Molecular Evolution and Ecology of *Calostoma* (Sclerodermatineae, Boletales).

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Ecology

Introduction - Previous analyses of the ecological role of *Calostoma* focused on *C. cinnabarinum* from Massachusetts[1]. This study was recently expanded to include two additional species of the genus. *Calostoma sarasinii* and *C. berkeleyi* from Malaysia were sampled in order to describe the nutrient acquisition and identify the potential host of tropical *Calostoma* species.

Molecular Methods - DNA was collected from ectomycorrhizal root tips using extraction protocols described in Cullings[6] with some modification. DNA was extracted from dried *Calostoma* fruiting bodies using the E.Z.N.A. fungal DNA Miniprep Kit. The polymerase chain reaction (PCR) and cycle sequencing were performed with primers ITS1F and ITS4B[8]. Plant specific primers ITS1-plant and ITS2-plant[9] were used for host plant identification.

Fungal nuclear ribosomal internal transcribed spacer 1 and 2 (nrITS) sequences were generated from ectomycorrhizal root tips collected from each sampling site and used as queries in BLAST searches of the GenBank[10] and UNITE[11,12] databases. The sequences obtained from these databases were aligned with ectomycorrhizal sequences using Clustal X 1.81[14] and MacClade v 4.03[15]. Distance analyses of DNA sequences used a neighbor-joining method that employed a Kimura two-parameter model of evolution. These analyses were performed using PAUP* v 4.0b[13]. Neighbor-joining bootstrap percentages were obtained using 1000 bootstrap replicates and the same distance analysis parameters described above.

Isotopic Methods - Saprotrophic and mycorrhizal fungi have been shown to differ in their ^{13}C and ^{15}N profiles[2,3]. The use of carbon and nitrogen isotopes to indicate the nutritional mode of a previously unknown fungus has been explored in recent studies[3, 4, 5]. For each of the represented *Calostoma* species ^{13}C and ^{15}N profiles were compared to that of co-occuring ectomycorrhizal and saprotrophic fungi. Stable isotope abundances are reported as: $\delta^{15}N$ or $\delta^{13}C$‰ = $(R_{sample}/R_{standard} - 1) \times 1000$. Where R is $^{15}N/^{14}N$ or $^{13}C/^{12}C$ of either the sample or the reference standard atmospheric N_2 for nitrogen, PeeDee belemnite for carbon.

Calostoma cinnabarinum Corda (1809) -

This species is native to eastern United States and parts of Central America. Isotopic analyses of *C. cinnabarinum* from Massachusetts show that carbon and nitrogen isotopes abundancies are ectomycorrhizal in its mode of nutrient acquisition (Fig 1b). ANOVA tests of $\delta^{13}C$ for *C. cinnabarinum* indicate that there was no significant difference compared to ectomycorrhizal fungi (P = 0.999) but there was compared to saprotrophic fungi (P = 0.019). No significant difference was found between fungi in regards to $\delta^{15}N$. However, *C. cinnabarinum* was observed to be ectomycorrhizal in its ^{15}N abundance.

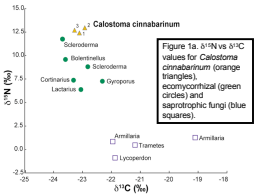


Figure 1a. $\delta^{15}N$ vs $\delta^{13}C$ values for *Calostoma cinnabarinum* (orange triangles), ecomycorrhizal (green circles) and saprotrophic fungi (blue squares).

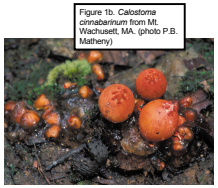


Figure 1b. *Calostoma cinnabarinum* from Mt. Wachusett, MA. (photo P.B. Matheny)

Calostoma sarasinii (Hennings) Van Overeem 1922 -

This species was collected in peninsular Malaysia in the dense cloud forests of Frazier's Hill, north east of Kuala Lumpur. The forests included several potential ectomycorrhizal hosts, including *Dipterocarpus*, *Castanopsis* and *Quercus*

Figure 2a. *Calostoma sarasinii* from Frazier's Hill, Malaysia (photo D.E. Desjardin)

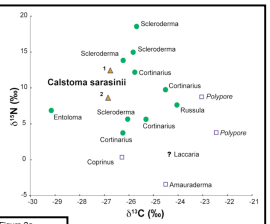


Figure 2b. $\delta^{15}N$ vs $\delta^{13}C$ values for *Calostoma sarasinii* (orange triangles), ecomycorrhizal (green circles) and saprotrophic fungi (blue squares).

Calostoma berkeleyi Massee 1888 -

This is another Malaysian species collected in Ulu Bendan recreational park, south of Kuala Lumpur.

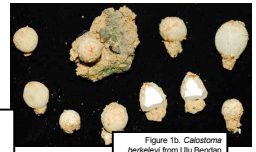


Figure 1b. *Calostoma berkeleyi* from Ulu Bendan recreational park, Kuala Pilah, Malaysia. (photo A. W. Wilson)

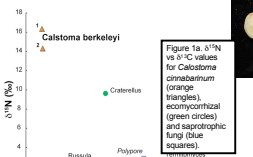
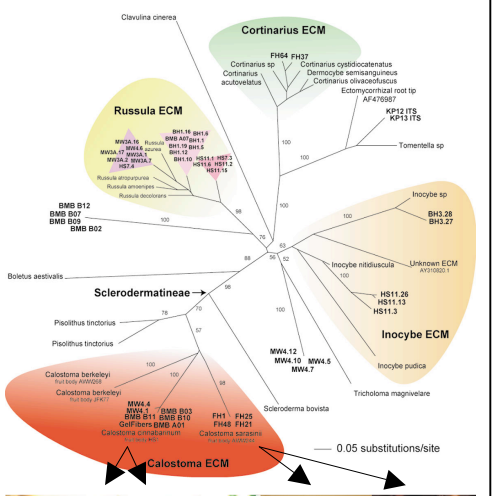


Figure 1a. $\delta^{15}N$ vs $\delta^{13}C$ values for *Calostoma cinnabarinum* (orange triangles), ecomycorrhizal (green circles) and saprotrophic fungi (blue squares).

Though no ectomycorrhizae were recovered from soil cores of *C. berkeleyi* fruiting bodies, isotopic analysis suggests an ectomycorrhizal habit. Potential hosts consist of *Dipterocarpus* and *Castanopsis*.

Figure 4a. Unrooted NJ tree of ectomycorrhizal root tip nrITS sequences and the nearest sequence matches from BLAST and UNITE databases. Ectomycorrhizal sequences in bold are from soil cores collected in Massachusetts (**HS, BMB, BH, MW**) and Malaysia (**FH, KP**). Identified basidiomycete groups include *Inocybe, Russula, Cortinarius* and the Sclerodermataceae featuring *Calostoma*. Numbers adjacent to branches represent statistical support determined by NJ Kimura 2 parameter bootstrap analysis using 1000 replicates.



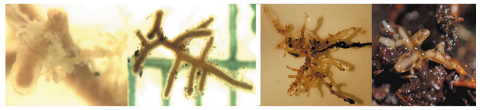


Figure 4b. Both *Calostoma cinnabarinum* (left) and *C. sarasinii* (right) ectomycorrhizae share a peculiar macromorphology. Both species form a thick gelatinous cuticle similar to the exoperidium produced in *C. cinnabarinum* fruiting bodies. Gelatinous rhizoids have been observed in the soil cores of *C. berkleyei*, similar to those produced by *C. cinnabarinum*.

Plant Hosts - Plant-specific primers used on *Calostoma cinnabarinum* ectomycorrhizae obtained ITS1, 5.8s and ITS2 sequences. The five plant sequences (DQ860279-DQ860283) were >98% similar in identity. BLAST results of Genbank for this sequence retrieved the top three hits of *Quercus incana* (AY456170), *Q. buckleyi* (AF174631) and *Q. rubra* (AF098478) indicating that *Quercus* is the host of *C. cinnabarinum* in this study. This is consistent with ecological observations of *Calostoma* from other locations: *C. cinnabarinum* in Costa Rica (Roy Halling, pers. comm.), *C. berkeleyii* in China (Zhu-Liang Yang, pers. comm.). A broader examination of the genus reveals a probable association with the order Fagales. In New Zealand, specimens of *C. fuscum* and *C. rodwayii* have been collected in *Nothofagus* forests. *Calostoma sarasinii* and *Calostoma berkeleyi* were recently collected by D. Desjardin and A. W. Wilson in forests dominated by *Castanopsis* and *Dipterocarpus* species.

Future Direction - Additional efforts will be made to identify the hosts of *C. sarasinii* and *C. berkeleyi* as well as other tropical and temporate species of *Calostoma*. This will aid in establishing patterns of host association for the genus and potentially link the evolution between fungi and plant host.

Evolution

Introduction - Recent studies of the Sclerodermatineae were limited to a single gene region[16] or multiple genes with limited taxa[17]. None of these studies displayed a well supported internal structure between genera, and both supported the inclusion of the boletoid (stipitate-pileate with tubular hymenophore) *Gyroporus* within the gasteroid Sclerodermatineae. This study shows preliminary results using an expanded data set and multiple gene regions attempting to address the following questions: 1) What taxonomic group is sister to *Calostoma*? 2) Is there suport for the the placement of the stipitate-pileate group *Gyroporus* within the gasteroid Sclerodermatineae?

Methods - Thirty-seven taxa are represented in the current the Sclerodermatineae study (Table 2). Twenty-nine of the taxa sampled, represent groups within and peripheral to the Sclerodermatineae. The other eight taxa have been sampled by the Assembling the Fungal Tree of Life (AFTOL) project[5] (Table 2). For the 29 non-AFTOL sampled taxa, the following nuclear ribosomal and protein coding genes have been collected: nuclear ribosomal large subunit (LSU), RNA polymerase II subunit 1 (RPB1), RNA polymerase II subunit 2 (RPB2). The AFTOL project has tested and developed numerous primers for these DNA regions. Additional primers wither greater specificity for the Sclerodermatineae were generated for this study. The primers used and statistical comparisons between genes are represented in Table 1.

Strong, single copy PCR products were sequenced directly. Weak PCR product and product showing multiple bands were cloned using TOPO TA cloning and TOPO TA One Shot kits. Colonies were screened and multiple colonies of the appropriate sized product were sequenced.

Sequences were cleaned and assembled using Sequencer v. 4.1[18]. Initial alignments were performed in Clustal X v. 1.81[14] and additional manual alignments were done with MacClade v. 4.07[15]. Parsimony and maximum likelihood analyses were performed using PAUP* 4.0b10[13]. Models of evolution used in likelihood analyses were chosen for each data set using Mr Model test v. 1.1b[19]

Analyses - Two sets of analyses were performed in an attempt to address the questions stated above. Analysis one (Fig. 5) consisted of a Bayesian analysis of 37 taxa and all gene regions(TABLE 2), using MrBayes 3.1.2[20] using the GTR+I+G model of evolution running 10 million generations and sampling every 100th tree. Analysis two (Table 3) used a limited data set of 20 taxa represented by all three gene regions and was subjected to maximum likelihood searches (GTR+I+G) under different topological constraints. A Shimodaira-Hasegawa[21] test (SH) of constraints was performed to compare the likelihood of different topologies.

Table 1 - Sequence comparisons for all 37 taxa with additional primers generated for sequencing Sclerodermatineae taxa.

	LSU	RPB1	RPB2	Combined
Regions	D1-D4	AC	5-7	
PCR primers	LR0R, LR5	RPB1-Af, RPB1-Cr	bRF, bRK2, bdF, bT-1R	
Designed primers		AAJFJWCTTRKCTCGTTTYCGACCTTC AAJFJGCCCTTCTTCTTCTTCTTCTTC	LT1_1F (CCRTTRYTTCCMAAGYTTTCCC) LT1_2F (CCRTTRYTTCCMAAGYTTTCCC) LT1_3F (CTTRKTTTCCMAAGYTTTCCC) LT1_4F (ATMACTTTCCTTCTTCTTCTTCTTC)	
Aligned length	977	1498	1238	3712
char excluded	N/A	508	128	636
char included	977	991	1108	3076
parsimony informative (PI)	268	387	419	1074
% PI	27.43	39.05	37.82	34.92
GC Frequency	0.548	0.5188	0.5018	0.526

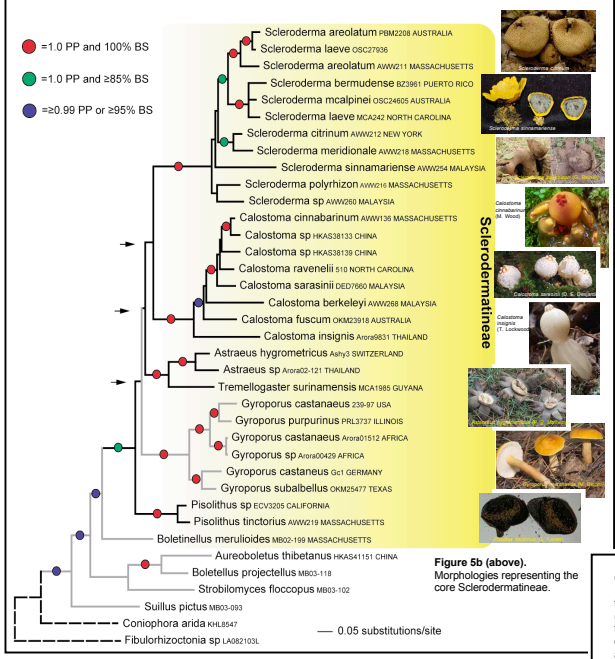


Figure 5a. Bayesian analysis using GTR+I+G model of evolution for thirty seven taxa represented by 28S, RPB1 and RPB2 gene regions. -ln L 40300.7223. Ngen=10,000,000, Sfreq=100, Burnin=15000. Ranges for posterior probabilities (PP) and parsimony bootstrap percentages (BS) are represented by the colored circles on the figure. Branches display morphologies gasteroid (black), stipitate-pileate (grey), and resupinate (dashed lines). Nodes indicated by black arrows illustrate the lack of support for any particular internal topology between Sclerodermatineae genera.

Figure 5b (above). Morphologies representing the core Sclerodermatineae.

Table 2 - Thirty-seven taxa sampled for study of the Sclerodermatineae. Columns 28S, RPB1 and RPB2 represent sequences obtained for represented taxa. Taxa from AFTOL and SH tests are also indicated.

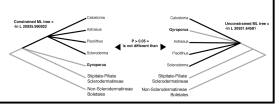
Genus	Species	ID	28S	RPB1	RPB2	AFTOL	SH test
Astraeus	hygrometricus	Ashy 3	x	x	x		x
	sp	Arora 02-121	x		x		
Boletinellus	meruliioides	MB02-199	x	x	x	x	
Calostoma	cinnabarinum	AWW136	x	x	x	x	x
	aff. sarasinii	AWW268	x	x	x		x
	fuscum	OKM 23918	x	x	x		
	insignis	Arora 9631	x		x		
	ravenelii	510	x	x	x		x
	sarasinii	DED7660	x	x	x		x
	sp	HKAS38139	x		x		
	sp	HKAS38133	x		x		
Gyroporus	castanaeus	Gc1	x	x	x		x
	castanaeus	Arora 01-512	x	x	x		
	castanaeus	239-97	x	x	x		x
	purpurinus	Leacock 3737	x	x			
	sp	Arora 00-429	x	x	x		x
	subalbellus	OKM25477	x	x	x		
Pisolithus	tinctorius	AWW219	x	x	x		x
	sp	ECV3205	x		x		
Scleroderma	areolatum	AWW211	x	x	x		x
	areolatum	PBM2208	x	x			
	bermudense	BZ3961	x	x			
	boviista (laeve)	MCA242	x	x	x		x
	citrinum	AWW212	x	x	x		
	laeve	OSC27936	x	x			
	mcalpinei	OSC 24605	x		x		
	meridionale	AWW218	x	x	x		x
	polyrhizum	AWW216	x	x	x		
	sinnamariense	AWW254	x	x	x		x
	sp. White	AWW260	x		x		
Tremellogaster	surinamensis	MCA 1985	x	x	x		
Aureoboletus	thibetanus	HKAS41151	x	x	x	x	x
Boletellus	projectellus	MB03-118	x	x	x	x	x
Coniophora	arida	KHL8547			x	x	
Fibularhizoctonia	sp	LA082103L	x	x	x	x	x
Strobilomyces	floccopus	MB03-102	x	x	x	x	x
Suillus	pictus	MB03-093	x	x	x	x	x

Table 3. Shimodaira-Hasegawa test (SH) of topological constraints analyzed 6 ML trees representing 20 taxa (14 Sclerodermatineae) (Table 1). Five ML trees were generated under the topological constraints listed. Results show no significant difference between any of the constrained trees vs. the optimal unconstrained tree.

Constraint	Likelihood	Difference	Probability
unconstrained tree	20931.64581	best	
Sclerodermatineae - Gyroporus	20935.99092	4.3451	0.479
Calostoma + Astraeus	20934.73396	3.08815	0.612
Calostoma + Gyroporus	20935.62070	3.97488	0.516
Calostoma + Pisolithus	20941.30251	9.6567	0.193
Calostoma + Scleroderma	20931.64581	0.00	1.00

Gyroporus + Sclerodermatineae? - *Gyroporus* continues to be resolved within the gasteroid Sclerodermatineae in both the Bayesian (Fig. 5). Though there is statistical support for this relationship in both PP and BS, an alternative topology that forces monophyly of the gasteroid Sclerodermatineae (to the exclusion of *Gyroporus*) could not be rejected in the SH analysis (Fig. 6; Table 3). Thus, there is no strong evidence that there was a reversal from a gasteroid to a boletoid fruiting body form in the Sclerodermatineae.

Fig. 6 - Results of the SH test state that the stiptate-pileate basidiome morphology (grey branches) evolving basal to the gasteroid morphology (black branches), is not significantly different from the stipitate-pileate *Gyroporus* evolving from gasteroid fungi. Below are representations of both kinds of constraints employed in the analysis.



Calostoma + ? - *Scleroderma* was resolved as the sister taxon to *Calostoma*. However, there was no statistical support (PP or BS) for this relationship, and alternative topologies could not be rejected (Table 3). Explanations for the absence of support for any particular topology could stem from the lack of appropriate taxon sampling, especially with *Astraeus* and *Pisolithus*. The genes used in this analysis may not be sufficient in resolving these questions about the generic relationships within the Sclerodermatineae.

Future Directions - This study will continue to answer the questions posed above by :

- Continued analysis of existing and new nucleotide and protein sequences.
- Expand representation of the ingroup taxa, especially *Astraeus* and *Pisolithus*.
- Analysis of more characters representing alternate genes: ITS 1 & 2, 5.8S, EF1a, mtATP6, etc....

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