. cystidiophora* is easy to distinguish from other *Tulasnella* species with globose to subglobose basidiospores by the gloeocystidia and unclamped hyphae. Sterigmata and gloeocystidia in *T. cystidiophora* are highly variable in length, 40 to 90 μm (Roberts 1994a). Due to a lack of molecular data from the type specimen, this species concept has not yet been confirmed by phylogenetic analyses. However, two sequences (accession no. AY585831 and AY856080) of one strain (Shefferson et al. 2005) are available for this species in the database GenBank NCBI.

Tulasnella danica Hauerslev, Friesia 11(5): 275 (1987) [1979]. Fig.12

Basidiomata resupinate, inconspicuous, grayish in dry fungarium material. Hyphae sinuous, with lateral branches in straight angles, septate, unclamped, 2–3 μm diam. Cystidia absent. Basidia globose to subglobose, basally thin, rarely sphaeropedunculate, mostly solitary or parallel to young basidia, 9–10(–11) × 8–9 μm. Sterigmata four per basidium, basally globose and apically tapered when young, fusiform to cylindrical at the central part with extended and tapered apex when mature, (15–)17–25 × 4–4.5 (–6) μm. Basidiospores allantoid, (10–)12–16 × 3–4 μm, Q-values 2.8–3.5(–5.3), smooth, germinating by repetition.

Specimen examined: EUROPE: **Belgium**, Walem, 20 Nov. 1994, Van de Put K. 94112004 (BR 6615400).

Tulasnella danica is morphologically similar to *T. allantospora* and *T. curvispora* because of allantoid basidiospores. *T. danica* has no clamps, although Roberts (1993) mentioned the presence of scattered clamp connections in the basal hyphae. This

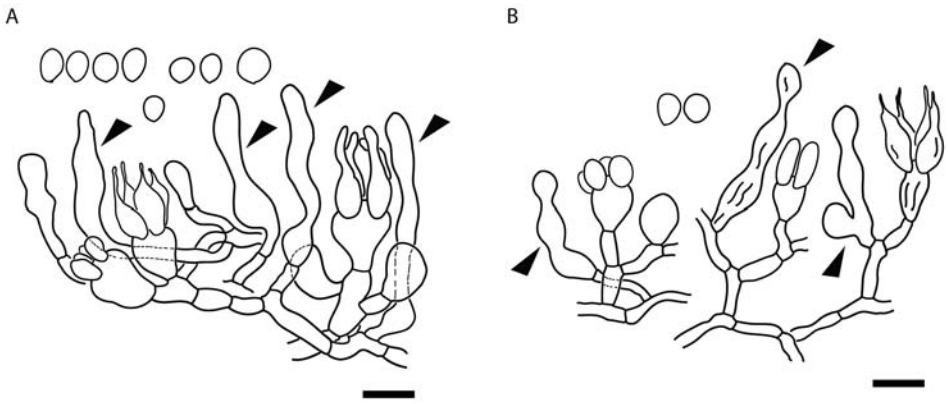

Fig. 11. *Tulasnella cystidiophora.* (A–B) Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition. The arrowheads indicate the gloeocystidia. (A) BR 6615396. (B) K 24627. Scale bars = 10 μm.

species also can be recognized by a "tangle-forming" hymenium (Roberts 1993). This characteristic, however, was not observed in our specimen. For this species, two sequences (accession no. AY373297 and AY382805) obtained from one strain (McCormick et al. 2004), isolated from a basidioma named as *Tulasnella danica* are available in GenBank NCBI.

Tulasnella deliquescens (Juel) Juel, Arch. für Botanik 14: 8 (1914); *Muciporus deliquescens* Juel, Bih. K. svenska VetenskAkad. Handl., Afd. 3 23(no. 10): 24 (1897); *Tulasnella rosella* Bourdot & Galzin, Bull. Soc. mycol. Fr. 39: 263 (1924). (Synonyms according to Roberts 1994a). Fig. 13

BASIDIOMATA inconspicuous, waxy, violaceous grey in dry material. HYPHAE undulate, forming long septate and unclamped cells, (2.5–)3–5(–6) μm diam., in some specimens some basal hyphae with thick walls. CYSTIDIA absent. BASIDIA calosporoid, similar to those in *T. calospora* with the upper part completely globose and the basal part thin, rarely sphaeropedunculate, (12–)17–28 × (10–)11–13 μm. STERIGMATA basally globose to sometimes fusiform, apical extended, (12–)14–25(–32) × 7–10 μm. BASIDIOSPORES elongated, mainly sigmoid and bacilliform when immature, tapered to both apices, (25–)30–37(–40) × 2.5–4(–5) μm, Q-values (5.6–)8.6–12(–13.3), hyaline, smooth, germinating by repetition.

SPECIMENS EXAMINED: EUROPE: **Belgium**, Zoersel, on *Quercus robur*, 02 Mar. 1991, Van de Put K. 91030205 (BR 6611962); same site, on *Quercus robur*, 04 Mar. 1995, Van de Put K. 95030411 (BR 6616713); same site, on *Salix* sp., 08 Mar. 1996, Van de Put K. 96030815 (BR 6620147). **Germany**, Hesse, Darmstadt-Eberstadt, Frankenstein, on decayed wood, 01 Mar. 1998, R.Kirschner 314.

Tulasnella deliquescens is similar to *T. calospora* because of the diameter of the hyphae, calosporoid basidia, and sterigmata. However, these species can be distinguished by the shape and the size of the basidiospores. In the case of *T. deliquescens*, they are

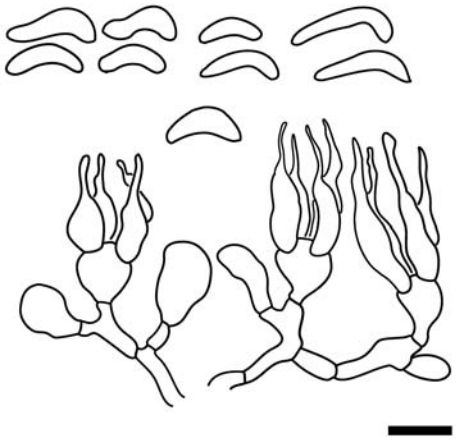

Fig. 12. *Tulasnella danica*. Hyphae, basidia, sterigmata in different developmental stages, and basidiospores. (BR 6615400). Scale bar = 10 μm.

mostly sigmoid and measure (25–)30–37(–40) × 2.5–4(–5) μm, whereas *T. calospora* has fusiform to subfusiform basidiospores measuring (15–)18–28(–35) × 4.5–6(–7) μm. Warcup (1967) described erroneously teleomorphic isolates from terrestrial orchids of *T. deliquescens* as "*T. calospora*". Roberts (1994a) noted this and changed the names. Two sequences in the databases as GenBank NCBI are named as *T. deliquescens* (i.e accession no. AY373291 for ITS-5.8 region and AY382798 for mtDNA, McCormick et al. 2004), however, due to a lack of molecular data from the type specimen, this species concept has not yet been confirmed by phylogenetic analyses. Similar taxonomic problems exist for *T. calospora*.

Tulasnella eichleriana Bres., Annls Mycol. 1: 113 (1903); *Tulasnella obscura* Bourdot & Galzin, Bull. Soc. Mycol. Fr. 39: 265 (1924) [1923]; *Tulasnella lactea* Bourdot & Calzin, Bull. Soc. Mycol. Fr. 39: 263 (1923); *Tulasnella microspora* Wakef. & A. Pearson, Trans. Br. Mycol. Soc. 8: 220 (1923). (Synonyms according to Roberts 1994b). Fig. 14

Basidiomata effused resupinate, violaceous grey or slightly pinkish, sometimes subgelatinous when fresh and grayish when dry. Hyphae unclamped, formed by short septate cells, 2–3(–4) μm diam., forming a hymenium with hyphae running parallel on the substrate, generating basidia close to each other. Cystidia absent. Basidia mostly clavate to subglobose and sometimes sphaeropedunculate, (7–)8–10(–12) × 5–6(–7) μm. Sterigmata fusiform, obclavate to globose at the base and finely extended at the apex, (7–)9–12(–20) × 3.5–5(–6) μm. Basidiospores generally globose to subglobose, variable to elliptical and oblong, 4–5(–6) × (3–)3.5–4 μm, Q-values 1–1.5(–1.8), hyaline, smooth and germinating by repetition. Oblong basidiospores rarely found 8 × 3–4 μm.

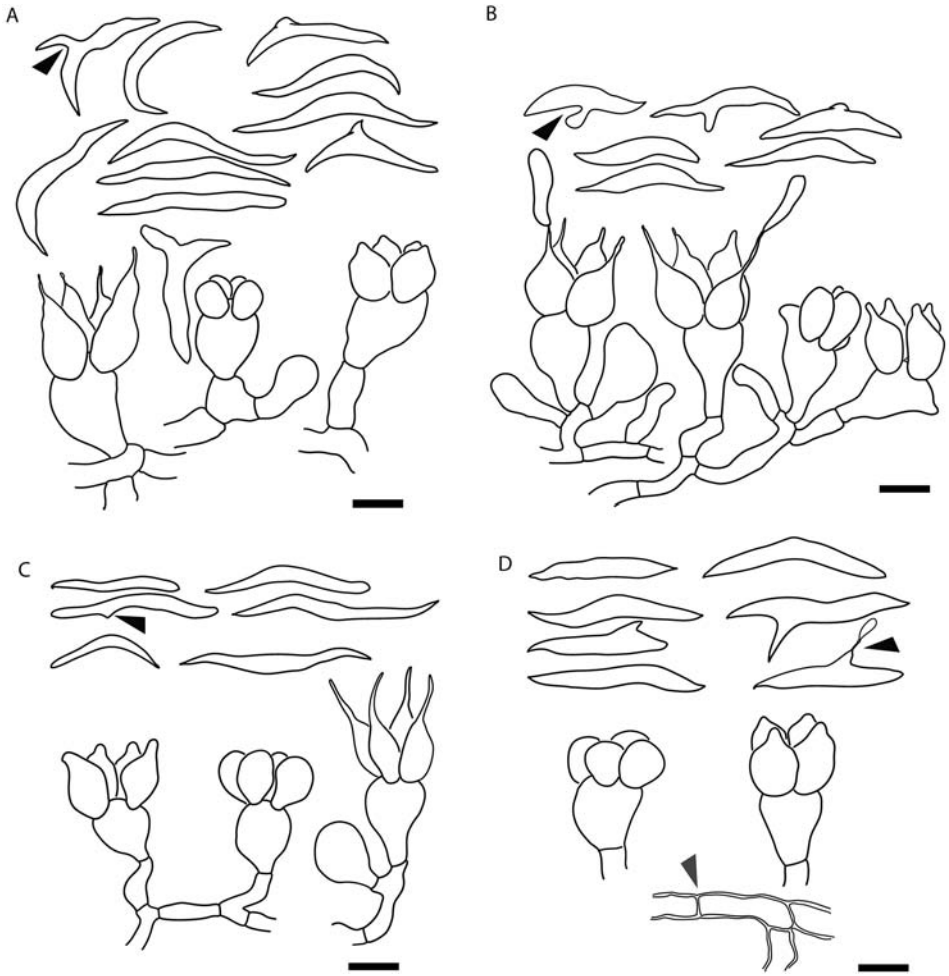

Fig. 13. *Tulasnella deliquescens*. (A–D) Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). The gray arrowhead indicates a thick-walled hypha. (A) BR 6611962. (B) BR 6616713. (C) BR 6620147. (D) R. Kirschner 314. Scale bars = 10 μm.

Specimens examined: SOUTH AMERICA: **Ecuador**, Zamora Chinchipe, Cordillera El Consuelo, forest on the border of Podocarpus National Park, km. 24 between Loja and Zamora road, on a decaying fallen branch, 16 Jul. 2009, D.Cruz 185; Reserva Biológica San Francisco, on fallen branch, 13 Nov. 2009, D.Cruz 262; same site, on fallen branch, 19 Nov. 2009, D.Cruz 271. **Venezuela**, Merida, La Mucuy, abobe Tabay, east of Merida, ± 2200 m, 25 Dec. 1968, F.Oberwinkler 14056; 25 Dec. 1968, F.Oberwinkler 14059. EUROPE: **Belgium**, Zoersel, on *Populus canadensis*, 31 Mar. 1990, Van de Put K. 90033103 (BR 6611154); same site, on decayed wood, 30 June 1990, Van de Put K. 90063016 (BR 6611457); same site, on *Salix* sp., 07 July 1990, Van de Put K. 90070701 (BR 6611558); same site, 10 Jan. 1992, Van de Put K. 92011004 (BR 6612467); same site, on *Quercus robur,* 01 Oct. 1992, Van de Put K. 92011002 (BR 6612366); same site, 02 Dec.

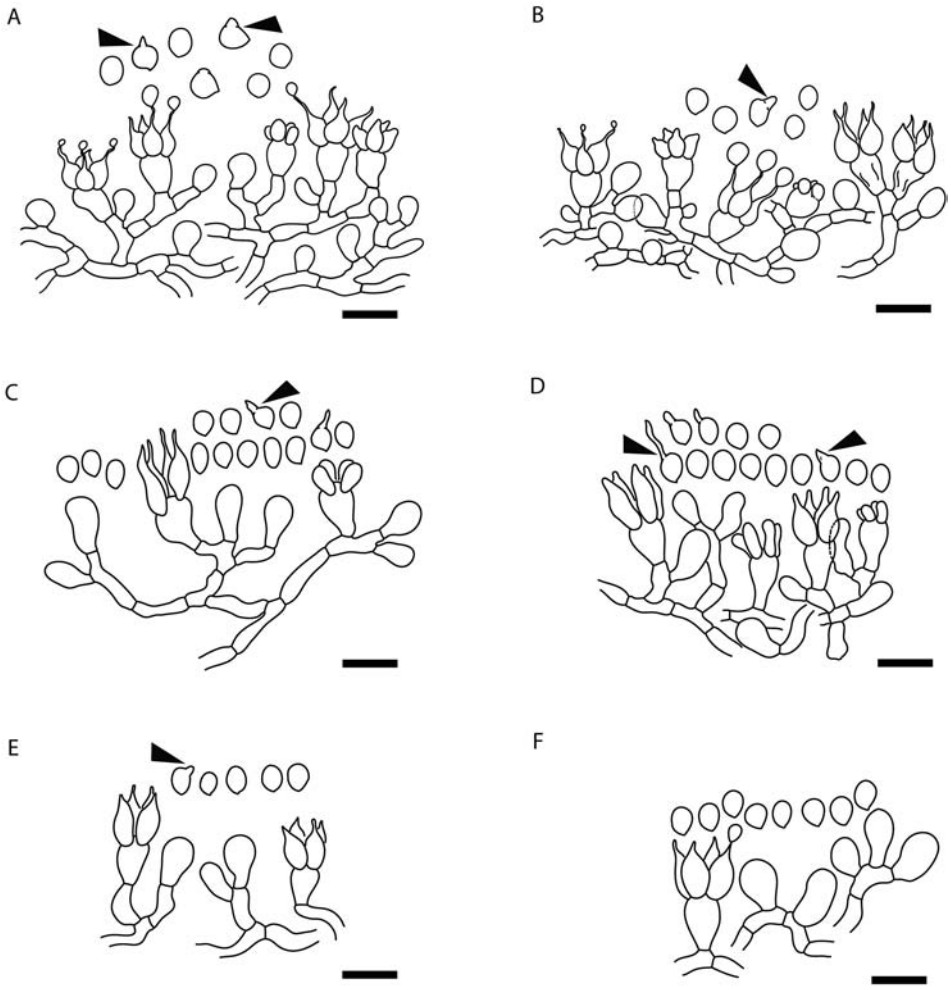

Fig. 14. *Tulasnella eichleriana.* (A–F) Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). (A) D. Cruz 262. (B) D. Cruz 271. (C) BR 6612467. (D) D. Cruz 294. (E) K 118140. (F) K 143600. Scale bars = 10 μm.

1994, Van de Put K. 94021207 (BR 6614790); same site, on *Salix* sp., 11 Mar. 1995, Van de Put K. 95031109 (BR 6616814); same site, on *Salix* sp., 09 Mar. 1996, Van de Put K. 96030902 (BR 6619844); same site, on *Salix* sp., 09 Mar. 1996, Van de Put K. 96030906 (BR 6619945); same site, on *Populus canadensis*, 13 Apr. 1996, Van de Put K. 96041308 (BR 6620652). **Germany**, Bayern, Allgäu, Oberjoch, 10 Oct. 1996, R. Kirschner 102; Hahnenfalz, Bavarian Forest, 15 Oct. 1996, R.Kirschner 49; Bayern, Allgäu, Pfeiffermühle at Oberjoch, 30 Sept. 1997, R.Kirschner 237; Tübingen, Baden-Württemberg, Heuberger Tor, on decayed wood, 09 Nov. 1999, R.Kirschner 614 as *Tulasnella albida*; Bavaria, Kirchholz in Bad Reichenhall, 500 m, in between a basidiomycete, 06 Sept. 1963, F.Oberwinkler 6323; Oberbayern between Seeshaupt and Gröben, Ostersee ±

600 m, on wood, 13 Oct. 1963, F.Oberwinkler 6939; Bayern, Oberbayern, München, Allacher Lohe, 520 m, 29 Apr. 1972, F.Oberwinkler 17884; Bayern, Allgäu, Oberjoch, Jochschrofen, south-facing slope in the spruce forest, 1200-1300 m, 19 Sept. 1981, F.Oberwinkler 31798; Bayern, Allgäu, Oberjoch, forest in the Kemnatsrieder Alp, 6 Oct. 1984, F.Oberwinkler 35715; Hesse, Frankfurt am Main, Stadtwald, Louisa forest, on decayed wood, 11 Apr. 2010, D.Cruz 294. **Norway**, Akershus, Leirfall, Skogreservat, on fallen wood, 16 Sept. 1993, P.Roberts 678 (K 26157). **Portugal**, Madeira; Faja da Nogueira, Power station Levada, 650–900 m, 31 Mar. 1984, F. Oberwinkler 35110; Madeira; path from Pico do Gato on the south side of Pico Ruivo, ca. 1550 m, 02 Apr. 1984, F. Oberwinkler 35218. **United Kingdom**, England, Surrey, East Horsley, Oldlands, on wood of decorticate fallen sapling trunk (*Acer pseudoplatanus*), 05 Dec. 2006, P.Roberts without number (K 143600). Wales, Breconshire, Cefn Bryn Uchaf, on fallen deciduous wood, 15 Oct. 2003, P. Roberts without number (K 118140). ASIA: **China**, Taiwan, Nantau, 8 Aug. 1999, R.Kirschner 563.

Tulasnella eichleriana is similar to *T. violea* due to globose basidiospores (Q-values 1–1.5) and to *T. albida* because sometimes elliptical or oblong basidiospores are present. Rogers (1933) erroneously synonymized *T. eichleriana* with *T. violea* according to this characteristic. However, the size of the basidiospores in *T. eichleriana* is usually between 4–5(–6) × (3–)3.5–4 µm whereas in case of *T. violea* it is (5–)6–9(–12) × (5–)6–8 µm. The measurements of basidiospores and other structures in these species are often overlapping. *T. eichleriana* appears similar to *T. tomaculum* by the hymenial systems.

Cruz et al. (2014) proposed one phylogenetic clade to correspond to *Tulasnella eichleriana* sensu stricto based on the congruence of morphological, phylogenetic, and geographic information. Most sequences clustering in that clade were obtained from teleomorphic basidiomata (e.g D. Cruz 294, K 143600 and K 118140) collected in European countries (Cruz et al. 2014). The type specimen (non vidi) collected in Poland, described by Bresadola (1903) and revised by Roberts (1994b) is closely related morphologically and geographically. The sequences from the specimens DC185, DC271, and DC262 (Table 1) collected in Ecuador, clustered in two other, different clades (Cruz et al. 2014). These clades were named *T.* sp. ECU4 in Group III (specimen DC271) and *T.* sp. ECU6 in Group IV (specimens DC185 and DC262). Because of the morphological similarity, all the specimens (Ecuadorian and European) were included in the description here. The three specimens from Ecuador present slight differences in the length of the basidia when compared with the European specimens. The basidia of the specimens from Ecuador are shorter, 7–9(–10) µm, compared to 8–10(–12) µm in the other specimens. An overlapping in size of basidia is, however, evident. In the specimen DC185 oblong basidiospores (8 × 3–4 µm) were found. It is evident that the morphological criteria appear insufficient to distinguish the phylogenetic species presented by Cruz et al. (2014). The phylogentic species *T.* sp. ECU4 (specimen DC271) and *T.* sp. ECU6 (specimens DC185 and DC262) remain without scientific names. However, the three specimens from Ecuador are provisionally considered as *Tulasnella* aff. *eichleriana* by morphology. This morphospecies represents a new record for Ecuador.

Tulasnella falcifera P.Roberts, Mycol. Res. 96(3): 236 (1992). Fig. 15

Basidiomata resupinate, effused, almost invisible in fungarium material. Hyphae straight, loosely attached to the substrate, forming long septate cells, completely unclamped, slightly branched before forming basidia, 1.5–2(–3) µm diam. Gloeocystidia elongated and undulate, solitary or close to the basidia, with central swollen parts and basally thin, (15–)22–24 × (3–)4–5 µm. Basidia globose, basally elongated, often with an apical

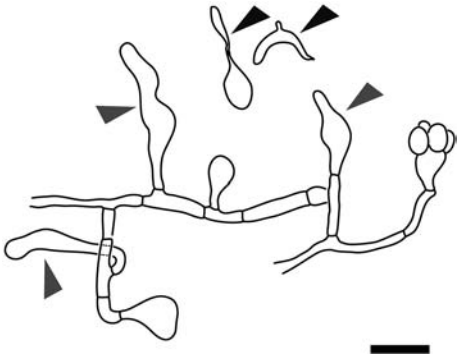

Fig. 15. *Tulasnella falcifera*. Hyphae and basidia with young sterigmata (holotype K 18539). The black arrowheads indicate one basidiospore partially germinating by repetition and one basidiospore attached to the sterigma. The gray arrowheads indicate gloeocystidia. Holotype K18539. Scale bar = 10 μm.

extension, 8–9 × 4–6 μm. Sterigmata four per basidium, basally globose and tapered to the apex, when immature globose, 10 × 4 μm. Basidiospores lunate, tapered and extended to the apiculus, 14 × 2 μm (Q-value 7, n = 1). Only one basidiospore could be found in the type specimen. Roberts (1992) described the shape of the basidiospores as similar to a question mark, similar to the shape of basidiospores of *T. interrogans* (11.4–16.8 × 1.8–3.0 μm).

Specimen examined: EUROPE: **United Kingdom**, England, South Hampshire, on decayed wood of *Salix* sp., mixed with *Scopuloides hydnoides*, 01 Apr. 1991, T.Læssøe 2309 (Holotype, K 18539).

The type specimen of *Tulasnella falcifera* revised here is degraded, so the description is insufficient and it is difficult to compare this species with other *Tulasnella* species with spiral basidiospores such as *T. interrogans*, *T. anguifera*, or *T. helicospora*. However, *T. falcifera* can be distinguished from *T. interrogans* or *T. anguifera* because these two species have clamp connections and lack gloeocystidia. *T. falcifera* is different from *T. helicospora* because *T. helicospora* lacks gloeocystidia and the basidiospores are larger, measuring (16–)20–26 × (2.5–)3 μm. Due to a lack of molecular data, this species concept has not yet been confirmed by phylogenetic analyses.

Tulasnella fuscoviolacea Bres., Fung. trident. 2(14): 98 (1900). Fig. 16

Basidiomata resupinate, effused and abundant, forming a grayish crust, very cracked when dry. Hyphae cylindrical, slightly branched forming an angle about 90°, lacking clamps and forming long septate cells, (3–)4–5(–6) μm diam. Cystidia absent. Basidia clavate, apically globose to rarely sphaeropedunculate, (13–)15–20 × 10–12 μm. Sterigmata four per basidium, obclavate, basally globose, tapered and extended to the apex, resembling to "pines of bowling", sometimes bifurcate to the apex, (13–)15–25(–30) × (6–)7–8 μm. Basidiospores mainly subcylindrical, tapered and slightly curved to the apiculus, rarely oblong or allantoid, (11–)12–16(–19) × (3–)4–5(–6) μm,

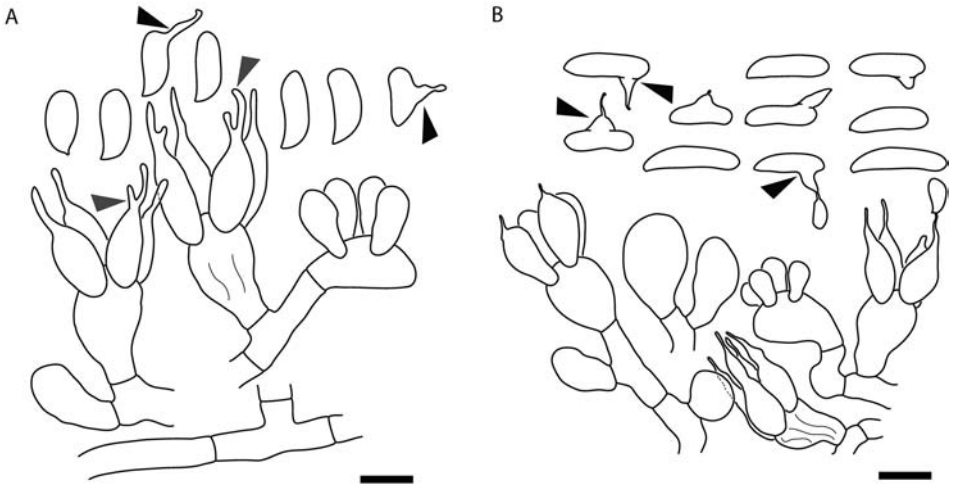

Fig. 16. *Tulasnella fuscoviolacea*. (A–B) Hyphae without clamps, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). The gray arrowheads indicate bifurcate sterigmata. (A) FO 35711. (B) TAA 54894. Scale bars = 10 μm.

Q-values (2–)2.6–4(–5.4), hyaline, smooth, thin-walled and germinating by repetition.

Specimens examined: EUROPE: **Germany**, Bayern, Allgäu, Oberjoch, forest in the Kemnatsrieder Alp, 06 Sept. 1984, F.Oberwinkler 35711; **Union of Soviet Socialist Republics** U.S.S.R, on *Picea abies*, 20 Oct. 1970, K.Wells, E.Parmasto TAA54894.

Tulasnella fuscoviolacea is rather similar to *T. pallidocremea* Jülich. Roberts (1994b) distinguished both species only by the width of the basidiospores when comparing the type specimens. The basidiospores in the type material of *T. pallidocremea* are narrower (10–12.5 × 2.5–3 μm) than the basidiospores [(7–)8.5–12 × 4–4.5 μm] in the type material of *T. fuscoviolacea* (Roberts 1994b).

The specimens TAA54894 and 35711 revised here are named *T. fuscoviolacea*. However, the specimen TAA54894 has narrower basidiospores [13–16(–19) × 3–4 μm] than the basidiospores [(11–)12–14(–15) × 5–6 μm] in the specimen TAA35711. Narrower basidiospores are considered here as insufficient to separate these specimens as different species. The variability in size could be higher and overlapping when comparing more specimens Roberts (1994b).

Tulasnella fuscoviolacea is also similar to *T. violea* by the shape and the size of hyphae and basidia. *T. fuscoviolacea* and *T. violea* are, however, completely different in the shape of their basidiospores. The basidiospores are subcylindric in *T. fuscoviolacea* and mostly globose in *T. violea*. Jülich and Jülich (1976) illustrated and described *T. fuscoviolacea* as similar to *T. brinkmannii*. However, *T. fuscoviolacea* with shorter [(11–)12–16(–19) × (3–)4–5(–6) μm] and subcylindrical basidiospores [Q-values (2–)2.6–4(–5.4)] can be differentiated from *T. brinkmannii* by larger [(13–)17–23(–25)

× 3–4(–5) µm] and subfusiform basidiospores [Q-values 4.2–6.3(–7.3)]. In addition to this, the sterigmata of *T. fuscoviolacea* are obclavate and basally globose, tapered and extended to the apex, resembling "pines of bowling", some are bifurcate at the apex. The sterigmata in *T. brinkmannii* are mostly subfusiform, tapered to the apex and cylindrical to slightly depressed at the ventral part. This species concept has not yet been confirmed by molecular phylogenetic analyses.

Tulasnella griseorubella Litsch., Svensk bot. Tidskr. 26: 448 (1933); *Gloeotulasnella griseorubella* (Litsch.) Pilát, Česká Mykol. 11: 80 (1957). (Synonym according to Roberts 1994b). Fig. 17

Basidiomata resupinate, effused, cracked and grayish in dry material. Hyphae tortuous, mostly narrow, completely septate and clamped, (2.5–)3–4 µm diam. Cystidia absent. Basidia clavate when young, and sphaeropedunculate to elongated when mature, (18–)20–27 × 6–8 µm. Basidia solitary or forming two clustered basidia resembling those of *T. pinicola.* Sterigmata four per basidium, mostly clavate to fusiform when young, and subfusiform, irregularly swollen to tapered and extending to the apex when mature, (16–)20–28 × 5–6 µm. Basidiospores mainly subcylindrical to oblong, occasionally pip-shaped, (8–)9–11 × 5(–6) µm, Q-values 1.6–1.8(–2.2), hyaline, smooth, and germinating by repetition.

Specimen examined: EUROPE: **Belgium**, Zoersel, 06 Feb. 1992, Van de Put K. 93020614 (BR 6613174).

Tulasnella griseorubella resembles *T. thelephorea* according to the clamped hyphae, absence of cystidia, and shape of basidiospores. The measurements of basidiospores in these species are overlapping, but basidiospores of *T. griseorubella* are mainly subcylindrical to oblong, occasionally pip-shaped [(8–)9–11 × 5(–6) µm]. Basidiospores are mainly elliptical to pip-shaped and rarely oblong [(6–)7–10 × (4–)5–8 µm] in the specimens of *T. thelephorea*. The arrangement of basidia in *T. griseorubella* is usually solitary or two clustered while in *T. thelephorea* the basidia are growing in clusters together with young basidia, resembling the arrangement in *T. albida.*

Roberts (1994b) compared the type specimens of both species and suggested that the length of the basidiospores is different in *T. thelephorea* and *T. griseorubella*. Based on all available information, the specimens revised here like *T. griseorubella* and *T. thelephorea* are retained as different species. These species concepts have not yet been confirmed by molecular phylogenetic analyses. Molecular data from type specimens are not available in the GenBank database.

Tulasnella helicospora (Raunk.) M.P.Christ., Dansk bot. Ark. 19(no. 2): 40 (1959). Fig. 18

Basidiomata resupinate, with sandy parts grayish to slightly blackish in fungarium material. Hyphae formed by long straight septate cells, unclamped, not abundant, and branched commonly in "T" shape forming an angle of about 90°, 3–4(4.5) µm diam. Cystidia absent. Basidia clavate, apically globose, (12–)14–17 × (5–)8–10(–12) µm, sometimes presenting an apical extension of the growing hyphae. Sterigmata four per basidium, obclavate and basally globose when young, mainly fusiform to lageniform

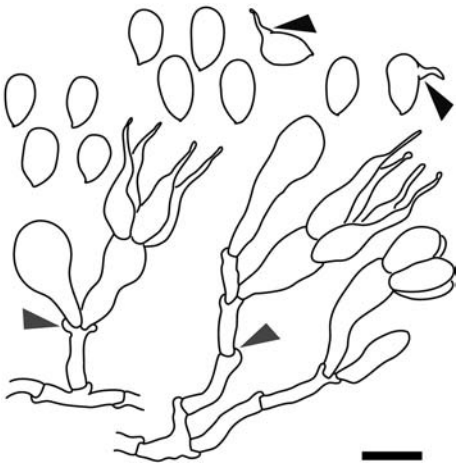

Fig. 17. *Tulasnella griseorubella*. Hyphae with clamps (indicated by the gray arrowheads), basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). BR 6613170. Scale bar = 10 μm.

with tapering apex when mature, (15–)16–23 × 7–8 μm. Basidiospores spiral, slightly curved to the apiculus and more straight at the central part, (16–)20–26 × (2.5–)3 μm, Q-values (5.3-)8-8.7, hyaline, smooth, and germinating by repetition.

Specimens examined: EUROPE: **Germany**, Bayern, Allgäu, Pfeiffermühle at Oberjoch, 01 Oct. 1996, R.Kirschner 74. **United Kingdom**, England, South Devon, on fallen branch of *Fraxinus excelsior*, 25 Nov. 1990, P.Roberts 113 (K 18544).

Tulasnella helicospora is similar to *T. interrogans* and *T. anguifera* by the spiral basidiospores. However, basidiospores of *T. helicospora* are slightly curved to the apiculus, more straight at the central part, and measure (16–)20–26 × (2.5–)3 μm. In contrast, basidiospores of *T. interrogans* are similar to question-marks, shorter and thinner, measuring (13–)15–17(–19) × 1.5–2 μm. *T. anguifera* has anguiform basidiospores resembling worms or snakes, measuring (12–)16–20 × 3–4 μm. In addition, hyphae of *T. helicospora* lack clamp connections. Due to a lack of molecular data, this species concept has not yet been confirmed by phylogenetic analyses.

Tulasnella hyalina Höhn. & Litsch., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 117: 1114 (1908) Wiesner Festschrift (Wien): 59 (1907); *Gloeotulasnella hyalina* (Höhn. & Litsch.) Juel, Arkiv Bot, 14: 8 (1914); *Gloeotulasnella metachroa* Bourdot & Galzin, Bull. Soc. mycol. Fr. 39: 265 (1924). (Synonyms according to Roberts 1994b). Fig. 19

Basidiomata inconspicuous, effused, slightly pruinose, adnate to the substrate and grayish to blackish in dry material. Hyphae tortuous, with intermediate swollen parts generating few lateral branches, hyphae septate and unclamped, (2–)3–5(–6) μm diam.

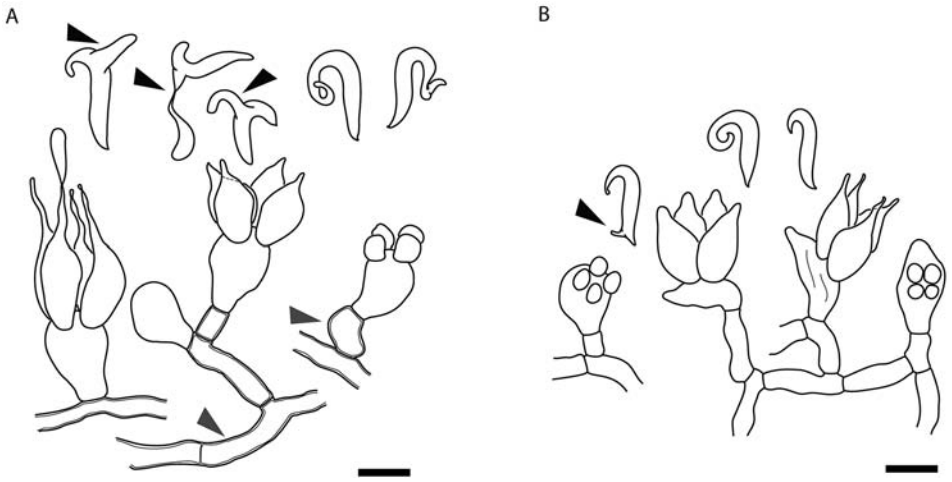

Fig. 18. *Tulasnella helicospora.* (A–B) Hyphae without clamps, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). The gray arrowheads indicate thick-walled hyphae. (A) K 18544. (B) R. Kirschner 74. Scale bars = 10 μm.

Gloeocystidia obclavate, some times fusiform or irregularly cylindrical with swollen parts, attenuate toward apex, (22–)32–45(–50) × (6–)7–10(–12) μm, usually growing together with basidia. Basidia clavate to sphaeropedunculate, sometimes solitary or forming two or three clusters, (12–)15–20 × (6–)7–9(–10) μm. Sterigmata four per basidium, ovoid to obpyriform when young, and obclavate, tapered and extended to the apex when mature, (12–)15–30(–35) × (5–)6–7 μm. Basidiospores mainly globose, sometimes subglobose to elliptical, (5–)6–8(–9) × (5–)6–8 μm, Q-values 1–1.3(–1.6), smooth and germinating by repetition.

Specimens examined: EUROPE: **Belgium**, Zoersel, on *Populus x canadensis*, 10 July 1997, Van de Put K. 93071009 (BR 6613780); Antwerpen; Linker-Oever, St. Annabos, 17 July 1993, Van de Put K. 93071701 (BR 6613982); same site, 17 July 1993, Van de Put K. 93071703 (BR 6614083); Walem, 25 Jan. 1994, Van de Put K. 94012502 (BR 6614588); same site, 20 Dec. 1994, Van de Put K. 94122001 (BR 6615602); same site, on *Pinus sylvestris*, 28 Mar. 1995, Van de Put K. 95032801 (BR 6617117); same site, 18 June 1995, Van de Put K. 95061801 (BR 6617824); same site, on *Pinus sylvestris*, 18 June 1995, Van de Put K. 95061803 (BR 6618026); Zoersel, on *Pinus sylvestris*, 17 Sept. 1995, Van de Put K. 95091709 (BR 6618127); same site, on *Salix* sp., 17 Sept. 1995, Van de Put K. 95091710 (BR 6618228); Walem, on *Pinus sylvestris*, 11 Jan. 1996, Van de Put K. 96011102 (BR 6618733). **United Kingdom**, England, South Devon, on fallen wood of *Picea* sp., and mixed with *Botryobasidium* sp. and *Tulasnella* sp., 17 Nov. 1991, P.Roberts 326 (K 26137).

Tulasnella hyalina is rather similar to *T. cystidiophora* by gloeocystidia and globose to subglobose basidiospores. However, the hyphae in *T. cystidiophora* are unclamped. *Tulasnella hyalina* is also similar to *T. subglobospora* and *T. violea* by the globose, subglobose, or elliptical basidiospores. However, *T. subglobospora* has clamp connections and lacks gloeocystidia, while *T. violea* lacks clamp connections and

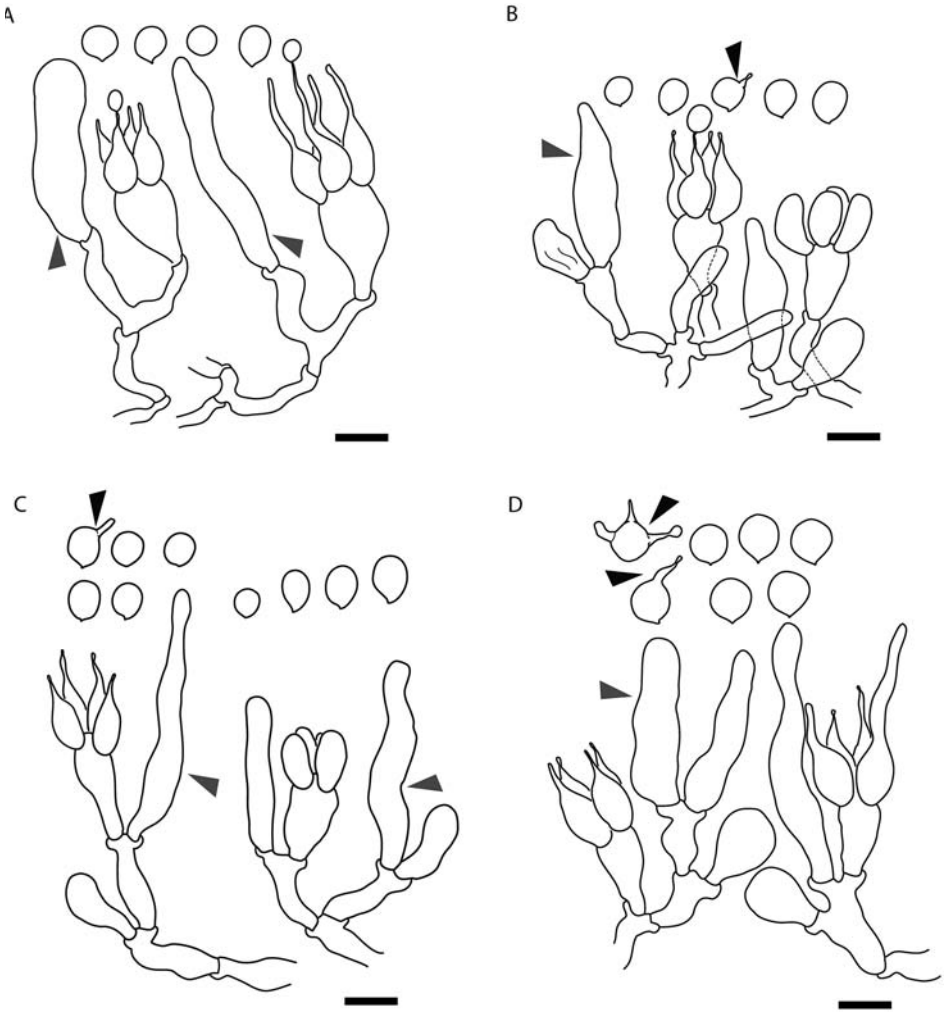

Fig. 19. *Tulasnella hyalina.* (A–D) Hyphae with clamps, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). The gray arrowheads indicate the gloeocystidia. (A) BR 6613780. (B) K 26137. (C) BR 6617117. (D) BR 6617824. Scale bars = 10 μm.

gloeocystidia. Molecular data from this species are lacking, consequentially the species concept of *T. hyalina* has not yet been confirmed by phylogenetic analyses.

Tulasnella interrogans P.Roberts, Mycol. Res. 96(3): 234 (1992). Fig. 20

BASIDIOMATA resupinate, inconspicuous but refracting the light in fungarium material. HYPHAE tortuous formed by long cells, slightly branching in "T" shape, hyphae septate

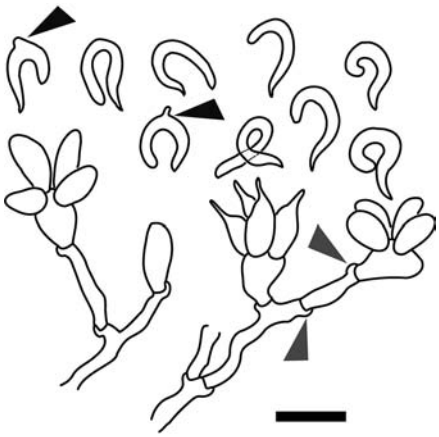

Fig. 20. *Tulasnella interrogans*. Hyphae with clamps (indicated by the gray arrowheads), basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). BR 6613679. Scale bar = 10 μm.

and unclamped, 2 μm diam. Cystidia absent. Basidia clavate to sphaeropedunculate, mostly solitary, 8–10 × 5–6 μm. Sterigmata four per basidium, oblong to fusiform when immature, and subfusiform to pyriform when mature, 10–11 × 5 μm. Basidiospores spiral, mainly with shape of question mark, (13–)15–17(–19) × 1.5–2 μm, Q-values 7.5–9(–12), smooth and germinating by repetition.

Specimen examined: EUROPE: **Belgium**, Zoersel, 26 June 1993, Van de Put K. 93062601 (BR 6613679).

Tulasnella interrogans can be easily differentiated from other species of *Tulasnella* with spiral basidiospores. *T. interrogans* lacks gloeocystidia while *T. falcifera* has elongated and undulate gloeocystidia. Furthermore *T. interrogans* has spiral basidiospores mainly in shape of question marks [(13–)15–17(–19) × 1.5–2 μm] while *T. anguifera* has mainly anguiform basidiospores resembling worms or snakes [(12–)16–20 × 3–4 μm]. Sequence data are not yet available for this species.

Tulasnella kirschneri D.Cruz, J.P.Suárez, & M.Piepenbr., **sp. nov.** Fig. 21

MycoBank: MB 814019

Etymology: This new species is dedicated to Roland Kirschner who contributed the type specimen as well as numerous further valuable specimens of *Tulasnella*.

Basidiomata resupinate, effused, abundant, spongy, grayish in dry material. Hyphae cylindrical, rarely branched forming an angle of up to ca. 90°, composed of short swollen cells without clamps, (4.5–)6–5 μm diam. The hyphae are thin- or thick-walled. Cystidia absent. Basidia clavate to sphaeropedunculate, apically globose, (12–)15–18 × 10–11 μm. Sterigmata four per basidium, obclavate when young, and mostly fusiform tapered to both apices when mature, 15–20(–23) × 6–7 μm. Basidiospores mainly

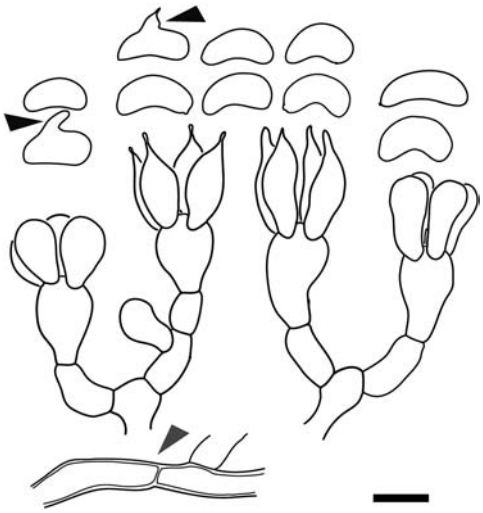

Fig. 21. *Tulasnella kirschneri*. Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). The gray arrowheads indicate thick-walled hyphae. R.Kirschner 923. Scale bar = 10 μm.

allantoid, sometimes subcylindrical, slightly tapered to the apiculus, (11–)12–15 × 5–6 μm, Q-values (2–)2.2–2.6(–3), hyaline, smooth, thin-walled and germinating by repetition.

HOLOTYPE: CHINA: Yunnan, Jian Chuan Shan, 28 July 2001, R.Kirschner 923 (KUN-HKAS).

Tulasnella kirschneri collected in China by R.Kirschner is morphologically related to *Tulasnella fuscoviolacea, T. pallida, T. subglobospora*, and *T. violea* according to similar hyphae, basidia, sterigmata, and hymenia. However, the basidiospores of this species of *Tulasnella* form China are mainly allantoid, sometimes subcylindrical [(11–)12–15 × 5–6 μm], while *T. fuscoviolacea* has basidiospores that are mostly subcylindrical and rarely oblong or allantoid [(11–)12–16(–19) × (3–)4–5(–6) μm]. *Tulasnella kirschneri* with allantoid basidiospores is distinct from *T. pallida* with fusiform basidiospores. *T. subglobospora* and *T. violea* are characterized by globose basidiospores, *T. subglobospora* has clamped hyphae.

Tulasnella kirschneri resembles *T. danica* or *T. saveloides* by the allantoid basidiospores and unclamped hyphae. However, in *Tulasnella kirschneri* all structures and microdimensions of hyphae, basidia, and sterigmata are larger than in *T. danica* or *T. saveloides*. In addition, *Tulasnella kirschneri* has wider basidiospores [(11–)12–15 × 5–6 μm] contrary to the narrower basidiospores in *T. danica* [(10–)12–16 × 3–4 μm] and in *T. saveloides* [(7–)8–9 × 3–3.5 μm]. *Tulasnella kirschneri* is proposed as a new species to science because it morphologically differs from all other known species. Inspite of several attemps, it was not possible to obtain sequence data from the type material, so the phylogenetic placement cannot be discussed.

Tulasnella pallida Bres., Annls mycol. 1(2): 122 (1903); *Tulasnella albolilacea* Bourdot & Galzin, Bull. Soc. mycol. Fr. 39: 264 (1924) [1923]; *Tulasnella violacea* (Johan-Olsen) Juel, Bihang till K.Svenska Vet.-Akad. Handl. 23, Afd III, 12: 22 (1897) [nom. *dub.; sensu auct. pro parte*]. (Synonyms according to Roberts 1994b and 2003). Fig. 22

BASIDIOMATA resupinate, effused and crusty, violaceous grey when dry. HYPHAE swollen and slightly tortuous, septate and unclamped, (3–)3.5–5(–6) µm diam. CYSTIDIA absent. BASIDIA globose to subglobose when young, and mostly clavate to sphaerandoculate when mature, (10–)15–22 × 5–7(–8) µm. STERIGMATA four per basidium, mainly obclavate, fusiform to elliptical, basally globose, tapered and extended to the apex, (14–)16–23(–25) × (5–)6–9(–10) µm, some specimens with bifurcate sterigmata. BASIDIOSPORES commonly fusiform to subfusiform, some occasionally oblong and elliptical, 8–12(–15) × (4–)5–6(–7) µm, Q-values (1.4–)1.8–2.5(–3), hyaline, smooth, germinating by repetition.

SPECIMENS EXAMINED: EUROPE: **Belgium**, Walem, 14 Dec. 1994, Van de Put K. 94121401 as *Tulasnella pinicola* (BR 6615501), same site, on *Pinus sylvestris*, 17 Jan. 1995, Van de Put K. 95011701 (BR 6615905); same site, on *Pinus sylvestris*, 30 Nov. 1995, Van de Put K. 95113001 (BR 6618632); same site, on *Pinus sylvestris*, 18 Jan. 1996, Van de Put K. 96011801 (BR 6618935); same site, on *Pinus sylvestris*, 05 Mar. 1996, Van de Put K. 96030502 (BR 6619743). **Germany**, Bayern, Schwaben, around Bergheim, southwest to Augsburg, ± 530 m, 23 Mar. 1968, B.Mayr & F.Oberwinkler 11999; same site, Bannacker at Bergheim, southwest Augsburg, ± 520 m, 23 Mar. 1970, B. & F.Oberwinkler 16207; Baden-Württemberg, Tübingen, Morgenstelle, 14 Feb. 1997, R.Kirschner 182 as *Tulasnella violaceae*; same site, 16 Feb. 1997, R.Kirschner 181 as *Tulasnella violaceae*; same site, 02 March 2000, R.Kirschner 621; **Spain**, Balearic Islands, Majorca, forest of Monnaber, on fallen wood, 13 Nov. 1992, P.Roberts 585 (K 26146).

Tulasnella pallida has been discussed and considered a synonym of *T. violacea* by Roberts (1994b, 2003). The latter species is considered a *nomen dubium* lacking an adequate description or type specimen (Roberts 1994b, 2003). Roberts (2003) thereby contradicts Van de Put (1996) who accepts *T. pallida* and *T. violacea* as separate species, based on the sizes and slight differences in the shape of the basidiospores.

Our revision of specimens named as *T. pallida* or "*T. violacea*" by Van de Put (1996) evidently show high variability in shapes and measurements of basidiospores confirming the observations made by Roberts (1994b, 2003). However, four of the specimens (BR 6615501; 6615905; 6618632 and 6618935) have rare bifurcated sterigmata and small elliptical and fusiform basidiospores [8–10(–12) × 5–6(–6.5) µm] different to other specimens without bifurcated sterigmata and larger fusiform basidiospores [10–12(–15) × (4–)5–6(–7) µm] (Fig. 22). Nevertheless, the presence of bifurcated sterigmata and the shapes or sizes of basidisopores are not constant within and among the specimens. Further specimens studied in the context of the present investigation can be identified as *T. pallida*. The specimens revised here are named *T. pallida* with *T. violacea* mentioned as a synonym according to Roberts (2003).

As molecular data from type specimens are not available, the species concepts for these species cannot be confirmed phylogenetic analyses.

Tulasnella pinicola Bres., Annls mycol. 1(2): 114 (1903); *Gloeotulasnella pinicola* (Bres.) D.P.Rogers, Annls mycol. 31(3): 199 (1933); *Tulasnella tremelloides* Wakef. &

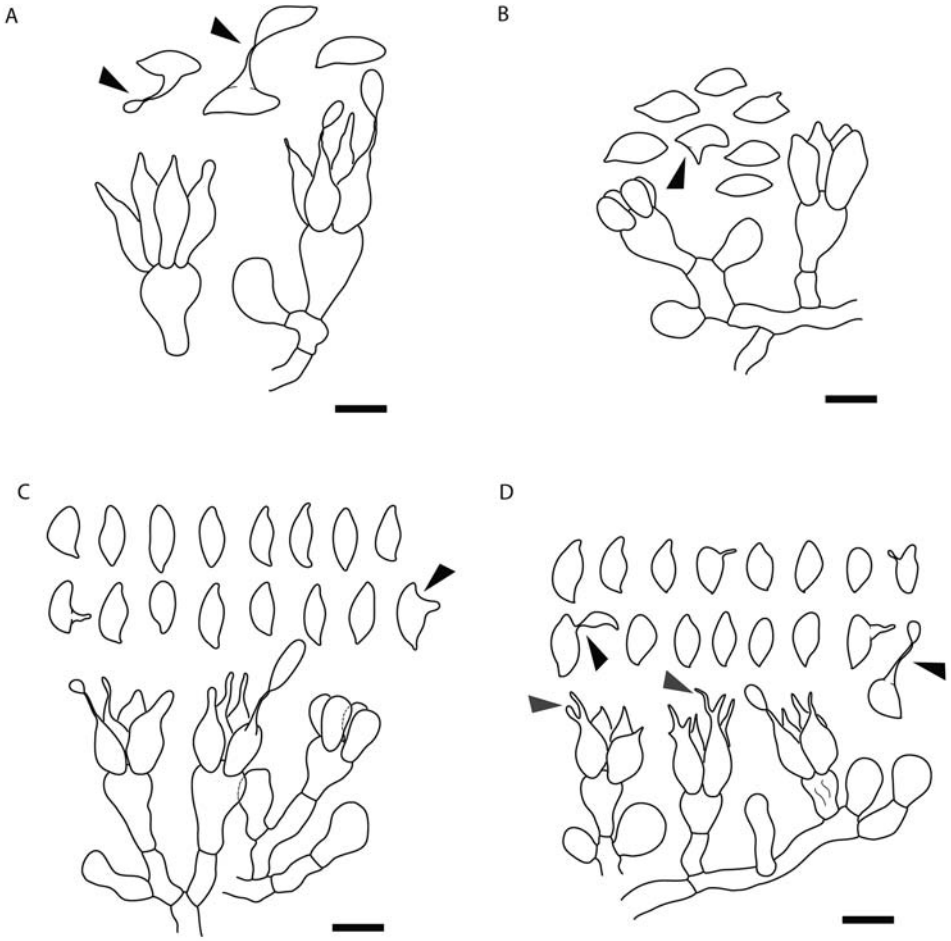

Fig. 22. *Tulasnella pallida.* (A–D) Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). The gray arrowheads indicate bifurcate sterigmata. (A) FO 11999. (B) K 26146. (C) BR 6619743. (D) BR 6615905. Scale bars = 10 μm.

A.Pearson, Trans. Br. mycol. Soc. 6: 70 (1917); *Gloeotulasnella tremelloides* (Wakef. & A.Pearson) D.P.Rogers, Annls mycol. 31: 201 (1933); *Tulasnella sordida* Bourdot & Galzin, Bull. Soc. mycol. Fr. 39: 265 (1923); *Gloeotulasnella sordida* (Bourdot & Galzin) M.P.Christ., Dansk bot. Ark. 19: 43 (1959). (Synonyms according to Roberts 1994b). Fig. 23

Basidiomata resupinate, effused, waxy to slightly gelatinous and pinkish when fresh, cracked and violaceous grey in dry material. Hyphae usually straight, sometimes swollen, forming long septate and unclamped cells, (2.5–)3–4 μm diam. Cystidia absent.

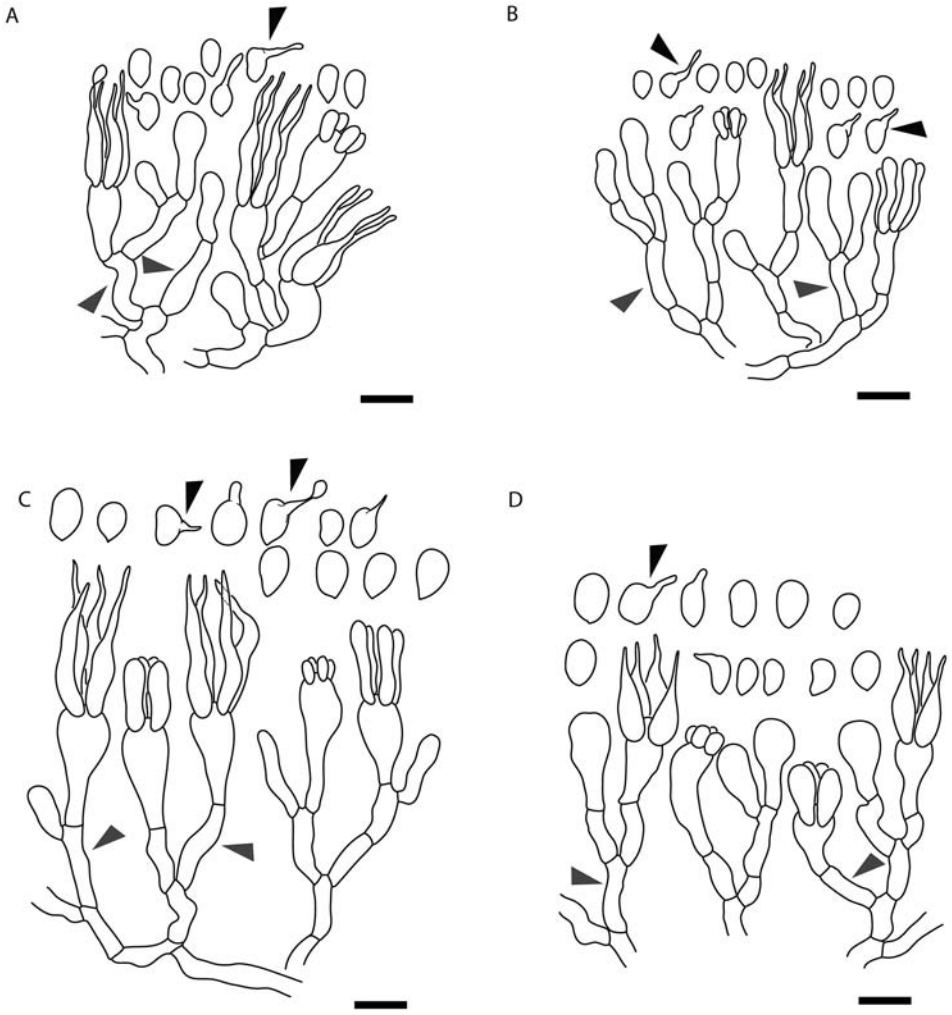

Fig. 23. *Tulasnella pinicola.* (A–D) Hyphae without clamps, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). The gray arrowheads indicate long basal cells. (A) FO 17289. (B) D. Cruz 309. (C) BR 6618531. (D) BR 6610851. Scale bars = 10 μm.

Basidia mostly sphaeropedunculate, forming a long stem at the base, (9–)14–23(–27) × (5–)7–9(–10) μm, usually solitary generated from a long basal hypha. Sterigmata four per basidium, obclavate when young, and cylindrical to subfusiform, longer and tapered to the apex when mature, (13–)15–33(–37) × 3–5(–6) μm. Basidiospores mainly oblong to elliptical, rarely pip-shaped or subcylindrical, (4–)7–9 × 4–6(–6.5) μm, Q-values (1.1–)1.3–1.8(–2.5), hyaline, smooth, germinating by repetition.

Specimens examined: EUROPE: **Belgium**, Zoersel, 18 Mar. 1989, Van de Put K. 89031802 (BR 6610851); Walem, on *Pinus sylvestris*, 28 Nov. 1995, Van de Put K. 95112801 (BR 6618531). **Germany**, Baden-Württemberg, Tübingen, Schönblick, 02 Mar. 1997, R.Kirschner 195; Bayer-Schwaben, north forest Reinhartshausen, southwest of Augsburg, 570–580 m, 26 Oct. 1970, F.Oberwinkler 17289; Baden-Württemberg, Schönbuch between Hagelloch (Bogentor) and Hohenentringen, 480 m, 06 Dec. 1984, F.Oberwinkler 36362; Frankfurt, Apfelbach, Grob-Gerau, on decayed wood, 27 Nov. 2010, 140 m, D.Cruz 309.

Tulasnella pinicola is similar to *T. asymmetrica* by the shape of basidiospores. Both species are considered as synonyms by Roberts (1999). However, the basidiospores of *T. pinicola* as presented here are mainly oblong to elliptical [(4–)7–9 × 4–6(–6.5) μm], whereas *T. asymmetrica* presents cylindrical and unilaterally flattened, sometimes curved basidiospores [6.5–9 × 3.5–4.5 μm] (Warcup & Talbot 1967). Furthermore, *T. pinicola* has mostly sphaeropedunculate basidia, forming a long stem at the base, measuring (9–)14–23(–27) × (5–)7–9(–10) μm, usually individually generated from a long basal hypha. *T. asymmetrica* has clavate basidia tapering distally into a thin-walled papilla, asymmetric with one side more curved than the other [10–15 × 5–7 μm] (Warcup & Talbot 1967). The teleomorph of *T. asymmetrica* is only available from the illustration in Warcup & Talbot (1967), so there is no type specimen for revision. Based on all information available we consider *T. pinicola* as different from *T. asymmetrica*. Cruz et al. (2014) assigned one clade to *Tulasnella* cf. *pinicola* by molecular phylogeny. The clade for *T.* cf. *pinicola* is distinct from clades containing *Tulasnella asymmetrica* especially from the clade that contains sequences obtained from the type culture [085 Warcup & Talbot (MAFF 305806)] (Cruz et al. 2014, Warcup & Talbot 1967).

The identification and phylogenetic position of *T.* cf. *pinicola* has been proposed by Cruz et al. (2014), however as there are no molecular data from the type specimen, this identification has not yet been confirmed.

Tulasnella pruinosa Bourdot & Galzin, Bull. Soc. Mycol. Fr. 39: 264 (1924) [1923]; *Tulasnella araneosa* Bourdot & Galzin, Bull. Soc. mycol. Fr. 39: 265 (1924) [1923]. (Synonym according to Roberts 1994b). Fig. 24

Basidiomata resupinate, effused, pruinose, grayish refracting the light when dry. Hyphae branched by "T" shape, forming angle of about 90°, hyaline, and rarely generating long septate and unclamped cells, (2–)2.5–3(–4) μm diam. Cystidia absent. Basidia subglobose to globose when immature, somewhat clavate when mature, (7–)9–12(–13) × (4–)5–6(–7) μm. Sterigmata four per basidium, globose to elliptical when young, and clavate, fusiform, sometimes pyriform or mitriform, slightly tapering to the apex when mature, (8–)9–12(–20) × (3–)4–5 μm. Basidiospores elliptical to oblong, often tapering towards the apiculus, occasionally phaseoliform or ventrally depressed, rarely subglobose, (3.5–)5–7(–7.5) × (2.5–)3–4(–5) μm, Q-values (1.4–)1.6–2.5(–2.8), hyaline, smooth, germinating by repetition.

Specimens examined: EUROPE: **Belgium**, Houwaart, Walembos, 03 Sept. 1993, Van de Put K. 93090304 (BR 6614184); Zoersel, on *Quercus robur*, 22 Jan. 1994, Van de Put K. 94012208 (BR 6614487); same site, on *Quercus robur*, 26 Feb. 1994, Van de Put K. 94022604 (BR 6614891); same site, on *Salix* sp., 08 Mar. 1996, Van de Put K. 96030811 (BR 6620046). **Germany**, Baden-Württemberg, Schönbuch, between Hagelloch and Hohenentringen, "Bruderhaus", ± 500 m, on bark of *Picea abies*, 17 Feb. 1976, B. and F. Oberwinkler 23473; Württemberg, Ostalbkreis Welzheimer forest at Durlangen-Tanau, 400 m, 30 Mar. 1976, F.Oberwinkler 23517. **United Kingdom**, England,

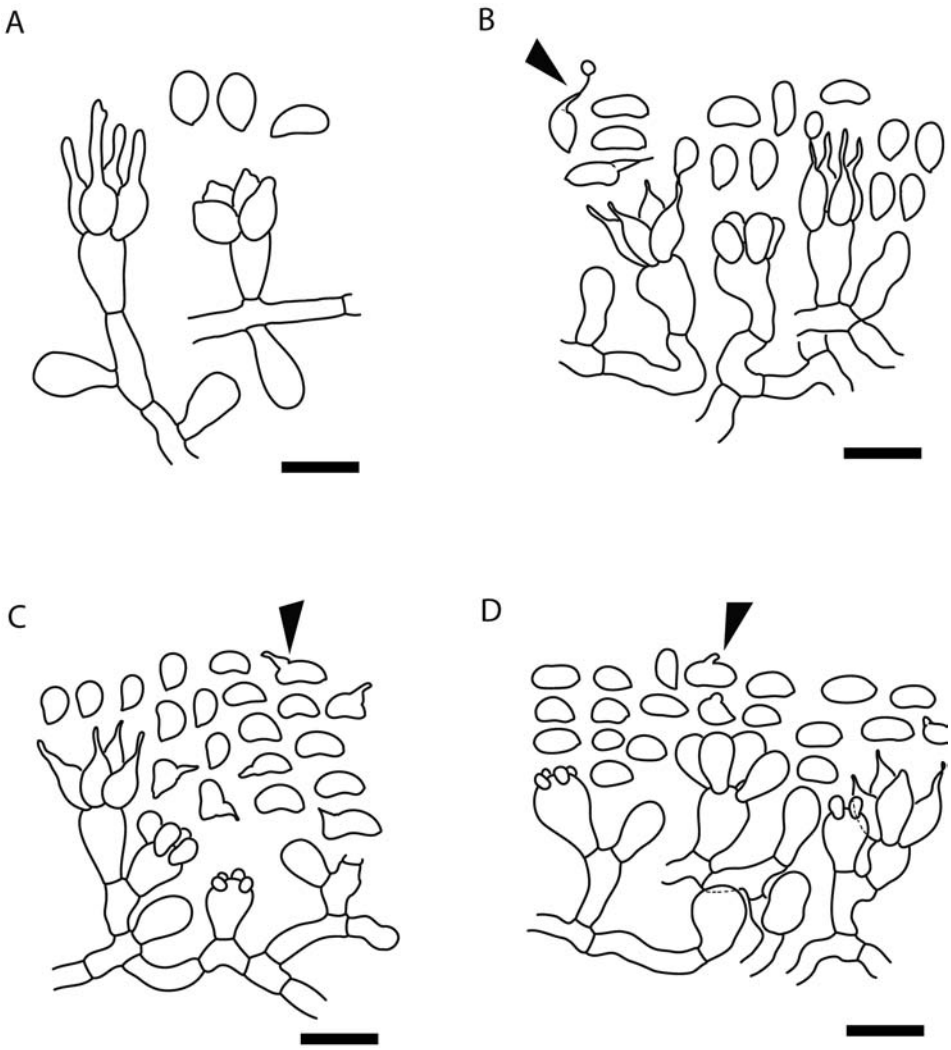

Fig. 24. *Tulasnella pruinosa.* (A–D) Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). (A) K 26130. (B) BR 66214891. (C) BR 6614184. (D) BR 6614487. Scale bars = 10 μm.

South Devon, Newton Abbot, Ipplepen, Orley Common, on *Quercus* sp., 30 Dec. 1990, P.Roberts 121 (K 26130).

Tulasnella pruinosa resembles *T. albida* and *T. pinicola* by elliptical and oblong basidiospores (Dueñas 2001, Jülich & Jülich 1976, Roberts 1994b, Van de Put 1996). The specimens of *T. pruinosa* show an extreme variability in size and shape of basidiospores, which can be occasionally phaseoliform, ventrally depressed, or rarely

subglobose [(3.5–)5–7(–7.5) × (2.5–)3–4(–5) µm; Q-values (1.4–)1.6–2.5(–2.8)], rendering a proper identification difficult. Roberts (1994b) considers this species to be uncertain and ill-defined or probably a deceptive form of *T. pinicola*.

Tulasnella pruinosa is distinct from *T. albida* as basidia are not growing in clusters together with young basidia. *T. pruinosa* produces shorter basidia [(7–)9–12(–13) × (4–)5–6(–7) µm] than *T. pinicola* [16–23(–27) × 7–9 µm]. *Tulasnella pruinosa*, *T. pinicola*, and *T. albida* are phylogenetically closely related (Group III, Fig. 2) according to molecular data of the ITS region available in GenBank, NCBI (Cruz et al. 2014). This phylogenetic relationship of these species is close to predictions made by Roberts (1994b), who suggested a morphological group of several species (i.e., *T. albida*, *T. pinicola*, *T. pruinosa*, and others) with a similar hymenial system that he described as "conventionally branching" (i.e., hymenium highly branched, short-celled, basidia-bearing hyphae arising from straighter, longer-celled, often thick-walled, basal hyphae running parallel to the substrate). *Tulasnella pruinosa* differs from *T. andina* as discussed above.

At least eleven sequences from the strain DAOM 17641 of *Tulasnella pruinosa* are available in the GenBank NCBI.

Tulasnella rubropallens Bourdot & Galzin, Bull. Soc. mycol. Fr. 39: 264 (1924) [1923]. Fig. 25

Basidiomata resupinate, effused, grayish to violaceous grey, inconspicuous in dried material. Hyphae slightly undulate, sometimes swollen forming short, unclamped cells, usually branched forming an angle of about. 90° with "T" shape, hyaline, hyphal diameter (2–)2.5–3.5(–4) µm. Cystidia absent. Basidia mostly clavate, apically globose to oblong in different specimens, sometimes sphaeropedunculate, (8–)9–13(–14) × (5–)6–7 µm. Sterigmata four per basidium, pyriform to fusiform, tapered and extended to the apex in mature state, 10–17(–20) × 4–5 µm. Basidiospores mostly subcylindrical, tapered and slightly curved at the apiculus, rarely oblong, allantoid, or phaseoliform, (5–)6–9(–10) × 2.5–3(–4) µm, Q-values (1.4–)2.3–3.3(–4), hyaline, smooth, producing secondary basidiospores by repetition.

Specimens examined: EUROPE: **Belgium**, Zoersel, on *Alnus glutinosa*, 27 Apr. 1991, Van de Put K. 91042715 (BR 6612063); same site, on *Salix* sp., 05 Mar. 1994, Van de Put K. 94030512 (BR 6614992); same site, no data, 01 Oct. 1994, Van de Put K. 94100107 (BR 6615194); same site, on *Salix* sp., 13 Apr. 1996, Van de Put K. 96041301 (BR 6620955); same site, on *Salix* sp., 13 Apr. 1996, Van de Put K. 96041303 (BR 6620854); same site, on *Betula* sp., 13 Apr. 1996, Van de Put K. 96041306 (BR 6620753); same site, on *Salix* sp., 13 Apr. 1996, Van de Put K. 96041309 (BR 6620551); same site, on *Salix* sp., 13 Apr. 1996, Van de Put K. 96041310 (BR 6620450); same site, on *Salix repens*, 10 July 1997, Van de Put K. 93071014 (BR 6613881).

Tulasnella rubropallens can be related to *T. andina* and *T. pruinosa* because of similar hyphae, basidia, and sterigmata arranged in the hymenium in a similar way. However, *T. rubropallens* has longer basidiospores, which are mostly subcylindrical, tapered and slightly curved to the apiculus, and rarely oblong, allantoid, or phaseoliform. *T. andina* has mostly shorter basidiospores that are phaseoliform to subcylindrical [(5–)6–8 × (2–)3–4(–5) µm, Q-values (1.5–)1.7–2.3(–2.7)]. The shape of the basidiospores in *T. rubropallens* appears to be more uniform than in *T. pruinosa*. The

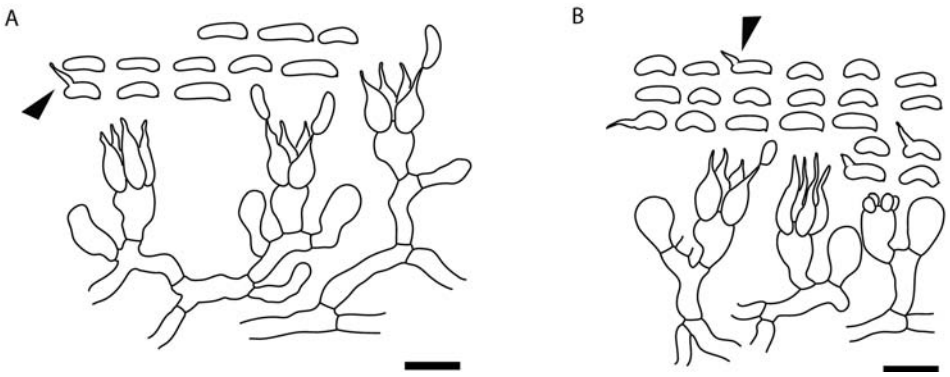

Fig. 25. *Tulasnella rubropallens.* (A–B) Hyphae without clamps, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). (A) BR 6620551. (B) BR 6612063. Scale bars = 10 μm.

latter species has basidiospores that are mainly elliptical to oblong, often tapering towards the apiculus, occasionally phaseoliform or ventrally depressed, rarely subglobose [(3.5–)5–7(–7.5) × (2.5–)3–4(–5) μm, Q-values (1.4–)1.6–2.5(–2.8)]. Bourdot and Galzin (1924) described *Tulasnella rubropallens* with scattered clamps and cylindrical and curved basidiospores similar to *T. allantospora* suggesting that both species are synonyms. Roberts (1994a) described *T. rubropallens* from the lectotype with totally unclamped hyphae. This data support the information presented in the revision of this species by Jülich (1984).

All the specimens revised here and named as *Tulasnella rubropallens* by Van de Put (1996) clearly represent a species different to *T. allantospora*. The latter species is characterized by always clamped hyphae and mainly allantoid basidiospores [(6–)7–10(–11) × 2.5–3.5(–4) μm, Q-values (2.3–)2.4–3.3(–4)]. This species concept is not yet confirmed by molecular phylogenetic analyses. Molecular data from type material are not available in the sequences databases.

Tulasnella saveloides P.Roberts, Mycol. Res. 97(2): 217 (1993). Fig. 26

Basidiomata resupinate, effused, inconspicuous in dry material. Hyphae slightly tortuous formed by short cells, septate without clamps, usually branched in "T" shape with angles of up to. 90°, hyaline, thick-walled, diameter 3–4 μm. Cystidia not seen. Basidia mostly clavate, sometimes apically globose, 10–15 × 7 μm, usually new basidia can be generated directly from the older basidia. The old and degraded basidia are present together with the young basidia. Sterigmata four per basidium, pyriform, globose at the basal part, tapered and extended to the apex in mature state, 14–23 × 6–5 μm. Conidia not seen. Basidiospores mostly allantoid, (7–)8–9 × 3–3.5 μm, Q-values (2.3)2.6–2.7(–3), hyaline, smooth, producing secondary basidiospores by repetition.

Specimen examined: EUROPE: **United Kingdom**, England, on fallen branch, 27 Dec. 1990, P.Roberts 114 (Paratype, K 21326).

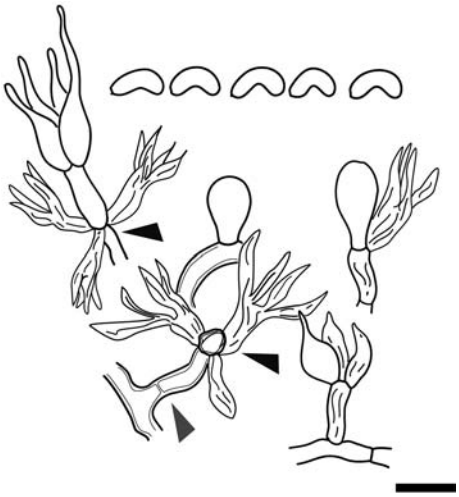

Fig. 26. *Tulasnella saveloides*. Hyphae, basidia, sterigmata in different developmental stages, and basidiospores. The black arrowheads show the older basidia generating young basidia. The arrowheads gray indicate thick-walled hyphae. K 21326. Scale bar = 10 μm.

The shape and measurements of the basidiospores of *Tulasnella saveloides* suggest that this species is distinct to *T. rubropallens* as defined by Van de Put (1996) and confirmed here. In addition, this specimen forms new basidia directly from older basidia, which are present together. However, Roberts (1993) mentioned that this characteristic can also be observed in other species of *Tulasnella*. In *Tulasnella saveloides* two additional but inconstant features are present, coffin-shaped conidia or conidia-like cells and gloeocystidia-like cells (Roberts 1993). *T. saveloides* differs from *T. allantospora* by these two characteristics and unclamped hyphae. *T. allantospora* has similar allantoid basidiospores but completely clamped hyphae. This species concept has not yet been confirmed by molecular phylogenetic analyses. Molecular data from specimens are not available in the GenBank databases.

Tulasnella subglobospora Hjortstam, Windahlia 12–13: 21 (1983) [1982]. Fig. 27

Basidiomata resupinate, effused, violaceous grey when dry. Hyphae tortuous, cylindrical and sometimes swollen, forming short cells, all septa with clamps, (2.5–)3–5(–6) μm diam. Cystidia absent. Basidia mostly clavate to subglobose, rarely sphaeropedunculate, (11–)14–20(–22) × (7–)9–10(–11) μm, usually solitary or sometimes forming groups of two basidia. Sterigmata ellipsoidal, sometimes fusiform, often swollen at central part and tapered to extended at the apex, (15–)18–23(–32) × 7–8 μm. Basidiospores globose to subglobose, rarely elliptical, (5–)7–9(–12) × 6–8 μm, Q-values 1–1.3(–1.4), hyaline, smooth, germinating by repetition.

Specimens examined: EUROPE: **Belgium**, Walem, on *Pinus sylvestris*, 22 Apr. 1995, Van de Put K. 95042204 (BR 6617319); **Germany**, Bavaria, Allgäu, Oberjoch-Hindelang, Wildbachgraben, 27 Sept.

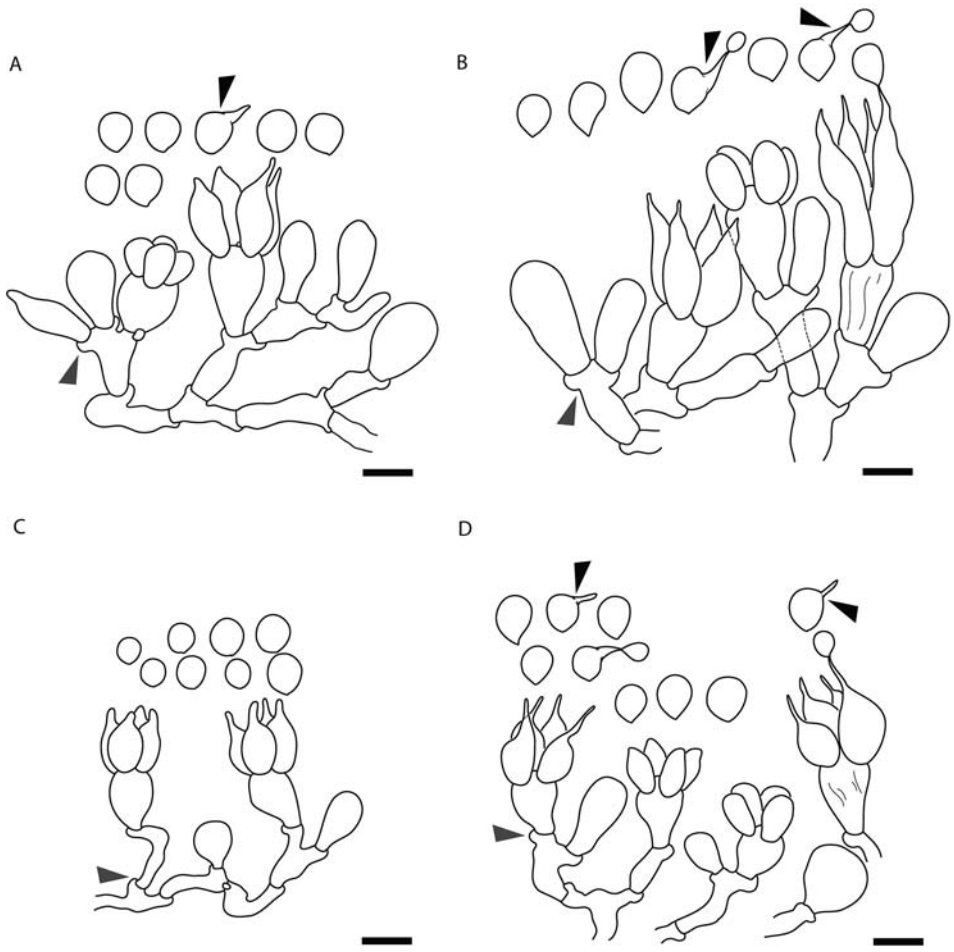

Fig. 27. *Tulasnella subglobospora.* (A–D) Hyphae with clamps (indicated by the gray arrowheads), basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). (A) K 26148. (B) FO 34704. (C) BR 6617319. (D) R.Kirschner 720. Scale bars = 10 μm.

1983, F.Oberwinkler 34704; Bavaria, Allgäu, Oberstdorf, close to Rohrmoos at Tiefenbach, 28 Sept. 2000, R.Kirschner 720; **Norway**, Oppland, on fallen wood, 14 Sept. 1993, P.Roberts 664 (K 26148).

Tulasnella subglobospora is morphologically similar to many other species of *Tulasnella* (i.e., *T. bourdotii*, *T. cystidiophora*, *T. eichleriana*, *T. hyalina*, *T. violea*) with globose to subglobose basidiospores. However, hyphae of *T. subglobospora* are completely clamped contrary to *T. eichleriana* or *T. violea* with hyphae lacking clamps. In addition, *T. subglobospora* lacks gloeocystidia unlike *T. cystidiophora* and *T. hyalina* that have long gloeocystidia.

Tulasnella subglobospora appears to be similar to *T. bourdotii* Jülich. Both species have clamps and globose to subglobose basidiospores. However, in *T. subglobospora* all microstructures are larger than in *T. bourdotii*. *T. subglobospora* has thick hyphae [(2.5–)3–5(–6) µm diam.] and basidiospores [(5–)7–9(–12) × 6–8 µm], while *T. bourdotii* has thin hyphae [1.5–2.5 µm] and basidiospores [4–6 × 3.5–5.5 µm] (Roberts 1994b). Molecular data from the type specimen are lacking, consequentially the species concept of *T. subglobospora* has not yet been confirmed by phylogenetic analyses.

Tulasnella thelephorea (Juel) Juel, Ark. Bot. 14: 8 (1914); *Gloeotulasnella inclusa* M.P.Christ., Dansk Bot. Arkiv 19: 41 (1959); *Tulasnella cremea* Jülich, Int. J. Mycol. Lichenol. 1(1): 122 (1982); *Tulasnella inclusa* (M.P.Christ.) Donk, Persoonia 4: 263 (1966). (Synonyms according to Roberts 1994b). Basionym: *Muciporus corticola* f. *thelephorea* Juel, Bih. K. Svenska Vetensk Akad. Handl., Afd. 3 23(no. 10): 23 (1897). Fig. 28

Basidiomata resupinate, effused, crusty and cracked, grey to darkish in dry material. Hyphae tortuous, with intermediate swollen parts, forming long septate cells, completely clamped, with few lateral branches, hyphae 2–4(–5) µm diam. Cystidia absent. Basidia mostly sphaeropedunculate, forming a long stem at the base, rarely globose or clavate, (10–)14–20(–21) × (6–)7–9(–10) µm, mostly solitary, rarely in clusters. Sterigmata four per basidium, clavate to obclavate when young, cylindrical to subfusiform, longer and tapering to the apex when mature, (13–)16–28(–30) × (4–)5–7 µm. Basidiospores mainly elliptical to pip-shaped, sometimes oblong, mostly subcylindrical, (6–)7–10 × (4–)5–8 µm, Q-values (1.3–)1.5–2.2(–2.5), hyaline, smooth, germinating by repetition.

Specimens examined: EUROPE: **Belgium**, Zoersel, 31 Mar. 1990, Van de Put K. 90033113 (BR 6611255); same site, 28 Mar. 1992, Van de Put K. 92032808 (BR 6612568); same site, 09 Jan. 1993, Van de Put K. 93010903 (BR 6612972); Walem, 03 Jan. 1995, Van de Put K. 95010301 (BR 6615804); Zoersel, on *Pinus sylvestris*, 17 Feb. 1996, Van de Put K. 96021701 (BR 6619036). **Germany**, Mecklenburg-Vorpommern, Mecklenburgische Schweiz, Neukalener Moorwiesen, 02 Sept. 2000, R.Kirschner 694. **Spain**, Tenerife, Laurel forest, 4–5 km west Erjos, ± 1000 m, 27 Mar. 1973, F.Oberwinkler 20359b.

Tulasnella thelephorea appears to be morphologically similar to *T. griseorubella* due to clamped hyphae, the absence of cystidia, and the similar shape of basidiospores. Following Roberts (1994b) the apparently main difference between both species is the length of the basidiospores. The type of *T. thelephorea* and the type specimens of synonyms presented by Roberts (1994b), have basidiospores with a maximal length of 10 µm. Basidiospores of the type of *T. griseorubella* are longer [11.5–13 × 6–7 µm] (Roberts 1994b sensu Hjortstam 1983).

The shape and measurements of basidiospores of the specimens named as *T. griseorubella* and *T. thelephorea* (revised here) are overlapping. Only small differences in the shape of basidiospores of *T. griseorubella* were found. Due to a lack of molecular data, these species concepts have not yet been confirmed by phylogenetic analyses.

Tulasnella tomaculum P.Roberts, Mycol. Res. 97(2): 215 (1993). Fig. 29

Basidiomata effused resupinate, violaceous grey to grayish when dry. Hyphae slightly undulate, formed by short septate cells, unclamped, often branched in angles of

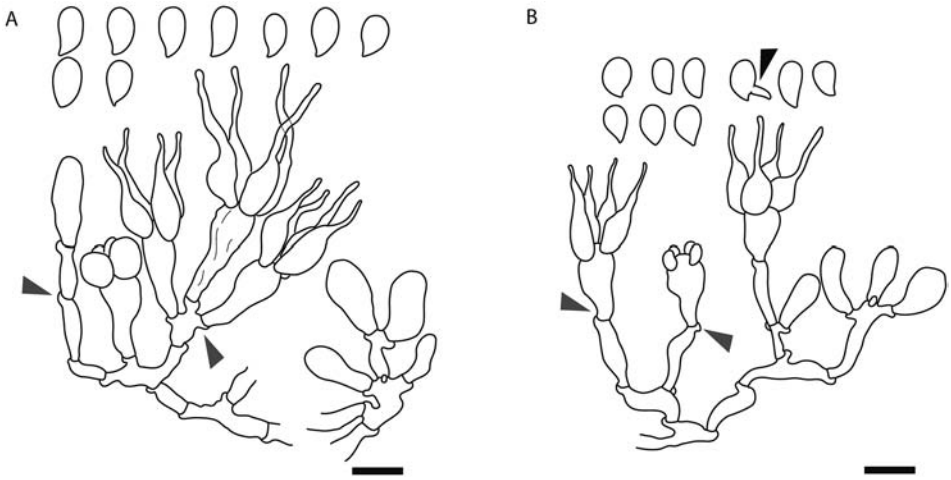

Fig. 28. *Tulasnella thelephorea.* (A–B) Hyphae with clamps indicated by the gray arrowheads, basidia, sterigmata in different developmental stages, and basidiospore partially germinating by repetition (indicated by the black arrowhead). (A) BR 6619036. (B) BR 661580. Scale bars = 10 μm.

ca. 90°, "T" shaped, hyaline, 2–3(–3.5) μm diam. CYSTIDIA absent. BASIDIA mostly clavate to subglobose, sometimes sphaeropedunculate, (7–)8–10(–11) × (4–)5–6(–7) μm. STERIGMATA fusiform, obclavate to globose at the base and finely extended at the apex, (9–)10–11(–13) × 3–4(–4.5) μm. BASIDIOSPORES mostly phaseoliform or "weakly allantoid" in mature state, elliptical when young, (4–)5–6(–8) × 2.5–3.5 μm, Q-values (1.2–)1.5–2(–2.7), hyaline, smooth, producing secondary basidiospores by repetition.

SPECIMENS EXAMINED: EUROPE: **Belgium**, Zoersel, on *Picea abies*, 18 Feb. 1995, Van de Put K. 95021808 (BR 6616410); De Panne, Oosthoek, on hardwood, 18 Feb. 1995, Van de Put K. 95060304 (BR 6617622); Zoersel, on *Alnus glutinosa*, 17 June 1995, Van de Put K. 95061705 (BR 6617723); Sinaai, Hernisse, on hardwood, mixed with *Tulasnella albida*, 23 Sept. 1995, Van de Put K. 95092305 (BR 6618329); Walem, on apple tree, 18 June 1995, Van de Put K. 95061802 (BR 6617925). **United Kingdom**, England, South Devon, Dartmoor Nat. Park, Fernworthy forest, on fallen wood of *Picea* sp., 01 Mar. 1992, P.Roberts 366 (Paratype, K 23190); same site, North-east Yorkshire, Scarborough near to Falling Foss, on rotten deciduous wood, 18 May 2004, K.Robinson without number (K 123675).

Following Roberts (1993a, 1993b, 2006) and Roberts & Piątek (2004), *Tulasnella tomaculum* is morphologically identical to *T. eichleriana* especially concerning the hymenium. The hyphae, basidia, and sterigmata of both species are similar in shape and dimensions. In addition, *T. tomaculum* can have elliptical immature basidiospores similar to the elliptical basidiospores of *T. eichleriana*, which was noted and reported by Roberts (1993b). However, the mature basidiospores of *T. tomaculum* are mostly phaseoliform or "weakly allantoid" [(4–)5–6(–8) × 2.5–3.5 μm, Q-values (1.2)1.5–2(–2.7)].

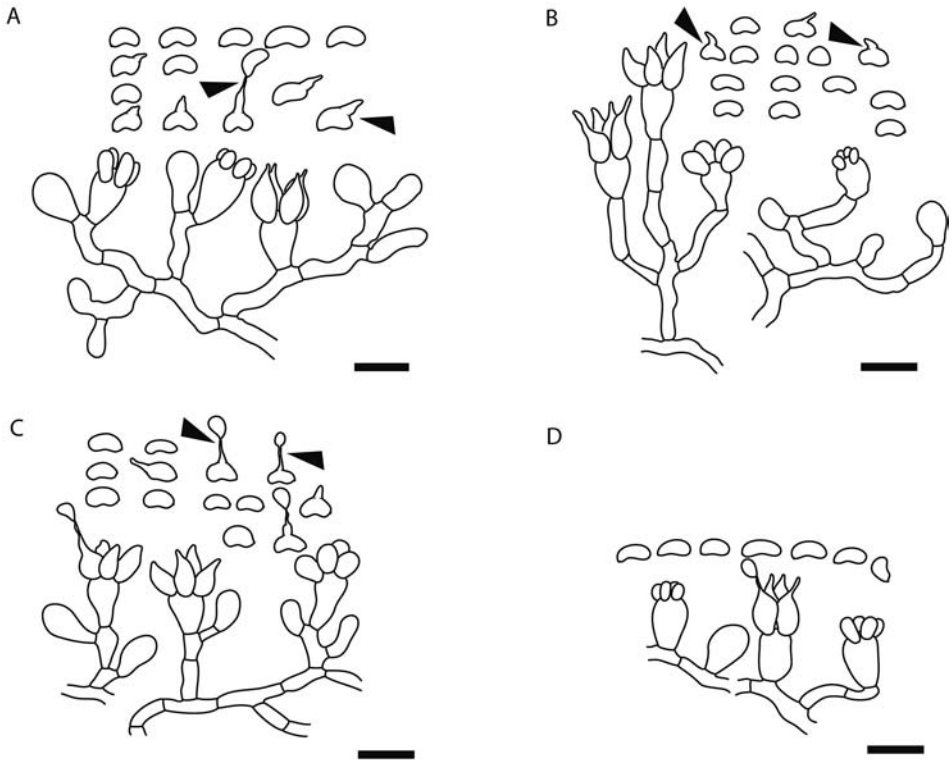

Fig. 29. *Tulasnella tomaculum*. (A–D) Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). (A) FO 35868. (B) BR 6617723. (C) BR 6617622. (D) Paratype K 23190. Scale bars = 10 μm.

Tulasnella tomaculum can be easily distinguished from other *Tulasnella* spp. by its short and narrow phaseoliform or weakly allantoid basidiospores. For instance, *T. tomaculum* differs from *T. saveloides* which has larger and mostly allantoid basidiospores [(7–)8–9 × 3–3.5 μm], and from *T. andina* with usually phaseoliform and subcylindrical basidiospores [(5–)6–8 × (2–)3–4(–5) μm]. Cruz et al. (2014) provided molecular data from basidiomata corresponding to the morphological species concept of *Tulasnella tomaculum*. However, molecular data from the type material are not available in order to confirm this species concept by phylogenetic analyses.

Tulasnella valentini Van de Put, Sterbeeckia 17: 64 (1997) [1996]. Fig. 30

Basidiomata resupinate, effused, thick and spongy, whitish to creamy in fungarium material. Hyphae often straight, usually with long cells, sometimes with swollen cells, septa unclamped, hyphae 3–5(–7.5) μm diam. Cystidia absent. Basidia mostly clavate, often solitary or sometimes two together, (12–)17–25 × 6–7 μm. Sterigmata four per basidium, obclavate when young, cylindrical, longer and tapered to the apex

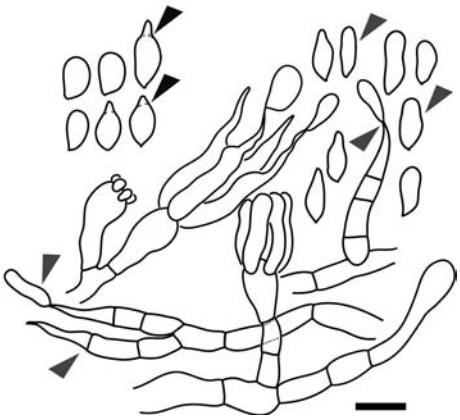

Fig. 30. *Tulasnella valentini*. Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). The gray arrowheads indicate conidiophore and conidia. Lectotype BR 6616006. Scale bar = 10 μm.

when mature, (20–)22–30 × 4–5 μm. BASIDIOSPORES mainly elliptical, sometimes oblong, 9–10(–13) × (3–)4–5.5 μm, Q-values (1.6–)1.8–2.5(–3.3), hyaline, smooth, producing secondary basidiospores by repetition. CONIDIOPHORES elongated, wide at the base, extended and tapered at the apex, septate, often the septa are present basally and through the conidiophores, 30–33 × 5 μm. Some specimens of *T. valentini* produce conidiophores without septa (Van de Put & Antonissen 1996). BLASTOCONIDIA fusiform to elliptical, formed by holoblastic conidiogenous cells generating a solitary conidium, 11–13 × 4 μm.

SPECIMEN EXAMINED: EUROPE: **Belgium**, Walem, on *Pinus sylvestris*, 14 Feb. 1995, Van de Put K. 95021402 (Lectotype, BR 6616006).

Tulasnella valentini with elliptical or oblong basidiospores is morphologically related to other *Tulasnella* species with similar basidiospores, e.g., *T. pinicola*. However, *T. valentini* is easily differentiated from other *Tulasnella* species by the presence of conidia produced by holoblastic conidiogenesis.

Tulasnella valentini and *Stilbotulasnella conidiophora* Bandoni & Oberwinkler (1982) both have elliptical basidiospores and conidia on long conidiophores. However, the basidiospores of *T. valentini* are shorter and narrower [9–10(–13) × (3–)4–5.5 μm] than the basidiospores of *S. conidiophora* [10–12 × 5–7 μm] (Bandoni & Oberwinkler 1982). The blastoconidia of *T. valentini* are produced individually, while in the case of *S. conidiophora* the blastoconidia are produced by annellidic conidiogenous loci (Bandoni & Oberwinkler 1982). *S. conidiophora* belongs to a different genus within Tulasnellaceae having dolipores and septa without parenthesomes. This characteristic was not revised for *T. valentini* (Van de Put & Antonissen 1996). This species concept has not yet been confirmed by molecular phylogenetic analyses.

Tulasnella violea (Quél.) Bourdot & Galzin, Bull. Soc. mycol. Fr. 25: 31 (1909); *Tulasnella tulasnei* (Pat.) Juel, Bih. Svensk. Vet. Akad. Handl. 23: 21 (1897); *Prototremella tulasnei* Pat., Journ. de Bot. 2: 270 (1888). (Synonyms according to Roberts 1994b). BASIONYM: *Hypochnus violeus* Quél., Ass. Fr. Av. Sci. 1882: 401 (1883). Fig. 31

BASIDIOMATA resupinate, effused to compact, ceraceous, violaceous grey or pink when fresh, sometimes without color when dry. HYPHAE unclamped, 3–5(–7) µm diam., basal hyphae thick-walled. CYSTIDIA absent. BASIDIA mostly clavate to subglobose, (8–)11–18(–20) × (6–)9–12 µm. STERIGMATA fusiform to ellipsoidal, (13–)15–26(–28) × (5–)6–8 µm. BASIDIOSPORES globose to subglobose, sometimes elliptical, (5–)6–9(–12) × (5–)6–8 µm, Q-values 1–1.5(–1.8) hyaline, smooth, germinating by repetition.

SPECIMENS EXAMINED: SOUTH AMERICA: **Ecuador**, Zamora, km 24 way to Zamora from Loja, on fallen branch, 27 Oct. 2009, D.Cruz 177. EUROPE: **Austria**, Steiermark, Niedere Tauern, Rottenmanner Tauren, East slopes of the Alpe Planner, 1600–1650 m, 04 July 1971, F.Oberwinkler 17476; Steiermark, Rottenmanner Tauern, Planner Alpe, southern slopes of Plannerseekarspitze between Edlinger Hütte and Plannersee, 1600-1700 m, on *Pinus mugo*, 04 July 1971, F.Oberwinkler 17403. **Belgium**, Zoersel, Van de Put K. s.n. (BR 6610750); same site, on *Picea abies*, 22 Dec. 1990, Van de Put K. 90122206 (BR 6611760); same site, on *Alnus glutinosa*, 12 Jan. 1991, Van de Put K. 91011201 (BR 6611861); same site, on *Quercus robur*, 23 Nov. 1991, Van de Put K. 91112308 (BR 6612164); Oostduinkerke, on *Salix* sp., 02 Nov. 1992, Van de Put K. 92110203 (BR 6612770); Walem, on *Pinus sylvestris*, 22 Dec. 1992, Van de Put K. 92122202 (BR 6612871); Zoersel, on *Populus x canadensis*, 30 Jan. 1993, Van de Put K. 93013015 (BR 6613073); same site, on *Populus x canadensis*, 21 Jan. 1995, Van de Put K. 95012107 (BR 6616107); same site, on *Pinus sylvestris*, 21 Jan. 1995, Van de Put K. 95012120 (BR 6616208); same site, on *Pinus sylvestris*, 04 Mar. 1995, Van de Put K. 95030404 (BR 6616612); same site, on *Salix* sp., 02 Mar. 1996, Van de Put K. 96030205 (BR 6619339); same site, on *Salix* sp., 02 Mar. 1996, Van de Put K. 96030212 (BR 6619440); same site, on *Salix* sp., 08 Mar. 1996, Van de Put K. 96030816 (BR 6620248); same site, 16 Mar. 1996, Van de Put K. 96031609 (BR 6620349). **Germany**, Hesse, Frankfurt am Main, Stadtwald, Louisa forest, on decayed wood, 11 Apr. 2010, D. Cruz 293; Bavaria, Wertach, Allgäu, on *Salix* sp., 03 Oct. 1996, R.Kirschner 72; Baden-Württemberg, Tübingen, Morgenstelle, on bark of *Pinus sylvestris*, 16 Feb. 1997, R.Kirschner 183; Bayer, Württemberg, Schönbuch between Hagelloch (Bogentor) and Hohenentringen, 480 m., 06 Dec. 1984, F.Oberwinkler 36358; Upper Bavaria, Bernrieder Forest between Holzhausen and Rottenried, south of Fürstenfeld-Bruck, 570 m, 20 Feb. 1972, F.Oberwinkler 17820; Bayerisch-Schwaben, Gut Bannacker at Bergheim, Southwest to Augsburg, ± 520 m, 20 Mar. 1970, F.Oberwinkler 16210; Baden-Württemberg, Black forest, Breitnau to Hinterzarten, 03 Sept. 1975, F.Oberwinkler 23078; Baden-Württemberg, Tübingen, Schönbuch, Tellerklinge, ± 400 m, 2 Dec. 1975, F.Oberwinkler 23395; Baden-Württemberg, Tübingen, Steinenberg, Neuhalde, 450 m, 9 Dec. 1975, F.Oberwinkler 23409; Bayern-Oberbayern, Gleibental in Deisenhofen, southeast München, 580 m, 09 Apr. 1972, F.Oberwinkler 17849; Bayern-Schwaben, Bergheim southwest of Augsburg, ± 530 m, 28 Nov. 1970, F.Oberwinkler 17339; Bayern-Schwaben, Bergheim southwest of Augsburg, ± 550 m, 03 June 1968, F.Oberwinkler 12070. **Norway**, Tomte, Nannestad, Akershus, on fallen wood, 17 Sept. 1993, P.Roberts 681 (K 26156). **United Kingdom**, England, Forest of Dean, Parkend (near), Churchill Inclosure, on underside of old log of *Fagus sylvatica*, 20 Sept. 2009, A.Henrici without number (K 164256); Wales, Brecon Beacons Nat. Park, Clydach, Cwm Clydach NNR, on log of *Fagus sylvatica*, 02 Jan. 2008, P.Roberts without number (K 155299).

The color of the basidiomata of *Tulasnella violea* varies within the same species and it is similar to the color of the basidiomata of other *Tulasnella* spp., e.g., *T. pinicola*. The specimen collected in Ecuador showed a violaceous grey color when fresh which disappeared upon drying. This color differs from the persistent pinkish color of *T. violea* from Europe (own observation).

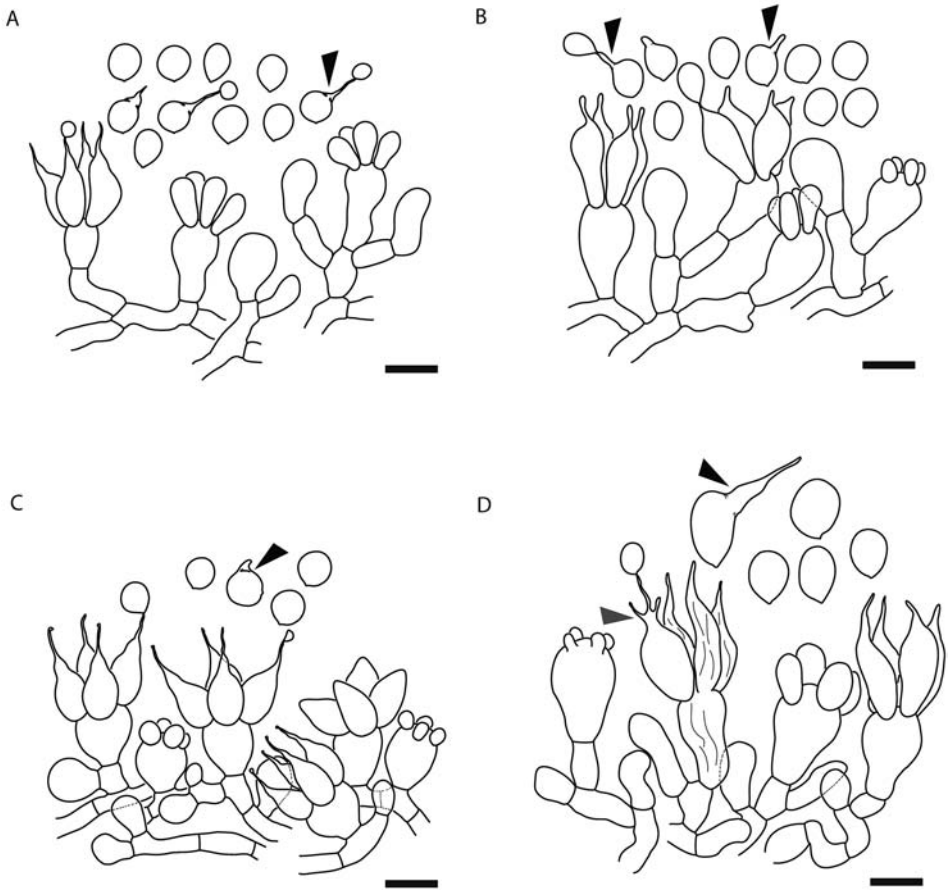

Fig. 31. *Tulasnella violea.* (A–D) Hyphae, basidia, sterigmata in different developmental stages, and basidiospores partially germinating by repetition (indicated by the black arrowheads). (A) BR 6616208. (B) D.Cruz 293. (C) D.Cruz 177. (D) FO 17820. Scale bars = 10 μm.

Tulasnella violea has characteristics similar to those of *T. eichleriana*, but *T. eichleriana* differs from *T. violea* by the smaller size of all microstructures. *T. albida* is another species similar to *T. violea*, but it has mostly oblong basidiospores. However, *T. violea* displays a high variability of basidiospore size, 8–11(–12) × 7–8(–9) μm in some specimens from Germany (FO 23078; FO 17820; FO 16210; FO 23078). Probably, in the studied material, more than one taxon is involved. The basidiospores of *T. violea* and *T. subglobospora* are impossible to distinguish, but as observed here and reported by Roberts (1994b), *T. subglobospora* differs from *T. violea* by the presence of clamp connections at all septa.

Cruz et al. (2014) presented a phylogenetic clade formed by sequences from five basidiomata assigned to *Tulasnella violea*. The corresponding specimens (DC177; DC292; DC293; K 155299; K 164256) are morphologically revised here and fit well in the morphological concept of *T. violea* (Bourdot & Galzin 1909; Roberts 1994b, 1999; Van de Put & Antonissen 1996). Despite the lack of molecular data from the type material, *T. violea* should be recognized as described here and phylogentically defined by Cruz et al. (2014).

Doubtful species

Tulasnella quasiflorens P.Roberts, Mycol. Res. 98(11): 1238 (1994).

Basidiomata resupinate, invisible to the naked eye in dry fungarium material. Hyphae sinuous, formed by long and thin cells, septate and unclamped, (2–)2.5 μm diam. Cystidia absent. Basidia mostly fusiform, tapered to the apex, usually solitary, 14 × 5–6 μm. Sterigmata four per basidium, globose at the base and forming an apical central appendage, sometimes mitriform, 12 × 8 μm, arranged on the basidia like four petals in flowers. Basidiospores not seen. Roberts (1993a) described the basidiospores of *T. quasiflorens* as elongated, curved, somewhat swollen or ventricose towards the apiculus, 19–25(–28) × 3–4 μm (holotype).

Specimen examined: EUROPE: Norway, Akershus, on fallen wood, growing together with *Ceratosebacina longispora*, 17 Sept. 1993, P.Roberts 683 (Paratype, K 26234).

The specimen P.Roberts 683 of *Tulasnella quasiflorens* revised here is degraded and the predominant basidioma observed in this material corresponds to *Ceratosebacina longispora* (Hauerslev) P.Roberts. Roberts (1994a) already mentioned this fact. Only hyphae, basidia, and the described sterigmata were found in this specimen of *T. quasiflorens*. This species concept is not yet confirmed by morphology and molecular phylogenetic analyses.

Phylogeny

The phylogenetic tree (Fig. 2) resulted after pruning the alignment by about 39% corresponding to 356 of originally 902 positions. The tree did not conflict with the tree obtained from the full alignment. This phylogram (Fig. 2) allows to evaluate the placement of the new species *Tulasnella andina* and the new records from Ecuador *T.* aff. *eichleriana* and *T. violea*. The clade of *T. andina* includes further sequences named as *Tulasnella* sp. obtained from orchid roots of the same area that most probably represent the new species. Some sequences named *Tulasnella bifrons*, *T. calospora*, and *T. deliquescens* fall in the same group showing inconsistencies in the concepts and taxonomy of these species.

Numerical analysis

Results obtained by bivariate analysis show seven groups separated by the shape of the basidiospores (Fig. 32). Clamp connections, cystidia, and other morphological characteristics are spread among the seven groups.

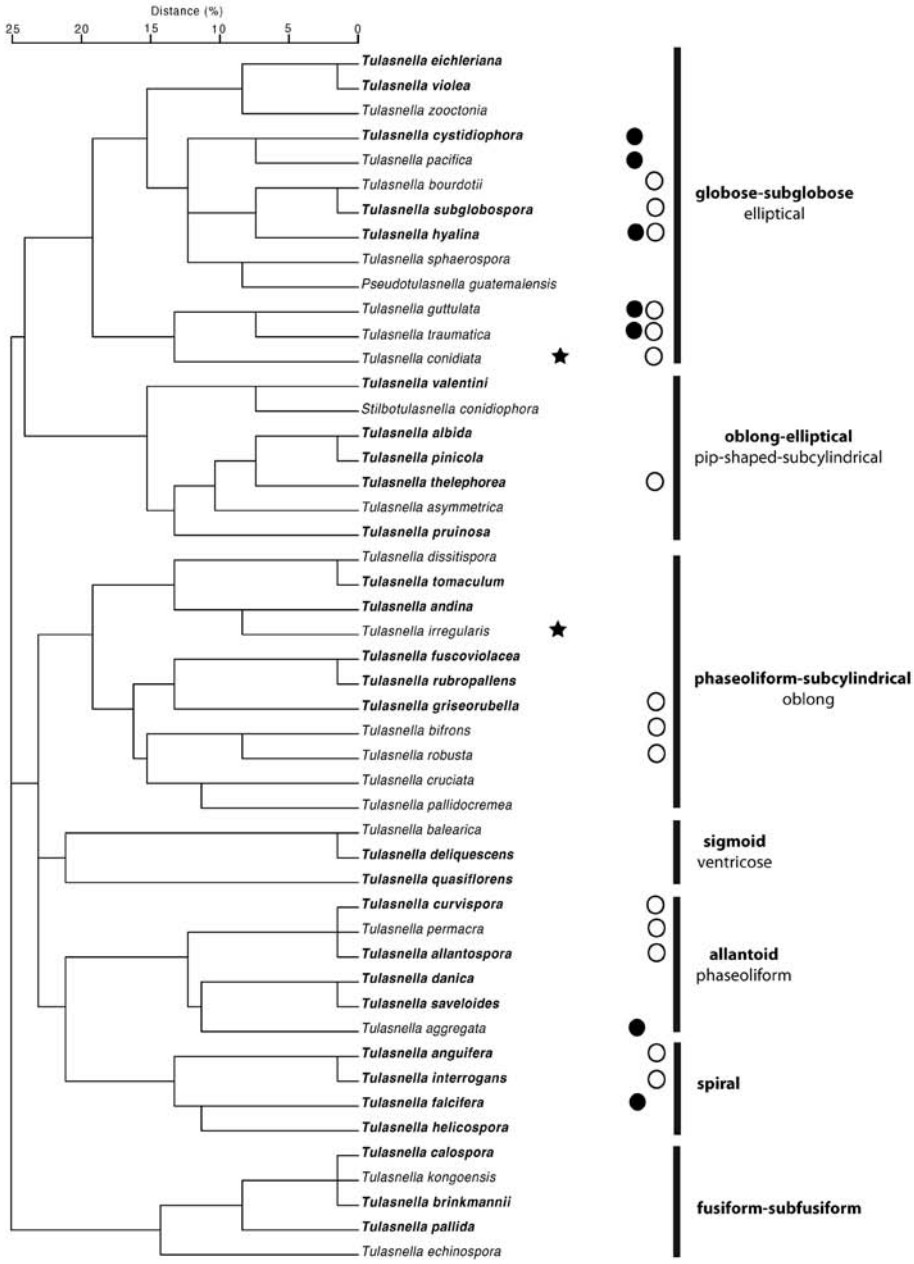

Fig. 32. Dendrogram of Tulasnellaceae inferred by Jaccard analysis of all available structures (see text) from 48 taxa (Index Fungorum) including the new species *Tulasnella andina*. Names of species presented in detail in the present publication are written in bold front. Seven groups are defined based on the main shapes of the basidiospores. Less common structures are indicated by symbols: clamp connections (o), cystidia (●), chlamydospores (★).

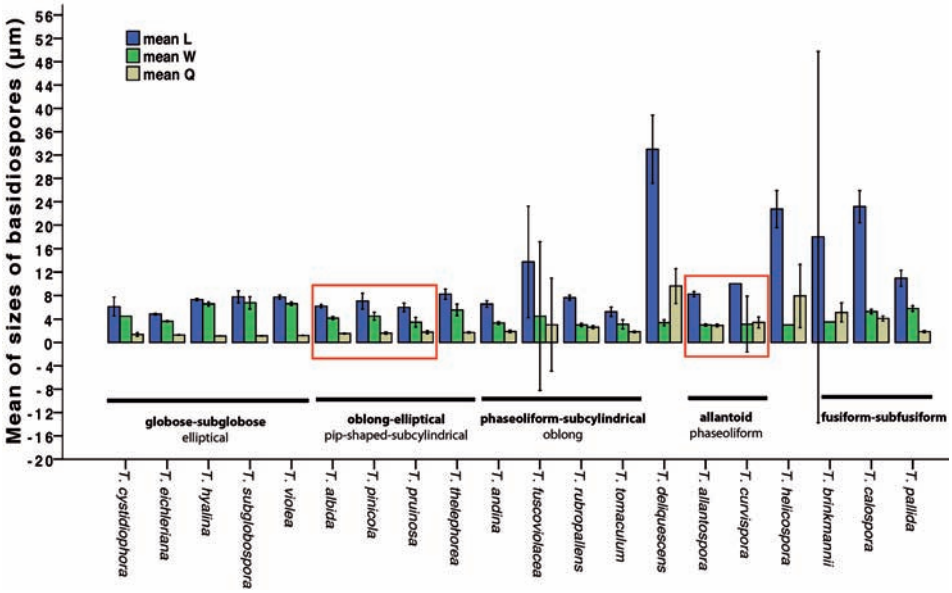

Fig. 33. Histogram showing the means of basidiospore length, width (both in µm), and Q-values (length/width) of 20 morphospecies of *Tulasnella* available by more than one specimen. Error bars on the histograms indicate 95% of confidence intervals. Groups of morphospecies that are conflictive by high similarity and without significant differences in length, width, or Q-values of basidiospores are highlighted in red. The shape of the basidiospores is indicated below the histograms.

Values of basidiospore length, width, and Q-values are compiled and related to spore shape in Fig. 33 for those taxa described above and represented by more than one specimen (Table 2). Statistical analysis (Tukey-tests; Table 3) does not separate the species into the groups of morphological similarity (morphospecies) as shown by bivariate analysis (Fig. 32). Pairwise comparison shows significant difference only for some morphospecies (Table 3).

Dichotomous key

In the following dichotomous key the shape and size of basidiospores are combined with other morphological characteristics in order to distinguish the three genera in Tulasnellaceae and the 30 taxa of genus *Tulasnella* described in the present publication. Measurements of basidiospores of *Tulasnella quasiflorens* correspond to data published by Roberts (1994a).

Table 2. Morphospecies analysed and defined in this study.

	Morphospecies name	Type specimen	Number of specimens
1	*Tulasnella albida* Bourdot & Galzin		10
2	*Tulasnella allantospora* Wakef. & A. Pearson		9
3	*Tulasnella andina* D.Cruz, J.P.Suárez & M.Piepenbr.	DC225 (holotype)	10
4	*Tulasnella anguifera* P.Roberts	K 18541 (holotype)	1
5	*Tulasnella brinkmannii* Bres.		2
6	*Tulasnella calospora* (Bourd.) Juel		6
7	*Tulasnella* aff. *cruciata* Warcup & P.H.B.Talbot		1
8	*Tulasnella curvispora* Donk		2
9	*Tulasnella cystidiophora* Honh. & Litsch.		2
10	*Tulasnella danica* Hauerslev		1
11	*Tulasnella deliquescens* (Juel) Juel		4
12	*Tulasnella eichleriana* Bres.		29
13	*Tulasnella falcifera* P. Roberts	K 18539 (holotype)	1
14	*Tulasnella fuscoviolacea* Bres		2
15	*Tulasnella griseorubella* Litsch.		1
16	*Tulasnella helicospora* Raunk.		2
17	*Tulasnella hyalina* Höhn. & Litsch.		12
18	*Tulasnella interrogans* P.Roberts		1
19	*Tulasnella kirschneri* D.Cruz, J.P.Suárez & M.Piepenbr.	R.Kirschner 923 (holotype)	1
20	*Tulasnella pallida* Bres.		11
21	*Tulasnella pinicola* Bres.		6
22	*Tulasnella pruinosa* Bourdot & Galzin		7
23	*Tulasnella rubropallens* Bourdot & Galzin		8
24	*Tulasnella saveloides* P.Roberts	Paratype P.Roberts 114 (K 21326)	1
25	*Tulasnella subglobospora* Hjortstam		4
26	*Tulasnella thelephorea* (Juel) Juel		7
27	*Tulasnella tomaculum* P.Roberts	Paratype P.Roberts 366 (K 23190)	7
28	*Tulasnella valentini* Van de Put	BR 6616006 (lectotype)	1
29	*Tulasnella violea* (Quél.) Bourdot & Galzin		31
Doubtful species			
30	*Tulasnella quasiflorens* P.Roberts	Paratype P.Roberts 683 (K 26234)	1
Total of specimens investigated:			**180**

Table 3. Significant differences of mean basidiospore sizes among morphospecies of *Tulasnella* (Tukey-test, $P < 0.05$, N 170). The capital letters (in bold), A mean spore length, B mean spore width, and C mean spore quotient (Q-value). In red are highlighted the most related and conflictive morphospecies without significant differences in the basidiospore measurements.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	*T. cystidiophora*																				
2	*T. eichleriana*																				
3	*T. hyalina*	B	AB																		
4	*T. subglobospora*	B	AB																		
5	*T. violea*	B	AB																		
6	*T. albida*			B	B	AB															
7	*T. pinicola*		A	B	B	B															
8	*T. pruinosa*		C	BC	B	BC															
9	*T. thelephorea*		AB	C		B	AB		AB												
10	*T. andina*		AC	BC	BC	BC		B		B											
11	*T. fuscoviolacea*	AC	AC	ABC	ABC	ABC	AC	AC	AC	AC	AC										
12	*T. rubropallens*	C	AC	BC	BC	BC	BC	BC	C	BC	C	A									
13	*T. tomaculum*		C	ABC	B	ABC	B	B		AB		AC	AC								
14	*T. deliquescens*	AC	AC	ABC	ABC	ABC	AC	AC	AC	ABC	AC	AC	AC	AC							
15	*T. allantospora*	C	AC	BC	BC	BC	ABC	BC	AC	BC	C	A		AC	AC						
16	*T. curvispora*	C	AC	BC	BC	BC	AC	C	AC	BC	AC			AC	AC						
17	*T. helicospora*	AC	AC	ABC	ABC	ABC	AC	AC	AC	ABC	AC	AC	AC	AC	AC	AC	AC				
18	*T. brinkmannii*	AC	AC	ABC	ABC	ABC	AC	AC	AC	ABC	AC	AC	AC	AC	AC	AC	AC	AC			
19	*T. calospora*	AC	ABC	ABC	ABC	ABC	ABC	AC	ABC	AC	ABC	AC	ABC	ABC	ABC	ABC	AB	BC	ABC		
20	*T. pallida*	A	ABC	AC	AC	ABC	AB	AB	AB	A	ABC	C	ABC	AB	ABC	ABC	BC	ABC	ABC	AC	

Key to genera in Tulasnellaceae

1 Tulasnelloid basidia aseptate 2
1 Tulasnelloid basidia partially septate ..... ***Pseudotulasnella***

2 Synnemata with blastic conidia absent ..... ***Tulasnella***
2 Synnemata with blastic conidia present ..... ***Stilbotulasnella***

Key to species in *Tulasnella*

1 Clamps present 2
1 Clamps absent 9

2 Basidiospores elliptical, globose, oblong, pip-shaped, subcylindrical, or subglobose 3
2 Basidiospores allantoid or spiral 6

3 Cystidia present and basidiospores globose to subglobose, 6–8 × 6–8 µm ... ***Tulasnella hyalina***
3 Cystidia absent 4

4 Basidiospores subcylindrical and rarely oblong or pip-shaped, 9–11 × 5 µm..... ..... ***Tulasnella griseorubella***
4 Basidiospores elliptical, globose, oblong, or subglobose.....5

5 Basidiospores globoses to subglobose, 7–9 × 6–8 µm ..... ***Tulasnella subglobospora***
5 Basidiospores oblong to elliptical and rarely pip-shaped, 7–10 × 5–8 µm..... ..... ***Tulasnella thelephorea***
6 Basidiospores allantoid.....7
6 Basidiospores spiral.....8

7 Basidiospores 7–10 × 2.5–3.5 µm ..... ***Tulasnella allantospora***
7 Basidiospores 9–11 × 3–4 µm ..... ***Tulasnella curvispora***

8 Basidiospores anguiform shaped like snake, 16–20 × 3–4 µm..... ***Tulasnella anguifera***
8 Basidiospores similar to question-mark, 15–17 × 1.5–2 µm ..... ***Tulasnella interrogans***

9 Basidiospores elliptical, globose, oblong, phaseoliform, weakly allantoid, pip-shaped, subcylindrical, or subglobose.....10
9 Basidiospores allantoid, fusiform, sigmoid, spiral, subfusiform, or ventricose 21

10 Cystidia present, basidiospores globose to subglobose, 5–7 × 4–5 µm..... ..... ***Tulasnella cystidiophora***
10 Cystidia absent.....11

11 Basidiospores elliptical, globose, oblong, pip-shaped, or subglobose 12
11 Basidiospores phaseoliform, weakly allantoid or subcylindrical 17

12 Conidiophore and conidia present, basidiospores elliptical to pip-shaped or rarely oblong, 9–10 × 4–5.5 µm..... ***Tulasnella valentini***
12 Conidiophore and conidia absent 13

13 Basidiospores globose to subglobose or rarely elliptical.....14
13 Basidiospores oblong, elliptical, pip-shaped, or phaseoliform.....15

14 Basidiospores 4–5 × 3.5–4 µm ..... ***Tulasnella eichleriana***
14 Basidiospores 6–9 × 6–8 µm ..... ***Tulasnella violea***

15 Basidia in clusters together with young basidia, basidiospores oblong to elliptical, or rarely pip-shaped, 6–7 × 4–5 µm..... ***Tulasnella albida***
15 Basidia solitary 16

16 Basidia sphaeropedunculate, 14–23 × 7–9 µm, with a long stem at the base, basidiospores oblong to elliptical, or rarely pip-shaped, 7–9 × 4–6 µm..... ***Tulasnella pinicola***
16 Basidia clavate, 9–12 × 5–6 µm, basidiospores oblong, elliptical to pip-shaped, or rarely phaseoliform, 5–7 × 3–4 µm..... ***Tulasnella pruinosa***

17 Length of basidiospores less than 9 µm.....18
17 Length of basidiospores more than 9 µm.....20

18 Basidiospores phaseoliform, weakly allantoid or rarely subcylindrical, 6–8 × 3–4 µm..... ..... ***Tulasnella andina***
18 Basidiospores phaseoliform, weakly allantoid, subcylindrical, and width less than 3 µm.....19

19 Basidiospores phaseoliform or weakly allantoid, 5–6 × 2.5–3 µm..... ..... ***Tulasnella tomaculum***
19 Basidiospores subcylindrical or rarely phaseoliform, 6–9 × 2.5–3 µm... ***Tulasnella rubropallens***

20 Adventitious septa in the sterigmata absent, basidiospores subcylindrical, 12–16 × 4–5 µm..... ..... ***Tulasnella fuscoviolacea***
20 Adventitious septa in the sterigmata present, basidiospores subcylindrical to phaseoliform or rarely allantoid, 12–15 × 3–4 µm..... ***Tulasnella* aff. *cruciata***

21 Cystidia present, basidiospores spiral similar to question-mark, 14 × 2 µm.... ***Tulasnella falcifera***
21 Cystidia absent.....22

22 Basidiospores sigmoid or ventricose 23
22 Basidiospores allantoid, fusiform, spiral or subfusiform 24

23 Basidiospores sigmoid, 30–37 × 2.5–4 µm..... ***Tulasnella deliquescens***
23 Basidiospores ventricose, 19–25 × 3–4 µm ..... ***Tulasnella quasiflorens***

24 Basidiospores allantoid..... 25
24 Basidiospores fusiform, spiral or subfusiform 27

25 Basidiospores 8–9 × 3–3.5 µm, basidia generated directly from the older basidia .... ***Tulasnella saveloides***
25 Length of basidiospores more than 10 µm..... 26

26 Hyphae 2–3 µm diam., thick walled basal hyphae absent, basidiospores 12–16 × 3–4 µm .... ***Tulasnella danica***
26 Hyphae 6–5 µm diam., thick walled basal hyphae present, basidiospores 12–15 × 5–6 µm..... ..... ***Tulasnella kirschneri***

27 Basidiospores spiral similar to question-mark, 20–26 × 3 µm ..... ***Tulasnella helicospora***
27 Basidiospores fusiform to subfusiform..... 28

28 Basidiospores fusiform, 8–12 × 5–6 µm..... ***Tulasnella pallida***
28 Length of basidiospores more than 15 µm..... 29

29 Basidiospores subfusiform and occasionally fusiform usually tapering and curved to both apices, 18–28 × 4.5–6 µm..... ***Tulasnella calospora***
29 Basidiospores subfusiform, with ventral concavity next to the apiculus and tapering to apex, 17–23 × 3–4 µm..... ***Tulasnella brinkmannii***

Discussion

Species in Tulasnellaceae were described from collections of basidiomata by many authors (Bandoni & Oberwinkler 1982, Bourdot & Galzin 1909, 1928, Dueñas 1988, 1989, Greslebin & Rajchenberg 2001, Jülich & Jülich 1976, Lowy 1964, Martin 1939, Olive 1957, Ordynets 2012, Roberts 1992, 1993a, 1993b, 1994a, 1994b, 1999, Rogers 1933, Van de Put & Antonissen 1996, Warcup & Talbot 1967, 1971, 1980). However, a recent compilation and comparison of all described morphospecies is missing. We reinvestigated available material from fungaria and studied specimens from personal collections to define 30 morphospecies (Table 2). Comparison of microstructures let us conclude that *Tulasnella andina* is new to the science. The delimitation of this species is supported by molecular phylogeny. To our knowledge it is the first species described in this genus based on morphological and molecular data. Further specimens recently collected in Ecuador were identified as *Tulasnella* aff. *eichleriana* and *T. violea*, and correspond to new records for Ecuador. In this country, 13 undetermined species of *Tulasnella* are listed according to molecular data (Liede-Schumann & Breckle 2008) and one *Tulasnella* sp. similar to *T. andina* is known from morphology and molecular data (Cruz et al. 2011). According to literature on the distribution of *Tulasnella* spp. in the neotropics (Cruz et al. 2011, Greslebin & Rajchenberg 2001, Lowy 1964, Martin 1939, Roberts 2006), these records are new for Ecuador.

Morphological data allowed us to define seven groups (Fig. 32) of *Tulasnella* spp. mostly differentiated by the shape of the basidiospores. However, only three of these groups (allantoid; globose-subglobose; spiral) are in concordance with Roberts´

concepts (1992, 1993b, 1994a, 1994b). Roberts published *Tulasnella* spp. in four papers mostly grouped by the shapes of basidiospores. Other morphological characteristics, e.g., the presence of clamp connections or cystidia, are spread among the seven groups (Fig. 32). Two morphological groups, oblong-elliptical and globose-subglobose, can be partially correlated to the phylogenetic groups III and IV respectively defined by Cruz et al. (2014). However, species like *Tulasnella* aff. *eichleriana* (with mostly globose-subglobose basidiospores) clustered into the phylogentic group III and *Tulasnella tomaculum* (with mostly phaseoliform or "weakly allantoid" basidiospores) clustered into the phylogentic group IV not allow to support the morphological groups by phylogenetic hyphothesis. The high rate of variability of morphological characteristics appears to be common in Tulasnellaceae (Cruz et al. 2014, Moncalvo et al. 2006, Hibbett et al. 2007).

However, basidiospore shapes, Q-values, and measurements previously have been considered as the main characteristics to separate species in this group. Nevertheless, statistically high variability of shape and size of basidiospores within and among species of *Tulasnella* is known and corroborated by our investigation and make species definition difficult. Morphologically distinct species like *Tulasnella allantospora* and *T. curvispora* (Figs 4, 10) or *T. albida* and *T. pinicola* (Figs. 3, 23) do not show significant difference (Table 3) when the basidiospores are analysed. By morphology most taxa cannot be defined and could be merged with other taxa. Molecular phylogenetic analyses, however, reveal many distinct groups of morphologically rather similar species as well as cryptic species (Cruz et al. 2014). Morphological problems because of high variability of microstructures occur also in other groups of Basidiomycota, e.g., in species of *Obba* and *Sebipora* (Miettinen & Rajchenberg 2012), *Phellinus* (Shell 2006), or Sebacinales (Oberwinkler et al. 2014). This problem appears to be ubiquitous in fungi, impeding morphological species recognition (Gazis et al. 2011), while morphologically defined higher taxa of fungi are often supported by molecular phylogenetic hypothesis (Hibbett et al. 2004, 2007).

Most DNA sequences of *Tulasnella* have been obtained by direct PCR amplification from orchid roots or liverworts (Kottke et al. 2003, McCormick et al. 2004, Suárez et al. 2006, Taylor & McCormick 2008, Preussing et al. 2010, Jacquemyn et al. 2010, 2011, Girlanda et al. 2011, Martos et al. 2012) or from axenic, anamorphic cultures (McCormick et al. 2004, Suárez et al. 2006). Sequencing of old fungarium specimens of *Tulasnella* spp. has been unsuccessful probably due to inappropriate conservation of DNA. We suspect that sequencing of the old type material will be difficult also in future even after improvement of techniques as discussed by Cruz et al. (2014). Methods to extract DNA from old fungarium samples were so far tested mainly for plants, liverworts, lichens, and macrofungi (Asif & Cannon 2005, Cubero et al. 2005, Jankowiak et al. 2005, Lister 2008, Góes-Neto et al. 1999).

Most of the OTUs presented in the phylogenetic tree (Fig. 2) cluster in three (II, III, IV) of four groups defined and discussed by Cruz et al. (2014). However, two sequences of *Tulasnella irregularis* form a different group not considered by Cruz et al. (2014) but suggested as CLADE III by Nontachaiyapoom et al. (2010). The phylogeny (Fig. 2) allows to delimit many species consistent with the morphological descriptions. The

clades of *Tulasnella andina* and *T.* aff. *andina* are discussed following their descriptions (see above). The clades formed by *Tulasnella albida*, *T. asymmetrica*, *T. eichleriana*, *T.* aff. *eichleriana* (ECU4 and ECU6), *T.* cf. *pinicola*, *T. tomaculum*, and *T. violea* are discussed concerning their molecular delimitation by Cruz et al. (2014). The clade of *Tulasnella irregularis* with a bootstrap value of 88% contains the sequence of the type (EU218889 McCormick et al. 2004 from strain type JHW 0632; Warcup and Talbot 1980). Other clades in Fig. 2 were generated with non-type sequences obtained from cultures isolated from basidiomata named as *Tulasnella bifrons* (AY373290, strain BPI 724849; McCormick et al. 2004), *T. danica* (AY373297, strain KC388, McCormick et al. 2004), and *T. pruinosa* (AY373295 and DQ457642, strain DAOM 17641=isolate AFTOL-ID 610, Matheny et al. 2006 and McCormick et al. 2004 respectively). In addition, the species definitions of *T. calospora* and *T. deliquescens* phylogentically present taxonomic inconsistences (Fig. 2) as previously discussed by Suárez et al. (2006) and Taylor and McCormick (2008). Their identity cannot be resolved here, because it was not possible to investigate the morphology of corresponding basidiomata in the context of the present study.

Further phylogenetic support of the described morphospecies will only be obtained by re-sampling and sequencing of fresh basidiomata. Further phylogenetic studies incorporating sequences and morphological studies from new collections of basidiomata of *Tulasnella* spp. are required in order solve the taxonomic problems. Our results including morphological descriptions, illustrations, and statistical analyses are but a first step to expand our knowledge on the intra- and inter-specific morphological variability of *Tulasnella* spp.

Acknowledgments

The authors thank Ingrid Kottke and Ralph Mangelsdorff for interesting discussions and help to improve the manuscript. We thank the curators of the herbaria of the National Botanic Garden of Belgium (BR) and Kew Royal Botanic Gardens (K) for answering requests and loans of specimens. We are grateful to Paulo Herrera for joining us during the fieldwork and Juan Sebastian Eguiguren for help and assistance in the laboratory in Ecuador. We also appreciate the help by Roland Kirschner who let us revise his personal collection of *Tulasnella* and Franz Oberwinkler who provided samples of *Tulasnella* collected by him, deposited at the Eberhard-Karls University of Tübingen, Germany. This investigation received institutional support by the Deutsche Forschungsgemeinschaft (DFG RU 816), the German Academic Exchange Service (DAAD), the Universidad Técnica Particular de Loja, and the LOEWE excellence initiative of the state of Hesse within the framework of the Cluster for Integrative Fungal Research (IPF). Nature and Culture International (NCI) provided research facilities at RBSF.

References

ANDERSEN, T.F. 1996: A comparative taxonomic study of *Rhizoctonia* sensu lato employing morphological, ultrastructural and molecular methods. – Mycol. Res. **100**: 1117–1128.

ASIF, M.J. & C.H. CANNON 2005: DNA extraction from processed wood: A case study for the identification of an endangered timber species (*Gonystylus bancanus*). – Plant Mol. Biol. Report. **23**: 185–192.

BANDONI, R.J. & F. OBERWINKLER 1982: *Stilbotulasnella*: a new genus in the Tulasnellaceae. – Canad. J. Bot. **60**: 1875–1879.

BOUGOURE, J.J., D.S. BOUGOURE, W.G. CAIRNEY & D.W. DEARNALEY 2005: ITS-RFLP and sequence analysis of endophytes from *Acianthus*, *Caladenia* and *Pterostylis* (Orchidaceae) in southeastern Queensland. – Mycol. Res. **109**: 452–460.

BOURDOT, H. & A. GALZIN 1923: Heterobasidieae nondum descriptae. – Bull. Soc. Mycol. Fr. **39**: 261–266.

BOURDOT, H. & A. GALZIN 1909: Hymenomycetes de France. I Heterobasidiés. – Bull. Soc. Mycol. Fr. **25**: 15–36.

BOURDOT, H. & A. GALZIN 1928: Hymenomycetes de France. Heterobasidiés. III Tulasnellaceae. – Biblioth. Mycol. Edited by J. Cramer, 23 (reprint, 1969).

CASTRESANA, J. 2000: Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. – Mol. Biol. Evol. **17**: 540–552.

CRUZ, D., J.P. SUÁREZ, I. KOTTKE, M. PIEPENBRING & F. OBERWINKER 2011: Defining species in *Tulasnella* by correlating morphology and nrDNA ITS-5.8s sequence data of basidiomata from a tropical Andean forest. – Mycol. Prog. **10**: 229–238.

CRUZ, D., J.P. SUÁREZ, I. KOTTKE & M. PIEPENBRING 2014: Cryptic species revealed by molecular phylogenetic analysis of sequences obtained from basidiomata of *Tulasnella*. – Mycologia **106**(4): 708–722.

CUBERO, O.F., A. CRESPO, J. FATEHI & P.D. BRIDGE 1999: DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. – Plant Syst. Evol. **216**(3-4): 243–249.

DUEÑAS, M. 1988: Fragmenta Chorologica Occidentalia, Fungi, 1282–1296. – 289. Anales Jard. Bot. Madrid **45**: 287

DUEÑAS, M. 1989: Phragmobasidiomycetes del norte de España. – Anales Jard. Bot. Madrid **46**(1): 229–232

DUEÑAS, M. 2001: Iberian intrahymenial species of Platygloeales, Tremellales, and Tulasnellales. – Nova Hedwigia **72**: 441–459.

FELSENSTEIN, J. 1985: Confidence limits on phylogenies: an approach using the bootstrap. – Evol. **39**(4): 783–791.

GAZIS, R., S. REHNER & P. CHAVERRI 2011: Species delimitation in fungal endophyte diversity studies and its implications in ecological and biogeographic inferences. – Mol. Ecol. **20**: 3001–3013.

GIRLANDA, M.R., SEGRETO, D. CAFASSO, H.B. LIEBEL, M. RODDA et al. 2011. Photosynthetic mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations. – Am. J. Bot. **98**: 1148–1163.

GÓES-NETO, A., C. LOGUERCIO-LEITE & R.T. GUERRERO 2005: DNA extraction from frozen field-collected and dehydrated fungarium fungal basidiomata: performance of SDS and CTAB-based methods. – Biotemas **18**: 19–32.

GRESLEBIN, G.A. & M. RAJCHENBERG 2001: The genus *Tulasnella* with a new species in the Patagonian Andes forests of Argentina. – Mycol. Res. **105**(9): 1149–1151.

HIBBETT, D. 2004: Trends in morphological evolution in Homobasi- diomycetes inferred using maximum likelihood: a comparison of binary and multistate approaches. – Syst. Biol. **53**: 889–903.

HIBBETT, D.S., M. BINDER, F. BISCHOFF, M. BLACKWELL, P.F. CANNON et al. 2007: A higher-level phylogenetic classification of the Fungi. – Mycol. Res. **111**: 509–547.

JACCARD, P. 1908: Nouvelles recherches sur la distribution florale. – Bull. Soc. Vaud. Sci. Nat. **44**: 223–270.

JACQUEMYN, H., O. HONNAY, B.P.A. CAMMUE, R. BRYS & B. LIEVENS 2010: Low specificity and nested subset structure characterize mycorrhizal associations in five closely related species of the genus *Orchis*. – Mol. Ecol. **19**: 4086–4095.

JACQUEMYN, H., V. MERCKX, R. BRYS, D. TYTECA, B.P.A. CAMMUE, O. HONNAY & B. LIEVENS 2011: Analysis of network architecture reveals phylogenetic constraints on mycorrhizal specificity in the genus *Orchis* (Orchidaceae). – New Phytol. **192**: 518–528.

JANKOWIAK, K., K. BUCZKOWSKA & Z. SZWEYKOWSKA-KULINSKA 2005: Successful extraction of DNA from 100-year-old herbarium specimens of the liverwort *Bazzania trilobata*. – Taxon **54**(2): 335–336.

JÜLICH, W. & U. JÜLICH 1976: A contribution towards a revision of the genus *Tulasnella*. – Persoonia **2**: 49–64.

KATOH, K., K. KUMA, H. TOH & T. MIYATA 2005: MAFFT version 5: improvement in accuracy of multiple sequence alignment. – Nucleic Acids Res. **33**: 511–518.

KIRK, P.M., P.F. CANNON, D.W. MINTER & J.A. STALPERS 2008: Dictionary of the Fungi, 10th ed., CABI Europe (Wallingford, UK).

KOTTKE, I. A. BEITER, M. WEISS, I. HAUG, F. OBERWINKLER & M. NEBEL 2003: Heterobasidiomycetes form symbiotic associations with hepatics: Jungermanniales have sebacinoid mycobionts while *Aneura pinguis* (Metzgeriales) is associated with a *Tulasnella* species. – Mycol. Res. **107**: 957–968.

KOTTKE, I., J.P. SUÁREZ, P. HERRERA, D. CRUZ, R. BAUER et al. 2010: Atractiellomycetes belonging to the 'rust' lineage (Pucciniomycotina) form mycorrhizae with terrestrial and epiphytic neotropical orchids. – Proc. R. Soc. London. B. **277**: 1289–1296.

LARGENT, D.L., D. JOHNSON & R. WATLING 1973: How to identify mushrooms to genus III: Microscopic features. – 1st Edition Eureka, CA: Mad River Press.

LIEDE-SCHUMANN, S. & S.W. BRECKLE 2008: Provisional checklist of flora and fauna of the San Francisco valley and its surroundings (Reserva Biológica San Francisco, Province Zamora-Chinchipe, southern Ecuador). – Ecotropical Monographs 4.

LISTER, D.L., M.A. BOWER, C.J. HOWE & M.K. JONES 2008: Extraction and amplification of nuclear DNA from fungarium specimens of emmer wheat: a method for assessing DNA preservation by maximum amplicon length recovery. – Taxon **57**: 254–258.

LOWY, B. 1964. A new genus of the Tulasnellaceae. – Mycologia **56**: 696–700.

MA, M., T.K. TAN & S.M. WONG 2003: Identification and molecular phylogeny of *Epulorhiza* isolates from tropical orchids. – Mycol. Res. **107**: 1041–1049.

MARTIN, G.W. 1939: New or noteworthy fungi from Panama and Colombia. III. – Mycologia **31**: 239–249.

MARTOS, F., F. MUNOZ, T. PAILLER, I. KOTTKE, C. GONNEAU & M.A. SELOSSE 2012: The role of epiphytism in architecture and evolutionary constraint within mycorrhizal networks of tropical orchids. – Mol. Ecol. **21**: 5098–5109.

MATHENY, P.B., J.M. CURTIS, V. HOFSTETTER, M.C. AIME, J.M. MONCALVO et al. 2006. Major clades of Agaricales: a multilocus phylogenetic overview. – Mycologia **98**: 984–997.

McCORMICK, M.K., D.F. WHIGHAM & J. O'NEILL 2004: Mycorrhizal diversity in photosynthetic terrestrial orchids. – New Phytol. **163**: 425–438.

MIETTINEN, O. & M. RAJCHENBERG 2012: *Obba* and *Sebipora*, new polypore genera related to *Cinereomyces* and *Gelatoporia* (Polyporales, Basidiomycota). – Mycol. Prog. **11**: 131–147.

MONCALVO, J.M., R.H. NILSSON, B. KOSTER, S.M. DUNHAM, T. BERNAUER et al. 2006: The cantharelloid clade: Dealing with incongruent gene trees and phylogenetic reconstruction methods. – Mycologia **98**: 937–948.

MOORE, R.T. 1996. The dolipore/parenthesome septum in modern taxonomy. – In: SNEH B., S. JABAJI-HARE, S. NEATE DIJST (eds.): *Rhizoctonia* species: taxonomy, molecular biology, ecology, pathology and disease control. Kluwer Academic Publishers, Dordrecht; 13–35.

NONTACHAIYAPOOM, S., S. SASIRAT, L. MANOCH 2010. Isolation and identification of Rhizoctonia-like fungi from roots of three orchid genera, *Paphiopedilum*, *Dendrobium* and *Cymbidium*, collected in Chiang Rai and Chiang Mai provinces of Thailand. – Mycorrhiza **20**: 459–471.

OBERWINKLER, F., K. RIESS, R. BAUER & S. GARNICA 2014: Morphology and molecules: the Sebacinales, a case study. – Mycol. Prog. **13**(3): 445–470.

OLIVE, L.S. 1957: Tulasnellaceae of Tahiti. A revision of the family. – Mycologia **49**: 663–679.

ORDYNETS, O. 2012: New records of corticioid fungi with heterobasidia from Ukraine. – Turk. J. Bot. **36**(5): 590–602.

PEREIRA, M., I. COELHO, R. VALADARES, F. OLIVEIRA, M. BOCAYUVA et al. 2014: Morphological and molecular characterization of *Tulasnella* spp. fungi isolated from the roots of Epidendrum secundum, a widespread Brazilian orchid. – Symbiosis **62**(2): 111–121.

PEREIRA, O.L., C.L. ROLLEMBERG, A.C. BORGES, K. MATSUOKA & M.C.M. KASUYA 2003: *Epulorhiza epiphytica* sp. nov. isolated from mycorrhizal roots of epiphytic orchids in Brazil. – Mycoscience **44**: 153–155.

PEREIRA, O.L., M.C.M KASUYA, A.C. BORGES & E.F. ARAUJO 2005: Morphological and molecular characterization of mycorrhizal fungi isolated from neotropical orchids in Brazil. – Can. J. Bot. **83**: 54–65.

PREUSSING, M., M. NEBEL, F. OBERWINKLER & M. WEISS 2010: Diverging diversity patterns in the *Tulasnella* (Basidiomycota, Tulasnellales) mycobionts of *Aneura pinguis* (Marchantiophyta, Metzgeriales) from Europe and Ecuador. – Mycorrhiza **20**(3):147–59.

RIOFRIO, M.L., D. CRUZ, E. TORRES, M. DE LA CRUZ, J.M. IRIONDO & J.P. SUÁREZ 2013: Mycorrhizal preferences and fine spatial structure of the epiphytic orchid *Epidendrum rhopalostele.* – Am. J. Bot. **100**(12): 1–10.

ROBERTS, P. & M. PIĄTEK 2004: Heterobasidiomycetes of the families Oliveoniaceae and Tulasnellaceae from Poland. – Polish Bot. J. **49**(1): 45–54.

ROBERTS, P. 1992: Spiral-spored *Tulasnella* species from Devon and the New Forest. – Mycol. Res. **96**(3): 233–236.

ROBERTS, P. 1993a: The genus *Tulasnella* in Norway. – Windahlia **20**: 67–74. Göteborg.

ROBERTS, P. 1993b: Allantoid-spored *Tulasnella* species from Devon. – Mycol. Res. **97**: 213–220.

ROBERTS, P. 1994a: Long-spored *Tulasnella* species from Devon, with additional notes on allantoid-spored species. – Mycol. Res. **98**: 1235–1244.

ROBERTS, P. 1994b: Globose and ellipsoid-spored *Tulasnella* species from Devon and Surrey, with a key to the genus in Europe. – Mycol. Res. **98**: 1431–1452

ROBERTS, P. 1997: New and unusual Scandinavian heterobasidiomycetes. – Windahlia **22**: 15–22. Coreborg.

ROBERTS, P. 1998: *Oliveonia* and the origin of the holobasidiomycetes. – Folia Cryptogamica Estonica **33**: 127–132.

ROBERTS, P. 1999: *Rhizoctonia*-forming Fungi: a taxonomic guide. – Royal Botanic Gardens, Kew.

ROBERTS, P. 2003: *Tulasnella echinospora:* an unusual new species from Great Britain and Sweden. – Cryptogamie Mycol. **25**(4): 23–27.

ROBERTS, P. 2006: Caribbean heterobasidiomycetes: 2. Jamaica. – Mycotaxon **96**: 83–107.

ROGERS, D.P. 1933: A taxonomic review of the Tulasnellaceae. – Annls. Mycol. **31**: 181–203.

SELL, I. 2006: Size and shape of basidiospores in the *Phellinus igniarius* group. – Agron. Res. **4**: 359–362.

SHEFFERSON, R.P., M. WEISS, T. KULL & D.L. TAYLOR 2005: High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. – Mol. Ecol. **14**: 613–626.

SUÁREZ, J.P., M. WEISS, A. ABELE, S. GARNICA, F. OBERWINKLER et al. 2006: Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. – Mycol. Res. **110**: 1257–1270.

TAMURA, K., D. PETERSON, N. PETERSON, G. STECHER, M. NEI et al. 2011: MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. – Mol. Biol. Evol. **28**: 2731–2739.

TAYLOR, D.L. & M.K. McCORMICK 2008: Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. – New Phytol. **177**: 1020–1033.

TULLOSS, R.E. & J.E. LINDGREN 2005: *Amanita aprica* a new toxic species from western North America. – Mycotaxon **91**: 193–205.

VAN DE PUT, K. & I. ANTONISSEN 1996: *Tulasnella's* uit Vlaanderen. – Sterbeeckia **17**: 44–69.

VAN DRIEL, K.G.A., B.M. HUMBEL, A.J., VERKLEIJ, J. STALPERS, W.H. MÜLLER et al. 2009: Septal pore complex morphology in the Agaricomycotina (Basidiomycota) with emphasis on the Cantharellales and Hymenochaetales. – Mycol. Res. **113**: 559–576.

VELDRE, V., K. ABARENKOV, M. BAHRAM, F. MARTOS, M-A. SELOSSE et al. 2013: Evolution of nutritional modes of Ceratobasidiaceae (Cantharellales, Basidiomycota) as revealed from publicly available ITS sequences. – Fungal Ecol. **6**: 256–268.

WARCUP, J.H. & P.H.B. TALBOT 1967: Perfect states of Rhizoctonias associated with orchids I. – New Phytol. **66**: 631–641.

WARCUP, J.H. & P.H.B. TALBOT 1971: Perfect states of Rhizoctonias associated with orchids II. – New Phytol. **76**: 35–40.

WARCUP, J.H. & P.H.B. TALBOT 1980: Perfect states of Rhizoctonias associated with orchids III. – New Phytol. **86**: 267–272.

Manuscript submitted March 25, 2015; accepted July 10, 2015.

The following page numbers of 708–722 are the page numbers of the following publication.

2.- Cruz DJ, Suarez JP, Kottke I, and Piepenbring M (2014) Cryptic species revealed by molecular phylogenetic analysis of sequences obtained from basidiomata of *Tulasnella*. *Mycologia* 106(4): 708–722

Status: published

Title of the magazine: Mycologia

Participating authors:

1.- DJC: Darío Javier Cruz

2.- JPS: Juan Pablo Suárez

3.- IK: Ingrid Kottke

4.- MP: Meike Piepenbring

To which extend did the doctoral candidate or the co-authors contribute individually to the dissertation?

(1) Development and planning

1.- DJC: 60%

2.- JPS: 10%

3.- IK: 10%

4.- MP: 20%

(2) Performance of individual research and experiments

1.- DJC: 85%

2.- JPS: 5%

3.- IK: 5%

4.- MP: 5%

DJC did all the sampling of basidiomata of *Tulasnella* spp. in a tropical rain forest in Ecuador and a regenerated forest in Germany. DJC did all the molecular work

(DNA isolation, PCR, electrophoresis and molecular cloning). Sequencing was carried out at Macrogen Inc. (Korea).

The standardized protocol of PCR and molecular cloning was improved by DJC, and in collaboration of JPS.

(3) Composition of data gathering and images

1.- DJC: 90%

2.- JPS: 5%

3.- IK: 0%

4.- MP: 5%

DJC did all of tables, phylograms, dendrograms, and other diagrams presented in this work. DJC compiled all the morphological information corresponding to measurements for the statistical analysis during this study. The main morphological characteristics were selected by DJC and following the suggestions of MP.

(4) Analysis and interpretation of the data

1.- DJC: 85%

2.- JPS: 5%

3.- IK: 5%

4.- MP: 5%

All the phylogenetic and statistical analyses were done by DJC and revised by JPS, IK, and MP. DJC improved these analyses and their interpretations according to recommendations given by JPS, IK, and MP.

(5) Preparation of the manuscript

1.- DJC: 80%

2.- JPS: 8%

3.- IK: 4%

4.- MP: 8%

Mycologia, 106(4), 2014, pp. 708–722. DOI: 10.3852/12-386

Cryptic species revealed by molecular phylogenetic analysis of sequences obtained from basidiomata of *Tulasnella*

Darío Cruz[1]
Juan Pablo Suárez
Departamento de Ciencias Naturales, Universidad Técnica Particular de Loja, San Cayetano Alto s/n C.P. 11 01 608, Loja, Ecuador

Ingrid Kottke
Departamento de Ciencias Naturales, Universidad Técnica Particular de Loja, San Cayetano Alto s/n C.P. 11 01 608, Loja, Ecuador
Institute of Evolution and Ecology, Evolutionary Ecology of Plants, Eberhard-Karls-University Tübingen, Auf der Morgenstelle 1, D-72076 Tübingen, Germany

Meike Piepenbring
Institute of Ecology, Evolution and Diversity, Goethe-University Frankfurt am Main, Max-von-Laue-Str. 13, D-60438 Frankfurt am Main, Germany

Abstract: Delimitation of species and the search for a proper threshold for defining phylogenetic species in fungi are under discussion. In this study, morphological and molecular data are correlated to delimit species of *Tulasnella,* the most important mycobionts of Orchidaceae, which suffer from poor taxonomy. Resupinate basidiomata of *Tulasnella* species were collected in Ecuador and Germany, and 11 specimens (seven from Ecuador, four from Germany) were assigned to traditional species concepts by use of morphological keys. The specimens were compared by micro-anatomical examination with 75 specimens of *Tulasnella* borrowed from fungaria to obtain better insights on variation of characters. Sequences of the ITS region (127) were obtained after cloning from the fresh basidiomata and from pure cultures. Proportional variability of ITS sequences was analyzed within and among the cultures and the specimens designated to different morphospecies. Results suggested an intragenomic variation of less than 2%, an intraspecific variation of up to 4% and an interspecific divergence of more than 9% in *Tulasnella*. Cryptic species in *Tulasnella*, mostly from Ecuador, were revealed by phylogenetic analyses with 4% intraspecific divergence as a minimum threshold for delimiting species. Conventional diagnostic morphological characters appeared insufficient for species characterization. Arguments are presented for molecular delimitation of the established species *Tulasnella albida*, *T. asymmetrica*, *T. eichleriana*, *T.* cf. *pinicola*, *T. tomaculum* and *T. violea*.

Key words: interspecific divergence, intragenomic variability, intraspecific variability, ITS-5.8S region, morphospecies, teleomorphic states

Submitted 9 Dec 2012; accepted for publication 6 Jan 2014.
[1] Corresponding author: djcruz@utpl.edu.ec

Introduction

Species concepts corresponding to phylogenetic units often combine morphological information as primary species hypotheses and molecular data (Walker et al. 2007, Stielow et al. 2011) or other evidence such as geographical or ecological data to reach conclusive secondary species hypotheses (Puillandre et al. 2012). However, in many groups of fungi morphological characters are difficult to define and are insufficient for species recognition (Gazis et al. 2011). Cryptic species thus may be frequently misclassified (Bickford et al. 2007). Species of Tulasnellaceae are especially difficult to organize taxonomically due to the ambiguous morphological and molecular characteristics (Moncalvo et al. 2006, Hibbett et al. 2007).

The genus *Tulasnella* J. Schröt. (1888) is globally distributed and includes species reported as saprotrophs on decayed wood (Roberts 1999, Greslebin and Rajchenberg 2001, Cruz et al. 2011) and mycobionts of Orchidaceae (Warcup and Talbot 1967, 1971, 1980; Cameron et al. 2006; Suárez et al. 2006; Smith and Read 2008; Jacquemyn et al. 2010, 2011; Martos et al. 2012) or liverworts (Kottke et al. 2003). Their inconspicuous basidiomata are difficult to find, and the few descriptions available, mostly from the northern hemisphere, mention lack of distinctive macromorphological characteristics and high variation in shape and overlapping sizes of microscopic structures (Roberts 1994b). Morphological descriptions of only 12 species of Tulasnellaceae are available from five Neotropical countries including Colombia (Martin 1939), Guatemala (Lowy 1964), Argentina (Greslebin and Rajchenberg 2001), Jamaica (Roberts 2006) and Ecuador (Cruz et al. 2011). Hitherto, most DNA sequences of *Tulasnella* were obtained by direct PCR amplification from orchid roots or liverworts (Kottke et al. 2003; McCormick et al. 2004; Suárez et al. 2006; Taylor and McCormick 2008; Preußing et al. 2010; Jacquemyn et al. 2010, 2011; Girlanda et al. 2011; Martos et al. 2012) or from axenic, anamorphic cultures (McCormick et al. 2004, Suárez et al. 2006). Corresponding morphological data of

teleomorphic stages are lacking, leading to inconsistencies among morphological and phylogenetic species concepts of *Tulasnella* (McCormick et al. 2004, Suárez et al. 2006, Taylor and McCormick 2008). *Tulasnella*, as with other cantharelloid fungi, exhibits exceptionally high molecular variability in nuclear ribosomal DNA regions (Moncalvo et al. 2006). The internal transcribed spacer (ITS), now the official marker for species delimitation in Fungi (Schoch et al. 2012), is exceptionally variable, impeding unambiguous sequence alignments (Suárez et al. 2006, Shefferson et al. 2007, Taylor and McCormick 2008, Nontachaiyapoom et al. 2010, Cruz et al. 2011). Beyond that sequence similarity thresholds delimiting phylogenetic species need to be evaluated, as has been accomplished by identifying the gap between intraspecific variation and interspecific divergence for other organisms (Meier et al. 2008, Ni et al. 2012, Puillandre et al. 2012). This has been named the ''barcode gap'' (Meyer and Paulay 2005). The species boundary on the ITS region in Fungi is typically defined at 3% sequence divergence (Nilsson et al. 2008), and 3–5% divergence was proposed for delimiting *Tulasnella* spp. based on molecular data from mycorrhiza isolates (Girlanda et al. 2011, Jacquemyn et al. 2011, Martos et al. 2012). Our own study of a limited number of basidiomata of one *Tulasnella* sp. collected in Ecuador revealed less than 1% variability among specimens except one clone of 5.1% divergence (Cruz et al. 2011).

The aim of the present study was to examine in more detail the variation within and divergence among species by combining molecular and morphological data of basidiomata of *Tulasnella* spp. newly collected in southern Ecuador and in temperate forests in Germany, including fungarium specimens and axenic, anamorphic cultures. Integration of the molecular and morphological data suggests improvement of the species concepts in *Tulasnella* and reveals several cryptic species.

MATERIALS AND METHODS

Sampling.—Sampling of resupinate fungi with tulasnelloid characteristics (Roberts 1999, Cruz et al. 2011) on decayed wood or fallen branches was carried out in the evergreen upper montane tropical rainforest in Ecuador and temperate forests near Frankfurt am Main, Germany, 2007–2010. Sampling sites in Ecuador include the Reserva Biológica San Francisco (RBSF), on the eastern slope of the Cordillera El Consuelo in the northern Andes, Zamora Chinchipe province (3°58′S, 79°04′W, 1900–2500 m), and a fragment of regenerated, approximately 35 y old forest on the eastern slope of the Cordillera Real in the northern Andes, Zamora-Chinchipe province (3°59′17″S, 79°6′4″W, 2280 m). The most species-rich plant families in these forests are Ericaceae, Melastomataceae, Orchidaceae and Rubiaceae (Homeier and Werner 2008). Details of the sampling area are in Beck et al. (2008). Sampling in Germany was carried out in regenerated forests in Frankfurt am Main, Ginnheim forest, (8°38′E, 50°08′N, 112 m), Stadtwald, Louisa forest, (8°40′E, 50°5′N, 149 m), and Apfelbach, Groß Gerau, (8°29′E, 49°55′N, 140 m).

Seven *Tulasnella* specimens were collected in Ecuador and four specimens in Germany. Names of species correspond to preliminary identifications based on morphology (TABLE I). All samples of *Tulasnella* spp. from Ecuador and Germany were dried and deposited in the Herbarium of the Universidad Técnica Particular de Loja (HUTPL), Loja, Ecuador.

Fungarium material.—*Tulasnella* specimens, 75 in total, were loaned from the following fungaria: specimens belonging to Franz Oberwinkler (FO) are deposited at Eberhard-Karls-Universität Tübingen (TUB). Further specimens are from National Botanic Garden of Belgium (BR) and Kew Royal Botanic Gardens (K). The specimens belonging of Roland Kirschner (RoKi) are deposited in the National Museum of Natural Science of Taiwan (TNM).

Cultures.—Six axenic cultures of *Tulasnella* spp. were analyzed; these include three isolates from Australia identified by Warcup and Talbot (1967) and Warcup (1973) as *Tulasnella asymmetrica* Warcup & P.H.B. Talbot (MAFF 305806, MAFF 305808, MAFF 305809) and three isolates from Germany contributed by Franz Oberwinkler, *Tulasnella* sp. FO 35532, *Tulasnella* sp. FO 24380a and *Tulasnella* sp. FO 24462a. All strains were obtained from the culture collection of the Institute of Ecology, Evolutionary Ecology of Plants, Eberhard-Karls-University Tübingen, Germany.

Two cultures were obtained in the context of the present study from fresh basidiomata sampled in Germany designated to *Tulasnella* cf. *pinicola* Bres., DC309 and *T. violea* (Quél.) Bourdot & Galzin, DC292. Isolation was achieved by fixing small pieces of basidiomata to the lid of Petri dishes where they released basidiospores onto solid malt extract agar (MEA) containing 0.1 g/L chloramphenicol for germination.

Morphology and numerical analysis of basidiomata.—Freehand sections of basidiomata of the fresh samples and fungarium material were stained with 1% phloxine. Sections were examined at 100–1000× magnification with a Leitz SM-LUX or Zeiss Axioskop 2 microscope. A provisional morphospecies designation was obtained by using the key provided by Roberts (1999). Morphological descriptions will be presented and discussed in detail elsewhere.

Hyphal diameter, length/width of basidia, sterigmata and spores were measured based on 30 spores and at least 25 other structures per specimen. The shapes of the basidiospores, globose, subglobose, elliptical, oblong, subcylindrical, allantoid, classified according to Largent et al. (1973), and the Q values (length/width of spores; Tulloss and Lindgren 2005) of each specimen were used to calculate a dendrogram based on Euclidean distances (Sokal and

TABLE I. Data on basidiomata for which new sequences (clones) were obtained

Specimen/ voucher	Location and date of collection by DC[a]	Substrate	Preliminary identification	Phylogenetic classification	Culture[b]
(DC157)	ECUADOR Cordillera Real, 16 Jul 2009	On decaying fallen branch	*Tulasnella* sp. ECU1	*T.* sp. ECU1 (3 molecular clones), and *T.* sp. ECU3 (2 clones)	No
(DC177)	ECUADOR Cordillera Real, 27 Oct. 2009	On fallen branch	*T. violea*	*T. violea* (3 molecular clones)	No
(DC185)	ECUADOR Cordillera Real, 16 Jul 2009	On decaying fallen branch	*T.* cf. *eichleriana*	*T.* sp. ECU6 (2 molecular clones)	No
(DC225)	ECUADOR RBSF, 27 Oct 2009	On decaying fallen branch	*Tulasnella* sp. ECU1	*T.* sp. ECU1 (6 molecular clones), and *T.* sp. ECU5 (2 clones)	No
(DC245)	ECUADOR RBSF, 11 Nov 2009	On decayed wood	*Tulasnella* sp. ECU1	*T.* sp. ECU1 (2 molecular clones)	No
(DC262)	ECUADOR RBSF, 13 Nov 2009	On fallen branch	*T.* cf. *eichleriana*	*T.* sp. ECU6 (9 molecular clones)	No
(DC271)	ECUADOR RBSF, 19 Nov 2009	On fallen branch	*T.* cf. *eichleriana*	*T.* sp. ECU4 (5 molecular clones)	No
(DC292)	GERMANY Ginnheim, 21 Mar 2010	On decaying wood	*T. violea*	*T. violea* (14 molecular clones)	Yes (7 molecular clones)
(DC293)	GERMANY Stadtwald, 11 Apr 2010	On decayed wood	*T. violea*	*T. violea* (3 molecular clones)	No
(DC294)	GERMANY Stadtwald, 11 Apr 2010	On decayed wood	*T. eichleriana*	*T. eichleriana* (8 molecular clones)	No
(DC309)	GERMANY Apfelbach, 27 Dec 2010	On decaying wood	*T.* cf. *pinicola*	*T.* cf. *pinicola*	Yes (8 molecular clones)

[a] DC = collector Darío Cruz.

[b] Two pure cultures isolated from basidiomata are indicated. The preliminary identification is based on morphology and the phylogenetic classification on molecular sequences obtained from clones.

Sneath 1963). Between-linkage clustering was applied, and all variables changed to z-scores to yield equal metrics and equal weighting (SUPPLEMENTARY FIG. 1). The means of the length and width of the basidiospores of each specimen were compared by analysis of correlation with simple linear regression (FIG. 1). All numerical analysis were performed with the statistical software SPSS 11.0 (IBM Corp., Somers, New York).

DNA isolation, PCR, cloning and sequencing.—DNA was extracted from fresh basidiomata, dried fungarium material and axenic cultures with a DNeasy Plant Mini Kit (QIAGEN). The DNA amplification, PCR, and cloning conditions are similar to those explained by Cruz et al. (2011), except that the number of colonies checked for cloned ITS amplicons was 10 or 20, (labeled C01–C20 per specimen from basidiomata and C01–C10 per axenic culture). A nested PCR was not necessary for DNA amplification from axenic cultures. The success of each PCR was visualized in 1% agarose containing GelRed 3× nucleic acid stain solution as recommended by the producer (Biotum, Hayward, California). Products were cloned with Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen). Sequencing was carried out with Big Dye Terminator 3.1 and analyzed by a 3500RUO Genetic Analyzer (Applied Biosystems). The sequences obtained in this study are available from GenBank (SUPPLEMENTARY TABLE II). Unfortunately it was not possible to obtain sequences from fungarium specimens, which were 20–40 y old and frequently in poor condition, with collapsed and degraded structures.

Alignment of DNA sequence data and phylogenetic analysis.—The sequence chromatograms were verified with the software Sequencher 4.6 (Gene Codes Corp., Ann Arbor, Michigan). Our 121 new sequences and six sequences obtained from basidiomata provided by M. Bidartondo (*Tulasnella albida* K[M]118140, K[M]120788; *T. eichleriana* K[M]143600; *T. tomaculum* K[M]123675 and *T. violea* K[M]155209, K[M]165206; all named according to their morphology) were aligned separately or with the most similar *Tulasnella* sequences available from GenBank (http://www.ncbi.nlm.nih.gov/). All ITS sequences were

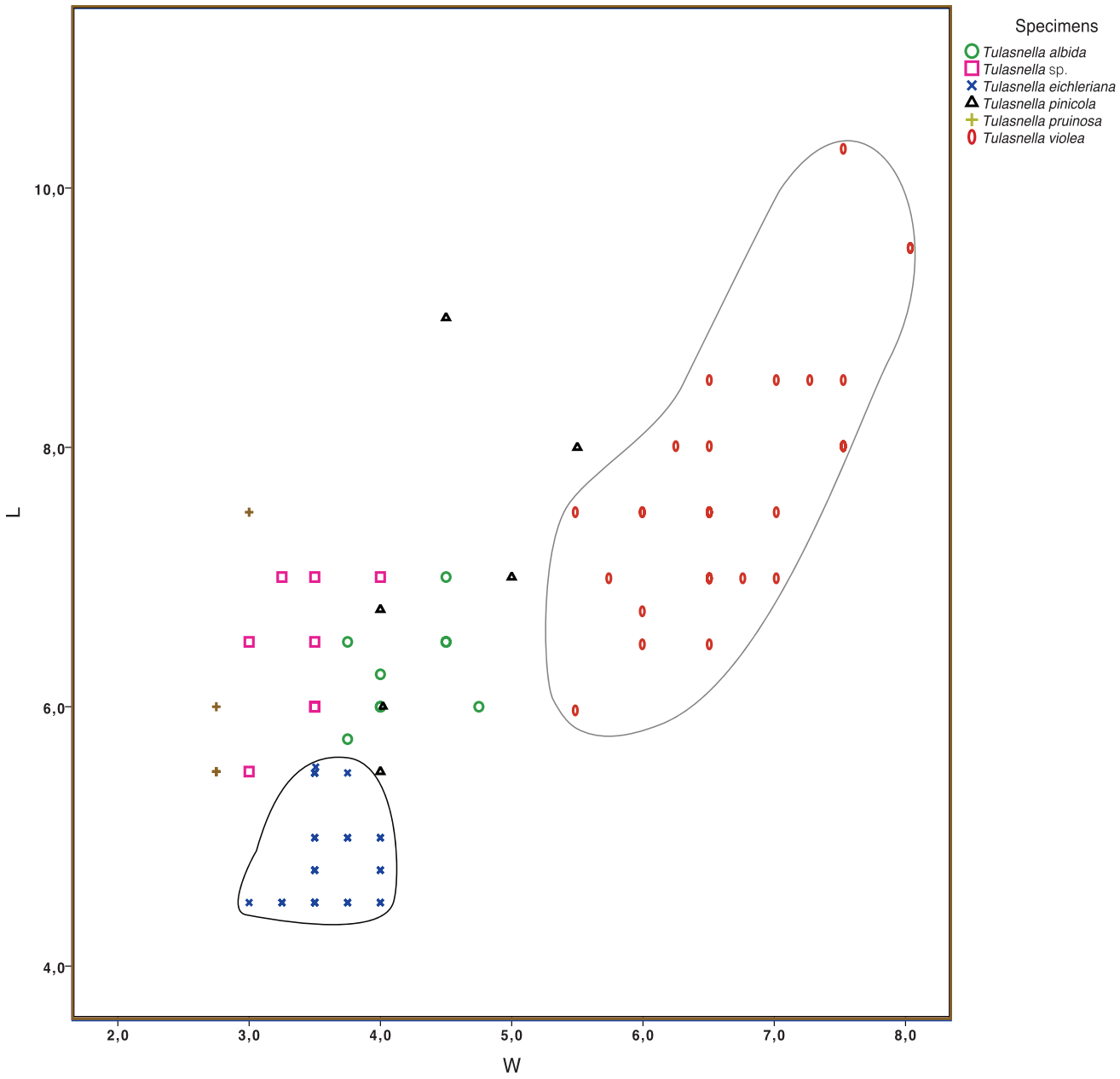

FIG. 1. Dispersion diagram of average dimensions of basidiospores from specimens of *Tulasnella* spp. Symbols represent species that specimens were assigned to based on morphology. Symbols of specimens assigned to *T. eichleriana* are grouped by black line; those of *T. violea* by gray line.

aligned following the multiple analysis method (Lee 2001), using G-INS-i strategy as implemented in MAFFT 5.667 (Katoh et al. 2002), Clustal W (Thompson et al. 1994), and MUSCLE 3.2 (Edgar 2004). The alignments were compared with the program SuiteMSA-1.2 (Anderson et al. 2011) to identify inconsistencies among MSAs (multiple sequence alignments) to choose the best alignment. Putative chimera sequences were analyzed in fragments of 100 bp (base pairs) with BLAST.

Two steps were necessary to calculate the phylogenetic trees because of the heterogeneity of *Tulasnella* sequences (Sharon et al. 2008). A similar method was successfully applied by Suárez et al. (2006), Shefferson et al. (2007), Taylor and McCormick (2008), Nontachaiyapoom et al. (2010) and Cruz et al. (2011). First, a phylogenetic tree was created with the data of the 5.8S region only, about 165 bp, including all new sequences plus six sequences of Cruz et al. (2011) (SUPPLEMENTARY FIG. 2). This tree was used to define well supported groups within which the entire ITS region (approximately 536–878 bp) was realigned. Four separate trees were calculated, including similar sequences from GenBank (FIGS. 2–5).

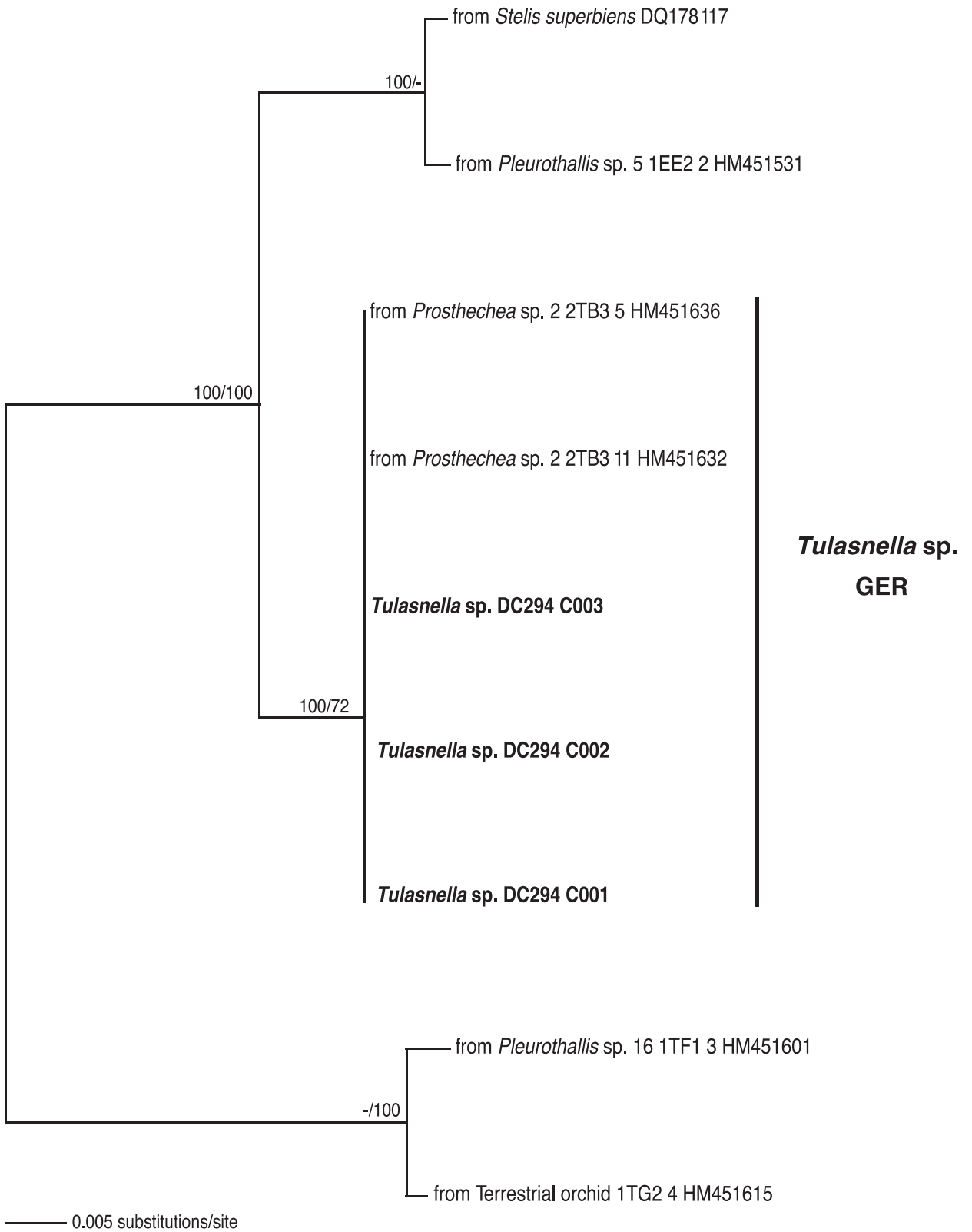

FIG. 2. Phylogenetic tree, group I, predefined by the 5.8S phylogeny (SUPPLEMENTARY FIG. 2). Phylogeny of the realigned ITS-5.8S region includes new sequences of *Tulasnella* spp. and the most similar sequences from GenBank (NCBI). References to new sequences with information about teleomorphic states are in boldface. Values close to the nodes correspond to maximum likelihood bootstrap values (left) and Bayesian posterior probabilities (right; converted to percent). Only values larger than 70% are shown. Tree is rooted at its midpoint. GER = Germany.

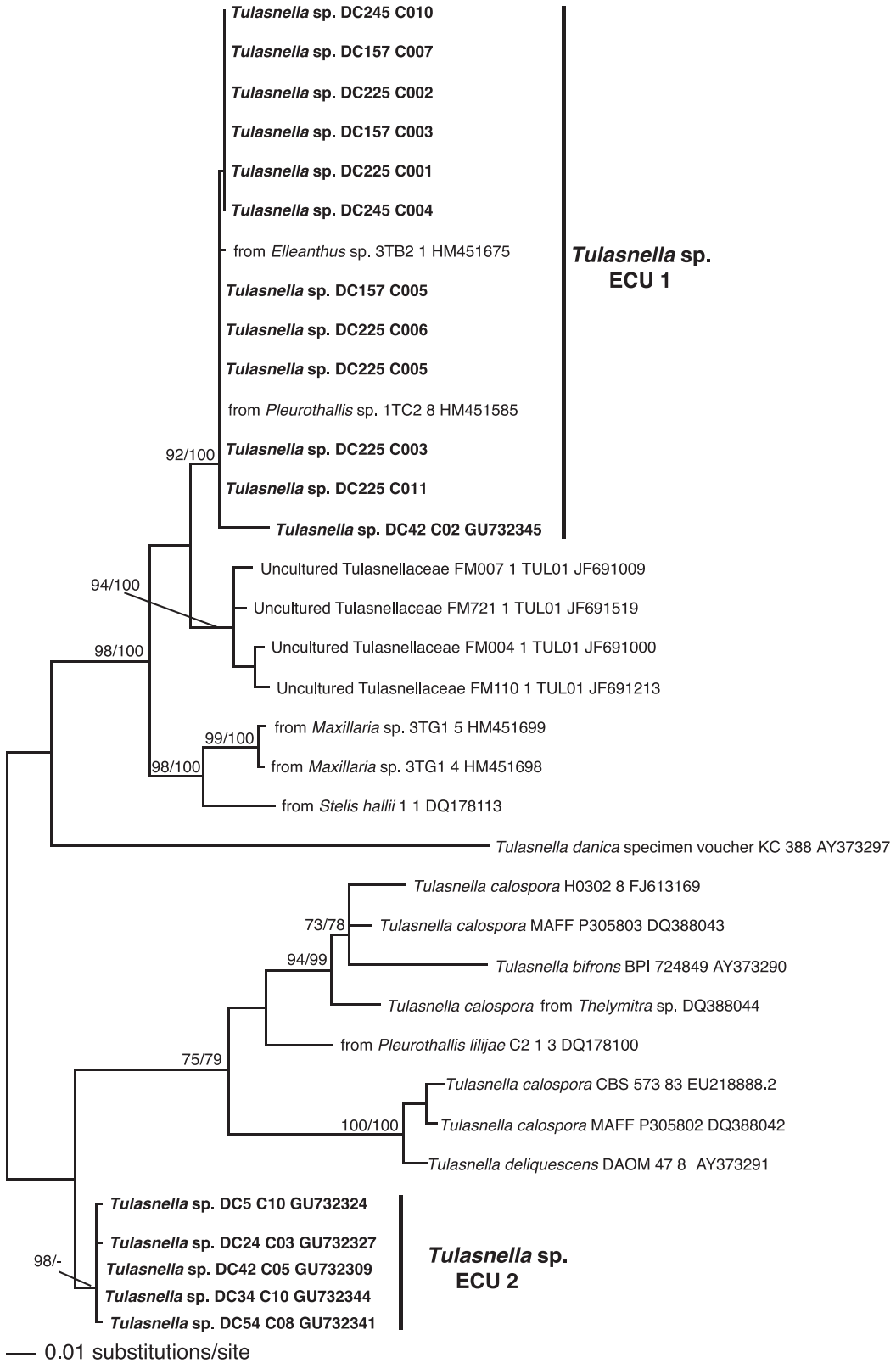

FIG. 3. Phylogenetic tree, group II, predefined by the 5.8S phylogeny (SUPPLEMENTARY FIG. 2). Phylogeny of the realigned ITS-5.8S region includes new sequences of *Tulasnella* spp. and the most similar sequences from GenBank (NCBI). References to new sequences with information about teleomorphic states are in boldface. Values close to the nodes correspond to maximum likelihood bootstrap values (left) and Bayesian posterior probabilities (right; converted to percent). Only values larger than 70% are shown. Tree is rooted at its midpoint. ECU = Ecuador.

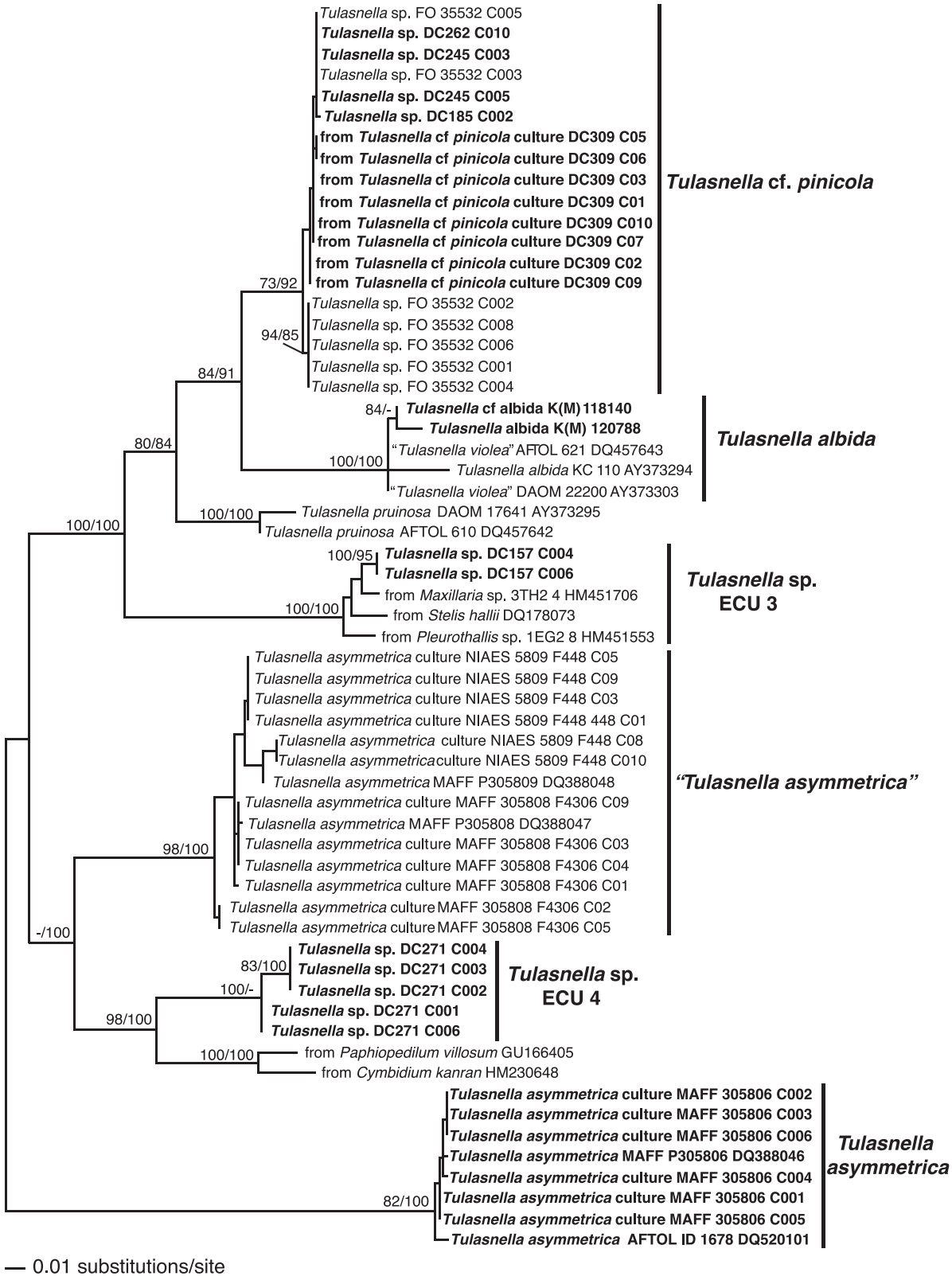

FIG. 4. Phylogenetic tree, group III, predefined by the 5.8S phylogeny (SUPPLEMENTARY FIG. 2). Phylogeny of the realigned ITS-5.8S region includes new sequences of *Tulasnella* spp. and the most similar sequences from GenBank (NCBI). References to new sequences with information about teleomorphic states are in boldface. Values close to the nodes correspond to

Phylogenetic calculations were performed by the programs RAxML 7.0.4 (Stamatakis 2006) and MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). Maximum likelihood (ML) analysis in RAxML used the GTRMIX DNA substitution model with 1000 bootstrap (BS) replicates (Felsenstein 1985). For the Bayesian posterior probability (PP) analysis, we used the most complex substitution model available (GTR+I+G) following Whelan et al. (2001), Douady et al. (2003) and Huelsenbeck and Rannala (2004), including two runs each involving four incrementally heated Markov chains over 4 000 000 generations and using random starting trees. Trees were sampled every 100 generations resulting in a total of 40 000 trees from which the last 24 000 were used to compute a 50% majority-rule consensus tree. Stationarity of the process and effective sample size (ESS) values were checked visually with the software Tracer 1.5 (Drummond and Rambaut 2007).

All trees presented here correspond to the best trees resulting from ML analysis. In total five phylogenetic trees (FIGS. 2–5, SUPPLEMENTARY FIG. 2) were calculated with 127 sequences consistently aligned in MAFFT (Katoh et al. 2002).

The pairwise differences of the ITS sequences were calculated with MEGA 5 software (Tamura et al. 2011) to evaluate the intragenomic variation from cultures as well as intraspecific and interspecific variation among specimens (basidiomata) and phylogenetic species. For genetic divergence estimation we employed the Kimura-2-parameter distances (Kimura 1980) with gap deletion. The ''barcode gap'' (Meyer and Paulay 2005) was estimated between the maximal intraspecific variation and the minimal interspecific variation (Meier et al. 2008).

RESULTS

Numerical analysis of spore shapes and sizes.—The 91 specimens were provisionally assigned to six species of *Tulasnella* (*T. albida*, *T. eichleriana*, *T. pinicola*, *T. pruinosa*, *T. violea*, *T.* sp.) based on morphology and the measurements of the structures (SUPPLEMENTARY TABLE I). The dendrogram based on shape and size of basidiospores revealed six groups, most of them heterogeneous with respect to the provisional, morphological species concepts (SUPPLEMENTARY FIG. 1). Classification of spore shapes is also largely inconsistent with the numerical classification in the dendrogram. Only the group of unnamed *Tulasnella* sp., consisting of the specimens DC5, DC24, DC34, DC42, DC54, DC157, DC225 and DC245 sampled in Ecuador, is consistent in shape and size for all specimens.

The dispersion diagram, based on correlation of width and length of basidiospores, separated *T. eichleriana* and *T. violea* specimens, but the others formed a mixed group (FIG. 1). Basidiospore size differences are obviously insufficient to distinguish clearly between *T. albida* Bourdot & Galzin, *T. pinicola*, *T. pruinosa* and *T.* sp.

Phylogenetic and pairwise distance analysis.—The phylogenetic tree based on 127 sequences of the conserved 5.8S region is composed of four groups (I–IV), well supported (73–100% BS and PP) by maximum likelihood bootstrap values and Bayesian posterior probabilities respectively (SUPPLEMENTARY FIG. 2). With Bayesian analysis we obtained ESS values of 8749.9 for Group I, 1196.7 for Group II, 364.6 for Group III and 370.9 for Group IV. The standard deviations of split frequencies, determined by MrBayes 3.1.2 at the end of the Bayesian analyses, were 0.001 for Group I, 0.003 for Group II, 0.009 for Group III and 0.01for Group IV. Within these groups, phylogenetic trees revealed well supported clades (FIGS. 2–5).

Group I (FIG. 2) called *Tulasnella* sp. GER includes sequences of three clones from specimen DC294 sampled in Germany and two sequences of mycobionts of *Prostechea* sp. (Orchidaceae) sampled in Ecuador. Further orchid mycobionts yielded closely related sequences. Other clones from the specimen DC294 are located in the *T. eichleriana* clade of Group IV (FIG. 5).

Group II (FIG. 3) contains two separated clades of specimens sampled in Ecuador, *Tulasnella* sp. ECU 1 and *T.* sp. ECU 2, without close affinities to known morphological or sequenced *Tulasnella* species. *Tulasnella* sp. ECU 1 also includes sequences of orchid mycobionts from Ecuador (*Elleanthus* sp. and *Pleurothallis* sp.). The clade *T.* sp. ECU 2 includes sequences from specimens of a *Tulasnella* sp. presented by Cruz et al. (2011), and it is closely related to sequences isolated from *Pleurothallis lilijae* Foldats and other orchids, as well as sequences of *Tulasnella* spp. named *T. bifrons* Bourdot & Galzin, *T. calospora* (Boud.) Juel and *T. deliquescens* (Juel) Juel.

Group III (FIG. 4) contains two distant clades for sequences obtained from cultures named *T. asymmetrica*. The clade of *Tulasnella* cf. *pinicola* is mainly formed by sequences obtained from a pure culture

←

maximum likelihood bootstrap values (left) and Bayesian posterior probabilities (right; converted to percent). Only values larger than 70% are shown. Dubious species names are between inverted commas. Tree is rooted at its midpoint. ECU = Ecuador.

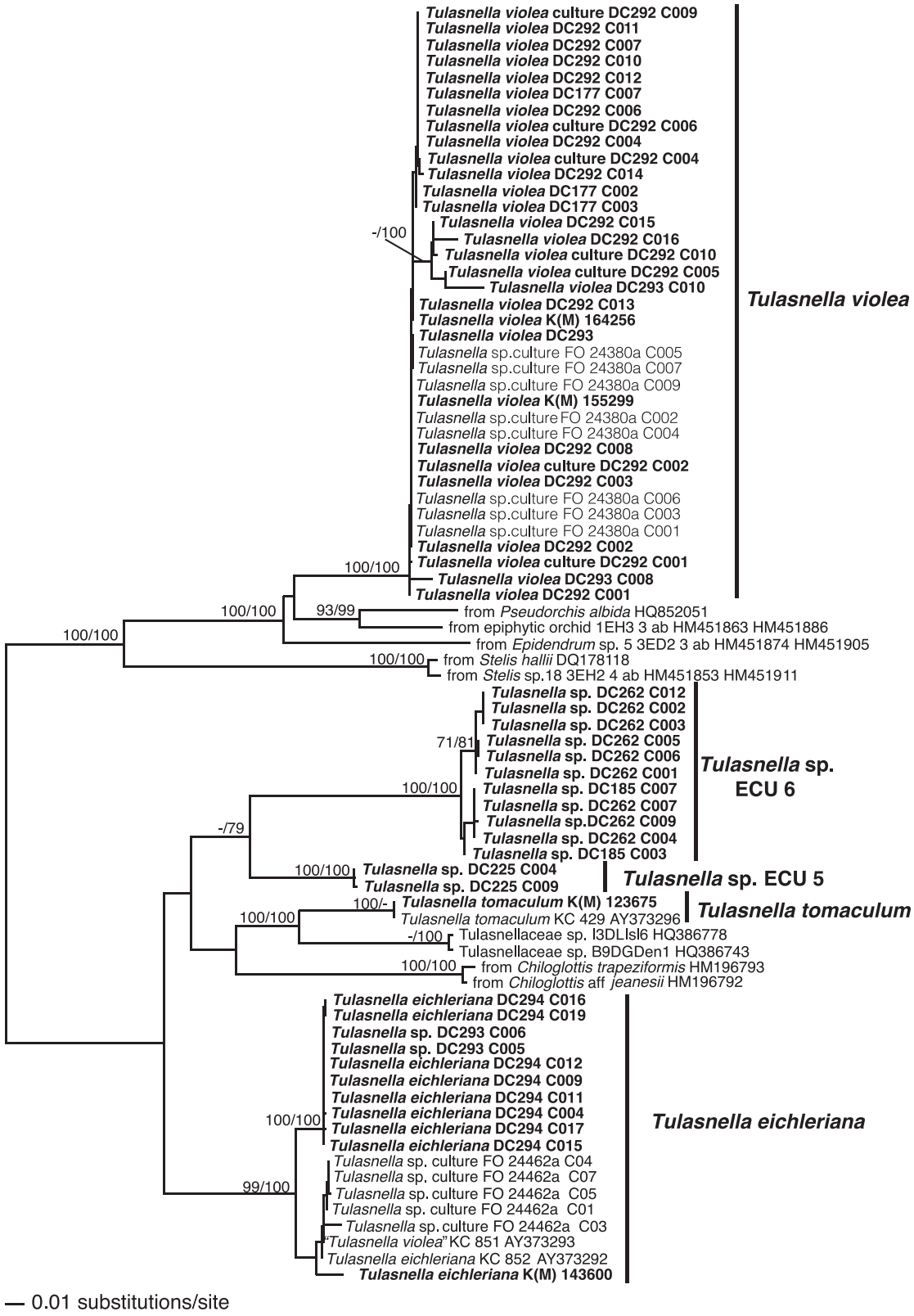

FIG. 5. Phylogenetic tree, group IV, predefined by the 5.8S phylogeny (SUPPLEMENTARY FIG. 2). Phylogeny of the realigned ITS-5.8S region includes new sequences of *Tulasnella* spp. and the most similar sequences from GenBank (NCBI). References to new sequences with information about teleomorphic states are in boldface. Values close to the nodes correspond to

TABLE II. Intragenomic variation in cultures, as well as intra- and interspecific sequence variability among specimens defined as morphospecies of *Tulasnella* within the respective groups. Pairwise distances were calculated in MEGA 5 software (Tamura et al. 2011) with Kimura-2-parameter distances (Kimura 1980) with gap deletion

Group	Name of the clade	No. of specimens in each clade	Length of ITS-5.8S in base pairs	Intragenomic difference	Intraspecific difference	Interspecific difference
I	*Tulasnella* sp. GER	1	536	—	—	—
II	*Tulasnella* sp. ECU 1	4	564	—	0–3%	9–13%
	Tulasnella sp. ECU 2	5		—	0–3%	
III	*Tulasnella albida*	2	582	—	0–1%	12–44%
	Tulasnella sp. ECU 3	1		—	—	
	Tulasnella asymmetrica	1		0–1%[a1]	0–1%	
	"Tulasnella asymmetrica"	2		0–2%[a2]	0–3%	
				0–2%[a3]		
	Tulasnella sp. ECU 4	1		—	—	
	Tulasnella cf. *pinicola*	3		0%[a4]	0–1%	
				0–1%[a5]		
IV	*Tulasnella* sp. ECU 5	1	878	—	—	11–43%
	Tulasnella eichleriana	3		0–1%[a6]	0–4%	
	Tulasnella sp. ECU 6	2		—	0–2%	
	Tulasnella tomaculum	1		—	—	
	Tulasnella violea	6		0%[a7]	0–3%	
				0–1%[a8]		

[a] Pure cultures: [1] MAFF 305806, [2] MAFF 305808, [3] MAFF 305809, [4] DC309, [5] FO 35532, [6] FO 24462a, [7] FO 24380a, [8] DC292.

isolated from a basidioma morphologically identified as *T.* cf. *pinicola* by us (DC309) and culture FO 35532 from a basidioma of *Tulasnella* sp. (non vid.) closely related to the clade *T. albida*. From basidioma DC309 no sequence was obtained directly. Two further clades, *T.* sp. ECU 3 and *T.* sp. ECU 4, are without known affinities. Clade *T.* sp. ECU 3 consists of two clones from specimen DC157 (C004, C006) and sequences obtained from species of Orchidaceae from Ecuador (*Maxillaria* sp., *Stelis hallii*, *Pleurothallis* sp.). Other clones from specimen DC157 form part of the clade *T.* sp. ECU 1 in Group II (FIG. 3). Specimen DC271 in clade *T.* sp. ECU 4 was determined provisionally to be *T. eichleriana* according to morphology.

Group IV (FIG. 5) contains sequences considered to represent *T. violea*, obtained from five basidiomata from Ecuador (DC177), Germany (DC292, DC293), England (K[M] 15529) or Wales (K[M] 164256), as well as sequences obtained from two pure cultures, one isolated from the basidioma *T. violea* DC292 and the culture FO 24380a isolated from a specimen of *Tulasnella* sp. (non vid.). The clade delegated to *T. eichleriana* consists of sequences from basidioma DC294 and culture FO 24462a obtained from a basidioma of *Tulasnella* sp. (non vid.). Clade *T.* sp. ECU6 with sequences from basidiomata lacks morphological identification, although specimen DC262 was preliminarily determined to be *T. eichleriana*. Clade *T.* sp. ECU 5 contains only two clones from specimen DC225 (C004, C009), but most of the clones from this specimen fall into clade *T.* sp. ECU 1 of Group II (FIG. 3).

Genetic variation, based on the new ITS sequences of fresh basidiomata and cultures and some sequences from basidiomata (Cruz et al. 2011) is provided (TABLE II). Intragenomic variability among sequences from individual cultures is 0–2%. The pairwise differences between sequences from specimens considered as a certain species have a sequence variability of 0–1% in *T. albida*, *T. asymmetrica* and *T. pinicola*, 0–3% in T. sp., ''*T. asymmetrica*'' and *T. violea*. The highest variation (4%) occurs in *T. eichleriana*. The interspecific divergences are at least 9% and can reach 44%. The lowest variation, 9–13%, occurs between the closely related *Tulasnella* spp. ECU1 and ECU2 from Ecuador (Group II).

←

maximum likelihood bootstrap values (left) and Bayesian posterior probabilities (right; converted to percent). Only values larger than 70% are shown. Dubious species names are between inverted commas. Tree is rooted at its midpoint. ECU = Ecuador.

The supported clades in each group containing sequences with up to 4% of proportional differences are considered phylogenetic species and are named according to the dominant species where possible. Clades lacking named species of *Tulasnella* were named according to the country of origin (ECU Ecuador, GER Germany).

DISCUSSION

Morphology.—Genus *Tulasnella* has been studied morphologically and illustrated by Bourdot and Galzin (1909, 1928); Rogers (1933); Olive (1957); Warcup and Talbot (1967, 1971, 1980); Jülich and Jülich (1976); Roberts (1992, 1993a, 1993b, 1994a, 1994b, 1999), van de Put and Antonissen (1996) and Ordynets (2012). Even with different species concepts (Bourdot and Galzin, 1909, 1928; Rogers 1933), size and shape of spores were emphasized as the principal criteria for delimiting species. Our data on the size and shape of hyphae, basidia, sterigmata and basidiospores, based on samples collected in Ecuador and Germany, distinguished *T. eichleriana*, *T.* cf. *pinicola*, *T.* sp. and *T. violea*. However, the morphological structures of *Tulasnella* often overlap in shape and size among species, making the correct identification problematic (Roberts 1994b). This situation is demonstrated here for basidiospores of *Tulasnella albida*, *T. pruinosa* and *T. pinicola*. Our analysis suggests that morphological characteristics alone are insufficient to delimit species of *Tulasnella*, a situation ubiquitous within the Basidiomycota.

Phylogeny.—The molecular data obtained from cultures and basidiomata of *Tulasnella* spp. let us identify genetic distances within and between species and to compare thresholds at different levels. Sequences of *Tulasnella* spp. obtained from clones of pure cultures in this study showed up to 2% intragenomic variation (TABLE II). The result is close to the 1.8% of intragenomic variation found for many isolates of *Tulasnella* spp. in consensus analysis of multiple loci (Linde et al. 2013). A similar and even higher variation was observed for species of *Laetiporus* after cloning the ITS region from pure cultures (Lindner and Banik 2011). The sequencing artifact produced during PCR was reported to be less than 1% (Acinas et al. 2005). We cannot discard Taq misreading during PCR amplification, which could inflate the intragenomic variation, but we used high-fidelity Taq to minimize PCR misreading (Simon and Weiss 2008); so this is unlikely to be the only source of variation.

The 4% of intraspecific variation indicates that variation among *Tulasnella* species can be higher than 3%, the widely accepted divergence threshold for defining phylogenetic species in other groups of fungi (Nilsson et al. 2008) or for estimating diversity, for example in Sebacinales (Setaro et al. 2011). They are, however, within the range of the less than 3–5% threshold for defining operational taxonomic units (OTUs) molecularly in *Tulasnella* as applied and discussed by Girlanda et al. (2011), Jacquemyn et al. (2011), Martos et al. (2012) and Linde et al. (2013). Here the so-called barcode gap used to delimit species in other organisms (Meyer and Paulay 2005, Meier et al. 2008, Ni et al. 2012, Puillandre et al. 2012) was detected between the maximum intraspecific variation of 4% (0.8% mean, SD 1.2%) and the minimum 9% interspecific divergence. If we apply the rule 10×, using the mean of intraspecific variation to find a threshold suitable to delimit species (Hebert et al. 2004), our threshold to delimit species in *Tulasnella* would be 8%, a value lower than the minimum 9% interspecific variation we observed. More extensive and detailed studies of other species of *Tulasnella* are necessary to determine whether a universal barcode gap exists in *Tulasnella*.

The phylogenetic trees revealed cryptic species of *Tulasnella*. *Tulasnella* sp. ECU 1 in Group II is indistinguishable morphologically from *T.* sp. ECU 2 (Cruz et al. 2011). *Tulasnella* sp. ECU 4 and *T.* sp. ECU 6 in groups III and IV were both determined to be *T. eichleriana* (FIG. 3). A further species is revealed from clones of a specimen sampled in Germany (*T.* sp. GER; FIG. 2).

Six of the seven specimens from Ecuador may represent new species or known species lacking molecular data. To our knowledge, molecular sequences including several genes are available in the NCBI nucleotide databases for no more than 13 described *Tulasnella* species (GenBank; http://www.ncbi.nlm.nih.gov/). Many sequences obtained from basidiomata of *Tulasnella* spp. in clades *T.* sp. GER (FIG. 2), *T.* sp. ECU 1 (FIG. 3) and *T.* sp. ECU 3 (FIG. 4) were grouped together with sequences obtained from orchid mycorrhiza sampled from the same site in Ecuador by Suárez et al. (2006) and P. Herrera (pers comm). These data suggest high mycorrhizal diversity within orchid-associated *Tulasnella* spp.. Considering the dependence of orchids on mycobionts, and *Tulasnella* in particular, many more *Tulasnella* species are expected for Ecuador and other regions that are species rich in the Orchidaceae. Suárez et al. (2008) already estimated a high number of *Tulasnella* species based on nucLSU D1/D2 sequence data.

In the present study we obtained several clades where our sequences clustered with sequences assigned to certain species of *Tulasnella*. We critically

examined these clades to decide about correct naming. The clade *T. eichleriana* supported with ML BS (99%) and Bayesian PP (100%) (FIG. 5) is thought to represent this species. All sequences in this clade were obtained from pure cultures or specimens isolated or collected in European countries. The strain *T. eichleriana* KC 852 (AY373292) from England (McCormick et al. 2004) was isolated from the basidioma identified as *T. eichleriana* K(M) 30990 (non vid.). New sequences were obtained from a basidioma DC294, from Germany, morphologically identified as *T. eichleriana* by us. The type material of *T. eichleriana* (non vid.) was collected in Poland and described by Bresadola (1903). The congruence of morphological, phylogenetic and geographic data supports our conclusion. The clade containing sequences of *T. asymmetrica* in Group III (FIG. 4), well supported by ML, BS (82%) and Bayesian PP (100%), is considered the correct clade for this species. The grouping of sequences from strains of culture 085 Warcup and Talbot (= MAFF 305806) designated type material in the description by Warcup and Talbot (1967) supports this assignment. The clade ''*Tulasnella asymmetrica*'' (Group III) is considered to be named erroneously. This clade differs from the correct *T. asymmetrica* clade (FIG. 4) by more than 12% sequence divergence with sequences obtained from the type culture of *T. asymmetrica* 085 Warcup and Talbot (= MAFF 305806). This clade includes sequences obtained from the pure cultures 0302 and 0591 Warcup and Talbot (= MAFF 305808 and MAFF 305809 respectively), which lack information on their teleomorphs. Warcup and Talbot (1967) did not include these cultures in the morphological description of the species. Specimens in the ''*T. asymmetrica*'' and *T. asymmetrica* clades are related to each other morphologically by similar growth of their anamorphs in culture (pers obs), so ''*T. asymmetrica*'' probably represents a cryptic species.

We propose a clade for *Tulasnella violea* in Group IV (FIG. 5), including sequences from five basidiomata that correspond to the morphological concept of this species (Bourdot and Galzin 1909; Roberts 1994b, 1999). However, a sequence obtained from a pure culture isolated from the basidioma named ''*T. violea*'' KC 851 AY373293 England by McCormick et al. (2004) is grouped within the clade of *T. eichleriana* (Group IV). Either the specimen was misidentified as *T. violea* or the culture from which the sequence was obtained by McCormick et al. (2004) does not correspond to the basidioma morphologically observed. The sequence ''*T. violea*'' KC 851 AY373293 is identical to sequence *T. eichleriana* AY373292 KC 852 (McCormick et al. 2004), as already noted by Sharon et al. (2008). The clades named *T. albida* and *T.* cf. *pinicola* in Group III and *T. tomaculum* in Group IV are designated with the same name according to sequences obtained from basidiomata assigned to these species and their affinity to sequences obtained from pure culture isolates from basidiomata named as these respective species (McCormick et al. 2004).

Several phylogenetic inconsistencies observed for sequences of *Tulasnella* species are not clarified by the data and interpretation compiled in this study. Some sequences obtained from one individual basidioma clustered in clades other than the recognized morphospecies. For example, sequences DC185 C002 and DC262 C010 obtained from two specimens of *Tulasnella* sp. ECU6 are grouped within the clade of *T.* cf. *pinicola* Group III (FIG. 4), sequences DC245 C003 and C005 obtained from a specimen *Tulasnella* sp. ECU 1 are grouped within the clade of *T.* cf. *pinicola* Group III (FIG. 4) and sequences DC293 C003 and C006 obtained from a specimen of *Tulasnella violea* are grouped within the clade *T. eichleriana* Group IV (FIG. 5). A potential explanation may be that several species of *Tulasnella* are growing intermixed. We observed several times that more than one morphospecies of *Tulasnella* can grow on the same piece of wood or the same substrate. Roberts (1993a, b; 1994a, b; 1999) described several specimens of *Tulasnella* where different species of *Tulasnella* or other resupinate fungi were growing intermixed on the same substrate. In tropical habitats, where orchids are abundant and harbor a multitude of *Tulasnella* mycobionts (Kottke et al. 2013), it is a realistic possibility that more than one species of *Tulasnella* establishes on the same substrate. When the total DNA is extracted from one extremely thin basidioma of *Tulasnella*, DNA of more than one species of *Tulasnella* is frequently found. Further studies incorporating, for example, the analysis of secondary structure of the ITS2 region as already analyzed in Lycoperdaceae (Basidiomycota) by Krüger and Cargas (2008) may help confirm the species concept and determine whether the sequences in different clades obtained in this study from the same basidioma are interrelated as conspecific. In addition, mating compatibility tests could be conducted (Lombard et al. 2010). However, both methods are difficult to perform with *Tulasnella* spp., the first because of the high variability of the ITS region (Suárez pers comm) and the second because of difficulties in isolation and slow growth of cultures.

The integration of morphological and molecular data as applied in this study highlights the genetic and morphological variability in *Tulasnella* spp., identified cryptic species and improved the interpretation of species based on morphology and phyloge-

netic concepts. The combination of morphological and molecular criteria lets us expand our vision toward a more realistic interpretation of diversity in this neglected group of fungi.

ACKNOWLEDGMENTS

The authors thank Martin Bidartondo for providing six sequences of *Tulasnella* included in the phylogenetic analysis. We also appreciate the help by Roland Kirschner who let us revise his personal collection of *Tulasnella* and Franz Oberwinkler who provided samples of *Tulasnella* collected by him, deposited at the Eberhard-Karls University of Tübingen, Germany. We are grateful for the help of Andreas Beck, Ralph Mangelsdorff for critical comments on the manuscript and Jascha Weisenborn for help and assistance in the laboratory in Germany. The research was generously supported by Deutsche Forschungsgemeinschaft (DFG RU 816) and German Academic Exchange Service (DAAD). Nature and Culture International (NCI) provided research facilities at RBSF.

LITERATURE CITED

Acinas SG, Sarma-Rupavtarm R, Klepac-Ceraj V, Polz MF. 2005. PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. Appl Environ Microbiol 71:966–8969, doi:10.1128/AEM.71.12.8966-8969.2005

Anderson C, Strope CL, Moriyama EN. 2011. Suite MSA: visual tools for multiple sequence alignment comparison and molecular sequence simulation. BMC Bioinformatics 12:184, doi:10.1186/1471-2105-12-184

Beck E, Makeschin F, Haubrich F, Richter M, Bendix J, Valarezo C. 2008. The ecosystem (Reserva Biológica San Francisco). In: Beck E, Bendix J, Kottke I, Makeschin F, Mosandl R, eds. Gradients in a tropical mountain ecosystem of Ecuador. Ecological studies No. 198. Berlin, Heidelberg, New York: Springer. p 1–14.

Bickford D, Lohman DL, Sodhi NS, Ng KL, Meier N, Winker K, Ingram KK, Das I. 2007. Cryptic species as a window on diversity and conservation. Trends Ecol Evol 22:148–155, doi:10.1016/j.tree.2006.11.004

Bourdot H, Galzin A. 1909. Hymenomycetes de France I. Heterobasidiés. Bull Soc Mycol Fr 25:15–36.

———, ———. 1928. Hymenomycetes de France. Heterobasidiés III. *Tulasnellaceae*. Bibliothèque Mycologique. J. Cramer, ed. Vol. 23 (reprint 1969).

Bresadola J. 1903. Fungi polonici. a cl. viro B. Eichler lecti. Ann Mycol 1:65–131.

Cameron DD, Leake JR, Read DJ. 2006. Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. New Phytol 171: 405–416, doi:10.1111/j.1469-8137.2006.01767.x

Cruz D, Suárez JP, Kottke I, Piepenbring M, Oberwinker F. 2011. Defining species in *Tulasnella* by correlating morphology and nrDNA ITS-5.8s sequence data of basidiomata from a tropical Andean forest. Mycol Prog 10:229–238, doi:10.1007/s11557-010-0692-3

Douady CJ, Delsuc F, Boucher Y, Doolittle WF, Douzery EJP. 2003. Comparison of Bayesian and maximum likelihood bootstrap measures of phylogenetic reliability. Mol Biol Evol 20:248–254, doi:10.1093/molbev/msg042

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214, doi:10.1186/1471-2148-7-214

Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinf 5:113, doi:10.1186/1471-2105-5-113

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791, doi:10.2307/2408678

Gazis R, Rehner S, Chaverri P. 2011. Species delimitation in fungal endophyte diversity studies and its implications in ecological and biogeographic inferences. Mol Ecol 20:3001–3013, doi:10.1111/j.1365-294X.2011.05110.x

Girlanda M, Segreto R, Cafasso D, Liebel HB, Rodda M, Ercole E, Cozzolino S, Gebauer G, Perotto AS. 2011. Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations. Am J Bot 98:1148–1163, doi:10.3732/ajb.1000486

Greslebin GA, Rajchenberg M. 2001. The genus *Tulasnella* with a new species in the Patagonian Andes forests of Argentina. Mycol Res 105:1149–1151, doi:10.1016/S0953-7562(08)61980-2

Hebert PDN, Stockle MY, Zemlak TS, Francis CM. 2004. Identification of birds through DNA barcodes. PLoS Biol 2:312, doi:10.1371/journal.pbio.0020312

Hibbett DS, Binder MJF, Bischoff M, Blackwell PF, Cannon OE, Eriksson S, Huhndorf et al. 2007. A higher-level phylogenetic classification of the Fungi. Mycol Res 111: 509–547, doi:10.1016/j.mycres.2007.03.004

Homeier J, Werner FA. 2008. Spermatophyta checklist—Reserva Biológica San Francisco (Prov. Zamora-Chinchipe, s. Ecuador). In: Liede-Schumann S, Breckle SW, eds. Provisional checklist of flora and fauna of the San Francisco Valley and its surroundings. Ecotropical monographs No. 4. Ulm, Germany, Society of Tropical Ecology. p 15–58.

Huelsenbeck JP, Rannala B. 2004. Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. Syst Biol 53:904–913, doi:10.1080/10635150490522629

———, Ronquist FR. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755, doi:10.1093/bioinformatics/17.8.754

Jacquemyn H, Honnay O, Cammue BPA, Brys R, Lievens B. 2010. Low specificity and nested subset structure characterize mycorrhizal associations in five closely related species of the genus *Orchis*. Mol Ecol 19:4086–4095, doi:10.1111/j.1365-294X.2010.04785.x

———, Merckx V, Brys R, Tyteca D, Cammue BPA, Honnay O, Lievens B. 2011. Analysis of network architecture reveals phylogenetic constraints on mycorrhizal speci-

ficity in the genus *Orchis* (Orchidaceae). New Phytol 192:518–528, doi:10.1111/j.1469-8137.2011.03796.x

Jülich W, Jülich U. 1976. A contribution toward a revision of the genus *Tulasnella*. Persoonia 2:49–64.

Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transformation. Nucleic Acids Res 30:3059–3066, doi:10.1093/nar/gkf436

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120, doi:10.1007/BF01731581

Kottke I, Beiter A, Weiß M, Haug I, Oberwinkler F, Nebel M. 2003. Heterobasidiomycetes form symbiotic associations with hepatics: Jungermanniales have sebacinoid mycobionts while *Aneura pinguis* (Metzgeriales) is associated with a *Tulasnella* species. Mycol Res 107: 957–968, doi:10.1017/S0953756203008141

———, Setaro S, Haug I, Herrera P, Cruz D, Fries A, Gawlik J, Homeier J, Werner F, Gerique A, Suárez JP. 2013. Mycorrhiza networks promote biodiversity and stabilize the tropical mountain rain forest ecosystem—perspectives for understanding complex communities. In: Ecosystem services, biodiversity and environmental change in a tropical mountain ecosystem of south Ecuador. Ecological studies. Heidelburg, Germany: Springer Verlag.

Krüger D, Gargas A. 2008. Secondary structure of ITS2 rRNA provides taxonomic characters for systematic studies—a case in Lycoperdaceae (Basidiomycota). Mycol Res 112:316–330.

Largent DL, Johnson D, Watling R. 1973. How to identify mushrooms to genus III: microscopic features. Eureka, California: Mad River Press. 148 p.

Lee MSY. 2001. Unalignable sequences and molecular evolution. Trends Ecol Evol 16:681–685, doi:10.1016/S0169-5347(01)02313-8

Linde CC, Phillips RD, Crisp MD, Peakall R. 2013. Congruent species delineation of *Tulasnella* using multiple loci and methods. New Phytol, doi:10.1111/nph.12492

Lindner DL, Banik MT. 2011. Intragenomic variation in the ITS rDNA region obscures phylogenetic relationships and inflates estimates of operational taxonomic units in genus *Laetiporus*. Mycologia 103:731–740, doi:10.3852/10-331

Lombard L, Crous PW, Wingfield BD, Wingfield MJ. 2010. Multigene phylogeny and mating tests reveal three cryptic species related to *Calonectria pauciramosa*. Stud Mycol 66:15–30, doi:10.3114/sim.2010.66.02

Lowy B. 1964. A new genus of the Tulasnellaceae. Mycologia 56:696–700, doi:10.2307/3756621

Martin GW. 1939. New or noteworthy fungi from Panama and Colombia III. Mycologia 31:239–249, doi:10.2307/3754518

Martos F, Munoz F, Pailler T, Kottke I, Gonneau C, Selosse MA. 2012. The role of epiphytism in architecture and evolutionary constraint within mycorrhizal networks of tropical orchids. Mol Ecol 21:5098–5109, doi:10.1111/j.1365-294X.2012.05692.x

McCormick MK, Whigham DF, O'Neill J. 2004. Mycorrhizal diversity in photosynthetic terrestrial orchids. New Phytol 163:425–438, doi:10.1111/j.1469-8137.2004.01114.x

Meier R, Zhang G, Ali F. 2008. The use of mean instead of smallest interspecific distances exaggerates the size of the ''barcoding gap'' and leads to misidentification. Syst Biol 57:809–813, doi:10.1080/10635150802406343

Meyer CP, Paulay G. 2005. DNA barcoding: error rates based on comprehensive sampling. PLoS Biol 3:2229–2238.

Moncalvo JM, Nilsson RH, Koster B, Dunham SM, Bernauer T, Matheny PB, Porter TM, et al. 2006. The cantharelloid clade: dealing with incongruent gene trees and phylogenetic reconstruction methods. Mycologia 98: 937–948, doi:10.3852/mycologia.98.6.937

Ni L, Li Q, Kong L, Huang S, Li L. 2012. DNA barcoding and phylogeny in the family Mactridae (Bivalvia: Heterodonta): evidence for cryptic species. Biochem Sys Ecol 44:164–172, doi:10.1016/j.bse.2012.05.008

Nilsson RH, Kristiansson E, Ryberg M, Hallenberg N, Larsson KH. 2008. Intraspecific ITS variability in the kingdom Fungi as expressed in the internal sequence databases and its implications for molecular species identification. Evol Bioinform 4:193–201.

Nontachaiyapoom S, Sasirat S, Manoch L. 2010. Isolation and identification of Rhizoctonia-like fungi from roots of three orchid genera, *Paphiopedilum*, *Dendrobium* and *Cymbidium*, collected in Chiang Rai and Chiang Mai provinces of Thailand. Mycorrhiza 20:459–471, doi:10.1007/s00572-010-0297-3

Olive LS. 1957. Tulasnellaceae of Tahiti. A revision of the family. Mycologia 49:663–679, doi:10.2307/3755985

Ordynets O. 2012. New records of corticioid fungi with heterobasidia from Ukraine. Turk J Bot, doi:10.3906/bot-1109-1

Preußing M, Nebel M, Oberwinkler F, Weiß M. 2010. Diverging diversity patterns in the *Tulasnella* (Basidiomycota, Tulasnellales) mycobionts of Aneura pinguis (Marchantiophyta, Metzgeriales) from Europe and Ecuador. Mycorrhiza 20:147–59, doi:10.1007/s00572-009-0275-9

Puillandre N, Modica MV, Zhang Y, Sirovich L, Boisselier MC, Cruaud C, Holford M, Samadi S. 2012. Large-scale species delimitation method for hyperdiverse groups. Mol Ecol 21:2671–2691, doi:10.1111/j.1365-294X.2012.05559.x

Roberts P. 1992. Spiral-spored *Tulasnella* species from Devon and the New Forest. Mycol Res 96:233–236, doi:10.1016/S0953-7562(09)80973-8

———. 1993a. The genus *Tulasnella* in Norway. Windahlia 20:67–73.

———. 1993b. Allantoid-spored *Tulasnella* species from Devon. Mycol Res 97:213–220, doi:10.1016/S0953-7562(09)80243-8

———. 1994a. Long-spored *Tulasnella* species from Devon, with additional notes on allantoid-spored species. Mycol Res 98:1235–1244, doi:10.1016/S0953-7562(09)80293-1

———. 1994b. Globose and ellipsoid-spored *Tulasnella* species from Devon and Surrey, with a key to the genus

in Europe. Mycol Res 98:1431–1452, doi:10.1016/S0953-7562(09)81075-7

———. 1999. Rhizoctonia-forming Fungi: a taxonomic guide. Kew, UK: Royal Botanic Gardens. 246 p.

———. 2006. Caribbean heterobasidiomycetes 2: Jamaica. Mycotaxon 96:83–107.

Rogers DP. 1933. A taxonomic review of the Tulasnellaceae. Ann Mycology 31:181–203.

Schoch C, Seifert K, Huhndorf S, Robert V, Spouge J, et al. 2012. The nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci USA 109:6241–6246, doi:10.1073/pnas.1117018109

Setaro S, Garnica S, Herrera PI, Suárez JP, Göker M. 2011. A clustering optimization strategy to estimate species richness of Sebacinales in the tropical Andes based on molecular sequences from distinct DNA regions. Biodivers Conserv, doi:10.1007/s10531-011-0205

Sharon M, Kuninaga S, Hyacumachi M, Naito S, Sneh B. 2008. Classification of *Rhizoctonia* spp. using rDNA-ITS sequence analysis supports the genetic basis of the classical anastomosis grouping. Mycoscience 49:93–114, doi:10.1007/S10267-007-0394-0

Shefferson RP, Taylor DL, Weiß M, Garnica S, McCormick MK, Adams S, Gray HM, McFarland JW, Kull T, Tali K, Yukawa T, Kawahara T, Miyoshi K, Lee Y. 2007. The evolutionary history of mycorrhizal specificity among lady's slipper orchids. Evolution 61:1380–1390, doi:10.1111/j.1558-5646.2007.00112.x

Simon UK, Weiss M. 2008. Intragenomic variation of fungal ribosomal genes is higher than previously thought. Mol Biol Evol 25:2251–2254, doi:10.1093/molbev/msn188

Smith S, Read D. 2008. Mycorrhizal symbiosis. 3rd ed. San Diego, California: Academic Press. 800 p.

Sokal RR, Sneath PHA. 1963. Principles of numerical taxonomy. San Francisco: WH Freeman & Co.

Stamatakis A. 2006. RAxML-VI-HPC: maximum Likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690, doi:10.1093/bioinformatics/btl446

Stielow B, Bratek Z, Orczán AKI, Rudnoy S, Hensel G, Hoffmann P, Klenk HP, Göker. 2011. Species delimitation in taxonomically difficult fungi: the case of *Hymenogaster*. PLoS ONE 6(1):e15614, doi:10.1371/journal.pone.0015614

Suárez JP, Weiss M, Abele A, Garnica S, Oberwinkler F, Kottke I. 2006. Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. Mycol Res 110:1257–1270, doi:10.1016/j.mycres.2006.08.004

———, ———, ———, Oberwinkler F, Kottke I. 2008. Members of Sebacinales subgroup B form mycorrhizae with epiphytic orchids in a Neotropical mountain rainforest. Mycol Prog 7:75–85, doi:10.1007/s11557-008-0554-4

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA 5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Mol Biol Evol 28:2731–2739, doi:10.1093/molbev/msr121

Taylor DL, McCormick MK. 2008. Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. New Phytol 177:1020–1033, doi:10.1111/j.1469-8137.2007.02320.x

Thompson JD, Higgins DG, Gibson TJ. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680, doi:10.1093/nar/22.22.4673

Tulloss RE, Lindgren JE. 2005. *Amanita aprica* a new toxic species from western North America. Mycotaxon 91:193–205.

van de Put K, Antonissen I. 1996. *Tulasnella*'s uit Vlaanderen. Sterbeeckia 17:44–69.

Walker C, Vestberg M, Demircik F, Stockinger H, Saito M, Sawaki H, Nishmura I, Schüßler A. 2007. Molecular phylogeny and new taxa in the Archaeosporales (Glomeromycota): *Ambispora fennica* gen. sp nov., Ambisporaceae fam. nov. and emendation of *Archaeospora* and Archaeosporaceae. Mycol Res 111:137–153, doi:10.1016/j.mycres.2006.11.008

Warcup, Talbot PHB. 1967. Perfect states of Rhizoctonias associated with orchids I. New Phytol 66:631–641, doi:10.1111/j.1469-8137.1967.tb05434.x

———, ———. 1971. Perfect states of Rhizoctonias associated with orchids II. New Phytol 76:35–40, doi:10.1111/j.1469-8137.1971.tb02506.x

———, ———. 1980. Perfect states of Rhizoctonias associated with orchids III. New Phytol 86:267–272, doi:10.1111/j.1469-8137.1980.tb00787.x

Warcup JH. 1973. Symbiotic germination of some Australian terrestrial orchids. New Phytol 72:387–392, doi:10.1111/j.1469-8137.1973.tb02046.x

Whelan S, Liò P, Goldman N. 2001. Molecular phylogenetics: state-of-the-art methods for looking into the past. Trends genet 17:262–272, doi:10.1016/S0168-9525(01)02272-7

The following page numbers of 1–10 are the page numbers of the following publication.

3.- Riofrio ML, Cruz DJ, Torres E, De La Cruz M, Iriondo JM, and Suarez JP (2013) Mycorrhizal preferences and fine spatial structure of the epiphytic orchid *Epidendrum rhopalostele*. *Am J Bot* 100(12): 1–10

Status: published

Title of the magazine: American Journal of Botany

Participating authors:

1.- MLR: María Lorena Riofrío

2.- DJC: Darío Javier Cruz

3.- ET: Elena Torres

4.- MDC: Marcelino De la Cruz

5.- JMI: José María Iriondo

6.- JPS: Juan Pablo Suárez

To which extend did the doctoral candidate or the co-authors contribute individually to the dissertation?

(1) Development and planning

1.- MLR: 50%

2.- DJC: 4%

3.- ET: 18%

4.-:MDC: 5%

5.- JMI: 18%

6.- JPS: 5%

(2) Performance of individual research and experiments

1.- MLR: 59%

2.- DJC: 7%

3.- ET: 12%

4.- MDC: 5%

5.- JMI: 12%

6.- JPS: 5%

DJC together with the first author performed the molecular work on fungi consisting of DNA isolation, PCR, electrophoresis, and molecular cloning.

(3) Composition of data gathering and images

1.- MLR: 50%

2.- DJC: 5%

3.- ET: 17%

4.- MDC: 6%

5.- JMI: 17%

6.- JPS: 5%

DJC helped the first author to do the consensus sequences and to build the alignments for the phylogenetic analysis. DJC improved the phylogenetic trees.

(4) Analysis and interpretation of the data

1.- MLR: 50%

2.- DJC: 6%

3.- ET: 17%

4.- MDC: 5%

5.- JMI: 17%

6.- JPS: 5%

DJC collaborated with the first author in the standardizing of the parameters to run the phylogenetic analyses. DJC also gave many comments and interpretations about the phylogenetic results.

(5) Preparation of the manuscript

1.- MLR: 50%

2.- DJC: 4%

3.- ET: 18%

4.- MDC: 6%

5.- JMI: 18%

6.- JPS: 5%

American Journal of Botany

American Journal of Botany 100(12): 000–000. 2013.

Mycorrhizal preferences and fine spatial structure of the epiphytic orchid *Epidendrum rhopalostele*[1]

María L. Riofrío[2,5], Dario Cruz[2], Elena Torres[3], Marcelino de la Cruz[3], José M. Iriondo[4], and Juan Pablo Suárez[2]

[2]Departamento de Ciencias Naturales, Universidad Técnica Particular de Loja, San Cayetano alto s/n, Loja, Ecuador; [3]Departamento de Biología Vegetal, Universidad Politécnica de Madrid, Madrid, Spain; and [4]Area de Biodiversidad y Conservación, Universidad Rey Juan Carlos, Móstoles, Spain

- *Premise of the study:* The presence of compatible fungi is necessary for epiphytic orchid recruitment. Thus, identifying associated mycorrhizal fungi at the population level is essential for orchid conservation. Recruitment patterns may also be conditioned by factors such as seed dispersal range and specific environmental characteristics.
- *Methods:* In a forest plot, all trees with a diameter at breast height >1 cm and all individuals of the epiphytic orchid *Epidendrum rhopalostele* were identified and mapped. Additionally, one flowering individual of *E. rhopalostele* per each host tree was randomly selected for root sampling and DNA extraction.
- *Key results:* A total of 239 *E. rhopalostele* individuals were located in 25 of the 714 potential host trees. Light microscopy of sampled roots showed mycorrhizal fungi in 22 of the 25 sampled orchids. Phylogenetic analysis of ITS1-5.8S-ITS2 sequences yielded two *Tulasnella* clades. In four cases, plants were found to be associated with both clades. The difference between univariate and bivariate *K* functions was consistent with the random labeling null model at all spatial scales, indicating that trees hosting clades A and B of *Tulasnella* are not spatially segregated. The analysis of the inhomogenous *K* function showed that host trees are not clustered, suggesting no limitations to population-scale dispersal. χ^2 analysis of contingency tables showed that *E. rhopalostele* is more frequent on dead trees than expected.
- *Conclusions: Epidendrum rhopalostele* establishes mycorrhizal associations with at least two different *Tulasnella* species. The analysis of the distribution patterns of this orchid suggests a microsite preference for dead trees and no seed dispersal limitation.

Key words: dead trees; *Epidendrum*; orchid mycorrhiza; Orchidaceae; preference; small-scale spatial distribution; *Tulasnella*.

The establishment and survival of epiphytic orchids depend on factors such as the environmental conditions of the forest that sustain them and, perhaps most importantly, on the presence of appropriate fungi. Because orchid seeds are minute and contain few stored reserves, colonization of a seed by a compatible fungus is essential for germination and early seedling development (Arditti and Ghani, 2000; Smith and Read, 2008). In epiphytic orchids, the stronger limitations to water and nutrients derived from their particular habitat may have increased the frequency of mycorrhizal associations that extend beyond the germination stage (Martos et al., 2012). Knowing the identity of associated mycorrhizal fungi thus becomes an important task in orchid conservation strategies (Batty et al., 2002; Dearnaley et al., 2012), especially when species require specific fungi (McCormick et al., 2004).

The identification of orchid mycorrhizal fungi has been studied for a long time, and more recently DNA-based molecular techniques have been used (Dearnaley et al., 2012). Orchids with different trophic strategies have been found to be associated with different groups of fungi (Rasmussen and Rasmussen, 2007, 2009). Mycoheterotrophic (MH) orchids are associated with a diverse group of Ascomycota or Basidiomycota fungi (Bidartondo et al., 2004; Girlanda et al., 2006; Martos et al., 2009). However, autotrophic orchids generally associate with a limited range of Basidiomycota, mainly Agaricomycotina including members of Tulasnellaceae (Suárez et al., 2006; Kottke et al., 2008), Sebacinales (Kottke et al., 2008; Suárez et al., 2008), and Ceratobasidiaceae (Otero et al., 2002, 2007b; Graham and Dearnaley, 2012). Nevertheless, Pucciniomycotina with members of Atractiellales have also been recently noted to form mycorrhizas (Kottke et al., 2010). Concerning the type of habitat, both epiphytic and photosynthetic terrestrial orchid species have been reported to be associated mainly to Tulasnellaceae (Martos et al., 2012).

Previous studies have shown that "specificity", understood as the phylogenetic diversity of the fungi associated with a particular plant species (Taylor et al., 2002), is difficult to predict. In the case of MH orchids, high specificity has been reported between them and their fungal partners (Taylor and Bruns, 1997; McKendrick et al., 2002; Selosse et al., 2002, 2004; Bidartondo and Read, 2008; Barrett et al., 2010), while specificity varies for photosynthetic orchids. Association with a narrow or even dominant group has been shown for many photosynthetic species, both terrestrial (McCormick et al., 2004, 2006; Shefferson et al., 2005, 2007, 2008; Roche et al., 2010; Swarts et al., 2010; Yuan et al., 2010) and epiphytic orchids (Otero

[1]Manuscript received 20 February 2013; revision accepted 3 September 2013.

The authors thank C. Naranjo for his field assistance, L. J. De Hond for her linguistic assistance, and the reviewers for valuable comments that helped to considerably improve the manuscript. This research was partially supported by the SENESCYT-ECUADOR. M. de la Cruz was supported by project grant CGL2009-13190-C03-02 of the Spanish Ministry of Science and Innovation.

[5]Author for correspondence: (email: mlriofrio@utpl.edu.ec)

doi:10.3732/ajb.1300069

et al., 2002, 2004, 2005; Suárez et al., 2008; Graham and Dearnaley, 2012). Nevertheless, associations between other photosynthetic species and a wide range of fungi (mycorrhizal generalists) have been described (Stark et al., 2009; Jacquemyn et al., 2010). Even closely related orchid species appear to have different degrees of specificity (Shefferson et al., 2007).

Degree of mycorrhizal specificity may have important consequences on orchid distribution and conservation. Thus, orchid rarity and vulnerability could be enhanced by their specificity for certain mycorrhizal fungi of rare or patchy distribution (e.g., Swarts et al., 2010). In contrast, orchid species associated with a broad range of fungi (or whose mycorrhiza are common and widespread) would be less predisposed to be endangered, as their seeds would have greater probability of encountering a compatible fungus after dispersal (Bonnardeaux et al., 2007). In any case, it is important to note that orchid distribution is limited not only by the presence of compatible mycorrhizal fungi, but also by its abundance (Diez, 2007; McCormick et al., 2012) and by other factors such as pollination (Pauw and Bond, 2011), seed dispersal (Jacquemyn et al., 2009; Winkler et al., 2009), or environmental conditions (Těšitelová et al., 2012).

Although many studies have been published regarding the identification of orchid mycorrhiza, little is still known about the intrapopulation variation in mycorrhizal associations. However, this information is important in orchid conservation as conservation actions are mainly undertaken at the population level. In the present work, we studied the mycorrhizal associations in one population of *Epidendrum rhopalostele* Hágsater & Dodson, an epiphytic orchid species of South America, and assessed the small-scale distribution of this orchid. Specifically, we asked the following questions: (1) Which fungi are associated with *E. rhopalostele*? If several species are detected, what are the phylogenetic relationships among them? (2) Do plants associate with one or more fungi simultaneously? (3) Do neighboring orchids share the same mycorrhizal fungus? (4) Are there additional factors affecting the fine spatial structure of *E. rhopalostele*? As found in other epiphythic orchids, we hypothesized that the mycorrhizal fungi of *E. rhopalostele* would fit in a narrow number of clades belonging to members of Tulasnellaceae, Sebacinales, and Ceratobasidiaceae. We also hypothesized that the small-scale spatial distribution of this orchid is conditioned by additional factors other than the availability of suitable mycorrhizal fungi, including seed dispersal limitations and specific environmental conditions.

MATERIALS AND METHODS

Study species and site—*Epidendrum rhopalostele* (Orchidaceae) is a photosynthetic, epiphytic species native to Ecuador, Peru and Bolivia in evergreen montane forests. It belongs to the alpicolum group, with a single racemose apical inflorescence of 10–25 light green flowers (Hágsater and Sánchez, 2001) (Fig. 1). It is very similar to *Epidendrum dialychilum* Hágsater & Dodson, both having the lip free from the column, but differs from the latter by its linear-lanceolate and acuminate lip; the long, filiform, acuminate petals; and the stigmatic cavity only in the apical third of the column. Its seed dispersal mechanism and reproductive biology have not been studied and are unknown.

The study site is located on the eastern slope of Cordillera Real in the Andes of southern Ecuador on the border of Podocarpus National Park along the Loja-Zamora Road in Zamora-Chinchipe province, at around 2250 m a.s.l. This regenerated forest, ~35-yr old and classified as evergreen, upper montane forest (Beck et al., 2008), covers an area of ~ 1 ha. The most diverse and abundant seed plant families are Orchidaceae, Melastomataceae, Ericaceae, and Rubiaceae. Mean annual precipitation is 2193 mm, and mean annual temperature is 20.8°C (4.7–25.5°C). A moderate rainy season typically extends from April to July. On average, there is 1 mo with less than 100 mm of rainfall during the driest part of the year, from October to January. Shorter dry spells of 1–2 wk are more frequent. Fog is common at this elevation and provides an additional water surplus of 9.6% (Bendix et al., 2008).

Fig. 1. Overview of a flowering plant of *Epidendrum rhopalostele*.

Sampling—One adult plant of *E. rhopalostele* was randomly selected on each host tree for root sampling between January and March 2009. Three to four roots per individual plant were packed in aluminum foil to prevent desiccation and transported to the laboratory on the sampling day. To ensure correct identification, we collected all orchid samples from plants in bloom.

Light microscopy—Light microscopy was used to select material with coils of hyphae or pelotons. Transversal sections were cut in the middle part of each root sample by hand using a razor blade. Sections were stained with a methyl blue (0.05% w/v) solution for 3 min and examined at 100× to 1000× (Zeiss

Axioskop 2). To reduce contamination with nonmycorrhizal fungi, we removed the velamen using a stereomicroscope, and the remaining cortical tissue was collected in microtubes for DNA isolation.

***DNA isolation, PCR, cloning, and sequencing*—**DNA was extracted from colonized root pieces of ~1–2 cm long using a Plant DNAeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

A PCR was conducted to amplify the ITS-5.8S rDNA region with the universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG-3′) (White et al., 1990) and TW14 (5′-GCT ATC CTG AGG GAA ACT TC-3′) (Cullings, 1994). This primer combination was chosen to increase the possibility of identifying the entire diversity of fungi within the root. PCR reactions were conducted in 20 μL reaction volume containing 0.5 pmol of each primer, 0.8 μg/μL of bovine serum albumin (SIGMA-USA) (Iotti and Zambonelli, 2006), and 10 μL of the Phusion High-Fidelity PCR Master Mix (Finnzymes, Espoo, Finland). In each PCR, a negative control of PCR mix without DNA template was included. The thermocycler program used for reactions was as follows: 98°C for 30 s (1 cycle); denaturation at 98°C for 10 s, annealing at 60°C for 20 s, and elongation at 72°C for 30 s (30 cycles); and a final extension at 72°C for 7 min. Success of PCR amplification was tested in 1% agarose gel.

PCR products were cloned with Zero Blunt TOPO PCR cloning kit (Invitrogen) following the manufacturer's protocol. To verify the DNA insert, we randomly selected 12 growing colonies for each sample, for a direct PCR with M13F and M13R primers modified according to Krüger et al. (2009). PCR conditions were as follows: 95°C for 5 min (1 cycle); denaturation at 94°C for 30 s, annealing 65°C for 30 s, elongation at 72°C for 2 min (35 cycles); and final extension at 72°C for 10 min. Success of the PCR was tested as mentioned before. The colonies with inserts were purified following S.N.A.P. miniprep kit (Invitrogen) protocol. Sequencing was carried out using primer M13F.

***Phylogenetic analyses of Epidendrum rhopalostele mycobionts*—**We used BLAST against the NCBI nucleotide database (GenBank; http://www.ncbi.nlm.nih.gov/) to detect published sequences with a high similarity. The majority of obtained sequences were closely related to Tulasnellaceae. Due to the heterogeneity of the Tulasnellaceae sequences, the ITS-5.8S rDNA region could not be aligned over the whole data set. Therefore, we used the 5.8S region (160 bp) to calculate a first phylogenetic tree of a wider phylogenetic spectrum. Subsequently, we carried out phylogenetic analyses for each subset of related sequences. The ITS-5.8S rDNA region was analyzed considering sequences derived from this study and other closely related sequences from GenBank. Sequences were aligned with G-INS-I strategy implemented in MAFFT version 5.667 (Katoh et al., 2005).

Phylogenetic analyses were performed with neighbor-joining (NJ), Bayesian likelihood, and maximum-likelihood (ML) analyses. Neighbor-joining was implemented in the program PAUP* (Swofford, 2002) using the BIONJ modification (Gascuel, 1997). Branch support was tested with 1000 bootstrap replicates. The Bayesian likelihood was based on Markov chain Monte Carlo (MCMC) method as implemented in MrBayes, version 3.1.2 (Huelsenbeck and Ronquist, 2001). We ran two independent MCMC analyses, each involving four incrementally heated Markov chains over 4 million generations and using random starting trees with GTR+I+G substitution model. Trees were sampled over 100 generations resulting in a total of 40 000 trees in each run from which 30 000 were used to compute a pooled majority rule consensus tree. For heuristic analysis, the program PHyML version 2.4.4 (Guindon and Gascuel, 2003; Guindon et al., 2005) was used with GTR+I+G DNA substitution model. Gamma distribution was approximated with four discrete rate categories; all model parameters were estimated using maximum likelihood. Branch support was inferred from 1000 bootstrap replicates.

***Spatial and association analyses of mycorrhizal fungi and orchids*—**All trees with a diameter at breast height (DBH) >1 cm in the study site were identified and mapped. All mapped trees were thoroughly inspected for *E. rhopalostele* individuals (seedlings, juveniles, or adults), and all individuals found were recorded. When the higher sections of the trunk and the higher branches of a tree could not be observed from the ground, we climbed the tree to search for *E. rhopalostele*. The shape and structure of the leaf and growth form were used as a reference when sampling seedlings. In addition, a genetic analysis with AFLPs that was performed on the same individuals (not included in this article) supported the identification because similar patterns were obtained. We also recorded whether each tree was dead or alive. Trees without branches or leaves, many of which showed clear signs of bark decomposition, and large branches fallen on the ground were classified as dead. All tree species were assigned to one of two functional categories (shade-tolerant or pioneer) according to literature (Finegan, 1992; Poorter et al., 2006).

Spatial point pattern analysis was used to describe the orchid spatial distribution in the site and its associated mycorrhizal fungi. To analyze spatial patterns, we employed univariate and bivariate versions of Ripley's K function (i.e., $K(r)$ and $K_{ij}(r)$; Ripley, 1976; Wiegand and Moloney, 2004). For a homogeneous point pattern, where λ is the intensity of the pattern, $\lambda K(r)$ is the expected number of points within a circle of radius r around an arbitrary point. Similarly, $\lambda_j K_{ij}(r)$ is defined as the expected number of type j points within distance r of an arbitrary type i point, where λ_j is the intensity of points of type j. We also employed the inhomogeneous K function [Kinhom(r)], which can be defined as the expected value of the sum of all terms $1/\lambda(x_j)$ over all points x_j within a circle of radius r around an arbitrary point. Here, $\lambda(x_j)$ is the intensity in the location of the point x_j (Baddeley and Turner, 2005). The inhomogeneous K reduces to the ordinary K function, if λ is constant.

In a first analysis, we employed the K function to analyze the spatial pattern of the orchid. As this analysis revealed that the distribution followed a heterogeneous pattern (i.e., the intensity was not constant and instead varied continuously throughout the plot), we shifted to the inhomogenous K function and estimated the intensity function $\lambda(x)$ with a Gaussian kernel with a bandwidth of 6.5 m. We tested the observed inhomogeneous function against a null model of weighted random labeling of the tree population. This new null model is a modified version of the "classical" random labeling null model (Wiegand and Moloney, 2004) that weights the probability of a tree being labeled (i.e., being assigned the presence of the orchid) according to the fitted intensity function.

We tested the spatial segregation of mycorrhiza clades by means of differences between univariate and bivariate K functions (Dixon, 2002). In this case, we tested the observed differences of K functions against the null model of random labeling, as we did not observe heterogeneity at the scale of the study.

Spatial analyses were carried out with the Kest and Kinhom functions of the package spatstat (Baddeley and Turner, 2005) and K1K2 of the package ecespa (de la Cruz et al., 2008) in the R environment (R Development Core Team, 2011).

Additionally, we employed contingency table analysis (Agresti, 2002) to test the possible association of *E. rhopalostele* with dead or live trees and with pioneer or shade-tolerant trees. Similarly, we employed contingency table analysis to test the association of the obtained mycorrhiza clades with dead or live trees and pioneer or shade-tolerant trees.

RESULTS

Light microscopy examination showed that the roots of 22 of the 25 sampled individuals of *E. rhopalostele* were colonized by mycorrhizal fungi forming coils. Vital and collapsed pelotons were present in the cortical cells, and abundant hyphae were found in the velamen (Fig. 2).

***Identification and phylogenetic analysis of* Epidendrum rhopalostele *mycobionts*—**PCR amplification of the ITS-5.8S rDNA region was successful for 19 of the 22 analyzed mycorrhizal samples. A total of 47 sequences were used; identical sequences from the same roots, chimeric sequences and low-quality sequences were discarded. BLAST searches in GenBank showed that 44 sequences were close to *Tulasnella*. The sequences obtained in this study are available in GenBank under accession numbers JF346765–JF346853. Additionally, two other sequences belonging to other Basidiomycota close to *Hyphoderma* (GenBank DQ873597.1) and *Infundibura* (GenBank AJ406404.1) and one sequence close to the Ascomycota *Hyalodendriella* (GenBank EU040232.1) were detected in the same root samples where *Tulasnella* was present. Finally, two identical sequences belonging to *E. rophalostele* were identified (GenBank KC165027, KC165028).

The phylogenetic analyses of the sole 5.8S region showed the sequences from *E. rhopalostele* mycobionts in two distinct clades within Tulasnellaceae DNA alignment (Appendix S1; see Supplemental Data with the online version of this article).

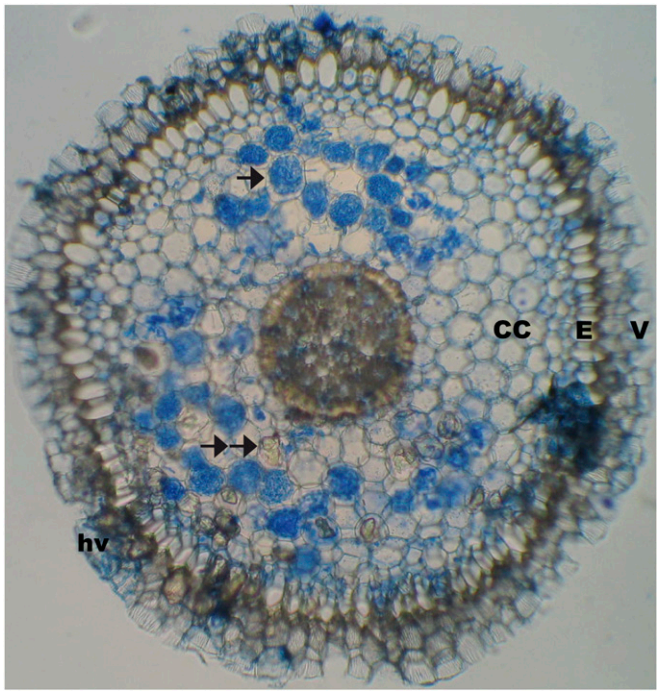

Fig. 2. Cross section of a mycorrhizal *E. rhopalostele* root stained with methyl blue (×100). Fungal hyphae (hv) traverse the velamen (V) and epidermis (E) and form tight coils called pelotons within cortical cells (CC). After a few days, pelotons lyse, and the lysed products are absorbed by the host cells. (Single arrow: vital peloton; double arrow: collapsed peloton).

The phylogenetic analyses of ITS1-5.8S-ITS2 for each subset increased phylogenetic resolution. Clade A (Fig. 3) was supported by 54/100/100 (MCMC / heuristic ML / BIONJ, respectively), and 63% of all sequences were found in this subset. Within clade A, sequences shared 99% similarity in the region ITS1-5.8S-ITS2, except in the case of clone JF346765 with just 98% similarity. The closest related sequences to clade A, supported by values 56/85/94 (MCMC / heuristic ML / BIONJ) (Fig. 3) were Tulasnellaceae from epiphytic orchids (subtribe Pleurothallidinae) *Stelis concinna* Lindl. and *Pleurothallis lilijae* Foldats, with 95% similarity, and *Stelis superbiens* Lindl. and *Stelis hallii* Lindl. with 84 to 95% similarity. Clade B was included in the second subset (Fig. 4) with a support of 94/99/100 (MCMC / heuristic ML / BIONJ), including 37% of *Tulasnella* sequences. All sequences of clade B shared a similarity of 99% in ITS1-5.8S-ITS2, except for the sequence JF346853 with 95% similarity compared to the rest. Sequences in clade B are close to *Tulasnella asymmetrica* Warcup & P.H.B.Talbot (DQ520101 and DQ388046.1, with 84% similarity), forming a clade supported by 100/99/100 (MCMC / heuristic ML / BIONJ).

Tulasnellaceae sequences displayed in clade A were present in nine orchid individuals, and Tulasnellaceae sequences from clade B were present in six orchids, whereas clades A and B were simultaneously found in four orchid individuals.

Spatial and association analyses of mycorrhizal fungi and orchids—Considering host and nonhost trees of *E. rhopalostele,* 714 trees with DBH > 1 cm were found and mapped at the study site. A total of 239 *E. rhopalostele* individuals were located in 25 of the 714 potential host trees in the forest plot. Orchids were found in the first 3 m of height of the tree hosts.

The inhomogeneous *K* function showed that *E. rhopalostele* host trees had a random distribution at all spatial scales (results not shown), suggesting that there are no limitations to population-scale dispersal.

On the other hand, the difference of univariate and bivariate *K* functions was consistent with the random labeling null model at all spatial scales (results not shown), indicating that the trees hosting clades A and B of *Tulasnella* are not spatially segregated.

Epidendrum rhopalostele was marginally more likely to be found on shade-tolerant trees than expected ($\chi^2 = 4.22$, df = 1, $p = 0.040$) and clearly more likely to be found on dead trees than expected ($\chi^2 = 28.4991$, df = 1, $p < 0.001$).

Clades A and B of *Tulasnella* showed no difference in their preference for shade-tolerant or pioneer trees ($\chi^2 = 0.127$, df = 1, $p = 0.722$) or for live or dead trees ($\chi^2 = 0.02$, df = 1, p value = 0.897).

DISCUSSION

Although a high number of epiphytic orchid species have been recorded, studies on their mycorrhizal fungi are still scarce (Dearnaley et al., 2012), and in most cases, only one or two individuals per population have been sampled. Our study provides a novel focus by assessing within-population variation of epiphytic orchid mycorrhizal interactions in a spatially explicit way. We thus found that *E. rhopalostele* can associate separately or simultaneously with two different clades of closely related *Tulasnella.* These clades are spatially randomly distributed showing no segregation patterns that would suggest a limited distribution of the fungi or competitive exclusion between clades. We have also found that this particular orchid is more likely than expected to be found on dead and fallen trees. Our results thus contribute to improving knowledge on epiphytic orchid species, providing relevant information on mycorrhizal fungi preference of *E. rhopalostele*, and identifying favorable environments at the population level.

***Identification and phylogenetic relationships of fungi associated with* E. rhopalostele—**Light microscopy examination showed that 22 of the 25 *E. rhopalostele* individuals were consistently colonized. Roots revealed the presence of vital and collapsed pelotons in the same tissue suggesting the possibility of subsequent reinfection in roots. Beyond the essential support that mycorrhizal fungi provide during seed germination, the presence of the fungi in adult orchid individuals may be useful to retain the fungus in the neighborhood to assure further seed germination, which could explain the high number of juveniles growing close to adults. The maintenance of this association in adult plants could also be advantageous in adverse seasons or under conditions of high shade, as plants could obtain part of organic carbon through their mycorrhizal fungi (Hudson, 1992; Dearnaley et al., 2012).

DNA sequence analysis showed the presence of *Tulasnella* in *E. rhopalostele* roots, which agrees with reports for other species of the same tribe (tribe Epidendreae). Zettler and Hofer (1998) and Pereira et al. (2003) identified the anamorphic genus *Epulorhiza* from mycorrhizal roots of *Epidendrum conopseum* R.Br. and *Epidendrum rigidum* Jacq., respectively, and Suárez et al. (2006, 2008) identified *Tulasnella* in *Stelis hallii*, *S. superbiens*, *S. concinna*, and *Pleurothallis lilijae.*

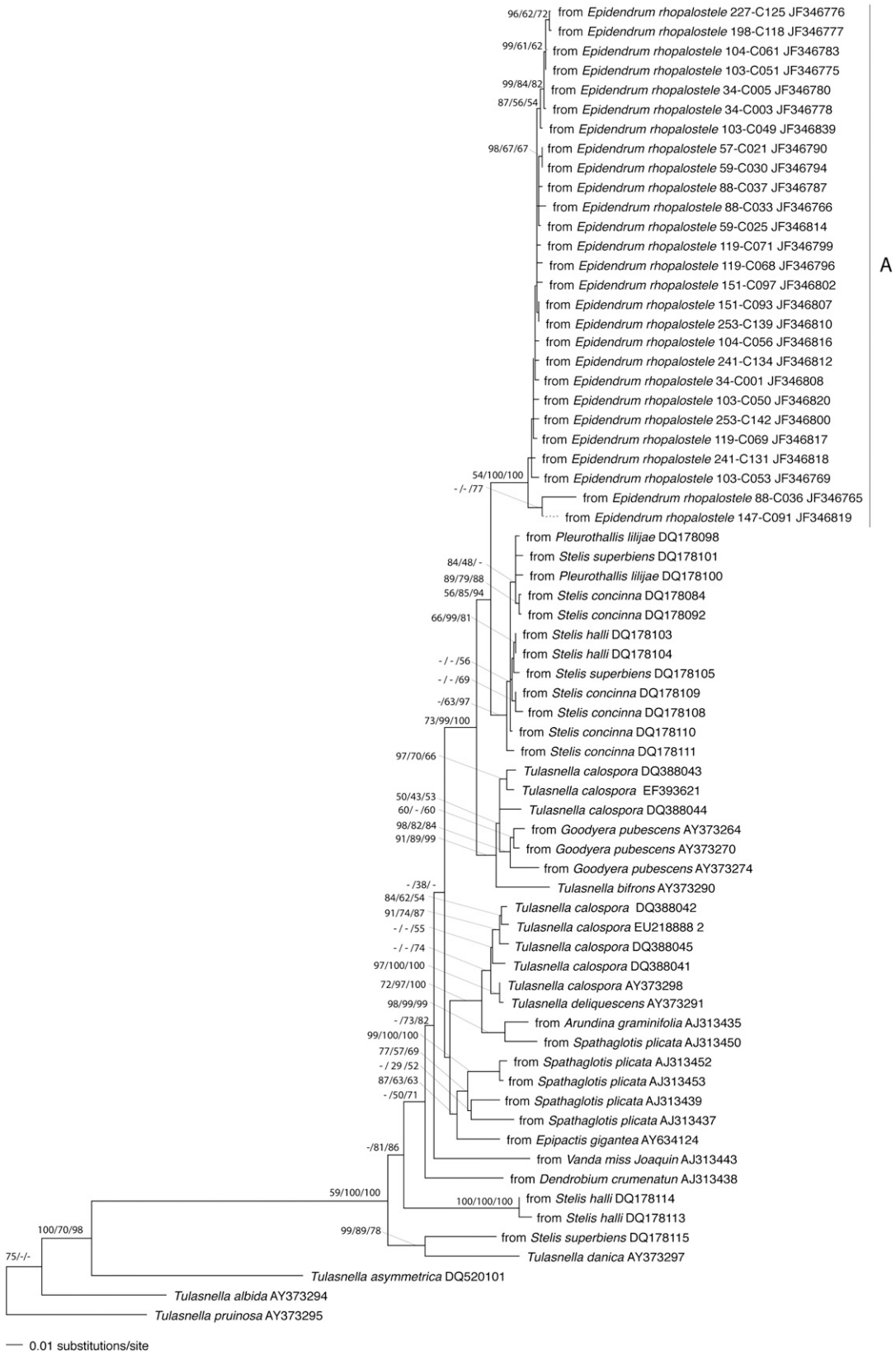

Fig. 3. Clade A of *Epidendrum rhopalostele* mycobionts. Phylogenetic placement of *Tulasnella* sequences, inferred by Markov chain Monte Carlo (MCMC) analysis of the ITS-5.8S region from nuclear rDNA sequences. Numbers on branches correspond to MCMC analysis/heuristic maximum likelihood bootstrap/neighbor-joining bootstrap (only values >50% are shown). Note that genetic distances cannot be directly correlated to branch lengths in the trees, since highly diverse alignment regions were excluded for tree calculation. The tree was rooted with *Tulasnella pruinosa* AY373295.

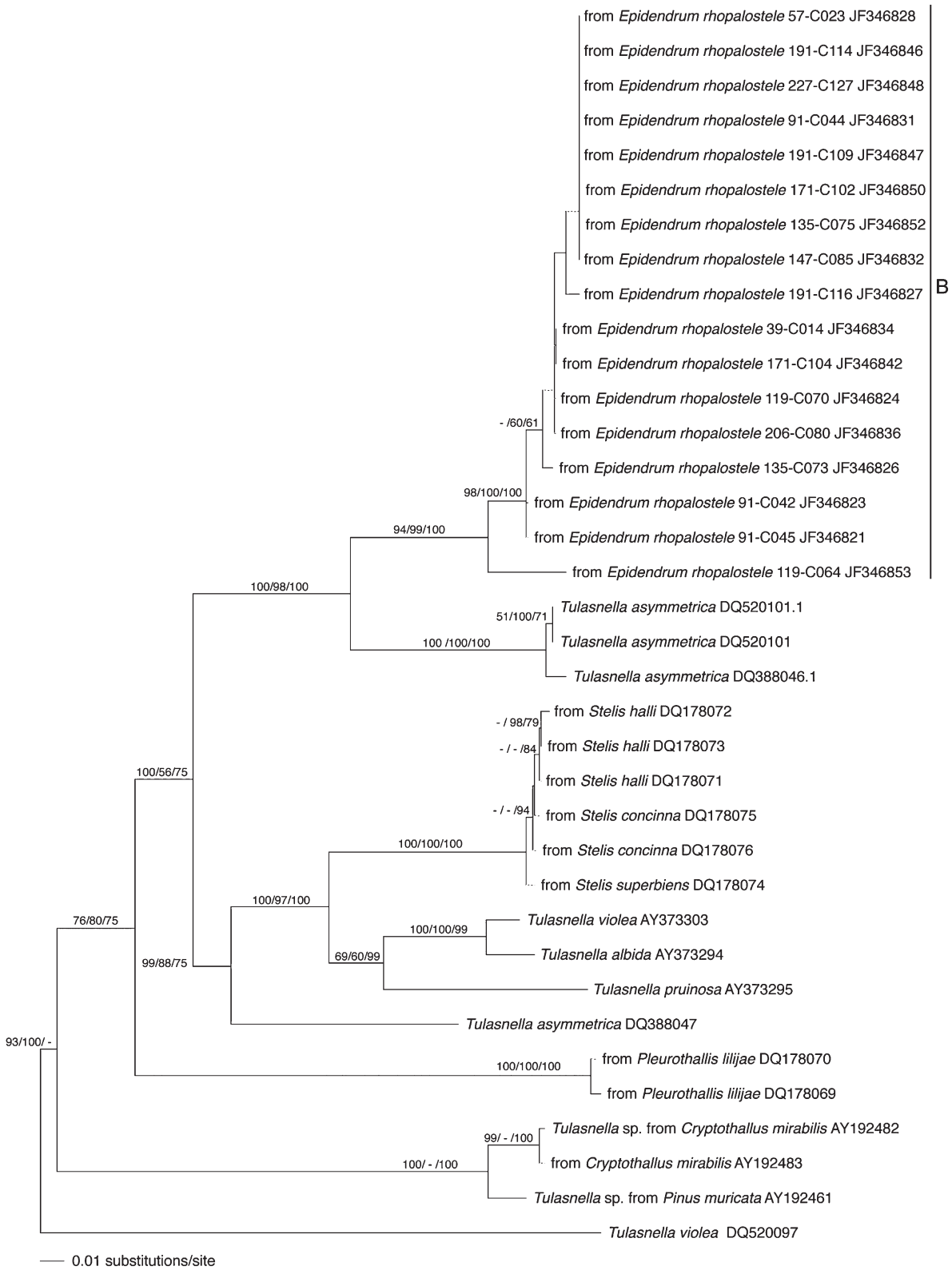

Fig. 4. Clade B of *Epidendrum rhopalostele* mycobionts. Phylogenetic placement of *Tulasnella* sequences, clade B from *Epidendrum rhopalostele*, inferred by Markov chain Monte Carlo (MCMC) analysis of ITS-5.8S region from nuclear rDNA. The values that support the nodes correspond to MCMC analysis/heuristic maximum likelihood bootstrap/neighbor-joining bootstrap (only values >50% are shown; hyphens indicate absence of these values for that analysis). Note that genetic distances cannot be directly correlated with the length of the branches on the trees, since the highly diverse alignment regions were excluded from the construction of the tree. The tree was rooted with *Tulasnella violea* DQ520097.

Our results showed that *E. rhopalostele* has a preference for two *Tulasnella* clades: (1) sequences of clade A were grouped with sequences of mycorrhizal fungi isolated from *Stelis* and *Pleurothallis* that correspond to clades A, B, and C reported by Suárez et al. (2006), all of which form a subset related to *Tulasnella calospora* Hadley, and (2) sequences of clade B were close to the clade of *Tulasnella asymmetrica* and to sequences from mycorrhizas isolated from *Stelis* and *Pleurothallis* that correspond to clades E and F of Suárez et al. (2006) and other *Tulasnella* species. Because the percentage of similarity between sequences of the same clade was over 95%, the mycobionts of each clade could be considered "genotypes" of the same *Tulasnella* species or operational taxonomic unit (OTU) (Lindner and Banik, 2011). These results suggest, therefore, that there is no absolute fungal specificity in *E. rhopalostele,* as it can form mycorrhizas with at least two *Tulasnella* species.

Concerning the similarity of the mycorrhizal sequences isolated for *E. rhopalostele* and those reported for *Stelis hallii*, *S. superbiens*, *S. concinna*, and *Pleurothallis lilijae*, it is important to note that the study by Suárez et al. (2006) was carried out in a nearby site with the same forest type as *E. rhopalostele*.

In addition to *Tulasnella* sequences, other Basidiomycota close to *Infundibura* and *Hyphoderma* and one Ascomycota close to *Hyalodendriella* were also found. Although Kottke et al. (2010) recently showed that other Atractiellales closely related to *Infundibura* formed mycorrhizas with several terrestrial and epiphytic orchid species, the presence of only one sequence does not provide enough evidence to conclude that these fungi form mycorrhizas with *E. rhopalostele*. In this sense, a highly diverse group of fungi has been reported to colonize only the velamen in epiphytic orchids, including mostly Ascomycota with members of Helotiales closest to *Hyalodendriella* as well as Polyporales such as *Hyphoderma* (Herrera et al., 2010). Thus, it would be interesting to extend this study to other known populations of *E. rhopalostele* occurring in Ecuador, Peru, and Bolivia to confirm genus-level specificity to *Tulasnella*.

Do plants associate with one or more fungi simultaneously? Do neighboring orchids share the same mycorrhizal fungus?—Four individuals of *E. rhopalostele* were found to be associated with two *Tulasnella* species at the same time, which supports the hypothesis of successive reinfections. Multiple fungal associations have also been described in some terrestrial orchid species, both photosynthetic (Jacquemyn et al., 2010, 2012; Martos et al., 2012) and mycoheterotrophic (Roy et al., 2009), and also in some epiphytic orchid species (Martos et al., 2012; Xing et al., 2013), although it is difficult to know if this is a common phenomenon because most studies do not provide this information. As suggested by Jacquemyn et al. (2012), associating with multiple fungi can provide an advantage if the associated fungi belong to different fungal lineages and have different nutritional and environmental requirements. Nevertheless, in our case, this is unlikely because the two mycorrhizal species found in the roots of *E. rhopalostele* are phylogenetically very close.

Because seed colonization by a compatible fungus is essential for germination and early seedling development, the spatial distribution of *E. rhopalostele* depends on the presence of the fungi, and therefore, it could potentially be influenced by the spatial distribution of the fungi. The detailed spatial distribution of the two *Tulasnella* species associated with *E. rhopalostele* is unknown in this forest, and there is virtually no information about patterns of fungal small-scale spatial distribution in the environment (Phillips et al., 2011). However, some conclusions can be derived from the bivariate spatial analysis, which found no trend in neighboring orchids to share the same mycorrhizal fungus. The existence of segregation between clade A and clade B of *Tulasnella* could have indicated a limited spatial distribution of fungi. The absence of such a pattern and the fact that both *Tulasnella* clades could be found in the same tree, and even in the same plant, provide no evidence in support of a limited distribution of *Tulasnella*.

***Additional factors affecting the fine spatial structure of* E. rhopalostele—**Previous studies about the factors affecting the epiphytic orchid distribution have been focused on the identity of the tree and its physical characteristics (e.g., size, age, architecture, bark roughness, cover of bark) (Bergstrom and Carter, 2008; Adhikari et al., 2012). The small number of trees hosting *E. rhopalostele* in the forest and high tree species richness made it impossible to assign a degree of preference for particular species, as some authors have accomplished in other epiphytic orchid species (Tremblay et al., 1998; Gowland et al., 2011). However, our results show that *E. rhopalostele* is more likely to grow on dead trees than expected. Epiphytic orchid species are relatively common on dead trees (Zimmerman, 1991; Ackerman et al., 1996; Gulledge et al., 2011), and preference for this type of substrate has been reported in some cases (Otero et al., 2007a; Cruz-Fernández et al., 2011). Because *Tulasnella* is mainly described as saprotrophic (Roberts, 1999), decomposing wood might offer better conditions for the growth and reproduction of these fungi. With a greater abundance of fungi, the probability of orchid seed infection in dead trees would also be greater. In this sense, it would be interesting to test whether the germination and establishment of *E. rhopalostele* can be enhanced by the presence of decomposing wood, as found in some terrestrial orchids (Rasmussen and Whigham, 1998).

In addition to the type of substrate, other environmental factors also seem to affect orchid distribution. Our analysis showed that the spatial pattern of *E. rhopalostele* was heterogeneous, that is, that the density of trees bearing *E. rhopalostele* was not constant and varied throughout the plot. This pattern is usually interpreted as a response to some environmental factor controlling the distribution of plants (Wiegand and Moloney, 2004). Because *E. rhopalostele* was mainly found at the base of trees near small ravines of the forest fragment, we hypothesize that high humidity could be a relevant environmental filter affecting the presence of this orchid species. In this sense, it is important to note that *E. rhopalostele* requires constant moisture because it lacks pseudobulbs and thick leaves. Because humidity is higher in the understory than in the canopy, this environmental difference might explain the fact that *E. rhopalostele* occurs only in the lower part of the trees. On the other hand, the small ravines could provide better conditions to *E. rhopalostele*; humidity in these areas is also greater, especially during the driest part of the year.

Contrary to our initial hypothesis, the random spatial distribution pattern of trees that had at least one adult plant of *E. rhopalostele* suggests no limitation to seed dispersal at the scale of the studied forest fragment, although seed dispersal mostly occurs within trees.

Final remarks—From a conservation perspective, the knowledge provided in this study may help detect recruitment sites and support management measures in the forest where the study took place. Thus, fallen and dead trees could be left

undisturbed to promote recruitment. Specific studies to characterize the spatial distribution of the two *Tulasnella* species in the forest and their ecological requirements is advisable because, in the end, the factors that influence the presence and abundance of these fungi indirectly affect *E. rhopalostele* establishment and survival. Furthermore, studies on other known populations of this species are needed to complete the characterization of microsite suitability at the species level and to confirm genus-level specificity to *Tulasnella*.

LITERATURE CITED

ACKERMAN, J. D., A. SABAT, AND J. K. ZIMMERMAN. 1996. Seedling establishment in an epiphytic orchid: An experimental study of seed limitation. *Oecologia* 106: 192–198.

ADHIKARI, Y. P., H. S. FISCHER, AND A. FISCHER. 2012. Host tree utilization by epiphytic orchids in different land-use intensities in Kathmandu Valley, Nepal. *Plant Ecology* 213: 1393–1412.

AGRESTI, A. 2002. Categorical data analysis, 2nd ed. Wiley, Hoboken, New Jersey, USA.

ARDITTI, J., AND A. K. A. GHANI. 2000. Numerical and physical properties of orchid seeds and their biological implications. *New Phytologist* 145: 367–421.

BADDELEY, A., AND R. TURNER. 2005. Spatsat: An R package for analyzing spatial point patterns. *Journal of Statistical Software* 12: 1–42.

BARRETT, C. F., J. V. FREUDENSTEIN, D. L. TAYLOR, AND U. KOLJALG. 2010. Rangewide analysis of fungal associations in the fully mycoheterotrophic *Corallorhiza striata* complex (Orchidaceae) reveals extreme specificity on ectomycorrhizal *Tomentella* (Thelephoraceae) across North America. *American Journal of Botany* 97: 628–643.

BATTY, A. L., K. W. DIXON, M. C. BRUNDRETT, AND K. SIVASITHAMPARAM. 2002. Orchid conservation and mycorrhizal associations. *In* K. Sivasithamparam, K. W. Dixon, and R. L. Barrett [eds.], Microorganisms in plant conservation and biodiversity, 195–226. Kluwer, Dordrecht, Netherlands.

BECK, E., F. MAKESCHIN, F. HAUBRICH, M. RICHTER, J. BENDIX, AND C. VALEREZO. 2008. The ecosystem (Reserva Biológica San Francisco). *In* E. Beck, J. Bendix, I. Kottke, F. Makenschin, and R. Mosandl [eds.], Gradients in a tropical mountain ecosystem of Ecuador. Ecological studies, 1–13. Springer-Verlag, Heidelberg, Germany.

BENDIX, J., R. ROLLENBECK, M. RICHTER, P. FABIAN, AND P. EMCK. 2008. Climate. *In* E. Beck, J. Bendix, I. Kottke, F. Makenschin, and R. Mosandl [eds.], Gradients in a tropical mountain ecosystem of Ecuador. Ecological studies, 63–73. Springer-Verlag, Heidelberg, Germany.

BERGSTROM, B. J., AND R. CARTER. 2008. Host-tree selection by an epiphytic orchid, *Epidendrum magnoliae* Muhl. (green fly orchid), in an inland hardwood hammock in Georgia. *Southeastern Naturalist* 7: 571–580.

BIDARTONDO, M. I., B. BURGHARDT, G. GEBAUER, T. D. BRUNS, AND D. J. READ. 2004. Changing partners in the dark: Isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings of the Royal Society of London, B, Biological Sciences* 271: 1799–1806.

BIDARTONDO, M., AND D. READ. 2008. Fungal specificity bottlenecks during orchid germination and development. *Molecular Ecology* 17: 3707–3716.

BONNARDEAUX, Y., M. BRUNDRETT, A. BATTY, K. DIXON, J. KOCH, AND K. SIVASITHAMPARAM. 2007. Diversity of mycorrhizal fungi of terrestrial orchids: Compatibility webs, brief encounters, lasting relationships and alien invasions. *Mycological Research* 111: 51–61.

CRUZ-FERNÁNDEZ, Q. T., M. L. ALQUICIRA-ARTEAGA, AND A. FLORES-PALACIOS. 2011. Is orchid species richness and abundance related to the conservation status of oak forest? *Plant Ecology* 212: 1091–1099.

CULLINGS, K. 1994. Molecular phylogeny of the Monotropoideae (Ericaeae) with a note on the placement of the Pyroloideae. *Journal of Evolutionary Biology* 7: 501–516.

DEARNALEY, J. D. W., F. MARTOS, AND M. A. SELOSSE. 2012. Orchid mycorrhizas: Molecular ecology, physiology, evolution and conservation aspects. *In* B. Hock [ed.], Fungal associations, The mycota, vol. 9, 207–230. Springer-Verlag, Berlin, Germany.

DE LA CRUZ, M., R. L. ROMAO, A. ESCUDERO, AND F. T. MAESTRE. 2008. Where do seedlings go? A spatio-temporal analysis of seedling mortality in a semi-arid gypsophyte. *Ecography* 31: 720–730.

DIEZ, J. M. 2007. Hierarchical patterns of symbiotic orchid germination linked to adult proximity and environmental gradients. *Journal of Ecology* 95: 159–170.

DIXON, P. 2002. Ripley's *K* function. *In* A. El-Shaarawi and W. Piergorsch [eds.], The encyclopedia of environmetrics, 1976–1803. Wiley, New York, New York, USA.

FINEGAN, B. 1992. Bases ecológicas para la silvicultura. Tema I. V. Curso internacional sobre silvicultura y manejo de bosques tropicales. Centro Agronómico Tropical de Investigación y Enseñanza, Turrialba, Costa Rica.

GASCUEL, O. 1997. BIONJ: An improved version of the NJ algorithm based on a simple model of sequence data. *Molecular Biology and Evolution* 14: 685–695.

GIRLANDA, M., M. A. SELOSSE, D. CAFASSO, F. BRILLI, S. DELFINE, R. FABBIAN, ET AL. 2006. Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae. *Molecular Ecology* 15: 491–504.

GOWLAND, K. M., J. WOOD, M. A. CLEMENTS, AND A. B. NICOTRA. 2011. Significant phorophyte (substrate) bias is not explained by fitness benefits in three epiphytic orchid species. *American Journal of Botany* 98: 197–206.

GRAHAM, R. R., AND J. D. W. DEARNALEY. 2012. The rare Australian epiphytic orchid *Sarcochilus weinthalii* associates with a single species of *Ceratobasidium. Fungal Diversity* 54: 31–37.

GUINDON, S., AND O. GASCUEL. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52: 696–704.

GUINDON, S., F. LETHIEC, P. DUROUX, AND O. GASCUEL. 2005. PHYML Online—A web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Research* 33: W557–W559.

GULLEDGE, K., A. F. JOHNSON, AND G. SCHULTZ. 2011. Rare plant survey of Chassahowitzka Wildlife Management Area, Hernando County, Florida. Report to the Florida Fish and Wildlife Conservation Commission. Florida Natural Areas Inventory, Tallahassee, Florida, USA.

HÁGSATER, E., AND L. SÁNCHEZ [eds.] 2001. Icones orchidacearum. The genus *Epidendrum.* A third century of new species in *Epidendrum*, fasc. 4, part 3. Herbario AMO, D.F. Mexico, Mexico.

HERRERA, P., J. P. SUÁREZ, AND I. KOTTKE. 2010. Orchids keep the ascomycetes outside: A highly diverse group of ascomycetes colonizing the velamen of epiphytic orchids from a tropical mountain rainforest in Southern Ecuador. *Mycology* 1: 262–268.

HUDSON, H. J. 1992. Fungi as mutualistic symbionts in endomycorrhizas. *In* H. J. Hudson [ed.], Fungal biology, 214–241. Cambridge University Press, Cambridge, UK.

HUELSENBECK, J. P., AND F. RONQUIST. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755.

IOTTI, M., AND A. ZAMBONELLI. 2006. A quick and precise technique for identifying ectomycorrhizas by PCR. *Mycological Research* 110: 60–65.

JACQUEMYN, H., A. DEJA, K. DE HERT, B. CACHAPA BAILATORE, AND B. LIEVENS. 2012. Variation in mycorrhizal associations with tulasnelloid fungi among populations of five *Dactylorhiza* species. *PLoS ONE* 7: e42212.

JACQUEMYN, H., O. HONNAY, B. P. A. CAMMUE, R. BRYS, AND B. LIEVENS. 2010. Low specificity and nested subset structure characterize mycorrhizal associations in five closely related species of the genus *Orchis. Molecular Ecology* 19: 4086–4095.

JACQUEMYN, H., T. WIEGAND, K. VANDEPITTE, R. BRYS, I. ROLDÁN-RUIZ, AND O. HONNAY. 2009. Multigenerational analysis of spatial structure in the terrestrial, food-deceptive orchid *Orchis mascula. Journal of Ecology* 97: 206–216.

KATOH, K., K. KUMA, H. TOH, AND T. MIYATA. 2005. MAFFT version 5: Improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research* 33: 511–518.

KOTTKE, I., I. HAUG, S. SETARO, J. P. SUÁREZ, M. WEISS, M. PREUSSING, M. NEBEL, AND F. OBERWINKLER. 2008. Guilds of mycorrhizal fungi and their relation to trees, ericads, orchids and liverworts in a neotropical mountain rain forest. *Basic and Applied Ecology* 9: 13–23.

KOTTKE, I., J. P. SUÁREZ, P. HERRERA, D. CRUZ, R. BAUER, H. INGEBORG, AND S. GARNICA. 2010. Atractiellomycetes belonging to the 'rust' lineage (Pucciniomycotina) form mycorrhizae with terrestrial and epiphytic neotropical orchids. *Proceedings of the Royal Society, B, Biological Sciences* 277: 1289–1298.

KRÜGER, M., H. STOCKINGER, C. KRÜGER, AND A. SCHÜSSLER. 2009. DNA-based species level detection of Glomeromycota: One PCR primer set for all arbuscular mycorrhizal fungi. *New Phytologist* 183: 212–223.

LINDNER, D. L., AND M. T. BANIK. 2011. Intra-genomic variation in the ITS rDNA region obscures phylogenetic relationships and inflates estimates of operational taxonomic units in genus *Laetiporus. Mycologia* 103: 731–740.

MARTOS, F., M. DULORMNE, T. PAILLER, P. BONFANTE, A. FACCIO, J. FOURNEL, M. P. DUBOIS, AND M. A. SELOSSE. 2009. Independent recruitment of saprotrophic fungi as mycorrhizal partners by tropical achlorophyllous orchids. *New Phytologist* 184: 668–681.

MARTOS, F., F. MUNOZ, T. PAILLER, I. KOTTKE, C. GONNEAU, AND M.-A. SELOSSE. 2012. The role of epiphytism in architecture and evolutionary constraint within mycorrhizal networks of tropical orchids. *Molecular Ecology* 21: 5098–5109.

MCCORMICK, M. K., D. F. WHIGHAM, AND J. O'NEILL. 2004. Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytologist* 163: 425–438.

MCCORMICK, M. K., D. F. WHIGHAM, D. SLOAN, K. O'MALLEY, AND B. HODKINSON. 2006. Orchid-fungus fidelity: A marriage meant to last? *Ecology* 87: 903–911.

MCCORMICK, M. K., D. LEE TAYLOR, K. JUHASZOVA, R. K. BURNETT, D. F. WHIGHAM, AND J. P. O'NEILL. 2012. Limitations on orchid recruitment: Not a simple picture. *Molecular Ecology* 21: 1511–1523.

MCKENDRICK, S. L., J. R. LEAKE, D. L. TAYLOR, AND D. J. READ. 2002. Symbiotic germination and development of the myco-heterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed *Sebacina* spp. *New Phytologist* 154: 233–247.

OTERO, J. T., J. D. ACKERMAN, AND P. BAYMAN. 2002. Diversity and host specificity of endophytic *Rhizoctonia*-like fungi from tropical orchids. *American Journal of Botany* 89: 1852–1858.

OTERO, J. T., J. D. ACKERMAN, AND P. BAYMAN. 2004. Differences in mycorrhizal preferences between two tropical orchids. *Molecular Ecology* 13: 2393–2404.

OTERO, J. T., S. ARAGÓN, AND J. D. ACKERMAN. 2007a. Site variation in spatial aggregation and phorophyte preference in *Psychilis monensis* (Orchidaceae). *Biotropica* 39: 227–231.

OTERO, J. T., P. BAYMAN, AND J. D. ACKERMAN. 2005. Variation in mycorrhizal performance in the epiphytic orchid *Tolumnia variegata in vitro*: The potential for natural selection. *Evolutionary Ecology* 19: 29–43.

OTERO, J. T., N. S. FLANAGAN, E. A. HERRE, J. D. ACKERMAN, AND P. BAYMAN. 2007b. Widespread mycorrhizal specificity correlates to mycorrhizal function in the neotropical, epiphytic orchid *Ionopsis utricularioides* (Orchidaceae). *American Journal of Botany* 94: 1944–1950.

PAUW, A., AND W. J. BOND. 2011. Mutualisms matter: Pollination rate limits the distribution of oil-secreting orchids. *Oikos* 120: 1531–1538.

PEREIRA, O. L., C. L. ROLLEMBERG, A. C. BORGES, K. MATSUOKA, AND M. C. M. KASUYA. 2003. *Epulorhiza epiphytica* sp. nov. isolated from mycorrhizal roots of epiphytic orchids in Brazil. *Mycoscience* 44: 153–155.

PHILLIPS, R. D., M. D. BARRET, K. W. DIXON, AND S. D. HOPPER. 2011. Do mycorrhizal symbioses cause rarity in orchids? *Journal of Ecology* 99: 858–869.

POORTER, L., L. BONGERS, AND F. BONGERS. 2006. Architecture of 54 moist-forest tree species: Traits, trade-offs, and functional groups. *Ecology* 87: 1289–1301.

R DEVELOPMENT CORE TEAM. 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Australia.

RASMUSSEN, H. N., AND F. N. RASMUSSEN. 2007. Trophic relationships in orchid mycorrhiza—Diversity and implications for conservation. *Lankesteriana* 7: 334–341.

RASMUSSEN, H. N., AND F. N. RASMUSSEN. 2009. Orchid mycorrhiza: Implications of a mycophagous life style. *Oikos* 118: 334–345.

RASMUSSEN, H. N., AND D. F. WHIGHAM. 1998. Importance of woody debris in seed germination of *Tipularia discolor* (Orchidaceae). *American Journal of Botany* 85: 829–834.

RIPLEY, B. D. 1976. The second order analysis of stationary point processes. *Journal of Applied Probability* 13: 255–266.

ROBERTS, P. 1999. Rhizoctonia-forming fungi: A taxonomic guide. Royal Botanical Garden, Kew, UK.

ROCHE, S. A., R. J. CARTER, R. PEAKALL, L. M. SMITH, M. R. WHITEHEAD, AND C. C. LINDE. 2010. A narrow group of monophyletic *Tulasnella* (Tulasnellaceae) symbiont lineages are associated with multiple species of *Chiloglottis* (Orchidaceae): Implications for orchid diversity. *American Journal of Botany* 97: 1313–1327.

ROY, M., S. WATTHANA, A. STIER, F. RICHARD, S. VESSABUTR, AND M.-A. SELOSSE. 2009. Two mycoheterotrophic orchids from Thailand tropical dipterocarpacean forests associate with a broad diversity of ectomycorrhizal fungi. *BMC Biology* 7: 51.

SELOSSE, M.-A., A. FACCIO, G. SCAPPATICCI, AND P. BOFANTE. 2004. Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal Septomycetes, including truffles. *Microbial Ecology* 47: 416–426.

SELOSSE, M.-A., M. WEISS, J.-L. JANY, AND A. TILLIER. 2002. Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) L.C.M. Rich. and neighbouring tree ectomycorrhizae. *Molecular Ecology* 11: 1831–1844.

SHEFFERSON, R. P., T. KULL, AND K. TALI. 2008. Mycorrhizal interactions of orchids colonizing Estonian mine tailings hills. *American Journal of Botany* 95: 156–164.

SHEFFERSON, R. P., D. L. TAYLOR, M. WEISS, S. GARNICA, M. K. MCCORMICK, S. ADAMS, H. M. GRAY, ET AL. 2007. The evolutionary history of mycorrhizal specificity among lady's slipper orchids. *Evolution* 61: 1380–1390.

SHEFFERSON, R. P., M. WEISS, T. KULL, AND D. L. TAYLOR. 2005. High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium. Molecular Ecology* 14: 613–626.

SMITH, S. D. AND D. READ. 2008. Mycorrhizal symbiosis, 3rd ed. Academic Press, New York, New York, USA.

STARK, C., W. BABIK, AND W. DURKA. 2009. Fungi from the roots of the common terrestrial orchid *Gymnadenia conopsea. Mycological Research* 113: 952–959.

SUÁREZ, J. P., M. WEIß, A. ABELE, S. GARNICA, F. OBERWINKLER, AND I. KOTTKE. 2006. Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. *Mycological Research* 110: 1257–1270.

SUÁREZ, J. P., M. WEIß, A. ABELE, F. OBERWINKLER, AND I. KOTTKE. 2008. Members of Sebacinales subgroup B form mycorrhizae with epiphytic orchids in a neotropical mountain rain forest. *Mycological Progress* 7: 75–85.

SWARTS, N. D., E. A. SINCLAIR, A. FRANCIS, AND K. W. DIXON. 2010. Ecological specialization in mycorrhizal symbiosis leads to rarity in an endangered orchid. *Molecular Ecology* 19: 3226–3242.

SWOFFORD, D. 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b10.0. Sinauer, Sunderland, Massachusetts, USA.

TAYLOR, D. L., AND T. D. BRUNS. 1997. Independent, specialized invasions of ectomycorrhizal mutualism by two nonphotosynthetic orchids. *Proceedings of the National Academy of Sciences, USA* 94: 4510–4515.

TAYLOR, D. L., T. D. BRUNS, J. R. LEAKE, AND D. J. READ. 2002. Mycorrhizal specificity and function in myco-heterotrophic plants. *In* M. G. A. van der Heijden and I. Sanders [eds.], Mycorrhizal ecology, 375–413. Springer-Verlag, Berlin, Germany.

TĚŠITELOVÁ, T., J. TĚŠITEL, J. JERSÁKOVÁ, G. ŘÍHOVÁ, AND M.-A. SELOSSE. 2012. Symbiotic germination capability of four *Epipactis* (Orchidaceae) is broader than expected from adult ecology. *American Journal of Botany* 99: 1020–1032.

TREMBLAY, R. L., J. K. ZIMMERMAN, L. LEBRÓN, P. BAYMAN, I. SASTRE, F. AXELROD, AND J. ALERS-GARCÍA. 1998. Host specificity and low reproductive success in the rare endemic Puerto Rican orchid *Lepanthes caritensis*. *Biological Conservation* 85: 297–304.

WHITE, T. J., T. BRUNS, S. LEE, AND J. W. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White [eds.], PCR protocols and applications: A laboratory manual, 315–322. Academic Press, San Diego, California, USA.

WIEGAND, T., AND K. A. MOLONEY. 2004. Rings, circles and null-models for point pattern analysis in ecology. *Oikos* 104: 209–229.

WINKLER, M., K. HÜLBER, AND P. HIETZ. 2009. Population dynamics of epiphytic orchids in a metapopulation context. *Annals of Botany* 104: 995–1004.

XING, X., X. MA, Z. DENG, J. CHEN, F. WU, AND S. GUO. 2013. Specificity and preference of mycorrhizal associations in two species of the genus *Dendrobium* (Orchidaceae). *Mycorrhiza* 23: 317–324.

YUAN, L., Z. L. YANG, S.-Y. LI, H. HU, AND J.-L. HUANG. 2010. Mycorrhizal specificity, preference, and plasticity of six slipper orchids from South Western China. *Mycorrhiza* 20: 559–568.

ZETTLER, L. W., AND C. J. HOFER. 1998. Propagation of the little club-spur orchid (*Platanthera clavellata*) by symbiotic seed germination and its ecological implications. *Environmental and Experimental Botany* 39: 189–195.

ZIMMERMAN, J. K. 1991. Ecological correlates of labile sex expression in the orchid *Catasetum viridiflavum*. *Ecology* 72: 597–608.

Curriculum vitae

Personal Information

Darío Javier Cruz Sarmiento

Date of birth: 04-04-1983

Place of birth: Loja-Ecuador

Nationality and Passport: Ecuadorian; 1104012016

Parents: Blanca Sarmiento and Arturo Cruz

E–mail: djcruz@utpl.edu.ec

Work address: San Cayetano Alto s/n. Loja, Ecuador; C.P. 11 01 608

Studies Information

Primary school:	1988-1994 “Alejandrino Velazco” Loja-Ecuador
High school:	1994-2000 “Adolfo Valarezo” Loja-Ecuador
Higher Education:	2000-2006 “Universidad Técnica Particular de Loja (UTPL)”. Biochemist and Pharmaceutical Engineer’s degree (2007) with thesis title “Collection and morphological characterization of resupinate Basidiomycetes with mycorrhizal potential of epiphytic orchids in the Biological Reserve in San Francisco (RBSF) ".

Work and research experience

- 2006 - 2007: Involved as a student in the Mycorrhiza research group (UTPL).
- December 2007 until June 2009: Employed in the UTPL “Centro de Biología Celular y Molecular (CBCM-UTPL)” as Technician and research assistant in the project “Mycorrhizal fungi for growth and rehabilitation of orchids of a

tropical mountain rain forest in southern Ecuador" funded by the DFG".

- June 2009 until nowadays: Employed in the UTPL as Researcher at the Museum of Biological Collections, Section of Basic and Applied Biology, Department of Natural Sciences. The current position allows teaching at the UTPL with flexible schedule in order to have more time to research.

- October 2010: Ph.D student of Prof. Dr. Meike Piepenbring at the Department of Mycology (Institute for Ecology, Evolution and Diversity) at the J.W. Goethe-University in Frankfurt am Main, Germany.

Mycological courses and congress

- February 2015: Course "Jornadas Micológicas" at the UTPL. Directed by the Mycologist, Ph.D. Pablo Perez from Spain – University of Cordoba.

- November 2014: Poster presentation "Especies crípticas de *Tulasnella* definidas molecularmente: Correlación entre morfología y secuencias de ADNrn (ITS-5.8S) desde basidiomas" at the VIII Congreso Latinoamericano de Micologia – Cali-Colombia.

- September 2012: Course "Systematics and diversity of orchids and cultivation of mycorrhizal fungi" at the UTPL. Directed by the Postdoc Ph.D Ralph Mangelsdorff from Germany – J.W.Goethe-University Frankfurt.

- March 2012: Course "Phylogenetic" at the UTPL. Directed by the Prof. Dietmar Quandt from Germany – University of Bonn.

- July 2011: Speech presentation "Definiendo especies de *Tulasnella* correlacionando morfología y secuencias de ADNrn (ITS-5.8S) desde basidiomas en un bosque Tropical Andino" at the VII Congreso Latinoamericano de Micología – San José de Costa Rica.

- November 2010: Master Module in Mycology at the J.W.Goethe-University Frankfurt am Main, Germany. Directed by the Mycologist, Prof. Dr. Meike Piepenbring.

- February-2009: David-Panamá: “The fungi in the highlands of western of Panamá” Directed by the Mycologist, Prof. Dr. Meike Piepenbring.

- August-2009: David-Panamá: “The pathogenic fungi in useful plants–Panamá” Directed by the Mycologist Prof. Meike Piepenbring and collaborators in Panamá.

Publications in International scientific journals

1. **Cruz DJ**, Suarez JP, and Piepenbring M (2016) Morphological revision of Tulasnellaceae, with two new species of *Tulasnella* and new records of *Tulasnella* spp. for Ecuador. *Nova Hedwigia* 102(3–4): 279–338(60)
2. Cartuche L, **Cruz DJ**, Ramírez MI, Bailón N, and Malagón O (2015) Antibacterial and cytotoxic activity from the extract and fractions of a marine derived bacterium from the *Streptomyces* genus. *Pharm Biol* DOI:10.3109/13880209.2015.1010739
3. **Cruz DJ**, Suarez JP, Kottke I, and Piepenbring M (2014) Cryptic species revealed by molecular phylogenetic analysis of sequences obtained from basidiomata of *Tulasnella*. *Mycologia* 106(4), 708–722. doi:10.3852/12-386
4. Riofrio ML, **Cruz DJ**, Torres E, De La Cruz M, Iriondo JM, and Suarez JP (2013) Mycorrhizal preferences and fine spatial structure of the epiphytic orchid *Epidendrum rhopalostele*. *Am J Bot* 100(12): 1–10
5. Setaro S, Suarez JP, Herrera P, **Cruz DJ,** and Kottke I (2013) Distinct but closely related Sebacinales from mycorrhizae with co-existing Ericaceae and Orchidaceae in a neotropical mountain area. In: Varma A, Kost G, Oelmu¨ller R (eds) Sebacinales. Forms, functions and biotechnological application. Springer, Berlin, pp 81–105
6. Kottke I, Setaro S, Haug I, Herrera P, **Cruz DJ,** et al. (2013) Mycorrhiza networks promote biodiversity and stabilize the tropical mountain rain forest ecosystem – Perspectives for understanding complex communities. In: Ecosystem Services, Biodiversity and Environmental Change in a Tropical

Mountain Ecosystem of South Ecuador, *Ecological Studies*, Springer Verlag, Heidelberg

7. **Cruz DJ**, Suarez JP, Kottke I, Piepenbring M, and Oberwinker F (2011) Defining species in *Tulasnella* by correlating morphology and nrDNA ITS-5.8s sequence data of basidiomata from a tropical Andean forest. *Mycol Prog* 10: 229-238

8. Piepenbring M, Camarena J, **Cruz DJ,** Gómez AK, Guerrero Y, Hofmann TA, et al. (2011) New records of pathogenic fungi on cultivated plants in Panama. *Mycotaxon* 115: 534-535

9. Kottke I, Suarez JP, Herrera P, **Cruz DJ,** Bauer R, Haug I, et al. (2010) Atractiellomycetes belonging to the 'rust' lineage (Pucciniomycotina) form mycorrhizae with terrestrial and epiphytic neotropical orchids. *Proc R Soc Lond [Biol]* 277: 1289–1296

9 Erklärung

Ich erkläre hiermit, dass ich die vorgelegte Dissertation selbstständig angefertigt habe und nur diejenigen Hilfen oder Hilfsmittel benutzt habe, die in der Dissertation angegeben werden. Ich habe die Grundsätze der guten wissenschaftlichen Praxis beachtet.

Loja-Ecuador, 12 jan. 2016.

Doktorand