

EVOLUTION IN ACTION: MOLECULAR EVIDENCE FOR RECENT EMERGENCE OF SECOTIOID GENERA *ENDOPTYCHUM*, *GYROPHRAGMIUM* AND *LONGULA* FROM *AGARICUS* ANCESTORS

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Phylogenetic analyses of 29 *Agaricus* species and one representative from genera *Endoptychum*, *Gyrophragmium* and *Longula* each were conducted based on sequence data of the entire internal transcribed spacers and partial large subunit of ribosomal DNA. The *Agaricus* species formed several distinct clades both confirming and challenging previous morphological sections in several cases. *Endoptychum depressum*, *Gyrophragmium dunalii* and *Longula texensis* were nested among species of the genus *Agaricus*. This study provides evidence for independent emergence of these secotioid fungi from *Agaricus* ancestors, most likely from species placed in the section *Arvenses* as inferred from molecular data.

Keywords: *Agaricus*, *Endoptychum*, *Gyrophragmium*, *Longula*, ITS, LSU, phylogeny, evolution, rDNA, secotioid fungi

Introduction

The homobasidiomycetes, including the mushroom-forming fungi that display an incredible diversity of fruiting body forms, contains over 90% of the species in the *Hymenomycetes*. Traditionally, taxonomy of homobasidiomycetes was based on morphological and anatomical characters of fruiting bodies. This group has been sampled intensively for molecular phylogenetic studies by fungal sys-

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tematists [1, 2, 3]. While many aspects of morphology-based classifications have been upheld, there have also been major rearrangements in systematics [3].

There is very limited information about the geologic history of the fungi, mostly due to a lack of good fossils. The fossil record for fungi is based on very few specimens compared to that for plants and animals. Therefore, analysis of DNA sequences has become an attractive and powerful tool for inferring evolutionary relationships and the times of origin for lineages of fungi. Revealing recent evolutionary processes via phylogenetic techniques is particularly of interest, because it can allow us to peek into the "black-box" of evolution, and understand the biological and ecological processes of past evolutionary events.

There are several examples where the rates of morphological and molecular evolution are different. One of these is when several cryptic species – distinguished by molecular divergence – are hidden behind a single morphological species. In other cases, some organisms have undergone accelerated morphological evolution, but little molecular divergence. Good examples of this latter are the genera specified in this study, or the genus *Rhizopogon* that is very different morphologically (being a gasteromycete) from its close relatives in the boletoid clade [4].

The genus *Agaricus* L.:Fr. contains numerous species and is the type genus of the family *Agaricaceae* [5]. The *Agaricus* species are saprophytes widely distributed over geographical areas from the tropics to the boreal regions. The genus is characterized by free, densely developed gills, becoming brown when the basidiome (and thus the spores) matures. The *Agaricus* species usually have a ring on the stipe as the residue of the *velum parziale*. The *velum universale* is highly variable; sometimes it is involved in the formation of the annulus or volva [5].

The genera *Endoptychum* Czern., *Gyrophragmium* Mont. and *Longula* Zeller consist of seven, five and one described species, respectively. They are secotioid fungi (epigeous gasteromycetes that resemble unopened mushrooms) and have been classified as "agaricoid gasteromycetes" in the family *Agaricaceae* [6, 7]. Species in the genus *Endoptychum* are decomposers in disturbed, usually xeric areas in the Northern Hemisphere and are characterized by 1–8 cm tall, 1–6 cm thick, oval or irregularly shaped fruiting bodies with very short stalk. Pileus white to light brown when young, becoming darker, shredded and splitting open in age. Gleba of irregular lamellate to anastomosing, intervenose plates, white when young to yellow-brown in age. Stipe-columella 1–3 cm long, 0.8–2 cm thick, white, sometimes bruises yellow. Spores 7.5–9.5 × 5–7 μm, ovoid, smooth, thick-walled, with a small apical pore, chocolate-brown. The *Gyrophragmium* species can be found in arid regions of the world. Their basidiocarps are round to

egg-shaped when young, 10–22 cm tall when fully expanded. Pileus convex, 3–3.5 cm broad, 1.5–2 cm thick, light to dark brown, margin smooth to torned, shredded from the separation of partial veil, which remains on the stalk as a ragged annulus. Gleba brown, gill-like, contorted and anastomosing, free from stalk. Stipe-columella 10–20 cm long, 7–22 cm thick, white to yellowish, with anulus and fragile, membranous volva. Spores 5–7 mm, globose to ovoid or irregular in shape, thick-walled, no germ pore, brown. The genus *Longula* is monotypic, containing *L. texensis* (Berk. & Curt.) Zeller as the only species, and can be found solitary or in small groups on dry, impoverished soils in the western United States. It is characterized by 5–18 cm tall fruiting bodies with a 5–7 cm broad, oval pileus, drying, splitting and shredding in age exposing the irregularly lamellate chocolate-brown gleba. Stipe-columella 4–15 cm long, 1–4 cm thick, white and fleshy at first, becoming woody in age and staining yellow when bruised. Schaeffer reaction is strong, positive on surface and flesh of the stalk. Spores 4–7 mm globose to subglobose, thick-walled, no germ pore, brown [7, 8].

Use of ribosomal DNA (rDNA) sequences to infer phylogenetic relationships among agaric fungi now is widely exploited [1–3, 9, 10]. For *Agaricus* research and phylogenetic analyses, researchers have used sequence variation found in rDNA [11–14], mitochondrial plasmid pEM [15] and the mitochondrial *atp6* gene [16]. However, these investigations were strictly limited to *Agaricus* species and the phylogenetic relationships of secotioid genera thought to be closely related to *Agaricus* remain unclear. The major goal of this work was to gain a better understanding of the evolutionary relationships among *Agaricus* and its sequestrate relatives.

Materials and methods

Isolates and DNA extraction

Twenty-nine *Agaricus* species and representatives from the genera *Endoptychum*, *Gyrophragmium*, *Longula*, and *Chlorophyllum* were included in this study (Table 1). Sequence data were generated for 25 *Agaricus* species, while sequences of three *Agaricus* and one species of *Endoptychum*, *Gyrophragmium*, *Longula*, and *Chlorophyllum* each were downloaded from GenBank. Isolates sequenced in this study were grown in potato dextrose yeast extract broth (50 ml) for 3 to 6 weeks depending on the growth rate of the mycelium. The mycelia were filtered from the broth and DNA was extracted using the PUREGENE[®] DNA Isolation Kit (Gentra Systems, Minneapolis, MN).

Table I

Species, isolate code and source of cultures included in this study

Species	Isolate code	Source, collector and geographic origin
<i>Agaricus abruptibulbus</i>	WC771	PSU Mushroom Culture Collection; ATCC 22035; Bohemia (Czech Republic)
<i>A. albolutescens</i>	WC907	PSU Mushroom Culture Collection; Mark G. Loftus, AA-0378; Monterey Bay Area, CA, U.S.A.
<i>A. arvensis</i>	WC848	PSU Mushroom Culture Collection; Mark G. Loftus, AA-0374; Monterey Bay Area, CA, U.S.A.
<i>A. augustus</i>	WC908	PSU Mushroom Culture Collection; Mark G. Loftus; Monterey Bay Area, CA, U.S.A.
<i>A. bernardii</i>	WC772	PSU Mushroom Culture Collection; ATCC #52974; MD, U.S.A.
<i>A. bisporus</i>	10104	Mushroom Culture Collection, Hungarian Museum of Natural History; Hortobágy, Hungary
<i>A. bitorquis</i>	B91	Korona Spawn Plant, commercial strain; Hungary
<i>A. blazei</i>	WC837	PSU Mushroom Culture Collection; ATCC #76739; Brazil
<i>A. californicus</i>	RWK1914	Richard W. Kerrigan; CA, U.S.A.
<i>A. campestris</i>	–	Sequences from Genbank: U85307, U85273
<i>A. cupreo-brunneus</i>	RWK1928	Richard W. Kerrigan; CA, U.S.A.
<i>A. devotensis</i>	–	Sequences from Genbank: AF059225, AJ418755
<i>A. dimittivus</i>	WC912	PSU Mushroom Culture Collection; Mark G. Loftus, AA-0379; Monterey Bay Area, CA, U.S.A.
<i>A. excellens</i>	RWK1929	Richard W. Kerrigan; CA, U.S.A.
<i>A. fissuratus</i>	WC777	PSU Mushroom Culture Collection; ATCC #56178; Denmark
<i>A. fusco-fibrillosus</i>	WC913	PSU Mushroom Culture Collection; Mark G. Loftus, AA-0369; Monterey Bay Area, CA, U.S.A.
<i>A. fuscovelatus</i>	WC914	PSU Mushroom Culture Collection; Mark G. Loftus, AA-0370; Monterey Bay Area, CA, U.S.A.
<i>A. hondensis</i>	WC915	PSU Mushroom Culture Collection; Mark G. Loftus, AA-0397; Monterey Bay Area, CA, U.S.A.
<i>A. langei</i>	WC784	PSU Mushroom Culture Collection; J.P. San Antonio, ATCC #56103
<i>A. liliceps</i>	WC916	PSU Mushroom Culture Collection; Mark G. Loftus; Monterey Bay Area, CA, U.S.A.
<i>A. macrocarpus</i>	WC778	PSU Mushroom Culture Collection; ATCC #56064
<i>A. macrosporus</i>	AmZ'	Korona Spawn Plant; Denjén, Hungary
<i>A. nivescens</i>	WC779	PSU Mushroom Culture Collection; ATCC #38034
<i>A. pocillator</i>	–	Sequences from Genbank: U85308, AF041542
<i>A. semotus</i>	–	Sequences from Genbank: AF059224, AJ133390
<i>A. subfloccosus</i>	WC721	PSU Mushroom Culture Collection; Richard W. Kerrigan; San Francisco, CA, U.S.A.
<i>A. subrufescens</i>	W17	PSU Mushroom Culture Collection; J.W. Sinden
<i>A. subrutilescens</i>	WC917	PSU Mushroom Culture Collection; Mark G. Loftus, AA-0398; Monterey Bay Area, CA, U.S.A.
<i>A. xanthoderma</i>	WC918	PSU Mushroom Culture Collection; Mark G. Loftus, AA-0396; Monterey Bay Area, CA, U.S.A.
<i>Chlorophyllum molybdites</i>	–	Sequences from Genbank: U85309, U85303
<i>Endopychum depressum</i>	–	Sequences from Genbank: AF482834, AF482878
<i>Gyrophragmium dunalii</i>	–	Sequence from Genbank: AF261478
<i>Longula texensis</i>	–	Sequence from Genbank: AF261479

PCR and PCR product purification

Internal transcribed spacer (ITS) regions were PCR-amplified using primers ITS5 and ITS4 [17], and newly constructed primers GJ1 (5' CCT AGT AAC TGC GAG TGA AGC G 3') and GJ2 (5' GCC ATT ATG CCA GCA TCC 3') were used to amplify a partial region of the LSU rRNA gene. Primers GJ1 and GJ2 were designed based on *Agaricus* spp. sequence data obtained from Genbank to amplify the region between the 36th and 613th base positions of LSU (based on *A. abruptibulbus* sequence AF059228). The ITS, and partial LSU rDNA regions were amplified in 25 μ l PCR reaction mixtures containing 1 unit of *Taq* DNA polymerase (Promega, Madison, WI), 0.2 mM of each dNTP, 2 mM MgCl₂, 0.1% Triton, and 0.5 μ M of forward primer and reverse primer for the certain region. PCR reactions were performed in a 96-well thermocycler (PTC-100 Programmable Thermal Controller, MJ Research, Inc.) with the following program: 94°C/2 min; 35 cycles of 94°C/15 sec, 57°C/30 sec, 72°C/45 sec; and 72°C/4 min. Amplification products were electrophoresed in a 1.0% agarose gel containing 0.03–0.1 μ g/ml ethidium bromide. Amplification products were purified directly from reactions using the Wizard[®] PCR Prep system (Promega, Madison, WI).

DNA sequencing

Purified amplification products were sequenced using the Applied Biosystems (ABI) BigDye terminator kit and an ABI 377 automated DNA sequencer (Perkin-Elmer, Foster City, CA). Each sample was sequenced in both directions with the same primers that were used for PCR.

Sequence data analysis

Sequence ends were trimmed, manually edited and assembled into contigs using the SeqMan™ II module in the Lasergene package (DNASTar Inc., Madison, WI). Sequences were then aligned using the Clustal W algorithm [18] of MegaAlign 4.03 (DNASTar Inc., Madison, WI) followed by visual corrections. Regions where alignments were judged to be ambiguous were excluded from the analyses. Phylogenetic analyses were conducted using PAUP* 4b10 [19] with both distance (neighbor-joining, NJ) and character-based (maximum likelihood, ML) methods to reconstruct phylogenies. Since these methods follow different

theories and algorithms, congruent features found in both types of analyses were considered meaningful. In order to maximize the objectivity of my analyses, I compared 56 evolutionary models starting with the simplest model (Jukes-Cantor model, number of substitution types=1, equal base frequencies, equal rate of evolution for variable sites, proportion of invariable sites=0) and progressed toward the most complex model (General Time-Reversible model, number of substitution types=6, estimated base frequencies, estimated rate matrix, rate of evolution for variable sites following a gamma distribution with an estimated alpha parameter, estimated proportion of invariable sites) using Modeltest 3.06 [20]. The likelihood scores corresponding to the different models were compared by the likelihood ratio test and the P-values were determined [21]. The likelihood ratio test statistic was calculated as twice the difference between the log likelihood scores of the two models contrasted. When the model representing the null hypothesis is a special case of the alternate model (as was the case in the models compared), this statistic fits a chi-square (χ^2) distribution with a number of degrees of freedom equal to the number of parameters that freely vary between the two models [20]. The best evolutionary model with the significantly highest likelihood score was selected to determine the settings of the subsequent ML and NJ analyses. ML analyses were carried out with the heuristic search option using the "tree bisection and reconnection" (TBR) algorithm. In order to find the overall optimum instead of local optima, searches were carried out with 50 random addition sequences and starting trees. To test the statistical reliability of the generated trees and the stability of clades the bootstrap test [22] was used with 1000 replicates for NJ. Bootstrap values with 100 replicates were obtained with "fast" stepwise-addition option for ML searches, because of the time consuming nature of this latter analysis. In order to root the trees, *Chlorophyllum* was designated as outgroup.

Results

The ITS/LSU alignment consisted of 1412 base pairs, of which 41 ambiguous positions were excluded from the analyses. The portion of variable sites within the alignment was 21.66%, with 148 parsimony-informative sites (10.80%), respectively. After testing 56 evolutionary models via hierarchical likelihood ratio tests, the Hasegawa-Kishino-Yano model was selected with calculated proportion of invariable sites and with rates of variable sites following the γ -distribution with an α -shape parameter (Table II). The best-fit model's settings were as follows: base frequencies (A=0.2553, C=0.2064, G=0.2523, T=0.2860), substitution

model (transition/transversion ratio=3.21), proportion of invariable sites ($I=0.6257$), and γ -distribution (G) shape parameter ($\alpha = 0.7326$). Both NJ and ML analyses – using settings according to the model chosen – resulted in one tree with a minimum evolution (ME) score of 0.2300 (NJ), and $-\ln L$ score of 5350.8686 (ML) (Figure 1). The NJ and ML trees had similar topologies.

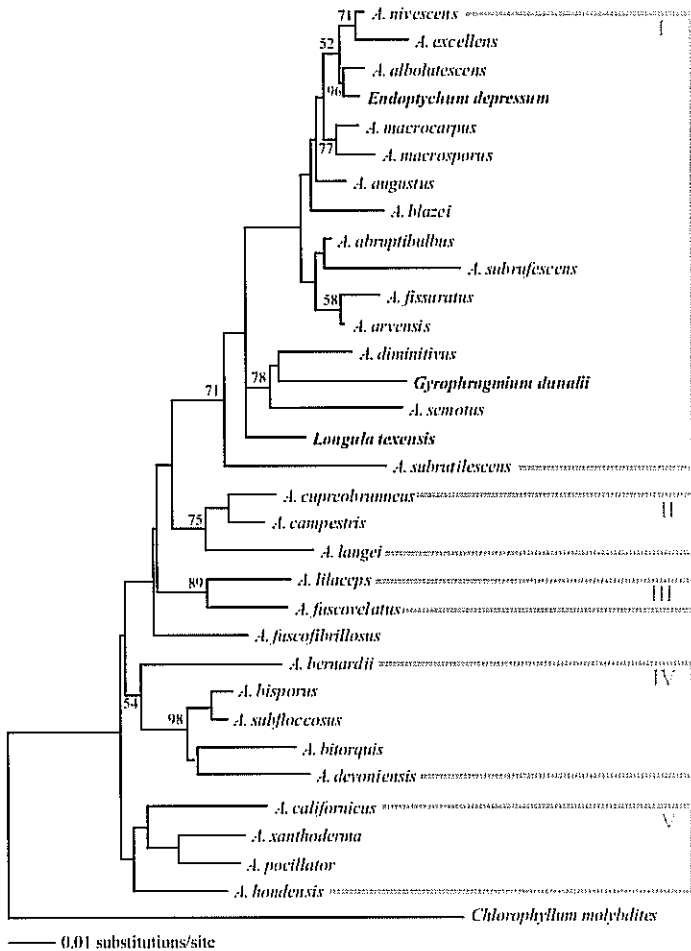


Figure 1. Phylogeny of *Agaricus*, *Endoptychum*, *Gyrophragmium*, and *Longula* species based on maximum likelihood ($-\ln L = 5350.8686$) analysis of the ITS/LSU rDNA dataset, showing clades described in the text. The tree is rooted with *Chlorophyllum* as outgroup. Only those bootstrap values exceeding 50% are shown. Bold names indicate secotioid species

Table II
 Determination of the best-fit evolutionary model for the ITS/LSU rDNA dataset by hierarchical likelihood ratio tests of 56 models.
 Only models with significantly better fit shown

Evolution model ^a	Number of substitution types	ti/tv ratio	Assumed base frequencies	Among site variation Invariable sites	Rates of variable sites ^b	Tree score -ln L	Degree of freedom	P-value
JC (starting model)	1	1	equal	-	-	5990.7144	-	-
F81	1	1	estimated	-	-	5984.1128	3	0.004217
HKY	2	estimated	estimated	-	-	5761.1392	1	<0.000001
HKY+G	2	estimated	estimated	-	estimated	5421.6572	1	<0.000001
HKY+I+G	2	estimated	estimated	estimated	estimated	5374.1128	1	<0.000001

^a Listed in order of increasing complexity. JC, Jukes-Cantor model [26]; F81, Felsenstein model [27]; HKY, Hasegawa-Kishino-Yano model [28] with or without proportion of invariable sites (I) and gamma distribution (G) shape α -parameter.

^b Variable sites assumed to follow a gamma distribution with a distribution shape α -parameter.

^c Compared with the best value previously calculated.

Several clades were identified within *Agaricus*, receiving varying levels of support. Clade I – with bootstrap values of 58% (NJ) and 71% (ML) – contained *A. abruptibulbus*, *A. albolutescens*, *A. arvensis*, *A. augustus*, *A. blazei*, *A. diminutivus*, *A. excellens*, *A. fissuratus*, *A. macrocarpus*, *A. macrosporus*, *A. nivescens*, *A. semotus*, *A. subrufescens*, *A. subrutilescens*, *Endoptychum depressum*, *Gyrophragmium dunalii* and *Longula texensis*. Clade II – with bootstrap values of 81% (NJ) and 75% (ML) – included *A. campestris*, *A. cupreo-brunneus*, *A. langei*, while Clade III – with bootstrap values of 86% (NJ) and 89% (ML) – consisted of *A. fuscovelatus* and *A. liliceps*. Clade IV – with bootstrap values of 58% (NJ) and 54% (ML) – contained *A. bernardii*, *A. bisporus*, *A. bitorquis*, *A. devoniensis*, *A. subfloccosus*. Although the bootstrap values of Clade V were low – 65% (NJ), <50% (ML) – isolates of *A. californicus*, *A. hondensis*, *A. pocillator*, *A. xanthoderma* grouped together in both types of analyses. Relationships of *A. fuscofibrillosus* within the genus could not be resolved, because different analyses showed different placing of the species, all of them receiving very weak support (<50% bootstrap values).

Discussion

Phylogenetic analyses showed that *Endoptychum depressum*, *Gyrophragmium dunalii* and *Longula texensis* are nested among species in *Agaricus* section *Arvenses sensu* Heinemann [23]. This observation is also supported by some morphological features, i.e. the yellow bruising of *Endoptychum*, long, *Agaricus*-like stipe with annulus and membranous volva in *Gyrophragmium*, the strong positive Schaeffer reaction on *Longula*, and brown spores and brown, free gill-like structures in all three genera. Both the molecular data and the morphological traits, i.e. distinct stipe, unopened, deformed mushroom-like appearance indicate that *Endoptychum*, *Gyrophragmium* and *Longula* diverged relatively recently from their ancestors and they still have not acquired all the characteristic gasteromycete features. However, despite their emergence in the same clade, these genera do not share one common ancestor, rather they evolved independently from different species of section *Arvenses*.

The most likely explanation for the emergence of these secotiid forms is the adaptation to xeric or at least seasonally dry environments. This theory is supported not only by the ecology of *Endoptychum*, *Gyrophragmium* and *Longula*, but also numerous other examples can be found in other xerophil secotiid genera, e.g. *Podaxis*, *Montagnea*, *Neosecotium*. The ancestors of *Endoptychum depres-*

sum, *Gyrophragmium dunalii* and *Longula texensis* were fleshy *Agaricus* species with open hymenium and active spore discharge. The early evolution of these sequestrate forms may have taken place in dry areas with enough moisture to initiate basidiocarp formation, but not enough for their full development. Sporocarps of fleshy mushrooms in xeric habitats often dry out and become arrested in their development before reaching maturity. There must have been a natural selection for basidiocarp types that offer protection against dry, warm environment, and epigeous and subepigeous secotioid forms with enclosed hymenium have arisen from ancestors with fully exposed hymenium. Perhaps this type of protection arose from the permanent establishment of arrested stages in sporocarp formation, as it was suggested, for example, in *Elasmomyces* and *Macowanites* [24]. The unopened pileus was able to maintain a higher humidity and to enhance spore production. Once the hymenium was no longer exposed, there was no selective advantage in the forcible discharge of basidiospores, thus the basidiospores became passively discharged.

As for future evolution of *Endoptychum*, *Gyrophragmium* and *Longula*, one can only speculate based on what is known about the evolution of other xerophil gasteromycetes. After the loss of ballistospory, the stipe offers no selective advantage, thus it is expected to disappear gradually, although the columella can persist for a much longer time within the gleba, as many of the hypogeous species still possess such structure [24]. The disappearance of the stipe allows the peridium to enclose the hymenophore, resulting in a gastroid type of basidiocarp, and with no stipe the sporocarp can gradually become hypogeous. This gastroid, hypogeous form can be considered as an additional adaptation to the dry environment. Once the spores are retained inside the fruit body, or kept underground, the problem of dispersal arises. The principal disseminating agent for the spores of secotioid fungi is no longer wind but animal vectors (mostly small mammals or insects) [24] or, to smaller extent, run-off water and rain-splash. Comparing the traits described above with the morphological structure of *Endoptychum*, *Gyrophragmium* and *Longula*, it seems that perhaps *Endoptychum depressum* has developed slightly further along the most likely evolutionary pathway, relative to the other two species. The short stipe, the sometimes subepigeous development, and the fact that small mammals, e.g. the North American flying squirrels (*Glaucomys sabrinus*) [25], has been reported to feed regularly on *Endoptychum depressum* sporocarps, might indicate a relatively fast evolution in morphological characters of this species.

This study provides phylogenetic information to characterize relationships among *Endoptychum*, *Gyrophragmium*, *Longula* and *Agaricus*. While the species

of these secotioid genera represented here clearly originated from *Agaricus* species, sequence data from additional *Endoptychum*, *Gyrophragmium* species are needed to test the monophyletic origin of these genera and to further elucidate their evolution. A better understanding of the evolutionary pathways in *Agaricus* and its close relatives may help to refine their classification and provide vital information about the diversity and biology of these organisms.

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