Phylogenetic Examination of Two Fungal Species, *Hygrocybe cantharellus* and *H. turunda,* by Microscopic and Molecular Methods

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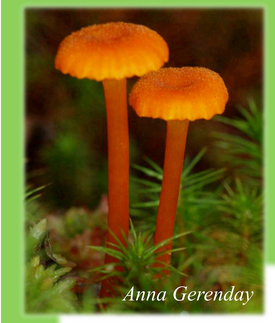
Anna Gerenday


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Introduction

Minnesotan *Sphagnum* bogs are under explored for their fungal diversity. This project sought to elucidate the relationship between two fungal species in the genus *Hygrocyb*e, known commonly as the "wax cap" mushrooms, which inhabit the *Sphagnum* bogs of Minnesota. *H. turunda* and *H. cantharellus* are specifically known as "scaly wax caps" in reference to the squamules on their pilei and are thus more formally placed into the Squamulose clade. These species were chosen because of their history of taxonomic revisions and suspicions from some current mycologists that they are in fact conspecific. *Hygrocybe* is known as a difficult genus to work with by mycologists because there are few microscopic characters that can help identify the mushroom to the species level. Spore size and pigmentation of the mushroom's scales are commonly reported by mycologists in describing these species. Fungal sequencing using the internal transcribed spacer region (ITS) as a barcode for species level identification and the use of phylogenetic trees are common modern tools to solve taxonomic issues in mycology (5).

Methods

Specimens were collected prior to this project by Anna Gerenday from Bernie's Bog, Warner Nature Center in Washington County, Minnesota. Three dried collections of mushrooms that were named *H.* cf. *turunda* and *H.* cf. *cantharellus* based on morphology were examined for pileipellis structure and spore character using a Olympus BH2 microscope. Sections of the cap cuticle were rehydrated following the methodology described by Largent et al. (3). Observations on the presence of clamp connections and the pigmentation of the terminal cells of the pileipellis were made under 40x and 100x magnification. Spore character was determined by measuring 10 spores from the gill of a mature sporocarp on each collection. Gills were sectioned under a stereoscope and rehydrated. A squash mount was prepared with 3% KOH and Congo Red stain . Observations on spore size and shape were made under 100x magnification in immersion oil. DNA extraction followed the "CTAB DNA minipreps of filamentous fungi" protocol from the R. Vilgalys Lab (6) with the exception that phenol: chloroform: isoamyl alcohol was used in place of chloroform: isoamyl alcohol. PCR amplification of the ITS region was accomplished using ITS5/ITS4 primers. Resulting sequences were edited manually and aligned using Clustal Omega. The PYLIP phylogeny suite was used to construct a Maximum Likelihood phenogram with 1000 bootstrap replicates. The gene sequences displayed on the phenogram were retrieved from Genbank, with the exception of those generated in this study.

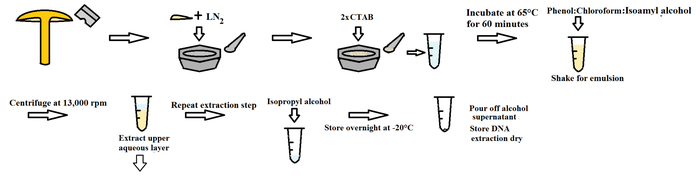

Figure 1: DNA extraction methodology

Results

Microscopic Character: Spores and Basidia

	H. turunda		*H. cantharellus*	
	Spore Size	Basidia	Spore Size	Basidia
Hesler and Smith (1963)	(9)10-14 x 5-9(10) µ	41-68 x 7-12 µ 4-spored	7-12 x 4-6 µ or 8-13 x 5-8 µ in 2-spored forms	(35)45-60 x 6.5-10 µ
Massimo Candusso (1997)	8.5-10.5(-12) x (4.8)5.6-6.5(7.5) µ Q_M=1.61-1.71	(45)53-65(70) x 6.5-8.5(11) µ	(8.5)9.5-11(13.5) x (5)6-7.5(8.5) µ Q_M=1.47-1.61	60-70 x 7-9 µ
Boertmann (1995)	(8)9.5-11.5(12.5) x (4.5)5.5-7(8) µ Q_M=1.70	40-60 x 8-10 µ mostly 4-spored	(7.5)9-10.5(11.5) x (5)5.5-7(9) µ Q_M=1.4-1.8	45-65 x 7-10 µ 4-spored, some 1-, 2-, 3-spored
Reilly (2014)	11-14(15) x (6)7-8(8.5) µ Q_M=1.5-1.88	(27)30-35 x 8-12 µ Mostly 4-spored, some 3-spored	(9)10-12 x 7 µ Q_M= 1.48	47.5-62.5 x 10-12.5 µ Mostly 4-spored, some 3-spored

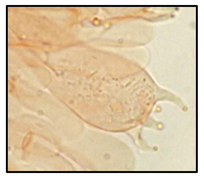

Table 1: A comparison of spore and basidia size among publications and this project. The basidia and spores collections of H. cf. turunda were smaller in this project than what has been found by Hesler and Smith (2), Candusso (4), and Boertmann (1). While there is little difference in spores of H. cf. cantharellus in this project, the basidia are slightly larger than what has been reported by the other authors.

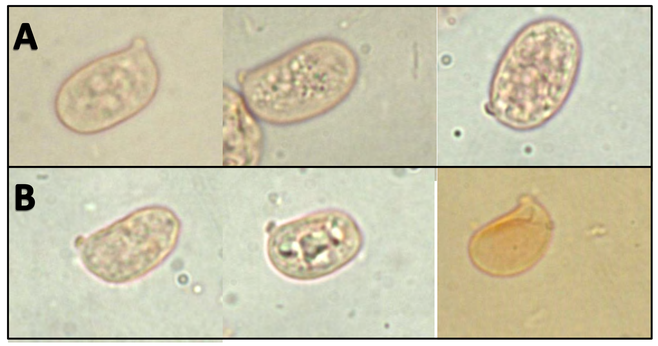

*Figure 1- Microscopic photographs displaying the larger spore size of H. cf. cantharellus when compared to those of H. cf. turunda A: H. cf. cantharellus spores of collection numbers AG20730, AG20714 and AG29174 respectively. **B** H. cf. turunda spores of collection numbers AG20420, AG20422 and AG20483 respectively.*

Sequence Analysis and Phylogeny

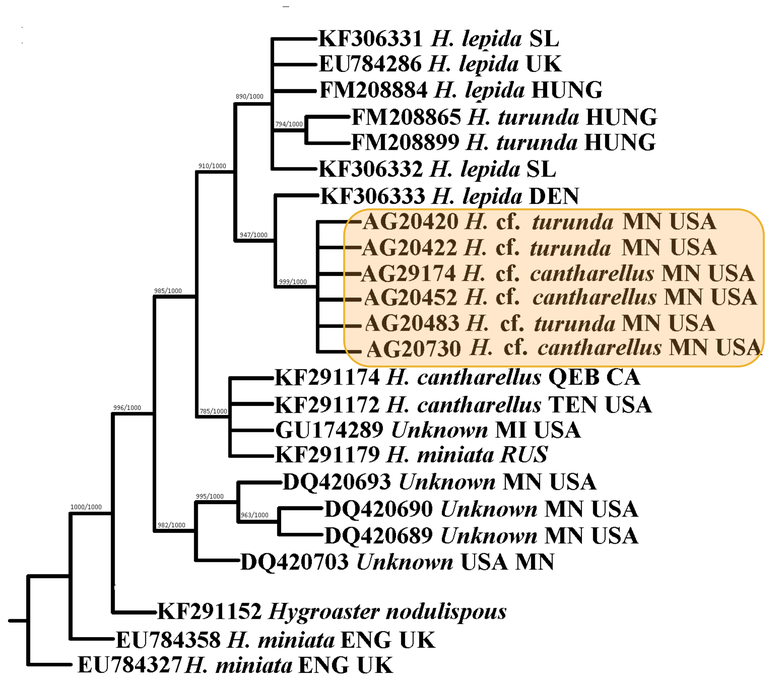

Figure 2- Maximum likelihood phenogram of selected Hygrocybe species, values based on a thousand bootstraps are given for branches with at least 75% support. The species contained in the orange box signify those sequences generated by this project.

Microscopic Character: A comparison of scales

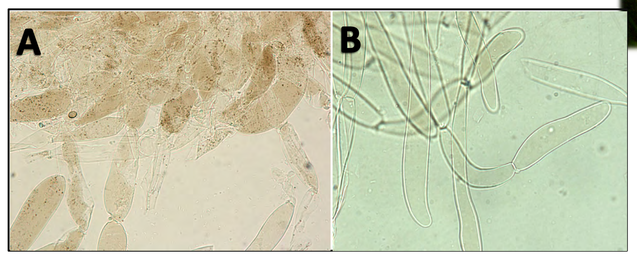

*Figure 3- Microscopic photos displaying the range of pileipellis pigmentation in H. cf. turunda. **A:** Pileipellis of collection number AG20483 displaying highly pigmented terminal cells **B:** Pileipellis of collection number AG20420 displaying low levels of hyphal pigmentation.*

Discussion

A literature review and collection of microscopic data show the difficulty in using microscopic character alone as a basis for distinguishing between these two species. Spore size differs between authors; this project found spores to be on the larger end of the spectrum for both *H.* cf. *turunda* and *H.* cf. *cantharellus*, noting that fewer spores were counted in this study than those listed in Table 1. Degree of pigmentation for the terminal cells of the pileipellis was found to vary widely in *H.* cf. *turunda* collections, where some that of some specimen's were nearly hyaline, but was always found to be not pigmented for *H.* cf. *cantharellus* collections. This suggests that terminal cell pigmentation of the pileipellis may not be a telling microscopic character for *H. turunda*. Based on microscopic evidence, our study could not conclusively support conspecificity and in line with the suspicion of mycologists that these species are not the same based on the studied microscopic characters.

ITS sequence analysis based on maximum likelihood methods shows 99% bootstrap support for *H.* cf. *turunda* and *H.* cf. *cantharellus* conspecificity from Minnesotan collections of the two species. These data could alternatively support, as Boertmann (1) suggests, that these species found in Minnesota may be an intermediate within the *Squamulose* clade, including *H. turunda, H. cantharellus*, and *H. lepida*. A multi-gene analysis should be done to conclusively determine the relationship of *H.* cf. *turunda* and *H.* cf. *cantharellus* to this clade.

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