CHAPTER 3

COLLECTING SITES, MATERIALS AND METHODS

Collecting Sites

Specimens of *Mycena* from South East Asia were collected over a four year period during field trips in forests throughout Thailand, Malaysia and Indonesia. The research was supported by funding from BIOTEC, Thailand (TRF/BRT-The Biodiversity Research and Training Program grant #R-45011 & #R-145010) to Thitiya Boonpratuang as part of her taxonomic research on the Mycenoid Fungi of Thailand. Additional funding to Thitiya Boonpratuang was provided by the National Science Foundation (USA) in grants to Prof. Dennis E. Desjardin (San Francisco State University, USA) as part of two separate projects: a PEET grant entitled "Monographic Studies of Southeast Asian Saprotrophic Agaricales" (grant # DEB-0118776) and a Systematics grant entitled "Molecular Systematics and Evolution of the Mycenoid Fungi" (grant # DEB-0542445).

Collecting Sites in Thailand

Collecting was conducted in a variety of habitats throughout Thailand, representing different national parks, wildlife sanctuaries, community forests and research field stations exhibiting a diversity of forest types and elevations. Details of the Thailand collecting sites that I sampled are provided in Table 1 and plotted on a map in Figure 3.

Table 1

Collecting Sites of Mycena from Thailand

Collecting Sites	Provinces	Forest Types	Approx. Elevation (m.)
1. Doi Inthanon National Park (DI)	Chiang Mai	DD*, DF*, HE*	600-2,500
2. Khao Yai National Park (KY)	Nakhon Nayok	MF*, DF*, TR*, HE*, SV*	200-1000
3. Bala Hala Wildlife sanctuary (BH)	Narathiwat	TR*, FS*	< 60
4. Chaloem Phrakiat Somdet Phra Thep Wildlife Sanctuary (Phru To Daeng) (Peet Swamp) (PS)	Narathiwat	TR*, FS*	< 60

*Forest types acronyms: TR = Tropical Rain Forest, DF = Dry Evergreen Forest, HE = Hill Evergreen Forest, CF = Coniferous Forest, FS = Fresh water Swamp Forest, MF = Mixed Deciduous Forest, DD = Deciduous Dipterocarp Forest, SV = Savanna Forest.

Figure 3 Distribution of collecting sites in Thailand.

Note. From Google Map by Google–Map data ©2009 Kingway. Retrieved May 20, 2009, from http://maps.google.com/maps?tab=ml

Collecting Site in Indonesia and Malaysia

Specimens of spinose species of *Mycena* from Indonesia and Malaysia were collected by Prof. Desjardin and his students over a period of five years. Details of the Indonesian and Malaysian collecting sites that were sampled are provided in Table 2 and plotted on a map in Figure 4.

Table 2

Collecting Sites of Mycena from Indonesia and Malaysia

Collecting Sites	Provinces	Approx. Elevation (m.)
Indonesia		
Bedugul Botanical Garden-Kebun Raya (IB)	Bali	< 50
Cibodas Botanical Garden (IC)	Java	< 1400
Halimun-Salak National Park (IH)	Bali	< 1,929
Mountain Catur, Lake Paratan (IL)	Bali	< 1262
Mountain Halimun National Park	Bali	< 539
Lake (Danau) Tamblingan	Bali	< 1351
Lake Brantan, Ulun Danu Bratan Temple	Bali	< 1291
Malaysia		
Cameron Highlands (MC)	Pahang	< 1900
Fraser Hill (Pine Tree Trail) (MF)	Pahang	< 1500
Gombak Field Station (the University of Malaya) (MG)	Kuala Lumpur	< 100

Figure 4 Distribution of collecting sites in Indonesia and Malaysia *Note.* From Google Map by Google–Map data © 2009 Kingway. Retrieved May 20, 2009, from http://maps.google.com/maps?tab=ml)

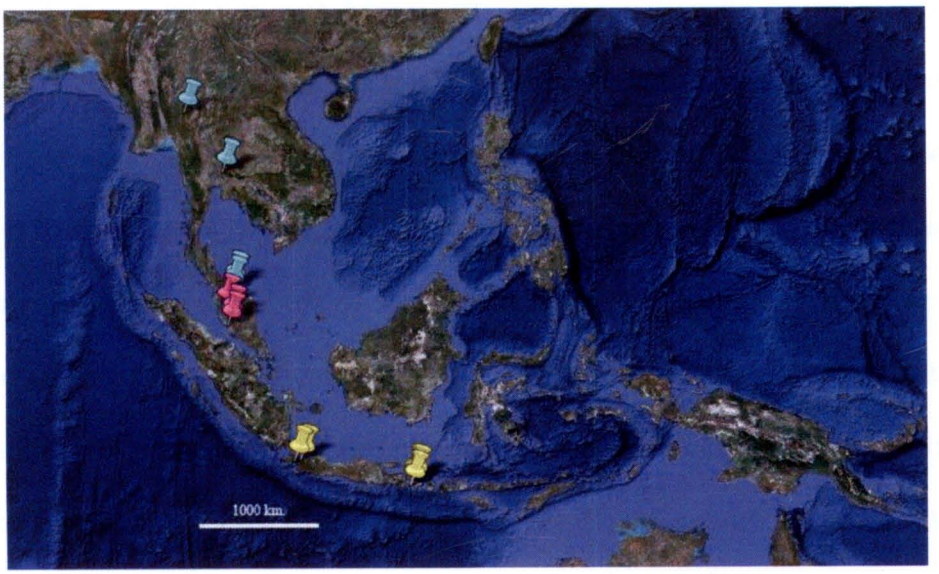

Figure 5 Distribution of collecting sites in Indonesia, Malaysia and Thailand *Note.* From Google Map by Google–Map data © 2009 Kingway. Retrieved May 20, 2009, from http://maps.google.com/maps?tab=ml)

Materials

Mushroom samples were collected and photographed when fresh in the field, then brought back to the field laboratory for description.

Field Collections from Thailand

More than 300 samples belonging to the genus *Mycena* were collected by the author from Thailand during the course of this research project. Of these, only 15 specimens represented species pertinent to the focus of this research. That is, 15 specimens possessed pileosetae or pileocystidia and would traditionally be accepted in *Mycena* sect. *Longisetae* as defined by Desjardin et al. (2002, p. 69). Many other "spinose" *Mycena* species were collected but excluded from this study because the pileus spines were not unicellular structures considered pileosetae or pileocystidia, but rather were formed from clusters of agglutinated hyphae, indicating that those species were not related to members of sect. *Longisetae*. These specimens were treated earlier in our publication wherein we described three new species from Thailand (Desjardin et al., 2003, pp. 8, 11, 13: *Mycena dermatogloea*, *M. mimicoseta* and *M. pseudoseta*). A list of the Thai specimens used in this study is presented in Table 3.

Table 3

Specimens from Thailand Examined in this Study

Spinose Species	Sites	Collections #	Specimen Examined	Date Collected
M. brunneisetosa	KY*, BH*	4	TBP0090	5 Jun. 2001
			TB00262	26 Jun 2001
			TB00421	9 Jul 2002
			TBP00782	5 May 2007
M. clavulifera	MS*, KY*	1	TB00311	14 Jan 2002
M. khonkhem	DS*, KY*	2	TB00117	4 Aug 2000
			TB00297	1 Nov 2001
M. longiseta	DI*, KY*	3	TBP0090	5 Jul 2000
			TB00262	26 Jun 2001
			TB00348	26 Jun 2002
M. palmicola	KY*	1	TB00254	26 Jun 2001
M. tenuisetosa	KY*	1	TBP00786	5 May 2007
M. variicystis sp. nov.	BH*	3	TB00176A	14 Feb 2001
			TB00176B	14 Feb 2001
			TB00176C	14 Feb 2001
Total		15		

*Collecting site acronyms: DI = Doi Inthanon, DS = Doi Suthep-Pui, MS = Mae Surin Waterfall, KY = Khao Yai, BH = Bala Hala.

Field Collections from Indonesia and Malaysia

A total of 18 specimens were collected from Indonesia and Malaysia by Prof. Desjardin and his students since 1998. Field macroscopic notes were made by Prof. Desjardin and used in this study. Microscopic notes and molecular sequences were generated by the author during her visit to the

Desjardin Lab in Aug 2007 - Jan 2008. A list of Indonesian and Malaysian specimens used in this study is presented in Table 4.

Table 4

Specimens from Indonesia and Malaysia Examined in this Study

Spinose Species	Collections #	Specimen Examined	Date Collected
M. amyloseta sp. nov.	1(MA*)	DED7640 (MA*)	11 Jan 2004
M. brunneisetosa	5(ID*), 2(MA*)	DED7903 (MA*)	8 Jan 2006
		DED7667 (MA*)	16 Jan 2004
		DED7112 (ID*)	14 Jan 2000
		DED6868 (ID*)	6 Jan 1999
		DED6813 (ID*)	17 Jan 1998
		DED6757 (ID*)	13 Jan 1998
		KPC99-45 (ID*)	15 Jan 1999
M. clavulifera	2(MA*)	DED7634 (MA*)	10 Jan 2004
		DED7651 (MA*)	15 Jan 2004
M. gracilipilosa sp. nov.	1 (ID*)	AR192 (ID*)	16 Jan 2000
M. gracilisetosa sp. nov.	3(ID*), 1(MA*)	DED7516 (MA*)	12 Jan 2003
		DED7140 (ID*)	16 Jan 2000
		DED6946 (ID*)	16 Jan 1999
		DED6885 (ID*)	7 Jan 1999
M. longiseta	2 (ID*)	DED7787 (ID*)	13 Jan 2005
		DED6883 (ID*)	7 Jan 1999
M. volvata	1 (MA*)	DED7628 (MA*)	9 Jan 2004
Total	18	11 (ID*) + 7 (MA*)	

*Collecting site acronyms: MA = Malaysia, ID = Indonesia

Methods

Morphological Studies

Macro- and micro-morphological information was generated for all specimens and used for species identification and classification using taxonomic keys, monographs or other related literature pertinent to *Mycena* taxonomy. The most important literature included the following: A world monograph of *Mycena* section *Sacchariferae* by Desjardin (1995, p. 2); *Mycena* and related genera from Papua New Guinea by Maas Geesteranus and Horak (1995, p. 144); *Mycena indica* from Southern India by Manimohan and Leelavathy (1988, p. 861); *Mycena* section *Longisetae* from Indonesia by Desjardin et al. (2002, p. 142); a world monograph of *Mycena* section *Longisetae* by Desjardin et al. (2002, p. 69); and new spinose species of *Mycena* from Thailand (Desjardin et al., 2003, p. 7).

Macromorphological Techniques

Macromorphological features were recorded from fresh material, documenting all aspects of basidiome (mushroom) size, shape, colour, surface, texture and odor (Hemmes & Desjardin, 2002, p. 16). Features of both young and mature basidiomes were documented (Figure 6). Colour of the pileus, lamellae and stipe were described in daylight by using the colour guide by (Kornerup & Wanscher, 1963, p. 1). Basidiomes of all specimens were illustrated with pencil, or with digital photographs when possible. Many

species form very tiny basidiomes and it was difficult to obtain quality photographs.

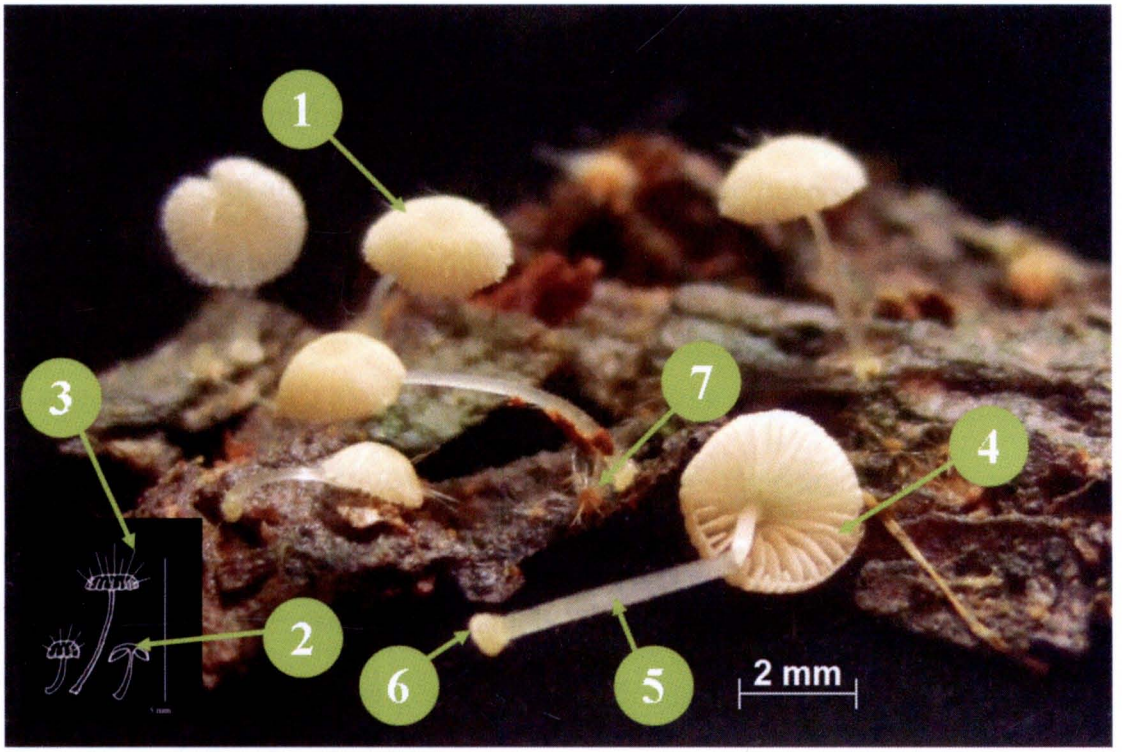

Figure 6 Basidiome structure of *Mycena brunneisetosa* (1) Pileus (cap), (2) Context of pileus, (3) Pileosetae (hairs), (4) Lamellae (gills), (5) Stipe (stem), (6) Basal Disc, (7) Primodia (young basidiome).

Taste and odor.

Taste was not documented in this group of *Mycena* because all basidiomes are very small and many collections consisted of only a few basidiomes. Tasting would have destroyed the specimens. Odor may be a taxonomically informative feature, since some *Mycena* have odors like chlorine, nitrous, or of radish. For example, *Mycena tenuisetosa* was reported

with a nitrous odor and this feature may be used to help distinguish the species from others.

Micromorphological Techniques

Dried specimens were used for analyses of micromorphological features, wherein all cell types and tissue types were described in detail. Pileus, lamellae, stipe and basal disc were dissected by using a very thin razor blade. The dissected portion was rehydrated in 95% ethanol followed by distilled water, 3% KOH or Melzer's reagent (See Appendix A) (Desjardin et al., 2002, pp. 70-71). Microscopic structures were measured with an ocular reticle and illustrated with aid of a drawing tube attached to an Olympus CX31 compound microscope (Figure 7). The size, shape, location, organization, and chemical reactions of each cell type and tissue were documented. For details of the terminology used for micromorphological features see (Desjardin, 1995, pp. 2-7).

Spore statistics include: x , the arithmetic mean of the spore length by spore width (± SD) for n spores measured in a single sample (specimen); x_{mr}, the range of spore means and x_{mm}, the mean of all spores measured (± SD) where more than one specimen was available; Q, the quotient of spore length and spore width in any one spore, indicated as a range of variation in n spores measured; Q, the mean of Q values in a single sample; Q_{mr}, the range of Q values and Q_{mm}, the mean of Q values where more than one specimen was available (Desjardin et al., 2003, p. 8).

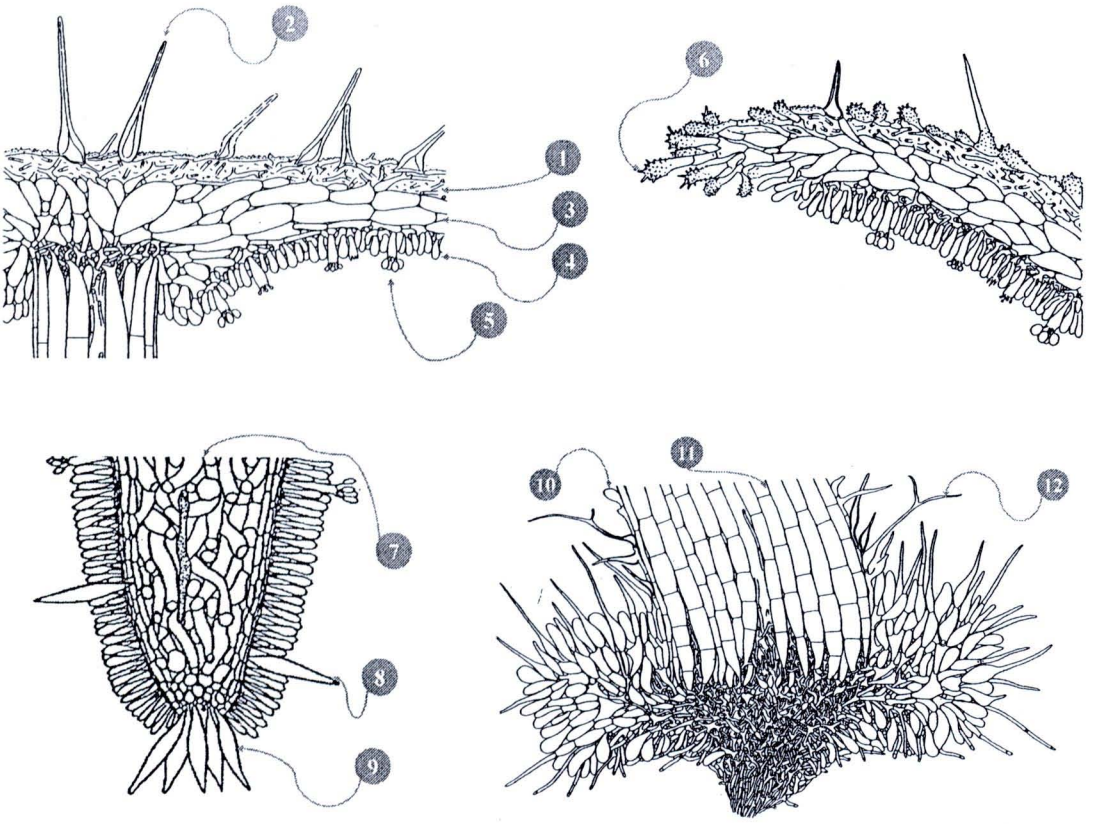

Figure 7 Anatomy of *Mycena* (1) Pileipellis; (2) Pileosetae; (3) Hypodermium and pileus trama; (4) Hymenium; (5) Basidium and basidiospores; (6) Marginal cystidia; (7) Lamellar trama; (8) Pleurocystidia; (9) Cheilocystidia; (10) Stipitipellis; (11) Stipe trama; (12) Caulocystidia

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Note. From "Agarics in Malesia I Tricholomatoid II Mycenoid, Nova Hedwigia" by E.J.H Corner, 1994, p. 171,176, 178, Germany: Cramer, J. Publisher.

Herbarium Protocols

Herbarium protocols followed the Royal Botanical Garden's herbarium handbook by Bridson and Forman (1998, pp. 8-52) for long-term preservation of biological collections. Specimens for herbarium preservation were

dehydrated by using a food drier at 45-50°C for (8-) 12-24 (-48) hours depending on the specimen size. Pests were controlled by freezing specimens at -20 °C for 48 hours. Specimens were stored in paper packets with label data created by the fungal database MIMSmy program designed by the Information System Laboratory, BIOTEC, Thailand. All specimens collected in Thailand were deposited at the BIOTEC Bangkok Herbarium-BBH (Pathumthani, Thailand). All specimens collected in Malaysia and Indonesia were deposited at the Thiers Herbarium-SFSU at San Francisco State University (California, U.S.A). BBH stores its herbarium specimens under controlled conditions of 18-22°C with 45-50% humidity.

Molecular Studies

Molecular sequence data are very important and useful in helping to understand relationships among species, and in helping to delimit species. Molecular techniques for this study mostly followed manufacturer protocols with modifications determined from experiments and from suggestions provided by Dr. Brian Perry and Dr. Frank Cipriano (Conservation Genetics Lab, SFSU) (Appendix B-E). The molecular protocol used for this study is presented in Figure 8.

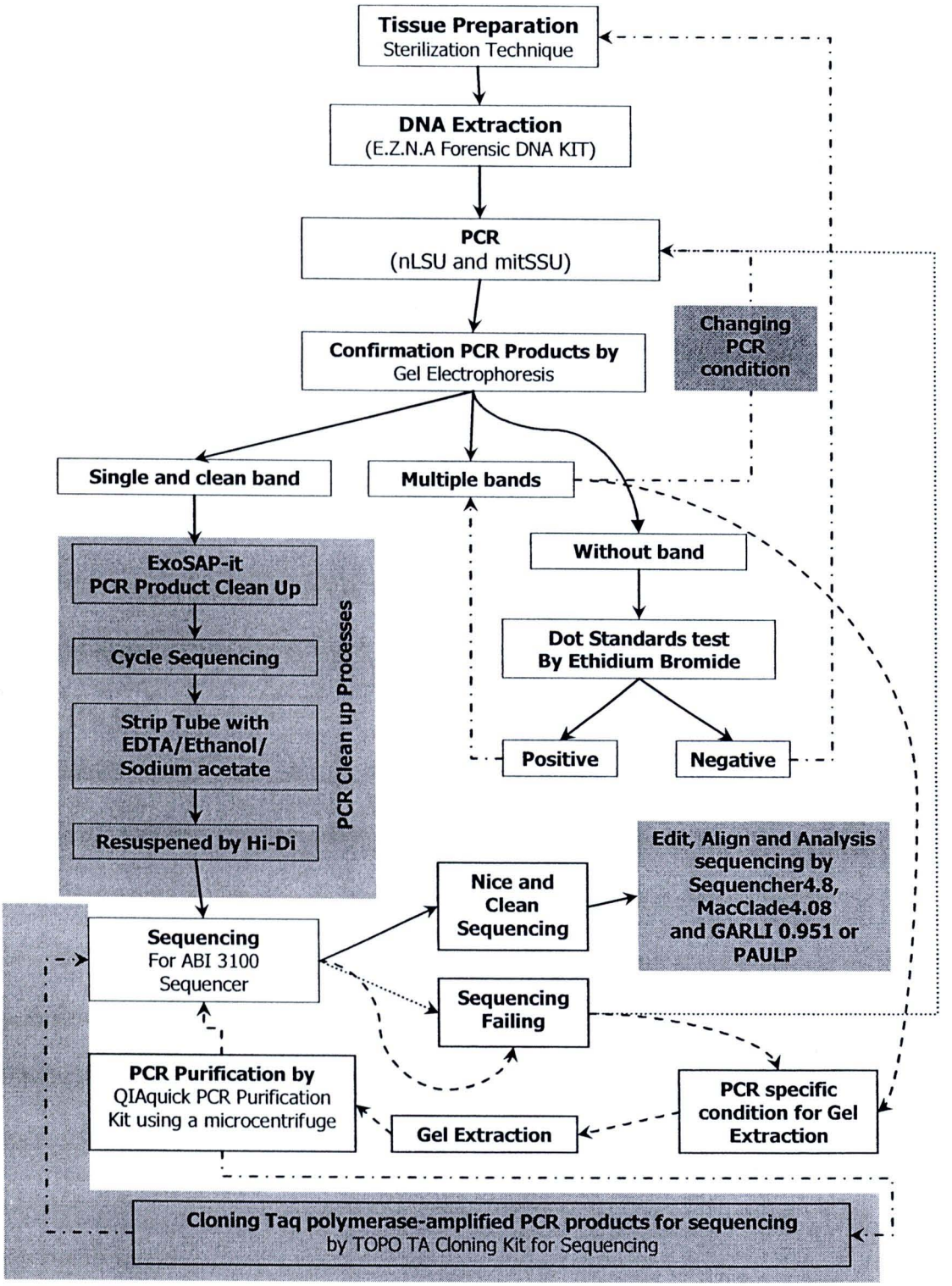

Figure 8 Molecular protocol followed in this study.

DNA preparation.

DNA was extracted from dried material. Small and whole basidiomes were ground in microfuge tubes (1 ml) with liquid nitrogen. DNA was extracted using E.Z.N.A.® Forensic DNA Extraction Kit (Omega Bio-tek Inc., Norcross, GA, USA) following the instructions given by the manufacturer (see Appendix B).

Polymerase chain reaction (PCR) and direct sequencing of the nLSU regions.

DNA fragments were amplified using the GeneAmp PCR System 9600 (Applied Biosystems®, Perkin-Elmer Corp., Nortwalk, CT, USA). The nLSU gene was amplified using the LROR, LR5 and LR21 primers. When amplification failed the primer LR4.8 was also used. For primer details see Appendix C.

The PCR programs were set specific for each gene region and primer as indicated in Table 5 (see Appendix D for information on the PCR MasterMix formula). PCR products were purified using modifications to the manufacturer's protocol of the ExoSAP-iT Kit (USB Corp., Cleveland, OH, USA) (see Appendix E). DNA sequencing reactions were performed with the ABI PRISM® BigDye Cycle Sequencing protocol and DNA precipitation with DTA/Ethanol/Sodium acetate protocol (Appendix F), and run on an ABI PRISM® 3100 Genetic Analyzer System (Applied Biosystems®, Foster City, CA, USA). Sequence editing was performed with Sequencher 4.2 software (GeneCodes Co., Ann Arbor, MI, USA). Edited sequences were deposited in GenBank (Table 6).

Table 5

Polymerase Chain Reaction (PCR) Program Settings Specific for nLSU and Primer set.

Region/ Primer	Initial Denature	Denature	Anneal	Extend	# of Cycles	Final Extension	Final Hold
nLSU							
LROR	95°C/300 secs	94°C/30 secs	58°C/30 secs	72°C/90 secs	35x	72°C/300 secs	
LR4.8	95°C/300 secs	94°C/30 secs	58°C/30 secs	72°C/90 secs	35x	72°C/300 secs	14°C/∞
LR5	95°C/300 secs	94°C/30 secs	52°C/30 secs	72°C/90 secs	35x	72°C/300 secs	

Table 6

Specimens used for the phylogenetic analyses.

	Species	Collection number	MycoBank number	GenBank accession number (nLSU)
1.	*Mycena adscendens*	–	–	✖
2.	*Mycena amicta*	–	–	DQ457692
3.	*Mycena amyloseta* sp. nov	DED7640 ✱	MB513209	FJ969544
4.	*Mycena aurantiomarginata*	–	–	AY207246
5.	*Mycena brunneisetosa*	DED7667_2I	–	FJ969545
6.	*Mycena brunneisetosa*	DED7667_2F	–	FJ969557
7.	*Mycena brunneisetosa*	DED7667_2C	–	FJ969555
8.	*Mycena brunneisetosa*	DED7667_2A	–	FJ969556
9.	*Mycena capillaripes*	–	–	AY207247
10.	*Mycena clavicularis*	–	–	AF042637
11.	*Mycena clavulifera*	DED7634	–	FJ969547
12.	*Mycena clavulifera*	DED7651	–	FJ969554
13.	*Mycena crocata*	–	–	✖

Table 6 (continued)

	Species	Collection number	MycoBank number	GenBank accession number (nLSU)
14.	*Mycena epipterygia*	–	–	AY207249
15.	*Mycena haematopus*	–	–	✖
16.	*Mycena galericulata*	–	–	AY207251
17.	*Mycena gohmaehn* sp. nov	TBP481	MB512329	FJ169960
18.	*Mycena gracilisetosa*	DED6885_1H	–	FJ969558
19.	*Mycena gracilisetosa*	DED6885_1F	–	FJ969559
20.	*Mycena gracilisetosa*	DED6885_1D	–	FJ969560
21.	*Mycena gracilisetosa*	DED6885_1B	–	FJ969561
22.	*Mycena gracilisetosa*	DED6946	–	FJ969548
23.	*Mycena gracilisetosa*	DED7140 ✸	MB513211	FJ969544
24.	*Mycena insignes*	–	–	✖
25.	*Mycena khonkhem*	TB177	–	FJ973102
26.	*Mycena leaiana*	–	–	AF261411
27.	*Mycena leptocephala*	–	–	✖
28.	*Mycena longiseta*	DED6883	–	FJ969552
29.	*Mycena longiseta*	DED7787	–	FJ969551
30.	*Mycena maculata*	–	–	AY207254
31.	*Mycena niveipes*	–	–	AY207242
32.	*Mycena olivaceomarginata*	–	–	AY207255
33.	*Mycena polygramma*	–	–	DQ071780
34.	*Mycena renati*	–	–	AY207256
35.	*Mycena rorida*	–	–	AY207298
36.	*Mycena rubromarginata*	–	–	AY207245
37.	*Mycena sanquinolenta*	–	–	✖
38.	*Mycena tenuisetosa*	TBP786	–	FJ969546
39.	*Mycena tintinnabulum*	–	–	AY207258
40.	*Mycena volvata*	DED7628 ✸	MB513213	FJ969550
41.	*Mycena zephirus*	–	–	AY207259

Table 6 (continued)

	Species	Collection number	MycoBank number	GenBank accession number (nLSU)
Non Mycena's Specimens used for the phylogenetic analyses.				
42.	*Agaricus bisporus*	–	–	U11911
43.	*Calocybe carnea*	–	–	✖
44.	*Clitocybe candicans*	–	–	AY207150
45.	*Collybia tuberosa*	–	–	AF261384
46.	*Coprinus comatus*	–	–	AF041529
47.	*Cystoderma amianthinum*	–	–	AF261473
48.	*Dictyopans pusillus*	–	–	AY014291
49.	*Entoloma prunuloides*	–	–	AY700180
50.	*Lepista nebularis*	–	–	AF223217
51.	*Lycoperdon sp 16*	–	–	EU522764
52.	*Macrolepiota dolichaula*	–	–	DQ411537
53.	*Myxomphalia maura*	–	–	AF261378
54.	*Nolanea sericea*	–	–	AF223170
55.	*Panellus stypticus*	–	–	AF261427
56.	*Prunulus pura*	–	–	AF261409
57.	*Prunulus pura*	–	–	AF261410
58.	*Resinomycena rhododendri*	–	–	AF261415
59.	*Tricholoma myomyces*	–	–	U76459

★= Holotype, ✖ = sequences from Dr. Brian A. Perry unpublished paper

Data analysis.

Phylogenetic analyses: Initial sequence alignment was performed with Clustal X (Thomson, Gibson, Plewniak, & Higgins, 1997, p. 4876) using the default settings, followed by manual alignment with MacClade 4.04 (Maddison & Maddison, 2000). Parsimony analyses were conducted using PAUP* 4.0b10 (Swofford, 2002). Searches employed a heuristic search

method with all characters weighted equally, gaps treated as missing data, random stepwise sequence addition, tree bisection reconnection (TBR), and collapse of zero length branches. Analyses were performed in two steps: first, 1,000 random sequence addition replicates were performed, saving no more than 5 trees per replicate, with MaxTrees set to auto increase. Second, with MaxTrees reset to 15 K, the most parsimonious trees resulting from the first step were used as starting trees and the analysis was allowed to swap to completion.

The appropriate model of sequence evolution for phylogenetic analyses was determined using the program MrModeltest v2.3 (Nylander, 2004). Maximum likelihood (ML) analyses were conducted in PAUP*, and emplyed the GTR+I+G model of sequence evolution. ML searches followed an iterative search strategy. First, a neighbor joining (NJ) tree was constructed using a Jukes-Cantor (JC) distance model. Next, with the search criterion reset to ML, the model parameters were estimated from the NJ tree, fixed, and an ML search was conducted. After this initial search was completed, the model parameters were re-estimated from the resulting ML tree(s), fixed, and another ML search was initiated with starting trees obtained via NJ. This step was completed a third and final time, for a total of three independent ML searches. Upon completion of the final search, trees resulting from all three iterations were compared to insure that the searches had converged on equally likely topologies. Maximum likelihood bootstrapping was performed in GARLI v0.951 (Zwickl, 2006, p. 2) with 500 replicates under the GTR+I+G

model of sequence evolution, with all parameter values estimated by the program and using the default run termination settings.

Bayesian phylogenetic analyses were carried out using Metropolis-coupled Markov chain Monte Carlo (MCMCMC) methods with MrBayes v3.1.2 (Huelsenbeck, Ronquist, Nielsen, & Bollback, 2001, p. 2311; Ronquist & Huelsenbeck, 2003, p. 1257), under a GTR+I+G model as determined above. The analyses consisted of two parallel searches, run for 5,000,000 generations, with six chains and random starting trees. Default settings in MrBayes were used for the incremental heating scheme of the chains (3 heated and 1 cold chain), unconstrained branch length (unconstrained: exponential (10.0)), and uninformative topology (uniform) priors. The chains were sampled every 1,000 generations. Trees sampled prior to searches reaching a split deviation frequency of 0.03 were discarded as the burn-in, and the resulting trees were used to calculate Bayesian posterior probabilities. Trees were viewed in TreeView (Roderic, 2002, p. 1257) and exported to graphics programs.