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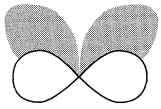

Shiitake mushrooms and molecular clocks: historical biogeography of *Lentinula*

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Abstract

Aim Fungi make up a large, ecologically important group of eukaryotes that has been neglected in historical biogeography. In this study, molecular phylogenies and molecular clock dating were used to infer historical biogeography of the shiitake genus, *Lentinula* Earle, which occurs in both the Old World and New World. The major goals of this study were to determine if the Old World and New World groups are monophyletic, and whether the Old World/New World disjunction is the result of vicariance or long distance dispersal.

Location Collections studied are from Asia, Australasia, the Gulf Coast region of North America, the Caribbean, Central America, and northern South America.

Methods Phylogenetic trees were inferred with nuclear large subunit (nuc-Isu) ribosomal DNA (rDNA) and internal transcribed spacer (ITS) sequences. Molecular clock dating was performed using trees derived from nuc-Isu rDNA and mitochondrial small-subunit (mt-ssu) rDNA sequences. Rate constancy was tested with maximum likelihood, and clocks were calibrated using evidence from fossils or other molecular clock studies, or according to expected ages under different historical biogeographic scenarios.

Results Analyses of ITS and nuc-Isu rDNA sequences suggest that there are seven species of *Lentinula* worldwide, which occur in two main clades, one in the New World the other in the Old World. Rate constancy cannot be rejected in nuc-Isu rDNA, but can be rejected in mt-ssu rDNA. Both genes suggest that the Old World/New World disjunction could be due to fragmentation of an ancient Laurasian range. An alternative Gondwanan hypothesis is not supported by the molecular clock age estimates.

Main conclusions Only one long distance dispersal event must be invoked in *Lentinula*, that being between Australia and New Zealand. Despite having airborne spores, it appears that long distance dispersal is rare in *Lentinula*. This may also be true in other fungi, which are therefore excellent candidates for historical biogeographic studies using molecular characters.

Keywords
Beringia, biogeography, fungi, *Lentinula*, maximum likelihood, molecular clock, phylogeny.

INTRODUCTION

One of the major goals of biogeography is to demonstrate common patterns in the historical movements of organisms. By integrating results from diverse taxa, it is possible to gain insight into the formation of ecological communities and the origins of global patterns of biodiversity. Plants and animals have been well studied by historical biogeographers, but fungi have been largely neglected. This is unfortunate not only because fungi make up a major clade of eukaryotes (with an estimated 1.5 million extant species; Hawksworth, 1991) but also they play pivotal ecological roles. Lack of knowledge regarding the movements of mycorrhizal, pathogenic, and saprotrophic fungi limits understanding of the evolution of terrestrial ecosystems.

Impediments to fungal historical biogeography have included poor knowledge of fungal phylogeny, the scanty fungal fossil record, and the perception that fungi, with their

airborne spores, have few barriers for dispersal. However, prospects are improving for historical biogeography of fungi (as the existence of this symposium indicates). This is primarily because of the growth of molecular systematics, which has provided phylogenetic resolution at all taxonomic levels in the fungi, as well as insight into dispersal abilities and patterns of gene flow. Finally, in conjunction with the slowly improving fungal fossil record, molecular characters have provided clues to the absolute ages of the major groups of fungi (Berbee & Taylor, 1993; Hibbett *et al*., 1997a). Recently, there has been an upsurge in the number of fungal phylogenetic studies that have a biogeographic focus, including work on *Pleurotus* (Vilgalys & Sun, 1994), and *Gibberella* (O'Donnell *et al*., 1998). In the present study, molecular characters were used to infer phylogenetic and historical biogeographic relationships in *Lentinula* Earle (Agaricales, Tricholomataceae, Collybieae), which includes shiitake mushrooms.

Shiitake mushrooms have been cultivated for many centuries in China and Japan (Chang & Miles, 1987) and are now grown on a commercial scale worldwide. Wild populations of shiitake mushrooms occur in the Old World and New World. In the Old World, *Lentinula* ranges from a northern limit in Japan and the Russian Far East, to South-east Asia and Australasia (including New Guinea, Australia, Tasmania, and New Zealand), and west to Nepal and India, in the foothills of the Himalayas. In the New World, *Lentinula* occurs in the Gulf Coast region of North America, the Caribbean, Central America, and northern South America (Pegler, 1983). *Lentinula* has not been recorded in Europe or Africa.

Broad disjunct distributions, like that of *Lentinula*, can result from long distance dispersal (LDD), fragmentation of ancestral ranges, or both. *Lentinula* reproduces by microscopic airborne spores, which at first glance might suggest that LDD could be common. However, several aspects of the ecology and life cycle of *Lentinula* suggest that LDD should be rare. *Lentinula* is heterothallic, meaning that it is obligately outcrossing, and it does not produce asexual spores or other mitotic propagules. In addition, *Lentinula* grows only on hardwood logs, which are patchy discrete resources, and it does not produce rhizomorphs or other somatic structures that would enable it to bridge gaps between logs. Consequently, for LDD of *Lentinula* to occur via spores, two mating compatible spores would have to colonize the very same log. Alternatively, *Lentinula* could be dispersed as dikaryons (the effectively diploid phase of the life cycle) by rafting inside hardwood logs that were already colonized.

Shiitake mushrooms are homobasidiomycetes. In addition to gilled mushrooms, homobasdiomycetes include puffballs, polypores, and other fleshy macrofungi. Homobasidiomycetes are nested within a more inclusive clade, the basidiomycetes, which also includes smuts, rusts, jelly fungi, and certain yeasts. The oldest unambiguous fossil basidiomycete is *Palaeancistrus martinii*, which is a hyphal fossil from a Pennsylvanian coal ball (≈ 300 Ma; Dennis, 1970). *Palaeancistrus* has clamp connections, indicating that it is a basidiomycete, but it lacks other characters that would be necessary to determine whether it is a homobasidiomycete or some other kind of basidiomycete. The oldest unambiguous homobasidiomycete fossil is *Archaeomarasmius leggetti*, which is a gilled mushroom from mid-Cretaceous amber (≈ 90 Ma; Hibbett *et al*., 1997a). Based on molecular characters, Berbee & Taylor (1993) estimated that the basidiomycetes are about 550 Myr BP and that the homobasidiomycetes are about 200 Myr BP.

Species-level taxonomy of *Lentinula* is controversial (Hibbett, 1992; Hibbett *et al*., 1995). The monograph of *Lentinula* by Pegler (1983) included five species that were recognized on the basis of morphology and geographical distribution. Three species of *Lentinula sensu* Pegler occur in the Old World: *L. edodes* (Berk.) Pegler, in North-east Asia, *L. lateritia* (Berk.) Pegler, in South-east Asia and Australasia (except New Zealand); and *L. novaezelandieae* (Stev.) Pegler, in New Zealand. These 'species' have been shown to be mating compatible, and consequently some authors have argued that there is only a single species of *Lentinula* in the Old World (Shimomura *et al*., 1992; Guzmán *et al*., 1997). Old World and New World isolates have so far proven to be incapable of mating (Guzmán *et al*., 1997). Two species of *Lentinula sensu* Pegler occur in the New World: *L. boryana* (Berk. & Mont.) Pegler and *L. guarapiensis* (Speg.) Pegler. *Lentinula guarapiensis* is known only from the type collection (from Paraguay) and its status as a member of *Lentinula* is controversial. In Pegler's monograph, all other New World populations of *Lentinula* are referred to *L. boryana*.

Molecular characters suggest that there are at least five species of *Lentinula* in the Old World, and at least two species in the New World (besides the questionable *L. guarapiensis*; Hibbett *et al*., 1995, 1998). The division of the New World '*L. boryana*' into two species is also suggested by anatomical characters and mating compatibility studies (Petersen *et al*., 1998). Of the five species recognized by Pegler (1983), only *L. novaezealandieae* appears to be monophyletic. The implications of the molecular phylogeny for conservation of genetic diversity in *Lentinula* were discussed by Hibbett & Donoghue (1996).

Higher-order relationships between the Old World and New World groups of *Lentinula* are not well resolved. The degree of divergence in ribosomal DNA (rDNA) and internal transcribed spacer sequences (ITS) between the two New World species of *Lentinula* are greater than that between any pair of Old World species (Hibbett *et al*., 1998), suggesting that the most recent common ancestor (MRCA) of the New World species could be older than the MRCA of the Old World species. One possibility suggested by the ITS data is that the New World group of *Lentinula* is paraphyletic. A recent study using β-tubulin sequences (Thon & Royse, 1999) failed to resolve whether the Old World and New World groups are monophyletic.

In this study, nuclear large subunit (nuc-lsu) rDNA sequences were used to test whether the Old World and New World groups of *Lentinula* are monophyletic. In addition, an expanded set of ITS sequences was used to assess the geographical distribution of phylogenetic species in

Lentinula. Finally, molecular clock analyses of partial nuc-lsu rDNA and mitochondrial small-subunit (mt-ssu) rDNA sequences were used to evaluate specific biogeographic scenarios that could explain the present distribution of *Lentinula*.

MATERIALS AND METHODS

All 43 *Lentinula* isolates studied by Hibbett *et al*. (1998), which should be consulted for locality information, were included in the present study. Five new *Lentinula* isolates were used in this study, including three individuals of *L.* '*boryana*' from Venezuela (isolate number LB4264), Brazil (sp834), and Puerto Rico (DSH 98-003), and two individuals of *L. 'edodes'* from the Russian Far East (BIN 1024) and India (B. Dhanuka s.*n*.). All isolates were collected from wild populations. Isolate numbers and other information for the remaining taxa included in this study are available from the author. DNA was extracted from cultured mycelia or dried fruiting bodies. Sequences were obtained by automated fluorescent dye terminator cycle sequencing (Applied Biosystems) of polymerase chain reaction (PCR) amplification products (except as noted, below). Protocols for DNA extraction, PCR, and DNA sequencing are standard and have been described elsewhere (White *et al*., 1990; Hibbett *et al*., 1995; Moncalvo *et al*., in press). Sequences were edited with Sequencher (GeneCodes Corp.) and aligned by hand. All data sets and new sequences have been deposited in TreeBASE (accession number S579) and GenBank (accession numbers AF356147–AF356173). Four sets of analyses were performed (described below). Analyses 1 and 2 were intended to resolve phylogenetic relationships of *Lentinula*, whereas analyses 3 and 4 were used for molecular clock dating.

Analysis 1 used nuc-lsu rDNA sequences from 17 *Lentinula* isolates (13 Old World, 4 New World) and nine species of collybioid homobasidiomycetes (*Collybia* and related taxa; one isolate per species). Based on the results of earlier studies (Hibbett & Vilgalys, 1993; Moncalvo *et al*., 2000), the collybioid taxa were used as outgroups. Characters for Analysis 1 were obtained from an approximately 900 base pair (bp) region at the 5′ end of the nuc-lsu rDNA, which was amplified using primers LR0R and LR5, and sequenced with these primers plus primers LR3, LR3R, and LR22 (Moncalvo *et al*., 2000). Sequences of the collybioid homobasidiomycetes were generously provided by Jean-Marc Moncalvo and Rytas Vilgalys (Duke University, Durham, North Carolina). The phylogenetic analysis used equally weighted parsimony implemented in PAUP* 4.0b2a (Swofford, 1999). A two-step search protocol was employed: First, a set of 1000 heuristic searches was performed, each with one random taxon addition sequence, and Tree bisection-reconnection (TBR) branch swapping, keeping only two trees per replicate. Second, the set of shortest trees found in step one was used as starting trees for TBR branch swapping with maximum number of trees (MAXTREES) set to 3000. Bootstrap analyses used 100 replicate heuristic searches, each with one random taxon addition sequence, TBR branch swapping, and MAXTREES set to 100.

Analysis 2 used ITS sequences from 48 isolates of *Lentinula*, including nine isolates from the New World (Brazil, Costa Rica, Mexico, Puerto Rico, Louisiana) and 38 isolates from the Old World (Australia, Borneo, China, India, Japan [including Okinawa], Nepal, New Guinea, New Zealand, North Korea, Russian Far East, Tasmania, Thailand). Most of these sequences were published previously (Hibbett *et al*., 1995, 1998). The new isolates included in this study are from Puerto Rico, Brazil, Venezuela, India, and Russia. The sequences of the isolates from Brazil and Venezuela were generated by Michael Thon and Daniel Royse (Pennsylvania State University), and were downloaded from GenBank (accession numbers AF079578 = isolate LB4264; AF079579 = isolate sp834). Based on the results of Analysis 1 (see below), the tree was rooted along the branch between the Old World and New World clades. The ITS region was amplified and sequenced using primers ITS3, ITS4, ITS5 and 5.8S, as described by Hibbett *et al*. (1995). PCR products of isolate DSH 98-003 (Puerto Rico) were cloned into pGEM T-easy (Promega) prior to sequencing, but all other sequences were obtained directly from PCR products. A phylogenetic analysis was performed using the same settings as in Analysis 1. An additional constrained analysis was performed that forced the monophyly of all isolates from Australasia (Australia, New Guinea, New Zealand, Tasmania), but that specified no other topological structure. The constraint tree was constructed in MacClade (Maddison & Maddison, 1992), and the analysis was run in PAUP*, using the same settings as the unconstrained analysis. Constrained and unconstrained trees were compared using the Wilcoxon signed rank (WSR) test in PAUP*.

Analysis 3 used nuc-lsu sequences from five isolates of *Lentinula* (two New World, three Old World), seven other species of homobasidiomycetes, and the heterobasidiomycete *Dacrymyces chrysospermus*, which was used for rooting purposes. The taxa in Analysis 3 represent seven of the eight major clades of homobasidiomycetes recognized by Hibbett & Thorn (2001). *Lentinula* and *Collybia* represent the 'euagarics clade', which also includes the mid-Cretaceous fossil *Archaeomarasmius* (Hibbett *et al*., 1997a). An equally weighted parsimony analysis was performed in PAUP* using the branch-and-bound algorithm, with gapped positions excluded.

Molecular clock analyses involved two operations: testing for rate heterogeneity and calibrating the clock. A maximum likelihood ratio test was used to test for evolutionary rate heterogeneity, as follows: Branch lengths were estimated with PAUP* on the tree generated in the parsimony analysis using the HKY-Ggr; model of sequence evolution, with and without the enforcement of a molecular clock (transition–transversion ratio set to two, empirical base frequencies, and among-site rate heterogeneity modelled on a 'discrete gamma' distribution with four rate classes). This resulted in two sets of branch length estimates and likelihood scores. The test statistic is equal to twice the ratio of log likelihood scores, which is χ^2 distributed with n-2 d.f., where n is the number of terminal taxa (Yang *et al*., 1995; Sanderson, 1998a).

After testing for rate heterogeneity, node ages and their standard errors were estimated with the maximum likelihood

program BASEML in the PAML 1.3 software package (Yang, 1997). Settings for BASEML used the HKY-Γ model, with four rate categories modelled on a discrete γ distribution, transition–transversion ratio set to 2, and molecular clock enforced. Absolute ages of nodes were estimated by fixing the age of one node at a time and using it to calibrate the ages of the remaining nodes. Four different calibrations were used (see Results for a description of the calibration points).

Analysis 4 used mt-ssu rDNA sequences from three isolates of *Lentinula* (one New World, two Old World), six other homobasidiomycetes, and *D. chrysospermus*. Except for the *Lentinula* sequences, the mt-ssu rDNA sequences were published elsewhere (Hibbett & Donoghue, 1995; Hibbett *et al.*, 1997b). PCR and sequencing were performed using primers MS1 and MS2 (White *et al.*, 1990). Phylogenetic estimation and molecular clock analyses were performed as in Analysis 3. Node ages were estimated using the same set of calibration points as in Analysis 3.

RESULTS

Analysis 1

Aligned sequences of nuc-lsu rDNA were 853 bp long, including 65 gapped positions (excluding gaps introduced at ends of sequences of uneven length) and 77 parsimony-informative positions. Phylogenetic analyses produced 37 trees of 287 steps with a consistency index (CI) of 0.752. The strict consensus tree and bootstrap analysis (Fig. 1) provide strong support for the view that *Lentinula* is monophyletic (bootstrap = 91%), and that the Old World and New World groups are also both monophyletic (bootstrap = 99 and 96%, respectively). In the Old World clade (13 isolates) there is only one resolved node, which includes two isolates from Japan and Thailand (Fig. 1). In the New World clade (four isolates) there is strong support for the existence of one clade that includes isolates from Louisiana and Puerto Rico and another clade that includes isolates from Costa Rica and Mexico (bootstrap = 100% for both groups; Fig. 1).

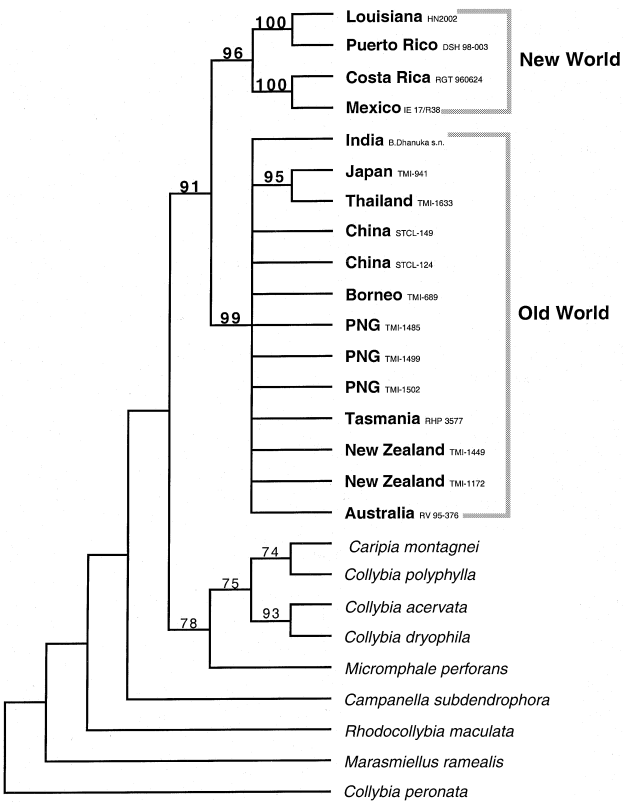

Figure 1 Phylogenetic relationships of *Lentinula* and collybioid homobasidiomycetes inferred from nuc-lsu rDNA sequences. Strict consensus of all 37 most parsimonious trees, 287 steps, CI = 0.752. Bootstrap values are shown above branches (values <70% are not shown). PNG = Papua New Guinea.

Analysis 2

Direct sequencing of PCR amplified ITS regions in isolate DSH 98-003 resulted in artefacts that suggested heterogeneity among amplified copies of ITS. Three unique clones were obtained, which differed by single base indels or point mutations at three nucleotide positions. All three unique ITS sequences from DSH 98-003 were included as terminal taxa in phylogenetic analyses. Aligned ITS sequences were 1057 bp long, including 498 gapped positions (excluding gaps introduced at ends of sequences of uneven length) and 168 parsimony-informative positions. The large number of gapped positions results from several highly variable regions that could only be aligned within closely related groups of isolates (these being the entire Old World clade and each of the two New World clades). In these hypervariable regions, blocks of similar sequences were aligned and offset from other sets of sequences. For examples of ITS alignments in *Lentinula*, see Hibbett *et al.* (1995, 1998; the alignments are also in TreeBASE).

Phylogenetic analyses produced over 3000 trees of 234 steps, CI = 0.861 (Fig. 2). The branch between the Old World and New World clades was strongly supported (bootstrap = 100%). In the Old World, the clades formerly called groups I–V (Hibbett *et al.*, 1995, 1998) were resolved: Group I (bootstrap = 77%) includes isolates from Japan (including Okinawa), Korea, the Russian Far East, Thailand, China, and Borneo. Group II (bootstrap = 97%) includes isolates from New Guinea, Australia (Queensland) and Tasmania. Group III (bootstrap = 86%) includes isolates from New Zealand. Group IV (bootstrap = 100%) includes isolates from New Guinea. Group V (bootstrap = 92%) includes isolates from India, Nepal, and China. Relationships among groups I–V are not resolved with confidence. There is a basal trichotomy in Old World clade in the strict consensus tree, which involves groups I, IV, and II/III/V. The group II/III/V clade is supported with a

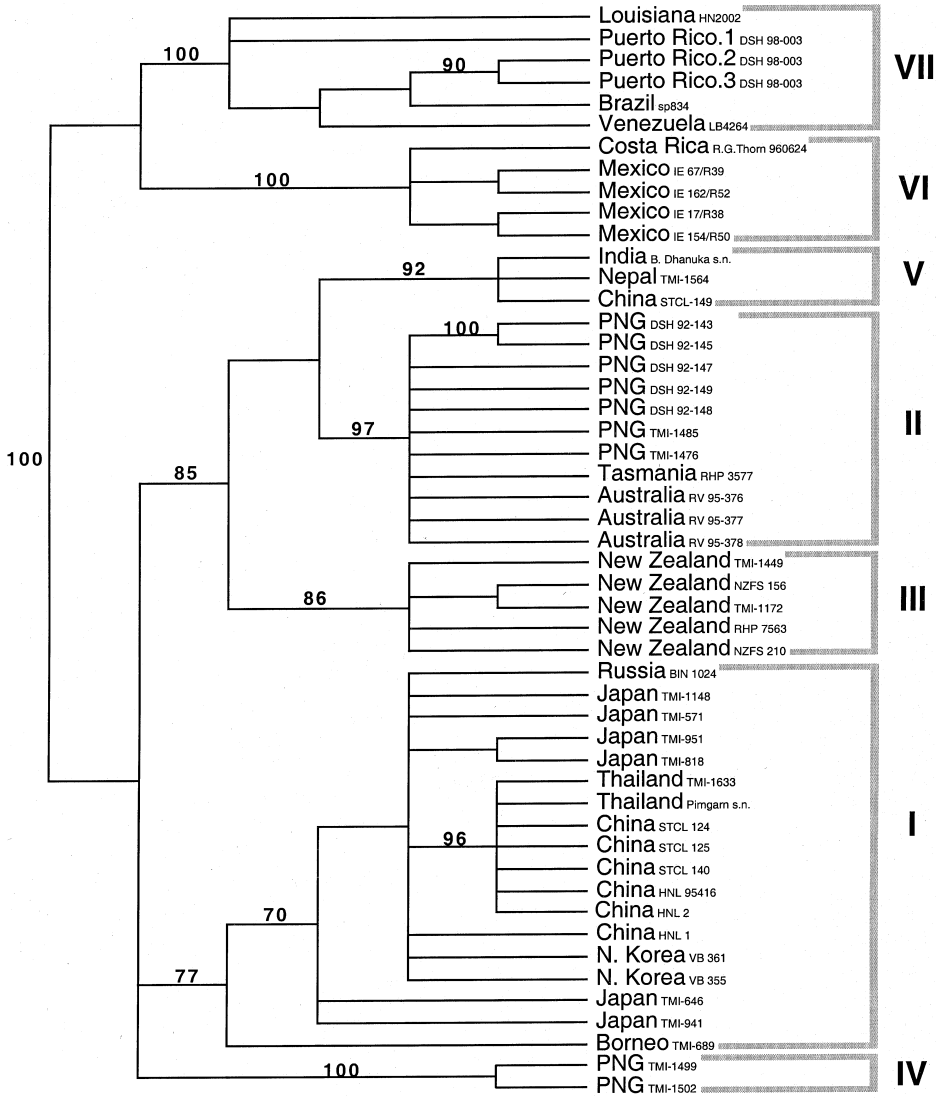

Figure 2 Phylogenetic relationships of *Lentinula* inferred from ITS sequences. Strict consensus of 3000 + most parsimonious trees, 234 steps, CI = 0.861. Bootstrap values are shown above branches (values < 70% are not shown). Numbered groups are phylogenetic species of *Lentinula* discussed in text. Three Puerto Rican terminals represent three unique cloned ITS sequences. PNG = Papua New Guinea.

bootstrap value of 85%, but the group II/V clade is supported at < 70%.

In the New World group there are two strongly supported clades, which we propose to call groups VI and VII (bootstrap = 100% for both groups). Group VI includes isolates from Mexico and Costa Rica, whereas group VII includes isolates from Brazil, Venezuela, Puerto Rico, and Louisiana. All three clones from DSH 98-003 are in group VII. Although they do not form a monophyletic group, there are no strongly supported nodes that contradict their monophyly.

Constrained analyses that forced the monophyly of the Australasian populations (i.e. groups II–IV) produced over 3000 trees of 238 steps (four steps longer than the unconstrained trees), CI = 0.845, which could not be rejected using the WSR test (Fig. 3).

Analysis 3

Aligned sequences of nuc-lsu rDNA for molecular clock analyses were 818 bp long, including 125 gapped positions (which were excluded from parsimony analyses and branch length estimation) and 158 ungapped parsimony-informative positions. Branch-and-bound produced a single tree of 534 steps, CI = 0.672 (Fig. 4). Likelihood of the tree with branch lengths optimized without enforcing a molecular clock was –log 3473.05877, whereas the likelihood with the clock enforced was –log 3479.62045. The difference in likelihood scores is insignificant according to the likelihood ratio test. Thus, the molecular clock could not be rejected.

Node ages in maximum likelihood units and absolute time are given in Table 1. All errors discussed below are equal to

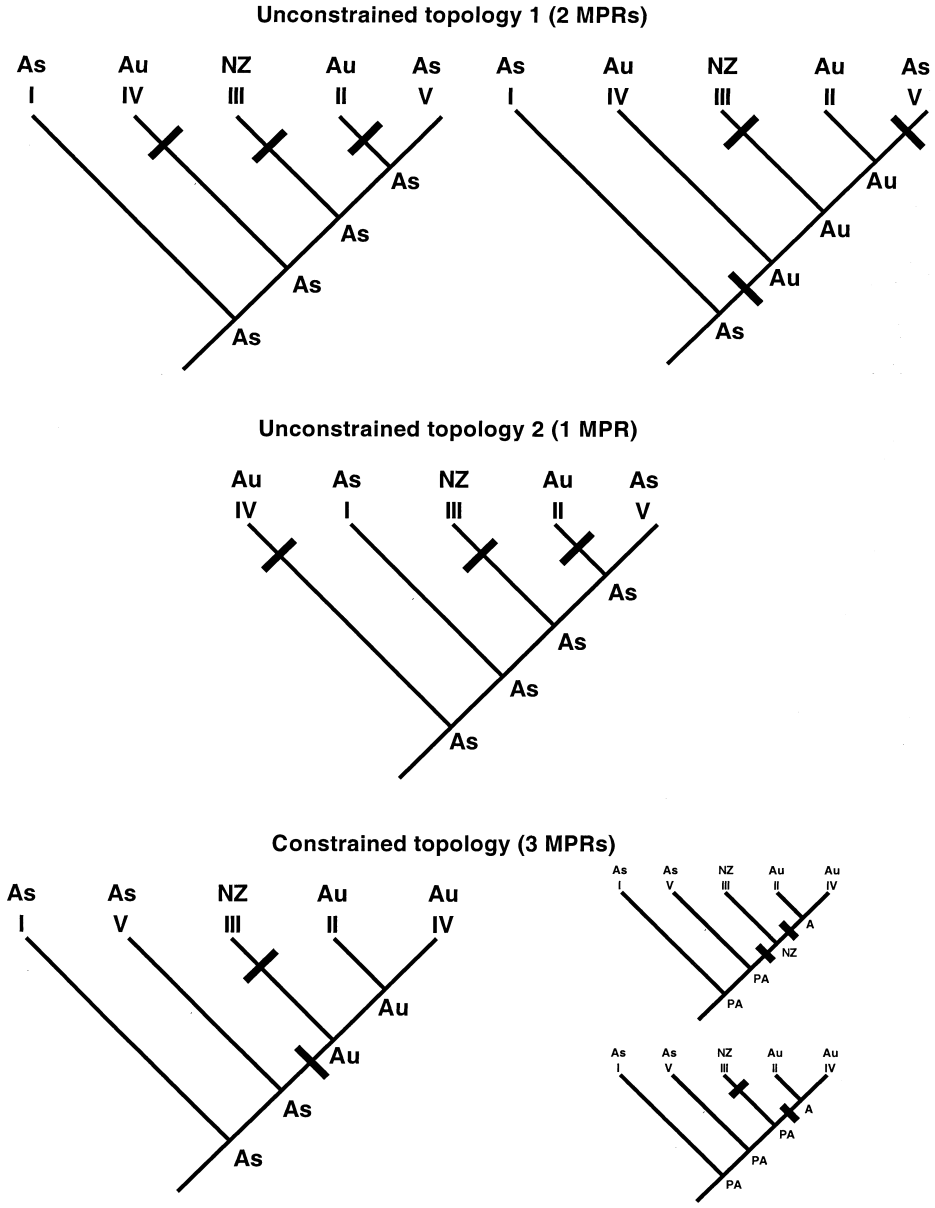

Figure 3 Simplified topologies of the Old World clade of *Lentinula* from unconstrained ITS analyses and constrained analyses that force monophyly of Australasian isolates. There are two general unconstrained topologies that have two and one MPRs of dispersal events, repsectively, and one general constrained topology that has three MPRs of dispersal events (these optimizations assume that the ancestral range of the Old World clade was in continental Asia). Bars on branches represent hypothesized dispersal events. As = Asia; Au = Australia-New Guinea-Tasmania; NZ = New Zealand.

two SE. Four nodes, labelled A–D (Fig. 4), were used as calibration points, yielding four sets of node age estimates:

(1) Node A is the root node of the homobasidiomycetes, which was calibrated at 200 Ma, based on the molecular clock estimates of Berbee & Taylor (1993). With this calibration point, the age of the MRCA of the euagarics clade and its nearest relatives (Node B) is estimated to be 169 Ma (± 37 Ma); the MRCA of the Old World and New World clades of *Lentinula* (Node C) is estimated to be 34 Ma (± 13 Ma); and, the MRCA of the Australian and New Zealand species of *Lentinula* (Node D) is estimated to be 8 Ma (± 6 Ma).

(2) Node B was calibrated at 90 Ma, based on *Archaeomarasmius*, which is thought to be a member of the euagarics clade (Hibbett *et al.*, 1997a). With this

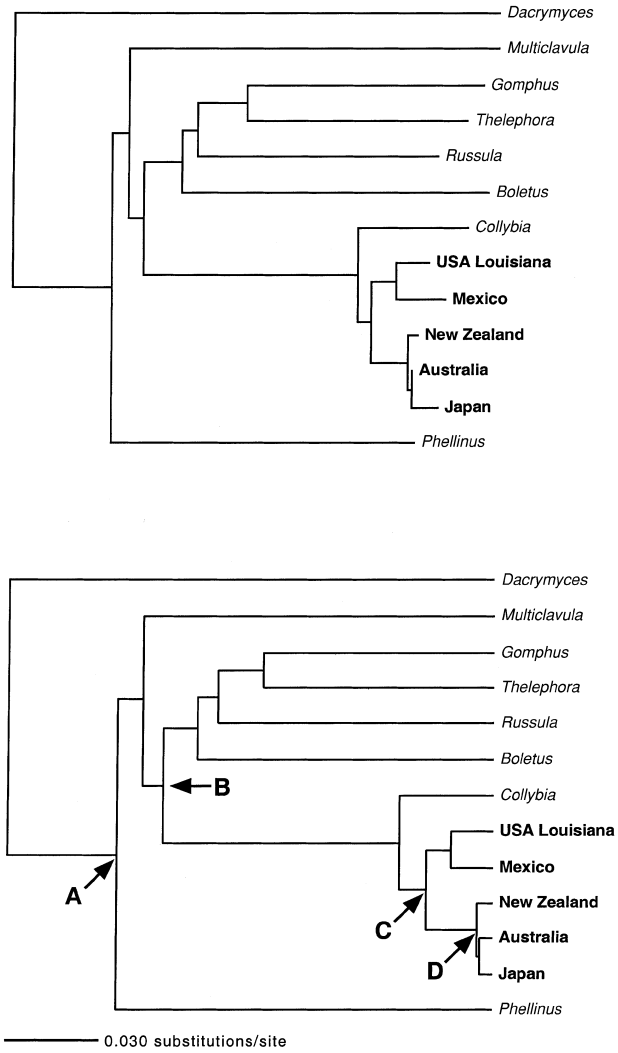

Figure 4 Phylogenetic relationships of *Lentinula* and other homobasidiomycetes inferred from nuc-lsu rDNA sequences. Single most parsimonious tree, 534 steps, CI = 0.672. Trees are phylograms showing branch lengths estimated with maximum likelihood with (below) and without (above) enforcement of a molecular clock. Labelled nodes A–D are discussed in the text and Table 1.

calibration point, the age of Node A is 107 Ma (± 25 Ma), Node C is 18 Ma (± 7 Ma), and Node D is 4 Ma (± 3 Ma).

(3) Node C was calibrated at 100 Ma, which is the expected age if the Old World/New World disjunction is the result of fragmentation of an ancient Gondwanan range (i.e. separation of South America and Australia). With this calibration point, Node A is 596 Ma (± 139 Ma), Node B is 503 Ma (± 110 Ma), and Node D is 25 Ma (± 17 Ma).

(4) Node D was calibrated at 80 Ma, which is the expected age if the Australian/New Zealand disjunction is the result of vicariance. With this calibration point, Node A is 1924 Ma (± 450 Ma), Node B is 1625 Ma (± 178 Ma), and Node C is 323 Ma (± 121 Ma).

Analysis 4

Aligned sequences of mt-ssu rDNA were 412 bp long, including 27 gapped positions (which were excluded from calculations) and 90 parsimony informative positions. Branch-and-bound produced four trees of 324 steps, CI = 0.769. Branch length estimation and likelihood ratio tests were performed on one of the trees, which was picked at random (Fig. 5). Likelihood of the tree with branch lengths optimized without enforcing a molecular clock was –log 1937.77460, whereas the likelihood with the clock enforced was –log 1951.26977. The difference in likelihood scores is significant according to the likelihood ratio test ($P < 0.001$). Thus, the molecular clock was rejected.

Despite rejection of the clock, the branch lengths inferred with the mt-ssu rDNA sequences could provide crude estimates of the ages of nodes. Therefore, the same four nodes used to calibrate node ages in the nuc-lsu rDNA tree were used to estimate node ages in the mt-ssu rDNA tree. Estimated node ages and their SEs are given in Table 1. Note that if Node C (the MRCA of the Old World and New World clades of *Lentinula*) is constrained to be 100 Ma, then Node A, the ancestor of the homobasidiomycetes is estimated at 3132 Ma (± 915 Ma). If Node D (the MRCA of the Old World clades of *Lentinula*) is constrained to be 80 Ma, then Node A is estimated to be 4017 Ma (± 1173 Ma).

DISCUSSION

Phylogenetic analysis of nuc-lsu rDNA sequences (Analysis 1) suggests that the New World and Old World groups of *Lentinula* are both monophyletic. The analysis of ITS sequences (Analysis 2) suggests that there are at least seven unique species in *Lentinula*, as found previously (Hibbett *et al.*, 1998). The five new isolates added to the ITS data set in this study refine understanding of the geographical distribution of *Lentinula* species. In the Old World, the placements of the new isolates are consistent with expectations: the isolate from the Russian Far East clustered with isolates from North-east Asia, Thailand, and Borneo (group I), and the isolate from India clustered with isolates from Nepal and China (group V). In the New World, the new isolates from Brazil, Venezuela, and Puerto Rico clustered with the isolate from Louisiana (group VII), to the exclusion of the Mexican and Costa Rican isolates (group VI). Based on these results, it appears that there are at least two species in the Americas: group VI, which has a Central American distribution, and group VII, which has a Gulf Coast-Caribbean-South American distribution. *Lentinula* is reported from several localities in the Caribbean that we did not sample, including Cuba, Hispaniola, Jamaica, Guadeloupe,

Table 1 Molecular clock age estimates of nodes in phylogenetic trees in Fig. 4 (based on nuc-lsu rDNA) and Fig. 5 (based on mt-ssu rDNA). Column 2 gives the node ages in maximum likelihood units (× 100) ± two SE. Columns 3–6 give absolute node age estimates in millions of years ± two SE. Columns 3–6 each represent a set of node ages obtained by fixing the age of one node (in bold) as a calibration point

	Node	ML units	Absolute age estimates			
nuc-lsu rDNA						
	A	127.69 ± 14.94	200	107 ± 25	596 ± 139	1924 ± 450
	B	107.88 ± 11.80	169 ± 37	90	503 ± 110	1625 ± 178
	C	21.44 ± 4.00	34 ± 13	18 ± 7	100	323 ± 121
	D	5.31 ± 1.83	8 ± 6	4 ± 3	25 ± 17	80
mt-ssu rDNA						
	A	131.56 ± 19.21	200	137 ± 20	3132 ± 915	4017 ± 1,173
	B	86.23 ± 12.39	131 ± 38	90	1642 ± 590	2633 ± 757
	C	4.20 ± 1.79	6 ± 5	4 ± 2	100	128 ± 109
	D	2.62 ± 1.43	4 ± 4	3 ± 3	62 ± 68	80

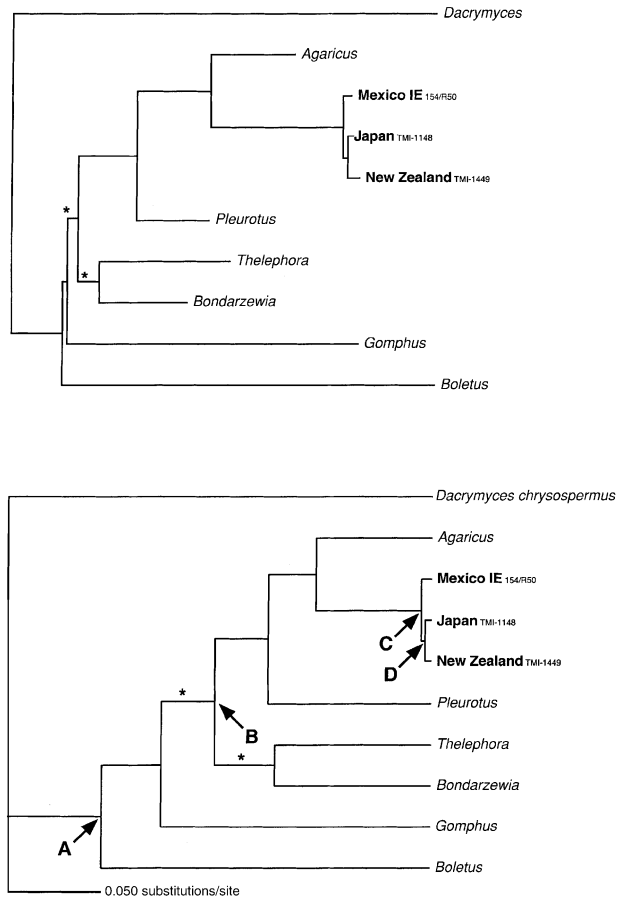

Figure 5 Phylogenetic relationships of *Lentinula* and other homobasidiomycetes inferred from mt-ssu rDNA sequences. One of four equally parsimonious trees, 324 steps, CI = 0.769. Branches that collapse in the strict consensus tree are marked with asterisks. Trees are phylograms showing branch lengths estimated with maximum likelihood with (below) and without (above) enforcement of a molecular clock. Labelled nodes A–D are discussed in the text and Table 1.

Martinique, and Trinidad (Pegler, 1983). We speculate that populations on these islands, as well as other localities in South America, will prove to be members of group VII. However, sampling of *Lentinula* in the Caribbean and northern South America is still quite fragmentary, and more field work is clearly needed.

The Old World/New World disjunction of *Lentinula* could be the result of vicariance or LDD. If it is the result of vicariance, then there are two competing hypotheses that need to be tested: a Gondwanan hypothesis and a Laurasian hypothesis (ignoring the possibility of a Pangean distribution). The Gondwanan hypothesis suggests that the ancestral range of *Lentinula* once spanned what are now South America and Australia. Separation of these land masses is estimated to have occurred about 100 Myr BP (Raven & Axelrod, 1974). Thus, the Gondwanan hypothesis requires that the MRCA of the Old World and New World clades of *Lentinula* lived at least 100 Myr BP. If the presence of *Lentinula* in New Zealand is also assumed to be the result of vicariance, then the MRCA of the Australian and New Zealand populations would have to have lived at least 80 Myr BP.

The alternative to the Gondwanan hypothesis is the Laurasian hypothesis, which suggests that the ancestral range of *Lentinula* once spanned what are now Asia and North America, and perhaps Europe. The history of connections and biotic exchanges between North America and Eurasia is complex and controversial. Exchanges between these regions could have taken place through the North Atlantic or through Beringia. The exact times when these routes were open, and had climates and forests suitable to *Lentinula* are unclear. Nevertheless, based on accounts by Tiffney (1985a,b) and Graham (1993), it appears that dispersal of *Lentinula* via the North Atlantic would have probably occurred in the late Palaeocene to Early Eocene, about 60–49 Ma. Dispersal via the Beringian route most likely would have occurred in the Early Eocene to Late Oligocene, or possibly Miocene, approximately 60–10 Ma. However, by the end of the Miocene the climate in Beringia probably would have been too cool for *Lentinula*. Thus, the Laurasian hypothesis requires that the MRCA of the Old World and New World clades is about 10–60 Myr BP.

Molecular clock analyses were used to test the Gondwanan and Laurasian hypotheses. Molecular clocks can have large associated errors (Hillis *et al*., 1996; Sanderson, 1998b). In this study, PAML was used to estimate SEs of node age estimates (Yang, 1997). Two genes were used for molecular clock analyses. Based on likelihood ratio tests, a constant-rate model of evolution could not be rejected for the nuc-lsu rDNA, but was rejected for the mt-ssu rDNA. Therefore, the age estimates obtained with the nuc-lsu rDNA are probably more accurate than those obtained with mt-ssu rDNA.

Initially, the nuc-lsu rDNA clock was calibrated with Berbee & Taylor (1993) estimate that the ancestor of the homobasidiomycetes (Node A) lived about 200 Myr BP. With this calibration point, the nuc-lsu rDNA clock suggests that the MRCA of the euagarics clade and its nearest relatives (Node B) lived about 169 Myr BP (± 37 Ma). This age estimate is consistent with the occurrence in the mid-Cretaceous (≈ 90 Ma) of *Archaeomarasmius*, which was not one of the fossils used as a calibration point by Berbee & Taylor (1993). If it is assumed that Node A is about 200 Myr BP, then the nuc-lsu rDNA clock suggests that the MRCA of the Old World and New World clades of *Lentinula* (Node C) is about 34 Ma (± 13 Ma). This age estimate is consistent with the Laurasian hypothesis, but it is too recent for the Gondwanan hypothesis.

The strength with which the nuc-lsu rDNA clock rejects the Gondwanan hypothesis can be assessed by considering the node ages that would be implied if the Gondwanan hypothesis was correct. A similar approach was employed by Baum (1998) in a study on biogeography of baobabs. If the Gondwanan hypothesis is correct, then Node C can be calibrated at 100 Ma. Using this calibration point, the nuc-lsu rDNA clock estimates Node A to be 596 Myr BP (± 139 Ma), which is almost three times older than the age estimate of the homobasidiomycetes by Berbee & Taylor (1993) and about twice as old as *Palaeancistrus*, the oldest known basidiomycete fossil. If Node D, the MRCA of the Australian and New Zealand clades, is used to calibrate the clock, then rejection of the Gondwanan hypothesis is even stronger; using this calibration point, the homobasidiomycetes are estimated to be about 1.9 Byr BP (± 450 Ma). These results suggest that the New Zealand population of *Lentinula* was established via LDD. Because the New Zealand population of *Lentinula* appears to be monophyletic, only one LDD event is suggested.

As noted previously, the mt-ssu rDNA data set fails the rate constancy test. Nevertheless, estimates of node ages based on the mt-ssu rDNA data should be considered because they provide the only independent corroboration of the nuc-lsu rDNA clock. Assuming that the homobasidiomycetes are about 200 million years old, the mt-ssu rDNA clock suggests that Node C was about 6 Ma (± 5 Ma), which may be old enough for dispersal via Beringia, but is probably too recent for dispersal via the North Atlantic (Table 1). In contrast, if the Gondwanan hypothesis is assumed to be correct, then the age of the homobasidiomycetes is estimated to be about 3.1 billion years (± 915 Ma; calibrated at Node C) or 4.0 billion years (± 1.2 billion years; calibrated at Node D). Thus, the mt-ssu rDNA clock is equivocal with regard to the Laurasian hypothesis, but it strongly rejects the Gondwanan hypothesis.

If the Laurasian hypothesis is correct, did dispersal of *Lentinula* between Asia and North America occur via Beringia or the North Atlantic? The nuc-lsu rDNA clock marginally favours Beringian dispersal, as does the mt-ssu rDNA clock. Dispersal via Beringia is also favoured because it does not require that extinction of *Lentinula* in Europe be invoked, whereas dispersal via the North Atlantic does imply extinction in Europe. Thus, dispersal via Beringia is the more parsimonious explanation, one that requires no ad hoc hypotheses.

There is no fossil record of *Lentinula* in either the Old World or New World. Consequently, the following hypotheses about possible movements of *Lentinula* in the Old World and New World are speculative. In the World, there are two species of *Lentinula*: group VI, with a Central American distribution, and group VII, with a Gulf Coast-Caribbean-South American distribution. It is plausible that *Lentinula* was driven out of Beringia at the same time that the Palaeotropical flora in North America retreated southward during the late Miocene to Pliocene (10–3 Ma; Graham, 1993). The subsequent expansion of arid regions in the west and south-west of North America may have created a barrier to gene flow between eastern and western populations, resulting in allopatric speciation. Similar patterns of movement have been inferred in plants, leading to the floristic similarities between eastern North America and eastern Mexico (Graham, 1993). Dispersal of group VII from North America to South America presumably occurred via island hopping through the Caribbean. Alternatively, group VII may have been dispersed overland through Central America, but this would require that extinction in Central America be invoked.

The situation is more complicated in the Old World than in the New World because there are five species of *Lentinula* in the Old World, not just two, some of which have overlapping ranges. Nevertheless, the same general pattern of dispersal from what are now temperate and boreal areas into the current temperate–tropical range probably occurred. At present, *Lentinula* occurs on many islands of South-east Asia and Australasia. However, during periods of glacial maxima in the Holocene, South-east Asia, Sumatra, Java, and Borneo were connected by land, as were Australia, New Guinea, and Tasmania (Pielou, 1991). Thus, dispersal through much of the Old World would require only diffusion over land and across limited stretches of water. Major water barriers for dispersal of *Lentinula* would have been between South-east Asia and Australasia, and between Australia-New Guinea-Tasmania and New Zealand.

The number of dispersal events between Asia and Australasia that must be invoked is dependent on the topology of the Old World clade, which is not resolved with confidence. Unconstrained trees have two general topologies; one topology shows group I to be the sister group of the rest of the Old World clade, whereas the other topology shows

group IV to be the sister group of the rest of the Old World clade (Fig. 3). Either topology implies a minimum of three dispersal events between Asia and Australasia (Fig. 3). However, alternative trees from the constrained analysis that require only two dispersal events (one from Asia to Australia-New Guinea-Tasmania, and a second from Australia-New Guinea-Tasmania to New Zealand) cannot be rejected based on the WSR test (Fig. 3).

CONCLUSIONS

Phylogenetic relationships of shiitake mushrooms are more complex than recent classifications would suggest (Pegler, 1983; Shimomura *et al*., 1992; Guzmán *et al*., 1997). Worldwide there are at least seven species of *Lentinula* that are divided into two major clades, one in the Old World and the other in New World. Molecular phylogenies and molecular clock age estimates are consistent with the view that the Old World/New World disjunction is the result of vicariance, probably involving fragmentation of an ancient trans-Beringian range. The present distribution of *Lentinula* is very broad, but for the most part the spread of *Lentinula* can be explained in terms of diffusion and limited island hopping. In its approximately 34 Myr history, only one LDD event must be invoked in *Lentinula*, that involving dispersal between Australia and New Zealand. These inferences are consistent with the view that LDD is rare in *Lentinula*. The same is probably true in other fungi, especially heterothallic species that lack long-lived asexual phases and that do not produce mitotic propagules. Such organisms, including many basidiomycetes, are excellent candidates for historical biogeographic studies using molecular characters. However, there will always be some degree of error in molecular estimates of tree topologies and node ages. The robustness of historical biogeographic hypotheses to error in phylogenetic estimation should always be assessed, using techniques such as bootstrapping and maximum likelihood ratio tests. As the number of rigorous phylogeny-based analyses of fungal biogeography increases, we may begin to perceive general patterns in the historical movements of fungi.

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