Short title for running head: DeLong-Duhon and Bagley: Species delimitation in Stereum

Phylogeny, morphology, and ecology resurrect previously synonymized species of North American *Stereum*

Sarah DeLong-Duhon[a] and Robin K. Bagley[b]

[a]*Department of Biology, University of Iowa, Iowa City, IA 52245;* [b]*Department of Evolution, Ecology, and Organismal Biology, The Ohio State University at Lima, Lima, OH*

Sarah DeLong-Duhon https://orcid.org/0000-0003-1865-2413 sarah-delong-duhon@uiowa.edu

Robin K. Bagley https://orcid.org/0000-0003-2209-0521

ABSTRACT

Stereum is a globally widespread genus of basidiomycete fungi with conspicuous shelf-like fruiting bodies. Several species have been extensively studied as pathogens and for medically relevant secondary metabolites, but prior to this publication the molecular phylogeny of *Stereum* had not been analyzed, despite pervasive morphological crypsis in the genus. Here, we provide a preliminary investigation into species boundaries among some North American *Stereum*. The nominal species *Stereum ostrea* has been referenced in field guides, textbooks, and scientific papers as a common fungus with a wide geographic range and even wider morphological variability. We use nuc rDNA ITS1-5.8S-ITS2 (ITS barcode) sequence data of specimens from midwestern and eastern North America, alongside morphological and ecological characters, to show that *Stereum ostrea* is a complex of at least three reproductively isolated species. Preliminary morphological analyses show that these three species correspond to three historical taxa that were previously synonymized with or confused for *S. ostrea*: *Stereum fasciatum*, *Stereum lobatum*, and *Stereum subtomentosum*. *Stereum hirsutum* ITS sequences taken from GenBank suggest that other *Stereum* species may also be species complexes. Future work should apply a multilocus approach and global sampling strategy to better resolve the taxonomy and evolutionary history of this important fungal genus.

KEY WORDS: Stereaceae; ITS; systematics; taxonomy; biodiversity

INTRODUCTION

The most recent estimate of fungal diversity predicts that there are 2.2 to 3.8 million species of fungi, but only ~120 000 have been formally described, and fewer than 23 000 have ITS sequences available on NCBI GenBank (Hawksworth and Lücking 2017; Vu et al. 2014). New species of fungi are often discovered as cryptic species lumped under single, well-established names, such as with *Amanita muscaria* and *Cantharellus cibarius* (Geml et al. 2006; Buyck and Hofstetter 2011). While the members of some species complexes may initially be difficult to separate morphologically, distinguishing features can become clear after phylogenetic analysis and trait-mapping, as was shown with *Fomes fomentarius* and its neglected sister species *F. inzengae* (Peintner et al. 2019). Critically, differences among cryptic or apparently cryptic species can be economically relevant, such as with the fungal plant pathogen *Magnaporthe grisea* and the morphologically indistinguishable *M. oryzae*, which infect crabgrass and rice, respectively (Couch and Kohn, 2002). Fungi are not only incredibly speciose, but are also morphologically and ecologically diverse, thus presenting fantastic systems to explore how divergent selection along a variety of ecological axes might influence the origin of new biodiversity. However, in the absence of robust taxonomic resources, it is difficult to even begin studying which ecological factors might drive divergence in fungi.

One understudied fungal group that may be a good candidate for future ecological speciation research is *Stereum*, a diverse genus of shelf-like wood-decay fungi common in wooded biomes throughout the world. Though *Stereum* has been the focus of extensive bioprospecting (Doljak et al. 2006; Hybelhauerová et al. 2008; Tian et al. 2020), and some economically important species are relatively well-researched (Stenlid and Vasiliauskas 1998; Čermák et al. 2004), the below-genus level taxonomy of *Stereum* has not been subjected to phylogenetic analysis. This disconnect between the slow progress toward molecular taxonomy in *Stereum* and interest in the potentially useful properties of *Stereum* species can be problematic, especially where species descriptions include uninformative or deceptive morphological characters that may lead to misidentification of study specimens.

Stereum ostrea, like many other *Stereum* treated carelessly by systematists, is an exemplar of a species with a fraught taxonomic history (Lloyd 1913). While the name *S. ostrea* has been applied to collections around the world, it is unlikely that these varied collections are from a single phylogenetic species given pre-Anthropocene obstacles to dispersal. First used to describe a collection from the island of Java in Indonesia, the name *S. ostrea* has been consistently applied to specimens from North America since a publication by Lentz (1955) placed it in synonymy with two other putative species, *S. fasciatum* and *S. lobatum*, with which Welden (1971) and Chamuris (1985, 1988) concurred. Even before this, *S. ostrea* was considered either a synonym of *S. fasciatum* by some authors (Burt 1920; Pilát 1930; Banerjee 1935; Hendrickx 1948) or of *S. lobatum* by other authors (Massee 1890; Cooke 1892; Hohnel and Litschauer 1907; Reinking 1920; Boedijn 1940). Critically, Demoulin (1985) argued in favor of *S. fasciatum* and *S. lobatum* as distinct species, outlining their differences, but the literature remained divided (Eicker and Louw 1998). Currently, *S. fasciatum* is considered a synonym of *S. ostrea* (according to Index Fungorum in October 2020, http://indexfungorum.org/), and despite being an accepted name *S. lobatum* is rarely used. Another similar species, *S. subtomentosum,* has both been confused with *S. fasciatum* and proposed to be in a complex with *S. hirsutum* (Pouzar 1964; Welden 1971; Chamuris 1988; Ginns and Lefebvre 1993).

We hypothesize that *S. ostrea* in North America is not a single species but a complex of several species. To test this, and to begin developing a more integrated approach to *Stereum* taxonomy, we collected over 50 *Stereum* specimens from eastern North America and used nuc rDNA ITS1-5.8S-ITS2 (ITS barcode) sequence data to create a phylogenetic tree. Because sequence information from a single gene is not always enough to reliably delineate species, we coupled our phylogenetic approach with morphological and ecological data. Following our hypothesis that *S. ostrea* is a species complex, we predicted that differences in ITS sequences would correlate with differences in morphology and ecology (e.g., substrate), and as such would reveal evidence of more than one reproductively isolated *Stereum* species.

MATERIALS AND METHODS

Collection and Identification. – We collected *Stereum* basidiocarps from locations in midwestern and eastern North America, with the most intensive sampling in Iowa and some supplemental collections made in Alabama and Florida (SUPPLEMENTARY TABLE 1). For identification we used the dichotomous key and morphological descriptions from Chamuris (1988), the most comprehensive and recent publication on *Stereum*. Based on morphology, we identified our collections as *S. ostrea*, *S. hirsutum*, *S. complicatum*, *S. gausapatum*, *S. sanguinolentum*, and *S. striatum*. We used iNaturalist to record photographs, dates of collection, and approximate GPS coordinates of collection location. We also recorded whether the substrate from which samples were collected was hardwood or conifer, and if the hymenium changed color when bruised. We air dried all collections and preserved samples in polyethylene plastic bags. In total, we collected and analyzed 49 samples, 36 of which are included in this paper (SUPPLEMENTARY TABLE 1).

All *Stereum* species in this study are found primarily on angiospermous wood, except for *S. sanguinolentum* which is primarily found on conifers. *Stereum* are occasionally reported on rare hosts and have been sequenced from atypical host species (Ginns and Lefebvre 1993; Jusino 2014). SD50 *S. complicatum* was found growing on a dead branch of loblolly pine still attached to the tree.

DNA Extraction, PCR and sequencing. – We used a CTAB and liquid nitrogen method adapted from Chen et al. (2010) to extract DNA from 3 x 3 mm pieces of basidiocarp from each collection, taking care to exclude as much dirt and debris as possible. We used DNA diluted 1:20 with molecular grade water for PCR amplification using ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) primers (Gardes and Bruns 1993; White et al. 1990), with the following thermocycler program: 3 min @ 94 C, 20-30 cycles (30 s @ 94 C, 30 s @ 55 C, 30 s @ 72 C), 2 min @ 72 C. We cleaned PCR products with Exo-SAP following manufacturer protocols. We sequenced PCR products in forward and reverse directions on an Applied Biosystems ABI 3730 DNA Analyzer (Thermo Fisher Scientific, Massachusetts) housed in the Roy J. Carver Center for Genomics in the University of Iowa Biology Department.

Phylogenetic Trees. – We used Geneious 8.1.7 (http://www.geneious.com/) for alignment of forward and reverse sequences for each collection, which we then manually checked and trimmed. We chose *Xylobolus subpileatus*, another member of the Stereaceae, as an outgroup. This specimen was collected in Florida by S.G.D. and sequenced by the Smith Lab at the University of Florida. We also obtained North American and Eurasian *S. hirsutum* sequences from BOLD and GenBank. We used MAFFT (Katoh et al. 2002) via the CIPRES server (Miller et al. 2010) to align chosen sequences, then generated a maximum likelihood tree using RAxML (Stamatakis 2014) via CIPRES. For computation of the Bayesian tree, we used MrBayes 3.2.7 (Ronquist et al. 2012) with a GTR+I+Γ substitution model for 200 000 generations, with sampling every 100 generations.

RESULTS

Maximum likelihood (FIG. 1) and Bayesian trees (SUPPLEMENTARY FIG. 1) were largely in agreement, except for the placement of the single *Stereum striatum* sequence. Both trees show *Stereum* sorting out into several clades. Specimens initially identified as *S. ostrea* were monophyletic but split into three well-differentiated clades (FIG. 1). Sequences within the same clade differed from one another by 1–3%, while sequences among the three clades differed from one another by 7–14%.

The "*S. ostrea*" specimens in the first clade (FIG. 1) all share features of the previously synonymized species *S. fasciatum*, as described by many authors including Burt (1920) and Demoulin (1985). These specimens feature a cap clothed in coarse hair that is resistant to wearing off in bands, and these hairs gather in individual clumps that are best observed with a hand lens or dissecting microscope (FIG. 2A–B). If bruised (when basidiocarps are fresh) or wetted (when dried) the hymenium (undersurface) does not stain color (FIG. 2A).

Similarly, the "*S. ostrea*" specimens in the second clade (FIG. 1) showed features consistent with the previously synonymized *S. lobatum* (Burt 1920; Demoulin 1985). The cap is clothed in matted, felted hairs that quickly begin wearing off, from the edge of the cap inwards, in concentric bands exposing the chestnut-brown context beneath (FIG. 2C–D). When bruised or wetted the hymenium stains a bright yellow color (FIG. 2B).

"*S. ostrea*" specimens in the third clade shared some features with both *S. fasciatum* and *S. lobatum*, however the basidiocarps were usually thicker, more irregular, and a richer brown color both cap and hymenium. The cap is covered clumping hairs that are typically longer and woolier than specimens of the first clade (*S. fasciatum*), and wear off in bands more readily but not to the extent of specimens in the second clade (*S. lobatum*) (FIG. 2E–F). When bruised or wetted the hymenium stains a bright yellow color like *S. lobatum* (FIG. 2B). Overall, the morphology agrees with descriptions of *S. subtomentosum* (Pouzar 1964; Chamuris 1988).

Specimens identified before sequencing as belonging to other species of *Stereum* formed clades with ITS sequences differing within clades by 15%. These included *S. complicatum*, *S. gausapatum*, and *S. sanguinolentum.* The single sequence of *S. striatum* was placed differently in the two trees but differed from all other sequences by more than 7%. *S. hirsutum* sequences acquired from GenBank and BOLD databases differed from one another by 1–6%, but only as much as 4% within the two geographically isolated (North America vs. Eurasia) clades.

DISCUSSION

Together, our results support the hypothesis that the North American fungi that have been lumped under the name "*S. ostrea*" are actually at least three distinct species, reproductively isolated and differing in morphology. The inference of reproductive isolation is based on the observation that while all three species can be found growing together on the same substrate, there is strong concordance between ITS sequence identity and morphology, such that they appear not to be hybridizing despite often living in sympatry. Demoulin (1985) argued that *S. fasciatum* and *S. lobatum* were distinct species based on differing morphology and lack of fusion while growing on the same substrate, and our results support this claim with new genetic evidence.

As a consequence of these new data, the *Stereum* in the three "*S. ostrea*" clades should be recognized as distinct species, and those in synonymy with *S. ostrea* should revert to their original names. Our FIGS. 2 and 3 along with images for each specimen on iNaturalist (SUPPLEMENTARY TABLE 1) are provided to assist future naturalists and researchers in distinguishing among these taxa. Most importantly, these three species in midwestern and eastern North America can be differentiated by 1) texture of cap hairs, 2) presence and extent of banding, and 3) color staining of the hymenium when bruised or wetted (FIGS. 2 and 3).

A more general conclusion of this research is that an integrative approach to *Stereum* taxonomy that includes DNA sequence data will be helpful in resolving species boundaries. In many cases, original descriptions are only a few sentences long or overly vague. Further, *Stereum* taxonomy has a long history of synonymization efforts such that descriptions of *Stereum* species from different authors are often conflicting. Historically, *Stereum* has been used as a genus name to describe many corticioid fungi, many of which have been transferred to other genera both in the Stereaceae and to other genetically distant families (Larsson and Larsson 2003). Many *Stereum* are accepted as synonyms of better-known *Stereum* species, but if our study is of any indication, some of those names may also need to be resurrected.

Our work here demonstrates a need to explore the rest of the *Stereum* genus, so that we may better understand their ecological roles, how they might be useful to us, and how they evolved. Molecular phylogenetic approaches will be necessary as a supplemental tool to delineate *Stereum* species, as morphology alone has proved inadequate. By using a multilocus approach and worldwide sampling strategy, we can achieve greater resolution within this genus and other members of the Stereaceae.

ACKNOWLEDGEMENTS

Financial support for this project was provided by awards to S.G.D. in the form of University of Iowa's Maureen Medberry Snell CLAS Award and two Iowa Center for Research by Undergraduates (ICRU) fellowships. We thank Dr. Andrew Forbes for his mentorship and contributions to the preparation of this manuscript, and Dr. Rosanne Healy for providing the outgroup sequence and her valuable advice. We are grateful to Dean Abel for his encouragement and donation of valuable literature on *Stereum*.

LITERATURE CITED

Banerjee SN. 1935. Thelephoraceae of Bengal: I. Journal of the Indian Botanical Society 14:13–48.

Boedijn KB. 1940. The Mycetozoa, fungi and lichens of the Krakatau group. Bulletin du Jardin Botanique de Buitenzorg 16:358–429

Burt EA. 1920. The Thelephoraceae of North America. XII. *Stereum*. Annals of the Missouri Botanical Garden 7:81–248.

Buyck B, Hofstetter V. 2011. The contribution of tef-1 sequences to species delimitation in the *Cantharellus cibarius* complex in the southeastern USA. Fungal Diversity 49:35–46.

Čermák P, Jankovský L, Glogar, J. 2004. Progress of spreading *Stereum sanguinolentum* (Alb. et Schw.: Fr.) Fr. wound rot and its impact on the stability of spruce stands. Journal of Forest Science 50:360–365.

Chamuris GP. 1985. On distinguishing *Stereum gausapatum* from the '*S. hirsutum*-Complex'. Mycotaxon 22:1–12.

Chamuris GP. 1988. The non-stipitate stereoid fungi in the north-eastern United States and adjacent Canada. Mycologia Memoirs 14:1–247.

Chen H, Rangasamy M, Tan SY, Wang H, Siegfried BD. 2010. Evaluation of Five Methods for Total DNA Extraction from Western Corn Rootworm Beetles. PLoS ONE 5: e11963.

Cooke MC. 1892. A Handbook of Australian Fungi. London: Williams and Norgate. 457 p.

Couch BC, Kohn LM. 2002. A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from M. grisea. Mycologia 94:683–693.

Demoulin V. 1985. *Stereum fasciatum* (Schw.) Fr. and *S. lobatum* (Kunze:Fr.) Fr.: Two Distinct Species. Mycotaxon 23:207–17.

Doljak B, Cateni F, Anderluh M, Procida G, Zilic J, Zacchigna M. 2006. Glycerolipids as selective thrombin inhibitors from the fungus *Stereum hirsutum*. Drug Development and Industrial Pharmacy 32:635–643.

Eicker A, Louw S. 1998. *Stereum* species (Stereaceae) of South Africa. South African Journal of Botany 64:30–37.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Molecular Ecology 2:113–118.

Geml J, Laursen GA, O’Neill K, Nusbaum HC, Taylor DL. 2006. Beringian origins and cryptic speciation events in the fly agaric (*Amanita muscaria*). Molecular Ecology 15:225–239.

Ginns J, Lefebvre MNL. 1993. Lignicolous corticioid fungi (Basidiomycota) of North America. Systematics, distribution, and ecology. Mycologia Memoirs. 19:1–247.

Hawksworth DL, Lücking R. 2017. Fungal diversity revisited: 2.2 to 3.8 million species. In: J. Heitman, B. Howlett, P. Crous, E. Stukenbrock, T. James, and N. Gow, eds. The Fungal Kingdom. Washington, DC: ASM Press. p. 79–95.

Hendrickx FL. 1948. Sylloge Fungorum Congensium. Publications de l'Institut Agronomique du Congo Belge. 35:1–216.

Höhnel FXR von, Litschauer V. 1907. Beiträge zur Kenntnis der Corticieen: II. Sitzungsberichte der Kaiserlichen Akademie der Wissenschaften Math.-naturw. Klasse Abt. I. 116:739–852.

Hybelhauerová S, Sejbal J, Dračínský M, Hahnová A, Kontek B. 2008. Chemical constituents of *Stereum subtomentosum* and two other birch-associated Basidiomycetes: An interspecies comparative study. Chemistry and Biodiversity, 5:743–750.

Jusino MA. 2014. The fungal communities associated with Red-cockaded Woodpeckers and their excavations: descriptive and experimental evidence of symbiosis [doctoral thesis]. Virginia Tech. 150 p.

Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast fourier transform. Nucleic Acids Research 30:3059–3066.

Larsson E, Larsson KH. 2003. Phylogenetic relationships of russuloid basidiomycetes with emphasis on aphyllophoralean taxa. Mycologia 95:1037–1065.

Lentz PL. 1955. *Stereum* and allied genera of fungi in the Upper Mississippi Valley. Washington, DC: United States Department of Agriculture. 74 p.

Lloyd CG. 1913. Synopsis of the Stipitate *Stereums*. Mycological Writings 4:13–44.

Massee G. 1890. A Monograph of the Thelephoreae - Part II. Journal of the Linnean Society of London Botany 27:95–205.

Miller MA, Pfeiffer W, Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop (GCE), 14 November 2010, New Orleans, Louisiana. New Orleans, LA: Institute of Electrical and Electronics Engineers. p. 1–8.

Miller SL, Larsson E, Larsson KH, Verbeken A, Nuytinck J. 2006. Perspectives in the new Russulales. Mycologia 98:960–970.

Peintner U, Kuhnert-Finkernagel R, Wille V, Biasioli F, Shiryaev A, Perini C. 2019. How to resolve cryptic species of polypores: An example in Fomes. IMA Fungus 10:1–21.

Pilát A. 1930. Monographie der europäischen Stereaceen. Hedwigia 70:10–132.

Pouzar Z. 1964. *Stereum subtomentosum* sp. nov. - pevnik plstnatÿ a jeho systematické vztahy. Česká Mykologie 18:147–156.

Reinking OA. 1920. Higher basidiomycetes from the Philippines and their hosts, II. Philippine Journal of Science 16:167–179.

Ronquist F, Teslenko M, van der Mark P, Ayres D, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61:539–542.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313.

Stenlid J, Vasiliauskas R. 1998. Genetic diversity within and among vegetative compatibility groups of *Stereum sanguinolentum* determined by arbitrary primed PCR. Molecular Ecology 7:1265–1274.

Tian M, Zhao P, Li G, Zhang K. 2020. In Depth Natural Product Discovery from the Basidiomycetes *Stereum* Species. Microorganisms 8:1049.

Vu D, Szöke S, Wiwie C, Baumbach J, Cardinali G, Röttger R, Robert V. 2015. Massive fungal biodiversity data re-annotation with multi-level clustering. Scientific Reports 4:6837.

Welden AL. 1971. An essay on *Stereum*. Mycologia 63:790–799.

White TJ, Bruns TD, Lee SB, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. New York: Academic Press. p. 315–322.

LEGENDS

Figure 1. Maximum likelihood phylogeny generated from ITS sequence data. Bootstrap values are above branches. Scale bar represents the number of nucleotide changes per site. The first three clades show strong support, suggesting *S. ostrea* in midwestern and eastern North America consists of three distinct species, which we identify as *S. fasciatum*, *S. lobatum*, and *S. subtomentosum*.

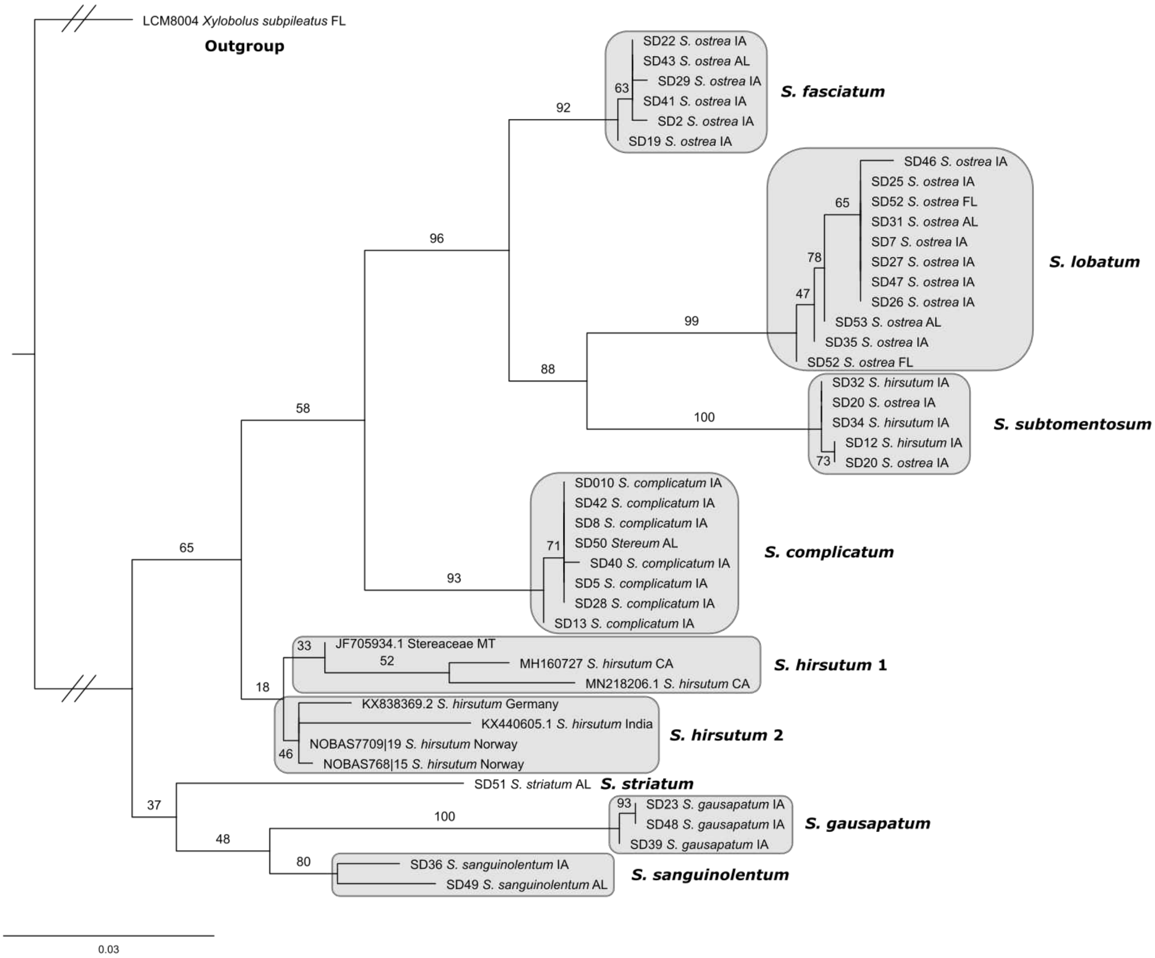
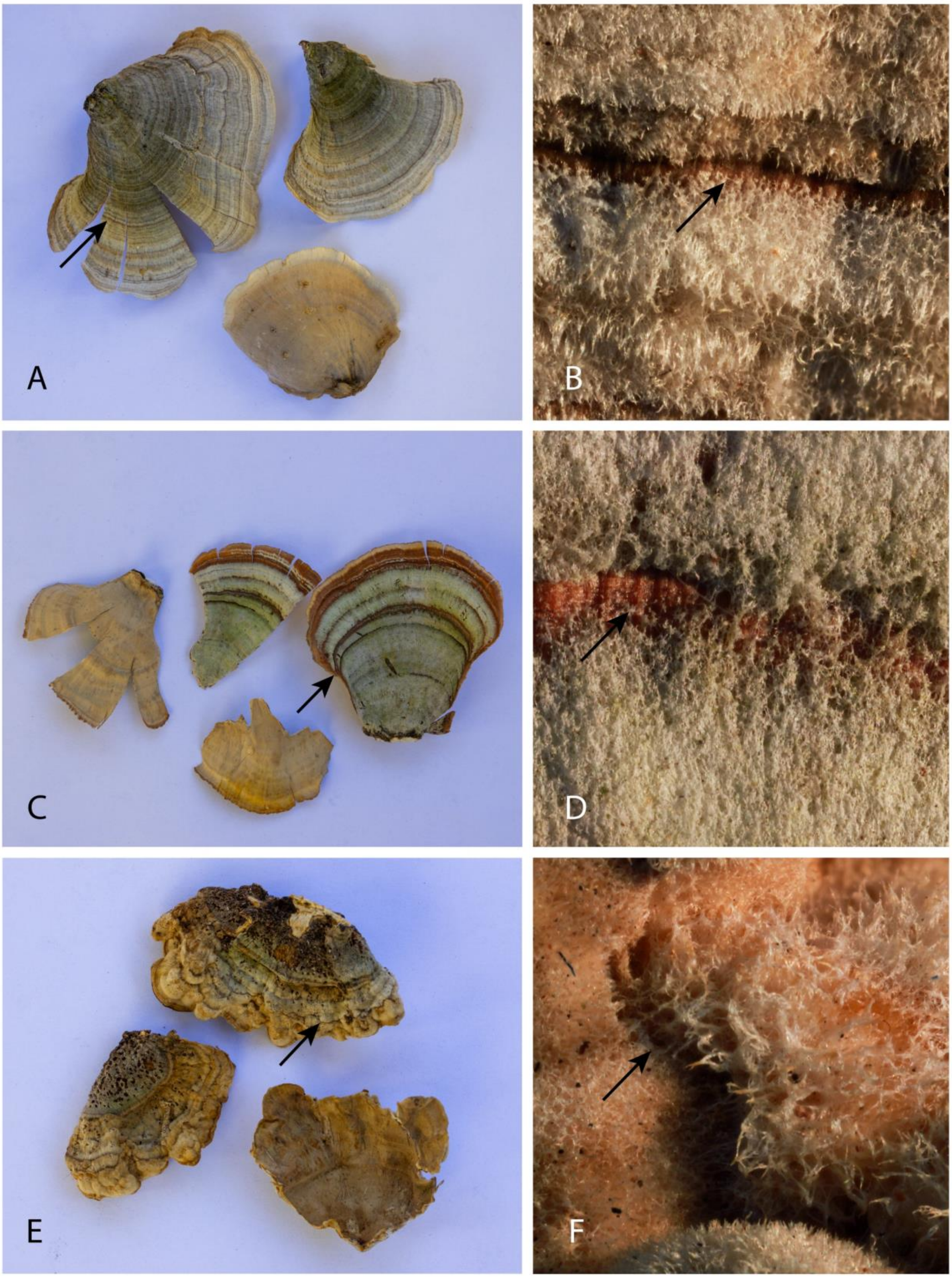


Figure 2. Morphological comparison of a-b. SD01 *Stereum fasciatum*, c-d. SD03 *S. lobatum*, e-f. SD12 *S. subtomentosum*. Arrows point to areas where hairs have worn away in concentric bands, exposing the chestnut-brown context; a. few very thin bands, c. wide bands, starting from cap edge, and e. irregular, uneven banding. The second column shows differences between species in cap hair texture; b. coarse and clumped in tufts, d. short, matted, and felted, and f. long, wooly and tufted.



309

310 **Figure 3**. Comparison of color staining between a. *S. fasciatum* and b. *S. lobatum*, which

311 show slight darkening, and bright yellow staining, respectively. *S. subtomentosum* (not

312 shown) exhibits the same bright yellow staining as *S. lobatum*. Note that both specimens

313 were dry, and re-wetted to investigate color staining.

314