Placement of the genus Angelina within Rhytismatales and observations of Angelina rufescens

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Abstract: Angelina rufescens is placed within the core clade of Rhytismatales (Leotiomycetes, Pezizomycotina, Ascomycota) based on analysis of LSU and mtSSU rDNA. The only species in the genus, it produces distinctive ascomata that reoccur annually on wood and on the remains of its own previous fructifications, forming dense conglomerations of interlocking longitudinally elongated apothecia with gray hymenia. Known collections and references of A. rufescens indicate that it is endemic to eastern and central United States. Morphological and cultural characters are described with notes on ascomatal development. No mitospores were observed in field collections or in culture. Lectotypes are designated for Hysterium rufescens and its synonym Ascobolus conglomeratus. Angelina rufescens is illustrated here for the first time in the taxonomic literature.

Key words: Hypoderma, lectotypification, Leotiomycetes, Lewis David von Schweinitz, Lophodermium, mitochondrial SSU, Nils Peter Angelin, nuclear LSU, ontogeny, Rhytismataceae

INTRODUCTION

A collection of Angelina rufescens (Schwein.) Duby was made in Carlisle, Massachusetts, United States 1 Apr 2012 from the underside of an oak log. The fungus had been observed at least 3 mo and had fruited yearly on the same log since it first was discovered five years earlier. Initially the identification of this fungus even to the family level proved elusive. It seemed to possess characters that were reminiscent of genera in both the Dermateaceae and Rhytismataceae, which we could not reconcile. We decided to sequence it, and at the same time Richard P. Korf identified the fungus as A. rufescens based on other material from New England provided by our colleague Lawrence Millman (Millman 2012). Korf noted that he had seen this fungus many years earlier around Ithaca, New York, and had issued it in his Discomycetes exsiccateae as No. 16 (Korf 1954). He referred us to a classic paper on this species by Durand (1902), which Korf also cited in his treatment of discomycetes (Korf 1973). Subsequent morphological and sequence analyses resolved the placement of this genus not in the Dermateaceae, where Durand (1902) and Korf (1973) had placed it, but instead among the core clade of Rhytismatales (Lantz et al. 2011).

MATERIALS AND METHODS

Collection.—Photographs were taken in situ and collections taken for cultural and morphological studies and molecular sequencing. The remaining portions were dried at low temperature in a home food dehydrator and accessioned into FH (Karakehian 12040101).

Culture.—A few hours after gathering A. rufescens, discharged spores were collected onto Difco PDA media in two 100 mm Petri dishes. Small sections of the substratum supporting 2–3 apothecia were removed from the collection and placed on moistened filter paper elevated on four glass slides. Plates were rotated 90 degrees every 20 min, and three regions of ejected ascospores were collected. The dishes were placed in a dark incubator at 23 C. Polysporous subcultures were made by excising 2 mm cubes of spore-bearing media and placing them at multiple points in 100 mm dishes of Difco PDA, which were maintained at 23 C in darkness.

Morphological observations.—Crush mounts from the living ascomata of A. rufescens were made in tap water or in 3% KOH and stained with phloxine in water and Congo red in ammonia. Dried material was rehydrated in sealed Petri dishes on water-dampened filter paper, transferred to dilute gum arabic for 12 h and sectioned to 20 μm on a freezing microtome. Dried material for crush mounts was rehydrated in 3% KOH or dipped in 70% ethanol, rehydrated in water and stained with Congo red. Ascospore sizes are given from the average of 30 spores discharged from living apothecia onto a cover slip and mounted in water. India ink was employed to detect the presence of gelatinous spore sheaths. Observations of ascomatal development were made from living field-collected material (Karakehian 12090801). Microscopic observations were made with Nomarski and brightfield optics on Olympus BH-2 and Olympus BX40 compound microscopes and an Olympus SZX9 stereomicroscope. Drawings were made by JMK with a Leitz Laborlux compound microscope fitted with a Leitz drawing tube.

DNA Isolation, PCR and sequencing techniques.—DNA samples were obtained from living fruiting bodies of A.
**RESULTS**

**Molecular phylogenetic analyses.**—Results from phylogenetic analyses of the combined LSU and mtSSU DNA sequences of 69 taxa, including *Angelina rufescens*, are presented (Fig. 1). Parsimony analysis produced 3441 most parsimonious trees 2491 long (504 sites were parsimony informative, 173 were parsimony uninformative and 1127 sites were constant). Results from ML, MP and Bayesian analyses placed *A. rufescens* with high support (95, 79, 100 ML, MP and Bayesian analyses respectively) as sister to all other taxa in a clade containing predominately species of *Hypoderma* and *Lophodermium*.

**Observations of ascomata development.**—Hemispherical primordia, 100–150 μm diam, develop superficially on the substratum. These are at first hyaline but soon darken to pale yellow, orange then red with their apices appearing granular, then tuberculate, from protruding cells (Fig. 2a, b). The primordia enlarge as hyaline hyphae emerge from their flanks and extend down, infiltrating crevices in the surrounding substratum (Fig. 2c). These hyphae are septate, 3.5–5.5 μm diam and sparsely encrusted with crystalline material. The primordia begin to elongate and enlarge laterally, becoming hysterioid and darkening to dark brown-black. Shallow apical sulci develop along their axes. The tissues surrounding the sulci and at the elongating ends remain red-brown (Fig. 2d). Along the flanks of the primordia subtle longitudinal striations develop and the hyphal extensions collapse forming a loose, flaky pale brown tissue and leaving a white tomentose halo of hyphal extensions only at the elongating ends (Fig. 2e).

In transverse section the sulci are composed of involute filamentous hyphae of the developing apothecial margins. Crystalline material accumulates around the hyphae, ultimately forming a distinct split (Fig. 2f). A thin pruinose layer of this material can be observed developing around the sulci on the outer surface of the primordia.

Ascosporangia mature before the opening of the apothecium. In moist conditions, the prosenchymatous regions of the excipulum swell, pushing the involute margins up and outward. Hymenial elements expand and asci extend upward through the hamathecium, pushing the apothecium open further. Where ascomata are densely gregarious, the flanks of the adjoining apothecia swell tightly against each other, thus appearing as interlocking gray hymenia. In senescent apothecia where the excipula are heavily carbonized, the apothecia tend to lose the ability to close.

**TAXONOMY**


**TYPE SPECIES:** *Ascobolus conglomeratus* Schwein.


**Lectotypes designated here:** *Hysterium rufescens* Schwein. (1822) PH, envelope No. 01104792, bar code No. 00064695, ex Collins Collection, packet...
Ascobolus conglomeratus Schwein. (1832) PH, envelope Number 01102746, bar code Number 00062099, “Beth” [Bethlehem, Pennsylvania].

Etymology of the generic name: In honor of the Swedish paleontologist and naturalist Nils Peter Angelin (1805–1876) (Fries 1849, Anonymous 1876).

Range: Maine south to northern Alabama and Georgia; Massachusetts west to Iowa.

Seasonality: Ascomata with mature ascospores are found from October through May.

Substratum: Surfaces of decorticated tree stumps and the cut ends and undersides of logs and branches of deciduous trees, particularly Castanea dentata and Quercus spp. The affected wood is reduced to a soft, fibrous state, creating a texture that appears fleshy and creamy white from mycelium and deposited crystalline material. Regions adjacent to the affected area remain hard and intact. At low magnification, dense white mats of appressed hyphae frequently are observed in the crevices of the wood or covering the dead, persistent remains of previous fruitings of A. rufescens. Apothecia develop on bare wood or upon layers of these remains, which after multiple seasons of fruiting and senescence in older colonies, accumulate into a spongy, heterogeneous layer of fungal material.
tissue, debris and crystalline material over the basal woody substratum.

Ascomata, macromorphological features. Sessile, superficial, elongated apothecial disks, flexuous, geniculate or trilaterally branched, never confluent, scattered or gregarious and appearing in mass as densely packed expanses of interlocking gray hymenial surfaces each bordered by white to brown margins, forming effuse masses several cm to dm in extent (FIG. 3a, b). Single ascomata 1–3 mm long × 0.5–1 mm wide × 0.4–0.6 mm thick. Hymenia flat, concave to slightly convex, slate gray, granular from asci projecting beyond the hymenial surface (FIG. 3c). Margins even with, or barely projecting above, the hymenium; fibrous, with a laciniate edge, white to pale cream to brown. Flanks stromatic, black, fleshy or somewhat coriaceous, smooth or longitudinally striate, sometimes encrusted with pale brown tissue. In dry conditions, the margins roll inward and the apothecia become nearly hysteriform (FIG. 3d).

Apothecial margins. In transverse section 50–85 μm thick, composed of hyaline-tipped filamentous hyphae originating within the excipulum, interspersed in a crystalline matrix composed of irregularly shaped granules and delicate fan-shaped fascicles of acicular crystals that disappear in KOH (FIGs. 4a–i, 5b). In older apothecia, the hyphae become darkened at the tips and the crystalline material may wear away, resulting in brown and eroded margins.

Excipulum. In transverse section 65–100 μm thick, stromatic, composed of prosenchymatous and pseudoparenchymatous regions with deposited extracellular pigments (FIGs. 4a–ii, 5a). The pseudoparenchymatous region is composed of indistinct isodiametric or interwoven elongate cells, golden brown, gray-brown or dark brown with a highly carbonized central region (FIGs. 4a–iia, 5c). Nearly the entire excipulum is carbonized in older apothecia. A prosenchymatous region, 15–26 μm thick is adjacent to the hymenium, composed of parallel, elongate, pale brown cells that originate in the intra-stromal matrix beneath the subhymenium and extend into the margin (FIGs. 4a–iib, 5d).

Intra-stromal matrix (after Sherwood, 1980). Located below the subhymenium and arising from the excipulum, 60–150 μm thick, composed of hyaline pseudoparenchyma with discrete pockets of gray-brown extracellular pigment, becoming prosenchymatous as cells form the subhymenium and extend into the margin (FIGs. 4a–iii, 5c).

Subhymenium. Composed of densely packed, hyaline, cuboidal, globular and curling ascogenous hyphae (FIG. 4a–iv).

Hymenium. 160–200 μm thick (FIGs. 4a–v, 5a).

Paraphyses. 1.5–2.5 μm wide, smooth, filiform, branching, septate from the base to the middle, the tips straight, sinuous or curled, interwoven to form an ephymenium, anastomosing near the bases to form H-shaped hyphal bridges (FIG. 4b).
Asci. Arising from croziers, clavate, thin-walled with a long stipe, 99–196 µm long from the base to the apex, 8–9 µm wide, apex acute, apical wall undifferentiated, no reaction with IKI or Meltzer’s reagent, dehiscence via a pore-like apical rupture. Asci develop sequentially. At maturity, apices project beyond the surface of the hymenium. Eight-spored, biseriate near the apex and uniseriate toward the base with the lowest few ascospores running singly down into the stipe (FIG. 4c).

Ascospores. Hyaline, smooth, without gelatinous sheaths. Spores observed within asci are dimorphic with four aseptate spores nearest to the apex followed by four one-septate spores. Aseptate ascospores cylindrical with blunt ends, straight or slightly curved, thin-walled, with refractive bodies at the poles, (14.1–) 16.7(–19.7) × (3.8–)4.4(–5.6) µm. One-septate ascospores cylindrical-fusoid, occasionally clavate with one cell attenuated, straight, relatively thicker-walled and slightly smaller than their aseptate counterparts at (11.3–)15.1(–18.8) × (2.8–)3.5(–4.7) µm, septum median, not constricted or occasionally slightly so, refractive bodies present at the poles of each cell (FIG. 4d).

Associated sterile structures. Solitary red bodies approximately 100 µm diam and adorned with short hyphal appendages are observed in many collections scattered on the substrata among mature ascomata (FIG. 6a). These are most easily observed on decorticated wood but are obscured within regions of dead apothecia and debris. In transverse section they are composed of a central region of densely packed hyaline plectenchyma surrounded by a rind of parallel-oriented pale brown hyphae (FIG. 6b). No spores were found associated with these structures.

Conidiomata. Not observed.

Culture. Ascospores germinated within 12 h (FIG. 4e). At 2 wk colonies 2–3 mm diam and white; the center tinged pink in reverse. Colonies appeared tomentose from dense growths of aerial hyphae, while the margins remained immersed in the media. Some hyphae contained small inclusions of red pigment. At 4 wk colonies were 6–10 mm diam, the pink cast in the media intensified to a pale red-orange or peach and became vivid purple-pink with some rust-brown spots at 7 wk (FIG. 7a). The margins remained white, and the hyphae in pigmented regions coalesced into short,
acicular, hyphal cords. In reverse the colonies were a dingy brown-pink. At this time three light and temperature regimes were established: i) 18 °C and a light/dark of 12 h with a 10 watt CFL bulb, ii) total darkness, iii) room temperature in diffuse daylight. In week 10, small regions near the margins in colonies exposed to light developed dense growth of mostly confluent red-brown globose bodies. Colonies kept in darkness did not develop these bodies. These bodies were approx. 150 μm and bore short hyphal cords at their apices or a sparse growth of short hyphae covering the entire body. In transverse section they were composed of an internal region of densely interwoven hyaline plectenchyma with an outer rind of pale brown hyphae (Fig. 7b). Morphologically they are similar to the red hemispherical bodies observed in natural collections. No spores were found associated with these structures. At 17 wk no further differentiation of tissues had occurred and pigmentation had not changed. The globose bodies continued to develop singly or in small groups. The fungus deposited noticeable amounts of crystalline material into the media. This was composed of irregularly shaped granules, fascicles of oblong granules and delicate feather-like fascicles of acicular granules (Fig. 8a–c). Nine months later, the largest colony was 2.5 cm diam and growth had stalled. Lowering the temperature to 18 °C had little effect on the rate of growth compared with colonies kept at room temperature. Samples from cultures were taken for sequencing and it was observed that the colonies were tough, rubbery and difficult to cut with a scalpel.


**Discussion**

**Previous treatments.**—Durand (1902) provides an excellent and concise account of the taxonomic history of A. rufescens. We add only in regard to the synonym Angelina conglomerata, the combination of which was not made by Fries when he erected the
genus *Angelina* in 1849 but in a subsequent study (Fries 1851).

**Phylogeny.**—In their phylogenetic analysis of Rhytismatales, Lantz et al. (2011) outlined a core clade that comprised mostly taxa of Rhytismataceae, Cudoniaceae and *Cryptomyces maximus* (Fr.) Rehm. Character mapping was employed, and the core clade was divided further into two strongly supported clades according to the plane of symmetry of the ascomata. To facilitate discussion in their study, informal designations of radiate and bilateral were assigned to the two clades. These terms describe taxa that possess circular ascomata that open in a radiate fashion, such as *Coccomyces* spp., and taxa in which the ascomata are elliptical or oblong and open along a single slit, such as those in *Lothodermium* and *Hypoderma* (Lantz et al. 2011). In our analysis, *Angelina rufescens* occupies its own branch within

**FIG. 5.** Apothecial tissues of *A. rufescens* (Karakehian 12040101) in transverse section; 20 μm sections in Congo red. a. Apothecium. Bar = 200 μm. b. Margin. Bar = 50 μm. c. Same apothecium showing pseudoparenchymatous tissue of the excipulum and detail of adjoining intra-stromal matrix. Bar = 100 μm. d. The same apothecium showing prosenchymatous tissue of the excipulum. Bar = 100 μm.

**FIG. 6.** Sterile red bodies associated with ascomata of *Angelina rufescens* on the surface of substratum (living) (Karakehian 12040101). a. Detail of two sterile bodies in profile. Bar = 100 μm. b. Transverse section of sterile body with encrusted hyphal extensions on the upper surface. Bar = 200 μm.
the bilateral clade. Although its ascomata share important morphological characters with other taxa in the core clade, such as opening along a single longitudinal slit, having undifferentiated, thin-walled ascus apices and hyaline ascospores, other characters diverge in varying degrees from those traditionally considered to circumscribe Rhytismataceae. Most notable are a superficial habit, elliptical ascospores that lack gelatinous sheaths and a cup-shaped excipulum; there is no discrete clypeal and basal layer as observed in *Lophodermium* and *Hypoderma*. Only in the earliest developmental stages do the primordia exhibit a complete peridium of pigmented hyphae. These tissues soon differentiate into the distinctive involute margins of the closed, immature apothecia—a preformed zone of dehiscence instead of an irregular splitting of covering layer tissues. There is no reduced excipulum lining the interior of the apothecia; ascogenous elements emerge out of the intra-stromal matrix within the centrum, and the hymenium is directly adjacent to the parallel oriented prosenchymatous tissue of the excipulum. These differences, along with those that are more conspicuous in members of the Cudoniaceae, further elucidate the diversity of ascomatal morphology within Rhytismataceae.

**Occurrence.**—We compiled a list of approximately 50 collections of *A. rufescens*. These are deposited at various herbaria dating to ca. 1822. With the exception of a few gaps of about 20 and 30 y, *A. rufescens* has been collected consistently but not frequently. The demise of the American chestnut in the eastern states caused by the chestnut blight fungus *Cryphonectria parasitica* (Murrill) M.E. Barr may be partly to blame. The fact that fruiting occurs through the winter when few collectors are afield may provide a further reason for the seeming rarity. Furthermore, the fungus is discrete and not brightly colored; although it may be conspicuous when fruiting in effuse masses. When immature it superficially resembles hysterioid genera of the Dothideomycetes, and when mature it may be confused with other apotheciod genera such as *Mollisia*. Until recently there have been no illustrations in any popular or taxonomic forum. However, once the fungus is correctly identified and the host substrata noted, it may be instantly recognizable in the field if encountered again or deliberately searched for. Furthermore, because the fungus fruits year after year on the same substratum, that location might be revisited for further observations and collections.

**Distribution.**—The collections and literature we examined indicate that the distribution of *A. rufescens* is restricted to eastern and central USA in the temperate northern hemisphere. Johnston (1997, 2001) notes that the order in the southern hemisphere is probably as diverse genetically, morphologically and biologically as it is in the northern hemisphere, with saprophytic, parasitic and endophytic species present. However, he notes that lignicolous taxa are more diverse in the northern hemisphere and less so in Australasia, where there is a greater diversity of leaf-inhabiting species. Furthermore, few species are shared between the two hemispheres (Johnston 2001). Most species of the Rhytismatales are restricted geographically, by host preference of a single species, genus or family and by tissue preference; that is leaves or wood (Johnston 1997). The fact that *Quercus* and *Castanea* are northern hemisphere taxa argue for a geographical restriction. A search of the literature for temperate Asian collections was fruitless.

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**LITERATURE CITED**


