

## Phylogenetic assessment of global *Suillus* ITS sequences supports morphologically defined species and reveals synonymous and undescribed taxa

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**Abstract:** The genus *Suillus* represents one of the most recognizable groups of mushrooms in conifer forests throughout the Northern Hemisphere. Although for decades the genus has been relatively well defined morphologically, previous molecular phylogenetic assessments have provided important yet preliminary insights into its evolutionary history. We present the first large-scale phylogenetic study of the boundaries of each species in the genus *Suillus* based on the most current internal transcribed spacer (ITS) barcode sequences available in public databases, as well as sequencing of 224 vouchered specimens and cultures, 15 of which were type specimens from North America. We found that species boundaries delimited by morphological data are broadly congruent with those based on ITS sequences. However, some species appear to have been described several times under different names, several species groups cannot be resolved by ITS sequences alone, and undescribed taxa are apparent, especially in Asia. Therefore, we elevated *S. tomentosus* var. *discolor* to *S. discolor*; proposed synonymies of *S. neoalbidipes* with *S. glandulosipes*, *S. borealis* with *S. brunnescens*, *Boletus serotinus* and *B. solidipes* with *Suillus elbensis*, *S. lactifluus* with *S. granulatus*, *S. himalayensis* with *S. americanus*; and proposed usage of the names *S. clintonianus* in the place of the North American *S. grevillei*, *S. weaverae* for North American *S. granulatus*, *S. ampliporus* in the place of the North American *S. cavipes*, and *S. elbensis* in place of the North American *S. viscidus*. We showed that the majority of *Suillus* species have strong affinities for particular host genera. Although deep node support was low, geographic differentiation was apparent, with species from North America, Eurasia, and Asia often forming their own clades.

Collectively, this comprehensive genus-level phylogenetic integration of currently available *Suillus* ITS molecular data and metadata will aid future taxonomic and ecological work on an important group of ectomycorrhizal fungi.

**Key words:** Boletales, bolete, geography, molecular phylogenetics, Suillaceae, suilloid, systematics, taxonomy

### INTRODUCTION

The genus *Suillus* Gray comprises approximately 100 mushroom-forming species that are widely distributed throughout the Northern Hemisphere. *Suillus* species associate almost exclusively with members of the family Pinaceae and exhibit strong patterns of host specificity among different genera (Smith and Thiers 1964a, Klofac 2013). They can also be found where hosts have been planted and have subsequently invaded in the Southern Hemisphere (Chapela et al. 2001, Dickie et al. 2010, Walbert et al. 2010, Hayward et al. 2015), as well as Pacific islands (Hynson et al. 2013). Given its broad distribution and notable host specificity patterns, the genus *Suillus* has emerged as a model system for studying linkages among ectomycorrhizal fungal diversity, ecosystem function, population genetics, and host-symbiont coevolution (Dahlberg and Finlay 1999, Wu et al. 2000, Branco et al. 2015).

Although many species need taxonomic revisions or descriptions, the genus *Suillus* is well circumscribed, and its species boundaries are seldom disputed (Smith and Thiers 1964a, 1971; Kretzer et al. 1996; Nguyen et al. 2012; Klofac 2013). The most comprehensive morphological work on the genus that focused on North American taxa was published over 50 y ago (Smith and Thiers 1964a). This work is still considered authoritative and has long served as the backbone for *Suillus* systematics. Recently, Klofac (2013) published an overview of the nomenclatural history of the genus, in which he extensively referenced the existing literature on *Suillus*, presented a list of taxa that he accepted, and provided keys to those taxa based mostly on morphological data. He accepted 94 species in *Suillus*. Since then, *S. indicus* B. Verma & M.S. Reddy, *S. triacicularis* B. Verma & M.S. Reddy, *S. himalayensis* B. Verma & M.S. Reddy, *S. marginielevatus* S. Sarwar, Khalid & Dentinger, *S. lariciphilus* K. Das, D. Chakr., K.P.D. Latha & Cotter, and *S. adhikarii* K. Das, D. Chakr. & Cotter have been described from Pakistan and the Himalayan regions of India and Nepal (Verma and Reddy 2014a, 2014b, 2015; Adamčík et al. 2015;

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Das et al. 2015) along with the very unusual *S. foetidus* Y. Li & L.L. Qi from northeastern China (Qi 2016). The description of many new species in a relatively short time suggests the possibility of a number of additional undescribed species in currently understudied areas.

Smith and Thiers (1964a) divided the genus *Suillus* into three sections, *Paragyrodon*, *Boletinus*, and *Suillus*, although they stated that these sections were recognized “partly for historical reasons and for convenience in dealing with the species in contrasting groups even if these are based mainly on a single character” (Smith and Thiers 1964a). Section *Paragyrodon* was monotypic and included only *Paragyrodon sphaerosporus* (Peck) Singer, a *Quercus*-associated species that Singer (1942) had previously placed in its own genus. An early mitochondrial mapping study subsequently confirmed that it did not belong in *Suillus* (Bruns and Palmer 1989). Section *Boletinus*, which was recognized as a distinct genus (Singer 1975), was included in *Suillus* and described as having either stipes with a fibrillose zone or a distinct membranous annulus or a distinctly fibrillose-squamulose pileus. Section *Suillus* was further broken down into series *Hirtellini* (misspelled as *Hirtellinii*) and *Suilli*. Species in series *Hirtellini* have a glandular-dotted stipe, although the glands often appear as smears rather than dots. The immature (button) pileus is covered by a tomentum that in fully expanded mature specimens aggregates into tufts or becomes glabrous in some species. For species of series *Suilli*, the veil forms a well developed annulus or it adheres to the pileus margin when it breaks open, leaving a “cottony roll” on the pileus margin; young specimens may have a distinct false veil (i.e. one not attached to the stipe but initially almost covering the tube cavity). Species with dark brown spores were excluded by Smith and Thiers (1964a) from *Suillus* and treated as members of a separate genus *Fuscoboletinus* Pomerl. & A.H. Sm., but mitochondrial mapping results disagreed with that segregation (Bruns and Palmer 1989).

The ability to sequence DNA started the modern era of molecular phylogenetic studies, with the first comprehensive molecular sequence-based work on *Suillus* conducted by Kretzer et al. (1996). Although that study was published 20 y ago and included only a limited sampling of species in the genus, it still represents the most comprehensive molecular phylogenetic study to date. It confirmed that *Fuscoboletinus* was part of *Suillus* and was neither a distinct nor a natural genus. Various other studies have used molecular phylogenetics to identify and support morphological taxonomy of species in their regional mycota, although species sampling across the genus was limited (Sarwar et al. 2011; Min et al. 2014; Sarwar and Khalid 2014;

Verma and Reddy 2014a, 2015; Qi et al. 2016). Sequencing of holotype specimens has been shown to be essential in assigning correct names to phylogenetic clades and delimiting species. For example, Nguyen et al. (2012) showed that by sequencing holotype specimens *S. ponderosus* A.H. Sm. & Thiers and *S. caeruleus* A.H. Sm. & Thiers each contained a variety of the species *S. imitatus* A.H. Sm. & Thiers, whereas Bruns et al. (2009) produced sequences from holotypes to indicate that *S. quiescens* T.D. Bruns & Vellinga indeed had not been described before. The widespread application of DNA sequencing has also produced numerous sequences from environmental samples (i.e. either ectomycorrhizal root tips or soil). These samples cannot be accurately assigned to species without the use of molecular phylogenetics, so placing these data in the context of a comprehensive global specimen-based phylogeny is necessary to assign taxonomic names properly (Bonito et al. 2010; Nguyen et al. 2012, 2013).

Molecular data has been useful in delineating and identifying *Suillus* species when macroscopic characters are plastic or hard to interpret (Nguyen et al. 2012). Most species distinctions are based on appearance of fresh collections, host associations, and geography. Microscopic differences in cystidial position and clustering, pileipellis structure, or spore color distinguish some major groups within the genus. However, distinctions among closely related species are generally limited to subtle differences in spore sizes that are not based on extensive measurements of multiple collections and rarely hold up to scrutiny. Although most species are distinct when young, many older specimens look similar to each other and their appearances are affected by environmental factors such as heavy rain, drying winds, or sun exposure that can lead to misidentifications. Developmental changes in color are also common. For example, some species always have white basidiocarps, whereas others start out white but darken to various colors, while a third group is made up of white variants of typically colored species. These variations often make identification based solely on morphology difficult to impossible. One frequently used aid to morphological identification is host association. In native environments, *Suillus* species co-occur with their hosts and are thus restrained by host biogeography. However, a few species have naturally broad geographic distribution and others have been introduced with novel host species in many regions where they were not native (Vellinga et al. 2009), further complicating correct identification.

Along with helping to address morphological and geographical ambiguities, DNA sequence data of *Suillus* specimens have also been used to examine patterns of host-symbiont co-evolution (Kretzer et al. 1996,

Wu et al. 2000). Based on field reports of host association, Kretzer et al. (1996) showed that *Suillus* clades (although some weakly supported) were associated with *Larix*, *Pseudotsuga*, or with different subgenera of *Pinus*, subgenus *Strobos* (hereafter referred to as soft pines), or subgenus *Pinus* (hereafter referred to as hard pines), as well as one species associating with *Quercus*. Wu et al. (2000) focused on a small group of the *Pinus*-associated species and found evidence of host specificity for two species groups from North America and China.

To further facilitate taxonomic, ecological, and evolutionary work on *Suillus*, we assembled all the available sequences of the nuclear rDNA internal transcribed spacers (ITS) for this genus and provided additional sequences for type material and vouchered specimens. We analyzed these data phylogenetically and used those analyses to discuss species boundaries and trends of host associations across the entire genus, with an emphasis on North American taxa. Whenever possible, we used holotype data to inform our assessments of taxonomic categorization, provided novel taxonomic treatments for various members of the genus, and pointed to species and species complexes where more taxonomic work is still needed.

#### MATERIALS AND METHODS

We sequenced freshly collected basidiocarp vouchers, existing collections deposited in herbaria, and cultures for this study. Loans of types and specimens deemed important were sent from DBG, MICH, and SFSU. Many of the fresh specimens from Colorado were contributed by the Pike's Peak Mycological Society and the Colorado Mycological Society on targeted forays to collect *Suillus*. Other specimens were contributed by citizen mycologists around the country. Specimens collected for this project were deposited in the University of California (UC) Herbarium (SUPPLEMENTARY TABLE I). Herbarium abbreviations are according to Thiers (2016 continuously updated).

DNA was extracted from dried or cultured tissue by using a modified Sigma Extract-N-Amp kit (Sigma Aldrich, St Louis, Missouri) for fresh or recently collected specimens. For those that have been stored under herbarium conditions, especially type specimens, DNA was extracted with a standard 2% CTAB buffer with 2% polyvinylpyrrolidone (PVP, mw 40 000) and chloroform, precipitated with isopropanol, followed by cleanup with ethanol. Standard PCR conditions were followed using the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) initially for all specimens. For those with degraded DNA (due to storage over decades), we used a combination of ITS1F and ITS2, ITS3, and ITS4 (Bruns et al. 2009) primers to individually amplify the nuclear rDNA ITS1-5.8S-ITS2 repeat. Amplified products were sequenced using standard BigDye® Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California).

Each automated sequence was manually interpreted and corrected for ambiguous base calling. The sequences generated

in this study were deposited in GenBank under accession Nos. KX213693–KX213835 and KX230573–KX230654 (SUPPLEMENTARY TABLE I). All ITS sequences of *Suillus* from GenBank or those that were released from UNITE (<https://unite.ut.ee>) were downloaded as of 1 Nov 2015. To find environmental sequences not classified under *Suillus*, representative sequences of vouchered species were matched to GenBank sequences with at least a 93% sequence similarity using BLAST. Sequences shorter than 150 bp were discarded, except in the case of a few species where these were the only sequences available. Locality and host metadata for each sequence were downloaded from GenBank using a custom Perl script.

All sequences were initially aligned using MAFFT and optimized manually: An initial phylogenetic tree was produced that allowed the sequences to be reorganized by clades, and sequences within each clade were realigned manually when necessary. Beginnings and ends of sequences were coded as missing when not available. Phylogenetic inference was made using the GTRGAMMA model in RAXML (Stamatakis et al. 2005). Maximum likelihood bootstrap support values were calculated from 1000 replications. Midpoint rooting was used for the tree, because the ITS sequences of taxa outside of *Suillus* (e.g. *Truncocollumella*, *Rhizopogon*) could not be aligned with confidence so no outgroup could be assigned. In cases where well supported taxa nested inside of a group of loosely defined taxa (*S. weaverae*, *S. subaureus*, *S. decipiens*, /suilloides clade), we used a subset of the main alignment for each taxon, realigned as necessary, and re-analyzed those sequences using Bayesian Inference implemented in MrBayes (Ronquist et al. 2012). Each subset of data was run for at least 1 000 000 generations until the trees converged and average split frequency was <0.01. The sequence alignment for this project is available under TreeBase study No. S19256. Clade notations are based on Moncalvo et al. (2002), i.e. a non-capitalized name preceded by a forward slash.

#### RESULTS

We successfully obtained ITS sequences for 15 type specimens and 209 sequences from vouchered specimens and cultures. Combined with environmental sequences, the final alignment contained 1029 sequences. The deeper phylogenetic nodes of the tree could not be reconstructed with confidence, but the terminal branches of the tree grouped into well defined and well supported clades (FIG. 1). Due to the large size of the tree, we have divided it into three major groups to facilitate interpretation and discussion: the *Spectabilis* group, the *Granulatus* group, and the *Tomentosus* group (this latter group includes the morphologically distinct *S. bovinus*). We readily acknowledge that these groups are not statistically supported but fit together using a combination of the ITS sequences and the morphological data reflecting Smith and Thiers's (1964a) sect. *Boletinus* plus *Fuscoboletinus* (≈*Spectabilis* group), series *Suilli* (≈*Granulatus* group), and series *Hirtellini* (≈*Tomentosus* group).

The three groups contain multiple clades that typically reflect morphological species, but each group also contains clades that require further taxonomic work. The *Spectabilis* group contains a suite of species that associate exclusively with hosts other than *Pinus* (i.e. *Larix*, *Pseudotsuga*, and possibly *Picea*). It contains two clades of *Larix*-associated species and one clade of *Pseudotsuga*-associated species; the /*grevillei* and /*lakei* clades are well supported (>97%), whereas the /*spectabilis* clade is not. The species status of the members of the /*lakei* and /*spectabilis* clades is taxonomically stable, but multiple entities appear that have been subsumed under the same name for the /*grevillei* clade. The *Granulatus* group represents the most diverse group of *Suillus* in this study. Many of the species have high statistical phylogenetic support, but some clades contain multiple taxa that could not be resolved (/ *placidus* and / *flavidus* clades) due to the lack of sequences from type specimens to anchor epithets. Other clades (e.g. / *acidus*, / *glandulosipes*, / *albivelatus*, and / *luteus* clades) were well resolved but each clade contained multiple holotypes. Compared to the other two groups, the *Tomentosus* group was held together with moderately high bootstrap support (89%), even though the data is based on a single gene region. Many of the recognized species within the *Tomentosus* group clustered into clades, although only a few were well supported.

*Species for which multiple names are applied.*—We identified three species that are currently accepted under multiple names with similar if not identical ITS sequences. These are species in the / *glandulosipes* clade, the / *albivelatus* clade, and the / *luteus* clade. The / *glandulosipes* clade contains two species, *S. glandulosipes* Thiers & A.H. Sm. and *S. neoalbidipes* M.E. Palm & E.L. Stewart, that have very similar morphological characteristics. In particular, these species share an abundant cottony veil that covers the tubes in young specimens but differ in that *S. glandulosipes* is thought to be a predominantly western species and to have more conspicuous clusters of caulocystidia (Palm and Stewart 1984). The holotype specimens of both species fall within one well defined clade with 100% bootstrap support. Therefore, we consider these two species as one, with *S. neoalbidipes* as a synonym of *S. glandulosipes*.

The / *albivelatus* clade includes sequences under four names: *S. albivelatus* A.H. Sm., Thiers & O.K. Mill., *S. pseudoalbivelatus* B. Ortiz & Lodge, *S. wasatchicus* Thiers and *S. volcanalis* Thiers. *Suillus albivelatus* was described from the Priest River area in northern Idaho (Smith et al. 1965); *S. wasatchicus* was described from the Wasatch Range of Utah (Thiers 1976), and *S. volcanalis* from Lassen County in Northern

California (Thiers 1967). *Suillus pseudoalbivelatus* is the only species in this clade not occurring in western North America; it is reported only from the Dominican Republic (Ortiz-Santana et al. 2007). All four holotypes are included in our phylogeny, and because no strong bootstrap support holds this clade together, we refrain from synonymizing these names. Should better data result in a well supported clade, the name *S. albivelatus* would supersede the other names.

The / *luteus* clade, although not statistically supported, holds all the specimens that have been identified as *S. luteus* (L.) Roussel, a common species that occurs with *P. sylvestris* in Europe and elsewhere where *P. sylvestris* and other *Pinus* species have been introduced. Two species thought of as North American endemics, *S. borealis* A.H. Sm. Thiers & O.K. Mill. and *S. brunnescens* A.H. Sm. & Thiers, nested within this clade. The name *S. borealis* A.H. Sm., Thiers & O.K. Mill. is illegitimate because of *S. borealis* (Peck) Kuntze described from Labrador, Canada. We are unable to confirm the identity of *S. borealis* (Peck) Kuntze, but the current concept of *S. borealis* is based on *S. borealis* A.H. Sm., Thiers & O.K. Mill. (Smith et al. 1965). This species has a strong persistent veil, similar to *S. luteus*, whereas *S. brunnescens* has a thin and membranous veil that disappears with age. The change in color from white to brown and a weak veil is used to distinguish *S. brunnescens* from the others, but our collections showed intermediate characteristics with nearly white basidiocarps that mature to brown with persistent veils. The relatedness of *S. luteus*, *S. borealis*, and *S. brunnescens* had not escaped the attention of Smith et al. (1965), who suggested that these species belong to stirps *Luteus*. However, we showed that the holotype ITS sequences of *S. borealis* and *S. brunnescens* are identical to each other. Therefore, we consider *S. borealis* a synonym of *S. brunnescens* due to their plastic morphological characters as well as identical ITS sequences. However, currently we do not consider this North American species to be conspecific with *S. luteus* because of its occurrence with soft pines (see DISCUSSION). Although we were not able to obtain holotype sequences for all the key members from several other clades, we think it is useful to discuss their potential taxonomic status below.

The / *salmonicolor* clade contained specimens that were identified as *S. subluteus* (Peck) Snell, *S. cothurnatus* Singer, and *S. salmonicolor* (Frost) Halling. This group of species has thin stipes, an annulus that is persistent, thick, often baggy and slimy, and salmon-colored flesh in the stipe. Halling (1983) considered *S. subluteus* and *S. pinorigidus* Snell & E.A. Dick synonyms of *S. salmonicolor* and Smith and Thiers (1964a) considered *S. cothurnatus* as a southern USA variant of *S. subluteus*. Currently, those who have collected

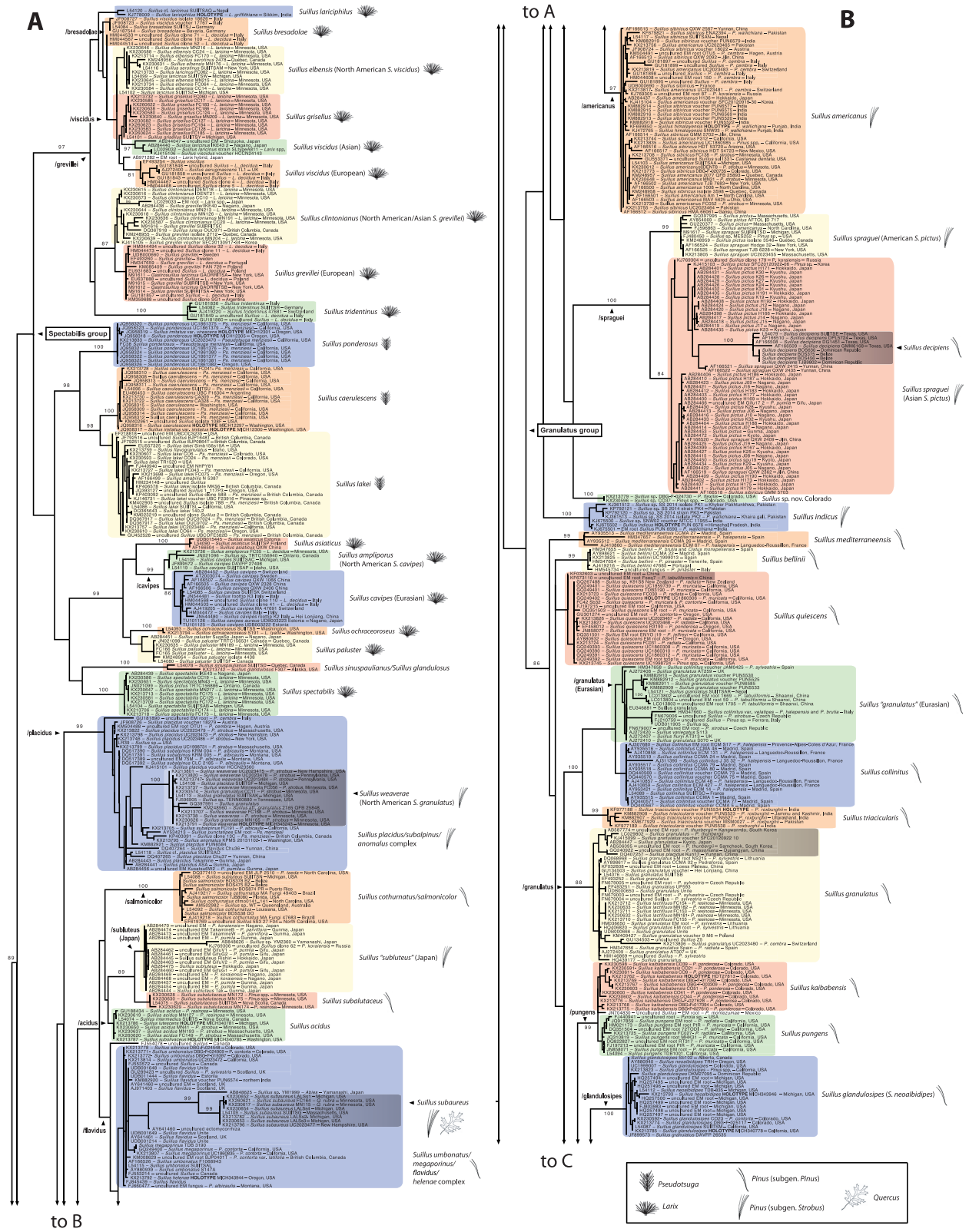


FIG. 1. ITS maximum likelihood tree of *Suillus* specimens and environmental sequences. Numbers on branches are maximum likelihood bootstrap values. Only bootstrap values >80% are shown. Each terminal leaf is annotated as accession number – species/sequence name – host – locality when they are available. Host abbreviations: Q = *Quercus*, P. = *Pinus*, Ps. = *Pseudotsuga*, L. = *Larix*. Clade names are preceded by a forward slash.



and identified *Suillus* suspect that *S. cothurnatus* and *S. salmonicolor* are synonymous, but there are no sequences available to support this hypothesis and the type collections for both of these species were made in the 19th century. If these two species were shown to be the same taxon, *S. salmonicolor* would be the oldest available name.

The /acidus clade contained sequences from the holotypes of *S. lutescens* A.H. Sm. & Thiers and *S. subolivaceus* A.H. Sm. & Thiers. We were unable to obtain a sequence for the type of *S. acidus* (Peck) Singer, but all specimens of what we identified as *S. acidus* based on morphology fit into this clade. (Smith and Thiers 1964a) thought that *S. lutescens* was closely related to *S. acidus*, and our sequence data support that relationship. To confirm the correct name for this clade, the holotype sequences for *S. acidus* will need to be obtained. Given the age of the holotype (before 1905), epitypification may be the best approach.

A similar problem exists within the /placidus and /flavidus clades, with no holotype sequences (except *S. helenae* Thiers & A.H. Sm. in the /flavidus clade) available to anchor species epithets. The /placidus clade was well supported (99%) and contained specimens from both North America and Europe; names such as *S. placidus* (Bonord.) Singer, *S. subalpinus* M.M. Moser, *S. punctatipes* (Snell & E.A. Dick) Singer, and *S. anomalus* T.J. Baroni, Largent & Thiers have all been applied to sequences and specimens in this clade. *Suillus placidus* has a white pileus that does not change color as the specimen ages; the species was described from Germany but is strictly associated with introduced North American *P. strobus* (Bonorden 1861). It is thought that this species was introduced to Europe with its North American *P. strobus* host. *Suillus subalpinus* is a high-altitude species that associates with soft pines. *Suillus anomalus* has pores that continue down the stipe, and although it morphologically appears to be distinct, its ITS sequence was not. The North American species generally called *S. granulatus* also belongs in this clade (see *The case of Suillus granulatus*) and forms a well supported taxon. The /flavidus clade contained specimens with names such as *S. umbonatus* E.A. Dick & Snell, *S. megaporinus* Snell & E.A. Dick, *S. flavidus* (Fr.: Fr.) J. Presl, and *S. helenae*. Members of this clade exhibit the largest diameter of pores in the genus (up to 1 cm). The name *S. flavidus* has been used for European specimens, whereas the name *S. umbonatus* is applied to the western North American specimens, and *S. megaporinus* is applied only to specimens collected in the Sierra Nevada of California. The holotype sequence of *S. helenae* (named for Helen Smith, Alexander Smith's wife) also fits within the group and was described from a single collection in Oregon. If holotypes of the aforementioned

species fit in this clade, *S. flavidus* is the oldest name and should be used for this species. Finally, *Suillus subaureus*, the lone angiosperm-associated species in this genus, was well supported (99%) and although nested, is separate from the rest of the sequences in the /flavidus clade.

*Suillus americanus* is the most naturally widespread *Suillus* species in the Northern Hemisphere. It is the only species found in North America, Eastern Europe through Pakistan, India, Russia, and Japan. Previously it was known under two names, *S. americanus* and *S. sibiricus*. Klofac (2013) subsumed these species under *S. americanus*. The lack of discernable phylogenetic structure for the /americanus clade agrees with this taxonomic change. The species currently has two varieties (var. *americanus*, var. *reticulipes*) and three formae (f. *americanus*, f. *helveticus*, and f. *sibiricus*). These within-species morphological variants are expected for such a widespread distribution. These forms are not easily distinguishable with the ITS, but inclusion of data from other genes may allow their separation. *Suillus himalayensis* B. Verma & M.S. Reddy was described and accepted as a species even though the two representative sequences did not form a well supported clade, and they were nested within *S. americanus* (Verma and Reddy 2014b). Our results from a much larger sampling of the species (FIG. 1B) show that the two sequences of *S. himalayensis* fit within *S. americanus*, and we consider the morphological differences as variations within *S. americanus*. We therefore consider *S. himalayensis* a synonym of *S. americanus*. Unfortunately, the epithet *americanus* takes precedence because the epithet *sibiricus* more clearly speaks to its distribution in higher latitudes or altitudes in the Northern Hemisphere.

*Undescribed taxa and new combinations.*—We think that the set of species discussed below has strong enough phylogenetic signal in our analysis to be considered new species. However, we note that in some cases, these species may have already been described under *Boletus*, and studies must be conducted to transfer those species to *Suillus*.

The /viscidus clade contains the currently accepted name *Suillus viscidus* (L.) Roussel, which has been applied broadly to three distinct clades of mushrooms occurring in North America, Europe, and Asia. The name *S. viscidus*, described as *Boletus viscidus* L. from Europe is best applied to the European clade, whereas the name *Suillus elbensis* (Peck) Kuntze best fits the North American taxon. Other North American names that would fit this taxon are *Boletus serotinus* Frost and *Boletus solidipes* Peck, but both names are younger than *Boletus elbensis* Peck. The clade from eastern Asia does not appear to have been critically compared to

related European and North American taxa and may require a new name. The consortium of sequences that we call the /bresadolae “clade” is loosely held together with specimens from Europe and India. Further studies and collection of Indian specimens will help determine whether they should be segregated from the European *S. bresadolae*.

*Suillus grevillei* (Klotzsch) Singer was monophyletic (with an 87% bootstrap support) although the clade was split between specimens from North America/Eurasia and those strictly from Europe. Korhonen et al. (1993) suggested that this group contained two species, where *S. grevillei* occurring mostly in Europe with lemon-yellow pileus, hyaline hyphae in the pileipellis, and smaller spores compared to *S. clintonianus* (Peck) Kuntze occurring mostly from Siberia to eastern Asia and in North America with dark reddish-brown pileus, mainly encrusted hyphae in the pileipellis and larger spores. Phylogenetic distance between these two groups, combined with morphological distinction, provide convincing evidence to recognize them as separate species. Therefore, we suggest that the name *S. grevillei* be reserved for the European taxon and *S. clintonianus* be applied to the North American/Asian taxon. The occurrence of *S. clintonianus* in North America and Asia observed by Korhonen et al. (1993) based on morphology and confirmed here with molecular data hints at a biogeographic pattern that may be linked to the distribution of their *Larix* hosts with which they fruit abundantly.

The /cavipes clade contained species that are mostly well defined morphologically and well supported by phylogeny (FIG. 1A). *Suillus cavipes* (Opat.) A.H. Sm. & Thiers itself may be separated into two species, one from Eurasia and the other from North America. It was originally described as *Boletus cavipes* Opat. from subalpine habitats in Styria, Austria (Opatowski 1836). Therefore, the name is best applied to the European/Asian clade. The name *Suillus ampliporus* (Peck) Kuntze is available for the North American taxon. Index Fungorum ([www.indexfungorum.org](http://www.indexfungorum.org)) erroneously lists *Boletinus porosus* (Berk.) Peck as a synonym of *S. cavipes*, but that species is considered a synonym of *Boletinellus merulioides* (Schwein.) Murrill (Both 1993). The Chinese species *S. cavipoides* (Z.S. Bi & G.Y. Zheng) Q.B. Wang & Y.J. Yao might be synonymous with the Eurasian *S. cavipes*. Unfortunately, no sequences of *S. cavipoides* were available for this study (Wang and Yao 2004).

The /subluteus (Japan) clade was sister to *S. subalutaceus* (A.H. Sm. & Thiers) A.H. Sm. & Thiers. It was a moderately supported clade of mostly environmental samples, with the majority of sequences from Japan and one from Russia. The clade contained three specimens that were identified as *S. subluteus* (Peck) Snell. These sequences from Japan clearly do not

represent *S. subluteus*, a species in the /salmonicolor clade. Instead, this represents an unnamed species native to East Asia.

The /spraguei clade was well supported (100%). Within it were several subclades separated by geography, with an American clade and a Japanese clade. The separation of these two clades reflects an earlier study by Burchhardt et al. (2011). Based on relative branch lengths of each of these clades compared to the rest of the genus, we propose that the Japanese clade does not belong to the same species as the North American *S. spraguei* (Berk. & M.A. Curtis) Kuntze. The North American species is better known as *S. pictus* (Peck) Kuntze, but that name is based on the illegitimate name *Boletus pictus* Peck. Both the American and Japanese species associate with soft pines. *Suillus decipiens* (Peck) Kuntze, an American species that associates with hard pines, nests within the Japanese clade (although Bayesian analysis showed that it was not nested, SUPPLEMENTARY FIG. 1). Related to the /spraguei clade are two collections that were identified as *S. sibiricus* (Singer) Singer. However, these are not closely related to true *S. americanus* (Peck) Snell (syn. *S. sibiricus*) and instead appear to be an unnamed species found at high elevation in the southern Rocky Mountains of Colorado, USA. This species occurs with soft pines, most likely *Pinus flexilis*. We plan to describe this species once more specimens become available.

*Suillus tomentosus* var. *discolor* A.H. Sm., Thiers & O.K. Mill. nested well within the *Tomentosus* group (100% clade support). Specimens identified as this variety grouped well together (97% clade support) and were separate from *S. tomentosus* (Kauffman) Singer. Furthermore, var. *discolor* associates with soft pines, whereas *S. tomentosus* associates with hard pines. Therefore, we elevate this variety to species as *Suillus discolor* (see TAXONOMY).

*The case of Suillus granulatus.*—Within the /placidus clade was a well supported clade (100%) of a well known North American species that has long been called *Suillus granulatus* (L.) Roussel. Whether this species is the same as the European species under that same name was fervently debated between Singer (1945) and Smith and Thiers (1964a, 1966). Singer (1945) described the North American specimens as *S. granulatus* ssp. *snellii* based on spore size, spore color (isabella), and an association with soft pines (*P. strobus*), which distinguishes the subspecies from the European taxon that associates with hard pines. As a result of this debate, no firm name had been accepted for general use, and the name *S. granulatus* has been in continual use in North America to the present. Our molecular evidence indicates that the



species in North America that associates with soft pines is different from the European *S. granulatus*. Surprisingly, the holotype sequence of *S. weaverae* described by Smith and Shaffer (1965) fits firmly within this clade. *Suillus weaverae* was described based on its “avellaneous to cacao brown” spore color, which does match specimens of *S. granulatus* ssp. *snellii* (cinnamon) as described by Singer. The photo of original material of *S. weaverae* shows a dark variant of *S. granulatus* sensu American authors with an infection of a *Hypomyces* ([http://iucn.ekoo.se/iucn/species\\_view/415953](http://iucn.ekoo.se/iucn/species_view/415953)). Unfortunately, we have not been able to obtain a holotype sequence for ssp. *snellii*, but even if the holotype of ssp. *snellii* fits firmly in this clade, the species level status of *S. weaverae* supersedes the subspecies level of *S. granulatus* ssp. *snellii* as the only available name for this clade. With morphological, photographic, and molecular evidence, we propose *S. weaverae* as the correct name for this species.

The /granulatus clade is the best fit for the true *S. granulatus*. Our phylogeny showed an Asian subclade, and although different, the branch length of this unsupported clade was not long enough to consider it a separate species without other evidence. North American specimens identified as *S. lactifluus* (With.) A.H. Sm. & Thiers also nested within this clade. It has long been suspected that this is an eastern North American introduction of the European *S. granulatus* (Murrill 1910, Singer 1965), but Smith and Thiers (1964b) thought that this species has “improperly been regarded as a synonym of *S. granulatus*”. Our data show that this species is indeed the same as the European *S. granulatus* or at least not the same species as the American *S. granulatus*. Although there is no type collection for *S. granulatus* (L.) Roussel, the concept of this species in Europe is clear.

The /granulatus (Eurasian) clade contains specimens called *S. granulatus*, *S. variegatus* (Sw.) Richon & Roze, *S. collinitus* (Fr.) Kuntze, and *S. fluryi* Huijsman. The names *S. variegatus* and *S. collinitus* were misapplied to the respective specimens because *S. variegatus* belongs in the Tomentosus group (see DISCUSSION) and *S. collinitus* is sister to the /granulatus (Eurasian) clade in consideration. Further studies will be necessary to determine the exact taxonomy of this clade.

*Recently diverged species and species complexes.*—The /pungens clade contained two species that appear recently diverged even though their morphologies are distinct and they can be separated easily. *Suillus pungens* Thiers & A.H. Sm. is a common species occurring with *P. muricata*, *P. radiata*, and introduced *P. pinea*, *P. pinaster*, and *P. halapensis* along the California coast (USA), whereas *S. kaibabensis* Thiers is a common

species occurring with *P. ponderosa* in the southwestern USA. One ectomycorrhizal root tip sequence from Mexico nested within this group, although it is unclear whether it best fits within *S. kaibabensis* or *S. pungens*. More material from Mexico may reveal that this complex has species that recently diverged through geographic and climatic separation between coastal California, Mexico, and the mountains of the southwestern USA.

The /pseudobrevipes clade contained four subclades or species that are similar morphologically and are difficult to distinguish due to evanescent veils often absent at maturity or in adverse weather. It included the widespread *S. albivelatus* (discussed above), *S. pseudobrevipes* that occurs with *P. ponderosa* in western North America, and *S. occidentalis* Thiers that occurs in the southwestern USA. The /sp. (Florida) clade might be the same taxon as *S. pseudogranulatus* (Murrill) A.H. Sm. & Thiers, but unfortunately we were not able to examine the fresh specimens from Florida to confirm the evanescent veil and color characteristics that would identify them as that species. *Suillus floridanus* Murrill is more closely related to *S. decipiens* and *S. spraguei*. Furthermore, one sequence from an ectomycorrhizal root tip found in Mexico may represent a distinct subclade of this complex. In this clade, morphological similarity appears to be reflected by ITS similarity. While no strong bootstrap support suggests that these four species could be collapsed into one, these names are well known, the species are widespread, and morphological characteristics can be used to differentiate them when observed carefully. We choose to retain the current nomenclature as a case of recent speciation.

#### TAXONOMY

Summarizing our results, we consider: (i) *S. neoalbidipes* M.E. Palm & E.L. Stewart a synonym of *S. glandulosipes* Thiers & A.H. Sm.; (ii) *S. borealis* A.H. Sm. Thiers & O.K. Miller is a synonym of *S. brunnescens* A.H. Sm. & Thiers; (iii) *Boletus serotinus* Frost and *Boletus solidipes* Peck synonyms of *Suillus elbensis* (Peck) Kuntze; (iv) *S. lactifluus* (With.) A.H. Sm. & Thiers a synonym of *S. granulatus* (L.) Roussel; and (v) *S. himalayensis* B. Verma & M.S. Reddy a synonym of *S. americanus* (Peck) Snell. Further, we propose usage of the names: (xi) *S. clintonianus* in the place of the North American *S. grevillei*; (xii) *S. ampliporus* in the place of the North American *S. cavipes*; (xiii) *S. elbensis* in place of the North American *S. viscidus*; and (xiv) *S. weaverae* in place of the North American *S. granulatus*.

**Suillus discolor** (A.H. Smith, Thiers & O.K. Miller)  
N.H. Nguyen, comb. & stat. nov. FIG. 2  
Mycobank MB816996

*Basionym:* *Suillus tomentosus* var. *discolor* A.H. Smith, Thiers & O.K. Miller, Lloydia 28:134. 1965.

*Typification:* USA: IDAHO. Reeder Bay, Priest Lake, Kaniksu National Forest between Priest Lake and Upper Priest Lake, 3 Oct 1964, A.H. Smith Sm-71001 (**holotype** MICH341813). GenBank ITS KX213790.

This taxon was separated from var. *tomentosus* based on the color of the dried specimens, having a vinaceous brown cap with no yellow and reddish tones present in and over the base of the stipe. However, *S. tomentosus* associates with hard pines (subgenus *Pinus*), whereas *S. discolor* associates with soft pines (subgenus *Strobus*, in particular *P. monticola* and *P. albicaulis* in western North America). We have observed these host patterns in the field, which has also been reported by Mohatt et al. (2008). Molecular data showed that *S. discolor* is closely related to *S. tomentosus* but is not nested within *S. tomentosus*, therefore further supporting its elevated species status. The species is reported from a broad range throughout western North America from Idaho, Montana, Colorado, New Mexico, Washington, Oregon, and California.

#### DISCUSSION

By combining all the sequences available from GenBank, our own specimens, and host and geographic metadata attached to each sequence, we assembled the newest species concepts for the genus *Suillus*. In particular, the availability of multiple sequences per species allowed us to infer genetic variations within and among species. In conjunction with sequencing of type specimens, this approach is a good start toward solving current taxonomic difficulties and connecting morphologically identified species with phylogenetic species. However, the weakness of a single gene study is concerning. Apparently, many of the species-level clades remain unresolved and recently diverged taxa could not be distinguished readily from each other. Combining the ITS region with other genes such as EF1- $\alpha$  from vouchered specimens represents a better approach to delimit species and detect biogeographic signals as shown by the global study of *Pluteus* (Justo et al. 2014). Unfortunately, sequencing only vouchered specimens would exclude most, if not all, of the environmental sequences that provided much useful associated host and geographic data seen in this study.

Despite those concerns, we think ITS still serves as a good barcoding gene region that mostly reflects species boundaries set by careful studies of morphological species in *Suillus*. Overall, we observed strong congruence between morphological species and ITS-defined phylogenetic species (FIG. 1). As expected, when multiple specimens or sequences were available for

comparison, they grouped well together and often apart from sister clades. In some cases, we observed that genetic distance of some sequences within a clade was larger than distance between clades (e.g. long branches). This may be due to the broad geographic nature of some species or poor quality sequences from environmental samples. Because those divergent sequences were submitted without their chromatograms, it was not possible for us to determine the quality of the nucleotide base calls. Long branches also occurred in some clades where it was necessary to include partial sequences of either the ITS1 or ITS2 regions. Despite these anomalies in the dataset, clades of one species tended to fall well apart from clades of other species.

In a few cases, well supported taxa nested inside of a group of loosely defined taxa (*S. weaverae*, *S. subaureus*, *S. decipiens*, /suilloides clade). Separating the alignments of those individual groups and re-analyzing them with maximum likelihood did not resolve their position, but Bayesian inference showed that *S. subaureus* and *S. decipiens* formed well supported un-nested clades, whereas *S. weaverae* and the *S. suilloides* clades remained nested within larger clades (SUPPLEMENTARY FIG. 1). Therefore, we attribute this inconsistency to the methodology and the minimal character information that the ITS gene region could provide. Addition of more loci will likely improve clade topology.

From sequences obtained from ectomycorrhizal root tips and field basidiocarp records, we observed that *Suillus* is associated with at least two of the four subfamilies of the Pinaceae: Pinoideae (*Pinus*), Laricoideae (*Larix*, *Pseudotsuga*), and possibly Piceoideae (*Picea*, *Cathaya*) but not Abietoideae (*Abies*, *Cedrus*, *Keteleeria*, *Tsuga*). Note that associations with *Picea* and *Cathaya* remain unconfirmed. Field records indicate that *Suillus glandulosus* (Peck) Singer occurred in mixed woods with *Picea mariana* and *Abies balsamea* and that *S. sinuspaulianus* (Pomerl. & A.H. Sm.) E.A. Dick & Snell occurred in areas with *P. glauca*, *P. mariana*, and *L. laricina*, but the occurrence of these two species on ectomycorrhizal root tips of *Picea* has not been confirmed. These host species overlap in habitat with *L. laricina*. Based on our phylogeny, we think that the primary host is *L. laricina* with *Picea* spp. or *A. balsamea* likely acting as secondary hosts. We did not find records of *Suillus* associating with the monotypic *Cathaya agrophylla* from southern China. Horton et al. (2005) studied roots of *Tsuga* and *Pseudotsuga* in the Pacific Northwest and found *S. punctatipes* (based on a 3-enzyme digest RFLP pattern) associating with *Pseudotsuga* but not *Tsuga*. Results from seedling inoculations of several *Suillus* species also confirmed that *S. tomentosus*, *S. clintonianus* (North American *S. grevillei*), and *S. caerulescens* colonize their respective

hosts but not *Tsuga* (SUPPLEMENTARY FIG. 2). These two lines of evidence suggest at the least that *Tsuga* is not a host for *Suillus*, and the lack of ectomycorrhizal root tips associated with *Abies*, *Cedrus*, and *Keteleeria* suggests that the subfamily Abietoideae is not associated with *Suillus*. *Suillus subaureus* from the midwestern USA and Ontario, Quebec, and Nova Scotia in Canada represents an exceptional case of host specificity, as it appears to have jumped to associating with angiosperm hosts in the genera *Quercus* and *Populus* (Smith and Thiers 1964a, 1971) and is able to produce basidiocarps with these hosts when *Pinus* individuals are locally absent.

Although deep-branch support was not obtainable based on data from a single gene region, our results do suggest some patterning of host association. Species that associate with *Pseudotsuga*, *Larix*, and perhaps *Picea* appear to cluster in the Spectabilis group. Whether these groups evolved as basal to the genus as stated in Kretzer et al. (1996) remains to be demonstrated with a multilocus phylogeny. *Pinus* is the most common host for *Suillus*, and there have been multiple switches between *Pinus* subgenus *Pinus* and subgenus *Strobus*. However, one large clade (contains species such as *S. mediterraneensis*, *S. brevipes*, and *S. luteus*) in the Granulatus group associates exclusively with hard pines, with the exception of *S. brunnescens* (FIG. 1C).

*Suillus luteus* is the most widespread species in the world, occurring in the Northern and Southern Hemisphere. It is native to Europe, where it associates with *P. sylvestris* and *P. nigra*, but it has also been introduced into North and South America, Asia, and Australasia with *P. sylvestris*. Vellinga et al. (2009) observed that it is able to grow with other *Pinus* species and has been repeatedly reported growing in New Zealand growing with *P. radiata* (Hedger 1986, Dunstan et al. 1998, Dickie et al. 2010). In addition, we collected specimens in two distant forests of pure *P. resinosa* in northern Minnesota. Our phylogeny showed that *S. luteus* was also found on ectomycorrhizal root tips of *P. tabuliformis* in Shaanxi, China as well as on *P. densiflora* in South Korea (FIG. 1C). This ability to associate with many different hosts is rare in the genus, where host jumps for *Suillus* species are often to a different host species within the same host subgenus. Specifically, the case of *S. brunnescens* in the /*luteus* clade is notable, as that species associates with soft pines within a large clade that associates exclusively with hard pines. Given this contrast in host associations, we think that the /*luteus* clade makes an excellent model to study biogeography (through recent introductions to new geographic areas) and the co-evolution of hosts and symbionts (i.e. determining the genomic mechanisms that allow host jumps across broad host phylogenetic distance).

The number of *Suillus* species in North America far exceeds that in Europe (Smith and Thiers 1964a, Klofac 2013), but exact numbers for Asia are not available, and it clearly has been undersampled. The number of unresolved taxonomic issues is also higher in North America than in Europe, but this may correlate with the amount of taxonomic efforts put into the genus. As seen repeatedly in fungal systematics, European names applied to other parts of the world mask regional diversity that could only be revealed through DNA sequencing and careful study of numerous specimens. Our combined analysis of the available sequences in public databases with sequencing of specimens and type material provide an enticing glimpse into the taxonomic problems and novelties present even within a well known group of ectomycorrhizal fungi. We have solved a few of those problems in the current study, but many more await the availability of holotype ITS sequences and genomic data from modern high-throughput sequencing. Ongoing and future work will contribute to *Suillus* as a well defined genus to the North American Mycoflora (<http://www.northamericanmycoflora.org/>).

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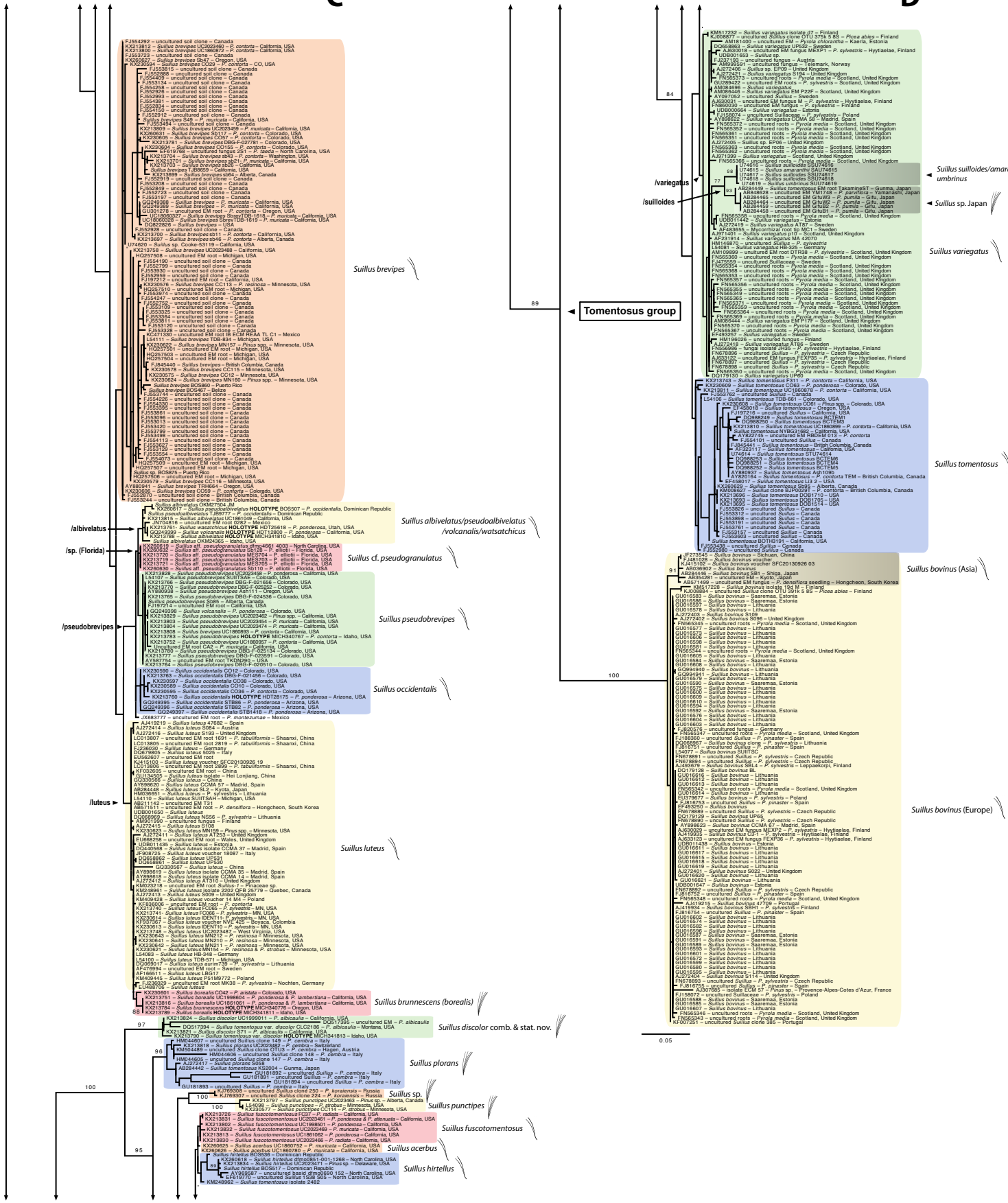
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