CHAPTER 5

DISCUSSION

The nLSU data indicate that *Mycena* as currently delimited is not monophyletic, as indicated earlier by Moncalvo et al. (2002, p. 370) and Matheny et al. (2006, p. 990). The data are not robust enough to determine the taxonomic boundaries of *Mycena sensu stricto*, although there is indication that the genera *Dictyopanus*, *Panellus*, *Cruentomycena* and *Resinomycena* are derived from within *Mycena s.s* and may represent taxonomic synonyms. Until further species and genes are added to the analyses, no formal taxonomic decisions at the generic rank will be made.

Mycena sect. *Longisetae*, as currently circumscribed is not monophyletic based on phylogenetic analyses of the nLSU dataset. *Mycena graciliseta*, with thin-walled pileocystidia, falls well outside of the clade containing all species with pileosetae (thick-walled cystidia). In addition, *Mycena adscendens* (= *M. tenerrima* [Berk.] Quél.), the type species of *Mycena* sect. *Sacchariferae* (see Desjardin 1995, p. 1) and a species lacking pileocystidia and pileosetae, is derived from within the clade containing seven spinose (setoid) species. This indicates that species with and without pileosetae belong to the same lineage. The oldest name available for this clade is sect. *Sacchariferae*. This result suggests that sect. *Longisetae* should be considered as a synonym of sect. *Sacchariferae*. Sequences of the three specimens of *M. graciliseta* were as different from each other (or more so)

than the differences between distinct species, as illustrated by the branch lengths in Figure 22. This is explained in part by the presence of insertions in DED 7140 and their absence in DED 6885 and DED 6946. Note also that it was necessary to clone DED 6885 (4 sequences included) because of the presence of different copies of the gene, and that the cloned sequences were as different from each other as the differences between other distinct species in the tree. The topology of the tree in Figure 22indicates that *M. gracilisetosa* belongs to a lineage different from all other spinose species and represents either a different infrageneric taxon or a different genus. Until a broader sampling of *Mycena* species is included in molecular phylogenetic analyses, the phylogenetic placement of *M. gracilisetosa* will remain elusive.

The two known species with clavate pileosetae, *M. clavulifera* and the new species, *M. volvata*, formed a monophyletic clade with 96% BS and 1.0 PP support, with *M. volvata* sister to the two specimens of *M. clavulifera*. In addition, DED 7634 (*M. clavulifera*) and DED 7628 (*M. volvata*) are sympatric populations, both collected at Cameron Highlands in central Malaysia. These data further support our recognition of *M. volvata* as a distinct species, albeit closely related to *M. clavulifera*.

Mycena amicta (A.H. Sm.) Maas Geest., the type species of sect. *Amictae*, is morphologically quite distinct from members of sections *Sacchariferae* and *Longisetae*. It forms a pileipellis composed of smooth (non-spinulose) hyphae embedded in a gelatinous matrix, has smooth cheilocystidia, and often forms a long, rooting stipe lacking a basal disc. These features are absent in all spinose *Mycena* species. The placement of *M.*

amicta as sister to the spinose species of *Mycena* included in this study with high statistical support (100% BS, 1.0 PP), in likely due to our limited sampling of the genus.

APENDIX A

CHEMICAL REAGENTS FOR MICROSCOPIC STUDY

All chemical reagent formulae are from How to identify mushrooms to genus III: Microscopic Feature by (Largent, Johnson, & Watling, 1977, pp. 23, 25-26).

Melzer's reagent

This reagent used for testing the chemical reaction of material for iodine. Three color reaction results are; a blue or black positive reaction for "Amyloid" material; a brownish to reddish-brown reaction for "Dextrinoid or Pseudoamyloid" material; and a yellow to hyaline reaction called "Inamyloid".

Formula:

Add iodine (1.5 gm), Potassium-Iodine (5.0 gm), and Chloral Hydrate (100 gm(ml)) to H_2O (100 ml) in warm condition but do not boil

Potassium Hydroxide (KOH): 3% aqueous solution

The main use for KOH is revive to hyphae of dried basidiocarps but chemical reactions with KOH may also occur. For example, it produces a yellow color in the chrysocystidia of *Hypholoma*, gray pileus hairs in of *Crinipellis* and grey cheilocystidia in a few *Mycena*.

Formula:

Dissolve 3 gm of potassium hydroxide in 97 ml water.

Congo Red: 1% aqueous solution; saturated solution in NH_4OH

Congo red uses always to observed hyphae walls because the red color of the stain gave good contrast and made it easier to examine fungal cells, especially basidiospore walls or their ornamentation.

Formula 1: always use with fresh material

Dissolve 1 gm of Congo Red in 99 ml water; filter the excess dye.

Formula 2: always use with dry material

Saturate concentrated ammonium hydroxide with Congo Red.

APENDIX B

MODIFIED DNA EXTRACTION PROTOCOL

Standard Forensic DNA Protocol (E.Z.N.A.® Forensic DNA Extraction Kit (Omega Bio-tek Inc., Norcross, GA, USA)): Protocol for Isolation of DNA from Dried Fungal Specimens (modification to manufacturer's protocol, B. Perry, December 08)

1. Cut dried fungal specimen or fungal herbarium specimen into small pieces under sterile conditions. Pieces shouldn't be bigger than 5 mm diam transfer to a 1 ml microfuge tube.

2. Add 100 µl STL buffer and grind the specimen, surrounded by liquid nitrogen for 1 min per round, 3 rounds.

3. Add another 100 µl STL buffer and incubate at 55°C for 15 min. Vortex every 2 min to mix.

4. Add 25 µl OB protease solution and mix by vortexing. Incubate for 45 minutes at 60°C with occasional mixing. Briefly centrifuge to remove any droplets from inside the lid.

5. Add 225 µl BL buffer and vortex to mix. Incubate at 60°C for 10 minutes. Briefly centrifuge to remove any droplets from inside the lid.

6. Add 225 µl absolute ethanol and mix thoroughly by vortexing. Briefly centrifuge to remove any droplets from inside the lid.

7. Insert each HiBind® DNA minicolumn into a 2 ml collection tube (kid provided). Transfer the entire sample from Step 6 into the column, including

any precipitate that may have formed. Centrifuge at 8,000 xg for 1 min to bind DNA Discard collection tube and flow-through liquid.

8. Prepare the elution buffer by heating at 60°C in a thermoblock.

9. Place each column into a second 2 ml tube and wash by pipetting 500 µl of HB buffer into the column. Centrifuge at 8,000 xg for 1 min. Dispose of flow-through liquid and re-use the collection tube.

10. Place each column into the same 2 ml tube from step 9 and wash by pipetting 750 µl of wash buffer diluted with ethanol into the column. Centrifuge at 8,000 xg for 1 min. Dispose of collection tube and flow-through liquid

11. Using a new collection tube, wash the column a second time with 750 µl of wash buffer and centrifuge as above. Discard the flow-through and reuse the collection tube.

12. Using the same 2 ml collection tube, centrifuge at maximum speed (> 10,000 xg) for 2 minutes to dry the column. This step is critical for removal of residual ethanol that might otherwise interfere with downstream applications.

13. Place the column into a nuclease-free 1.5 ml microfuge tube and add 50 µl of elution buffer preheated to 70°C. Allow the tube to sit for 3 minutes at room temperature.

14. To elute DNA from the column (use the same column), centrifuge at 8,000 xg for 1 min. Repeat the elution with a second volume of 50 µl elution buffer.

APENDIX C

PRIMER DETAILS

All primer information from this study are from Assembling the Fungal Tree of Life (AFTOL© 2005)

Primer sequences: Nuclear Large Subunit ribosomal DNA

Primer Name:	LR0R	LR4	LR5	LR7	LR21
added by:	Frank Kauff	Frank Kauff	Frank Kauff	Frank Kauff	Frank Kauff
Locus:	nuclsu	nuclsu	nuclsu	nuclsu	nuclsu
Primer id:	56	62	63	67	83
Sequence:	GTACCCGCT GAACTTAAG C	ACCAGAGTT TCCTCTGG	ATCCTGAGG GAAACTTC	TACTACCAC CAAGATCT	ACTTCAAGC GTTTCCCTT T
Direction:	forward	reverse	reverse	reverse	reverse

Primer sequences : Nuclear Small Subunit ribosomal DNA

Primer Name:	MS1	MS2
added by:	Conrad Schoch	Conrad Schoch
Locus:	mitssu	mitssu
Primer id:	135	136
Sequence:	CAGCAGTCAAGA ATATTAGTCAATG	GCGGATTATCG AATTAAATAAC
Direction:	forward	reverse

APENDIX D

POLYMERASE CHAIN REACTION (PCR)

MasterMix (MM) preparation sheet for PCR

	Vol/Samp (µl)	❶ x(n+2)	❷ =MM vol. (µl)
1. water	7.0	13	91.0
2. buffer (10 x)	1.25		16.25
3. dNTP (∑10mM)	1.0		13
4. MgC_{l2} (25mM)	–		–
5. 10 µm Primer LROR	0.625		8.125
6. 10 µm Primer LR4.8 (or 5)	0.625		8.125
7. DMSO (1%)	–		–
8. glycerol (1%)	–		–
9. other	–		–
10. Taq	0.0625		0.8125
11. DNA template	2.0		
∑ volumn	12.56		

Note: Example calculation sheet of MasterMix for PCR from nLSU gene of 13 samples. Changing volume in ❶ and ❷ only for new calculation!

APENDIX E

ExoSAP-it PROTOCOL

ExoSAP-it PCR Product Cleanup (modified manufacturer's protocol, F. Cipriano, 6/26/06)

In order to sequence PCR products, excess nucleotides (dNTPs) and primers remaining from the PCR reaction must be removed. Exonuclease I (Exol) digests single stranded DNA into free nucleotides and shrimp alkaline phosphatase (SAP) dephosphorylates free nucleotides making them unavailable for polymerization. These enzyme (and a proprietary buffer) are supplied in the USB ExoSAP-it kit, premixed and ready to use.

Protocol

1. Label a 200 µl tube (or strip tube) for each of your PCR products to be purified. Please label it clearly and make sure you keep to the correct orientation.
2. Transfer 5 µl of each PCR product from the PCR tube to the ExoSAP-it tube/strip tube.
3. Add 1.0 µl of the ExoSAP-it and mix to each well of the ExoSAP-it strip tube and mix well. (most products are cleaned sufficiently with only 1.0 µl of ExoSAP-it, some can even be cleaned with 0.5 µl ExoSAP-it).
4. Place/close the lid(s) onto the tube/strip tubes and place in a thermal cycler block. Incubate with the following parameters:

a. 37°C 30 min to perform the digestion

b. 80°C10 min inactivate the enzymes

5. Once the ExoSAP-it reaction is finished, store your cleaned PCR product (frozen) until ready to set up a cycle-sequencing reaction. Even with the 80°C inactivation step, your cleaned PCR product will degrade over time, so do not purify more than you will need for cycle-sequencing.

APENDIX F

DNA PRECIPITATION PROTOCOL

Strip tube With EDTA/Ethanol/Sodium Acetate Protocol for the 3100 Sequencer (by Conservation and Genetic Laboratory, SFSU, 9/11/06)

1. Add 5 uL of 125 mM EDTA to each tube.
2. Then add 50 uL of 0.11 M sodium acetate in ethanol to each tube, cap and vortex tubes.
3. Spin 45 min at 4,000 rpm (program 1).
4. Dump (carefully) onto paper towel, invert spin 1 min/700 rpm (program 2).
5. Spin 5 min at 4,000 rpm (program 3).
6. Dump (carefully) onto paper towel, invert spin 1 min/700 rpm (program 2).
7. Dry in "Speed-Vac" (vacuum concentrator) for 5 min – no heat.
8. Resuspened in 15 uL Hi-Di.
9. Denature 95 degrees/2 min in 96-well thermocycler hot block.

Note: The protocol should be stopped if the sample isn't used for the sequencing matching on the same day, by following 1-7 steps and then stopping the protocol by cooling the sample tube/strip tube at 4°C (refrigerator).