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Variation in Modes and Rates of Evolution in Nuclear and Mitochondrial Ribosomal DNA in the Mushroom Genus *Amanita* (Agaricales, Basidiomycota): Phylogenetic Implications

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Modes and rates of molecular evolution, and congruence and combinability for phylogenetic reconstruction, of portions of the nuclear large ribosomal subunit (nLSU-rDNA) and mitochondrial small subunit (mtSSU-rDNA) genes were investigated in the mushroom genus *Amanita*. The AT content was higher in the mtSSU-rDNA than in the nLSU-rDNA. A transition bias in which AT substitutions were as frequent as transitions was present in the mtSSU-rDNA but not in the nLSU-rDNA. Among-sites rate variation in nucleotide substitutions at variable sites was present in the nLSU-rDNA but not in the mtSSU-rDNA. Likelihood ratio tests indicated very different models of evolution for the two molecules. A molecular clock could be rejected for both data sets. Rates of molecular evolution in the two molecules were uncoupled: faster evolutionary rates in the mtSSU-rDNA and nLSU-rDNA were not observed for the same taxa. In separate phylogenetic analyses, the nLSU-rDNA data set had higher phylogenetic resolution. The partition homogeneity test and statistical bootstrap support for branches indicated absence of conflict in the phylogenetic signal in the two data sets; however, tree topologies produced from the separate data sets were not congruent. Heterogeneity in modes and rates of evolution in the two molecules pose difficulties for a combined analysis of the two data sets: the use of equally weighted parsimony is not fully satisfactory when rate heterogeneity is present, and it is impractical to determine a model for maximum-likelihood analysis that fits simultaneously two heterogeneous data sets. Overall topologies produced from either the separated or the combined analyses using various tree reconstruction methods were identical for nearly all statistically significant branches.

INTRODUCTION

The best understanding of organismal phylogenies can be gained by simultaneous analyses of different genes, for at least two reasons: (1) a gene tree is not necessarily a species tree, and phylogenetic trees inferred from the sequences of different genes can be contradictory (Felsenstein, 1978; Doyle, 1992; Maddison, 1997); (2) several empirical and theoretical studies have shown that phylogenetic accuracy increases when more characters are added in the analysis (Huelsenbeck, 1995; Poe and Swofford, 1999). Congruence between multiple data sets and combinability of data sets from different origins have been a source of major debates in the systematic literature (Bull *et al.*, 1993; de Queiroz, 1993; Chippindale and Wiens, 1994; Miyamoto and Fitch, 1995; Huelsenbeck *et al.*, 1996; Cunningham, 1997a,b). Different data sets can be incongruent for multiple reasons, including differences in their power of phylogenetic resolution, discordance in rates or modes of evolution (Bull *et al.*, 1993), or differences in their phylogenetic histories (Doyle, 1992). Various statistical tests have been proposed to examine the significance of incongruence between data sets of different origins (Templeton, 1983; Kishino and Hasegawa, 1989; Rodrigo *et al.*, 1993; Farris *et al.*, 1994, 1995; Huelsenbeck and Bull, 1996); when incongruence exists, some authors suggest that the data sets should be analyzed separately only (Miyamoto and Fitch, 1995), whereas others argue that they should always be combined ("total evidence" approach; Kluge, 1989). The choice and performance of different tree-building methods have been debated (Huelsenbeck, 1995; Russo *et al.*, 1996), and it remains unclear which methods are preferable when data sets with potentially different phylogenetic signals are combined.

Molecular phylogenetics in mushrooms have been largely based on parsimony analysis of sequence data from nuclear ribosomal genes (Moncalvo *et al.*, 1993, 1995, 2000; Hibbett and Vilgalys, 1993; Chapela *et al.*, 1994; Vilgalys and Sun, 1994; Hibbett *et al.*, 1995; Binder *et al.*, 1997; Kretzer and Bruns, 1997; Liu *et al.*, 1997; Lutzoni, 1997; Lutzoni and Pagel, 1997; Johnson and Vilgalys, 1998; Pegler *et al.*, 1998; Hopple and Vilgalys, 1999). Sequence data from mitochondrial ribosomal genes have been used in a limited number of

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studies (Hibbett and Donoghue, 1995; Cullings *et al.*, 1996; Bruns *et al.*, 1998), and in only a few cases in combination with data from nuclear ribosomal genes (Bruns and Szaro, 1992; Hibbett *et al.*, 1997a; Johnson, 1999). As a consequence, little is known about congruence of sequence data from these two unlinked loci in phylogenetic reconstruction or about modes and rates of evolution of these two genes in mushrooms.

In this study we sequenced portions of the nuclear large ribosomal subunit RNA gene (25–28S rDNA, or nLSU-rDNA) and the mitochondrial small ribosomal subunit RNA gene (12S rDNA, or mtSSU-rDNA) for 20 species representative of all known lineages in the mushroom genus *Amanita*. We examined modes and rates of evolution of these two unlinked loci, tested for their congruence and combinability in phylogenetic reconstruction, and conducted separated and combined phylogenetic analyses under three optimization criteria: equally weighted and weighted parsimony and maximum-likelihood. These two loci were chosen for two reasons: (1) they are unlinked, and nuclear and mitochondrial genes might have different phylogenetic histories; (2) nLSU-rDNA and mtSSU-rDNA data have been largely used in fungal systematics but rarely in combination.

The mushroom genus *Amanita* includes several species known to everyone. For instance, the hallucinogenic fly-agaric, *Amanita muscaria*, is perhaps the most popular of all mushrooms: it has been depicted in many fairy tales and used ritually by several societies. *Amanita* is also well known for several deadly poisonous (e.g., *A. phalloides* and *A. virosa*) and edible (e.g., *A. rubescens* and *A. caesareae*) species. Most *Amanita* species are obligatorily ectomycorrhizal and play a significant role in forest ecosystems. Based on characters derived from morphology, *Amanita* has always been held to be a natural group. It is distinguished by several conspicuous characters, including free or subfree lamellae, divergent lamellar trama, hemiangiocarpic development, and a white spore print. Monophyly of *Amanita* is also supported by molecular data (Moncalvo *et al.*, 2000). Within *Amanita*, two subgenera are commonly recognized: subgenus *Amanita*, with inamyloid spores and a striate, sulcate, or pectinate pileus margin, and subgenus *Lepidella*, with amyloid spores and a smooth pileus margin (Bas, 1969; Jenkins, 1986; Singer, 1986; Tulloss *et al.*, 1995). Singer (1986) recognized four sections within subgenus *Amanita* (*Amanita, Ovigerae, Vaginatae,* and *Caesareae*) and five within subgenus *Lepidella* (*Lepidella, Mappae, Phalloideae, Amidellae,* and *Validae*). Two recent molecular studies (Weiss *et al.*, 1998; Drehmel *et al.*, 1999) using sequence data from the nLSU-rDNA gene showed support for Singer's (1986) divisions of *Amanita*, although some basal relationships remained unknown. Based on nLSU-rDNA evidence, the second study supported monophyly of subgenera *Amanita* and *Lepidella*, resolved several relationships between Singer's (1986) sections, and proposed a novel, phylogenetically based classification which recognizes two subgenera, four sections, seven subsections, and two series (Table 1).

Because the existence of natural groups at several taxonomic levels in *Amanita* (subgenera, sections) is supported by both morphological (Singer, 1986) and nLSU-rDNA nucleotide sequence (Weiss *et al.*, 1998; Drehmel *et al.*, 1999) data, the genus provides an opportunity to examine congruence among multigene phylogenies in mushroom systematics.

MATERIAL AND METHODS

Sequence Data

Nucleotide sequence data were produced from the 12S mitochondrial RNA genes (mtSSU-rDNA) and the 25S nuclear RNA genes (nLSU-rDNA) for 20 *Amanita* species (Table 1). We sampled at least one member from each terminal clade in the nLSU-rDNA phylogeny of Drehmel *et al.* (1999) (which corresponds with Singer's (1986) sections). A member of the genus *Limacella* was chosen as outgroup for rooting purposes; *Limacella* is proposed as the sister genus of *Amanita*, based on both conventional taxonomy (Singer, 1986) and molecular phylogeny (Moncalvo *et al.*, 2000). DNA was isolated from fresh fruit bodies or dried herbarium material using miniprep procedures employing either CTAB (Zolan and Pukkila, 1986) or SDS (Lee and Taylor, 1990) lysis buffers. PCR amplification followed Vilgalys and Hester (1990). Amplified PCR products were purified by microfiltration using Ultrafree-MC filters (Millipore) and sequenced using fluorescent dye terminator chemistries (Perkin–Elmer) on an automated sequencer (ABI 373 or ABI 377). Primers used for PCR amplification of the nLSU-rDNA were 5.8SR and LR7, and sequencing primers were LR0R, LR3R, LR5, and LR16 (Hopple and Vilgalys, 1999). Primers for both PCR amplification and sequencing of the mtSSU rDNA were MS1 and MS2 (White *et al.*, 1990). Assembly and correction of raw sequence data were performed using Sequencher 3.0 software (Gene Codes Corp.).

Phylogenetic Analyses

Nucleotide sequences were aligned by eye using PAUP* (Swofford, 1998). Gap regions were excluded from the analyses. Phylogenetic analyses were conducted in PAUP* with a Power Macintosh 8600/300 MHz, and used maximum-parsimony and maximum-likelihood to search for optimal trees.

Maximum-parsimony analyses were conducted with characters weighted equally (MP) and differentially (WP). WP was employed to compensate for nucleotide substitution biases, using step matrices derived from maximum-likelihood estimates of nucleotide substitu-

TABLE 1

Organisms Used and GenBank Accession Nos.

Taxa	Collection No.	Clade[a]	GenBank Accession Nos. nLSU	GenBank Accession Nos. mtSSU
Subgenus *Amanita*				
Amanita muscaria var. *persicina*	JM96/63	*Amanita*[b]	AF097367	AF159064
Amanita roseitincta	RV94/163	*Amanita*[b]	AF097369	AF159065
Amanita gemmata	JM96/62	*Amanita*[b]	AF097371	AF159067
Amanita farinosa	RV96/104	*Ovigerae*[c]	AF097370	AF159066
Amanita ceciliae	RV6Jul94	*Vaginatae*[d]	AF097372	AF159068
Amanita fulva	RV97/34	*Vaginatae*[d]	AF097373	AF159069
Amanita vaginata	RV4Aug96	*Vaginatae*[d]	AF097375	AF159070
Amanita jacksonii	TV96/1	*Caesareae*[e]	AF097376	AF159071
Subgenus *Lepidella*				
Amanita virosa	JM97/42	*Phalloideae*[f]	AF159086	AF159084 AF159085
Amanita flavoconia	RV5Aug96	*Validae*[g]	AF042609	AF159074
Amanita flavorubescens	RV96/102	*Validae*[g]	AF097380	AF159075
Amanita franchetii	JM96/27	*Validae*[g]	AF097381	AF159076
Amanita rubescens	JM96/53	*Validae*[g]	AF097382	AF159077
Amanita citrina	JM96/61	*Mappae*[h]	AF097378	AF159072
Amanita brunnescens	JS94/2	*Mappae*[h]	AF097379	AF159073
Amanita peckiana	RV94/143	*Amidellae*[i]	AF042608	AF159078
Amanita volvata	RV97/24	*Amidellae*[i]	AF097388	AF159079
Amanita solitariiformis	DD97/12	*Lepidella*[j]	AF097390	AF159080
Amanita rhoadsii	DD97/13	*Lepidella*[j]	AF097391	AF159081
Amanita rhopalopus	JM97/20	*Lepidella*[j]	AF097393	AF159082
Limacella glischra	VTGB505	(outgroup)	U85301	AF159083

[a] Following Drehmel *et al.* (1999).
[b] Section *Amanita* Subsection *Amanita.*
[c] Section *Amanita* Subsection *Ovigerae.*
[d] Section *Vaginatae* Subsection *Vaginatae.*
[e] Section *Vaginatae* Subsection *Caesareae.*
[f] Section *Phalloideae* Subsection *Phalloideae.*
[g] Section *Phalloideae* Subsection *Validae* Series *Validae.*
[h] Section *Phalloideae* Subsection *Validae* Series *Mappae.*
[i] Section *Phalloideae* Subsection *Amidellae.*
[j] Section *Lepidella.*

tion rates; in the combined WP analyses of the nLSU-rDNA and mtSSU-rDNA data, the two data sets were partitioned (using the CHARSETS option in PAUP*) to allow differential weighting of nucleotide substitutions in the two molecules. MP and WP analyses were performed using 100 heuristic searches employing tree bisection–reconnection (TBR) branch swapping and random addition sequence, with the following settings: all characters of type unordered, multistate taxa interpreted as uncertainty, one tree held at each step during stepwise addition, steepest descent option not in effect, branches collapsed (creating polytomies) if minimum branch length = 0, MAXTREES unlimited, and MULPARS option in effect. To evaluate branch robustness in the parsimony trees, bootstrap (Felsenstein, 1985) analyses were conducted. Bootstrap supports (BS) were evaluated using 100 bootstrap replicates, each replicate consisting of 10 heuristic searches with random addition sequences and TBR branch swapping.

Maximum-likelihood ratio tests (LRT) (Goldman, 1993; Huelsenbeck and Rannala, 1997) were employed to identify a simple and robust substitution model for each data set (the "best-fit model;" Cunningham *et al.*, 1998). LRT were conducted from trees obtained in the MP analyses. Maximum-likelihood (ML) heuristic searches employed the settings suggested by the best-fit model and used the "asis" addition sequence in PAUP* with TBR branch swapping. Because maximum-likelihood searches are time expensive, bootstrap support for branches in trees obtained in ML searches were estimated with the "fast bootstrap" option in PAUP* (MULPARS off and no branch swapping).

Evolution of the nLSU and mtSSU-rDNA Genes

Base composition, transition/transversions ratios, rates of nucleotide substitutions, and variation of rates among sites in the nLSU and mtSSU-rDNA genes were examined from maximum-likelihood estimates and the option "dinucleotide frequencies" in PAUP*. A statistical test of the molecular clock for various data sets was

conducted using maximum-likelihood. To do such a test we compared trees produced with heuristic searches using the "enforce molecular clock" option in PAUP* with trees obtained without molecular clock enforcement, using the χ^2 test with $n - 2$ degrees of freedom (Felsenstein, 1993). To test for equality of evolutionary rates of change in DNA sequences for pairs of taxa within each data set, in general and restricted to transitions or transversions, we used the program NUCRATES of Muse and Weir (1992). This program employed a likelihood ratio test for comparing rates of evolutionary change in two species with reference to an outgroup sequence by comparing the likelihood of sequences in the three species under the situations of no constraint and the constraint of equal rates of changes.

Congruence and Combinability Tests

Data congruence between the nLSU-rDNA and the mtSSU-rDNA sequences was evaluated with the use of the incongruence length difference (ILD) test of Farris *et al.* (1994), also known as the partition homogeneity test. Topological congruence between trees produced using different data sets and tree reconstruction methods was evaluated with the Templeton (1993) test under the MP criterion and the Kishino–Hasegawa (1989) test under the ML criterion.

RESULTS AND DISCUSSION

nLSU Analyses

Nucleotide sequences of the nLSU-rDNA were produced for the region located between primers LR0R and LR5. They were aligned across all taxa for 1061 positions. Missing data at the 5′ and 3′ ends of the aligned sequences or regions with ambiguous alignment were removed from the analyses (198 positions). Of the remaining 863 characters, 613 characters were constant, 96 variable characters were parsimony uninformative, and 154 were parsimony informative.

MP analyses yielded a single most-parsimonious tree 554 steps in length (consistency index [CI] = 0.534; retention index [RI] = 0.614; rescaled consistency index [RC] = 0.331). This tree is depicted in Fig. 1a. It is similar to that in Drehmel *et al.* (1999) and is in agreement with the taxonomy presented in Table 1.

The step matrix for WP analyses was derived from maximum-likelihood estimates of rates of nucleotide substitutions (see Table 2 and below) that suggested the presence of three categories of nucleotide substitutions. The most common substitution type (CT transitions) was weighted "1," the rarest types (all transversions) were weighted "3," and the intermediate type (AG transitions) was weighted "2." The WP searches yielded a single most-parsimonious tree 1011 steps in length (CI = 0.572; RI = 0.642; RC = 0.367); under the criterion of equally weighted parsimony this tree is 1 step longer (555 steps) than the MP tree (554 steps). The two trees differ only in the placement of *A. virosa*, which is the sister group of subsection *Amidellae* in the MP tree (with lack of BS, however; Fig. 1a) and is basal to subsection *Validae* in the WP tree with moderately high support (78% BS; Fig. 1b).

LRT tests indicated that the Tamura–Nei (Tamura and Nei, 1993) (TN) model of nucleotide substitution fits the nLSU data set best (Table 2). This model has three substitution classes: two classes for transitions and one for transversions. Likelihood scores were significantly improved when the TN model also took into account the proportions of invariable sites (estimated to be 0.470) and allowed the rates for variable sites to follow a gamma distribution with a shape parameter $\Gamma = 0.620$ (estimated via maximum-likelihood) with four rate categories (Table 2). These parameters were therefore included in the TN model. With these settings, an heuristic search with TBR branch swapping produced a single tree of score 3858.756 (Fig. 2a). This tree is congruent with the WP tree (Fig. 1b) in the placement of *A. virosa* as basal to subsection *Validae* (73% BS) instead of as sister group to subsection *Amidellae* (MP tree, Fig. 1a), but differs from both the MP and the WP trees in the placement of *A. farinosa* (subsection *Ovigerae*) as close to *A. muscaria*, making subsection *Amanita* paraphyletic. Based on both the Templeton and the Kishino–Hasegawa tests, the ML, WP, and MP trees produced from the nLSU data set are not significantly different from each other (Table 3).

mtSSU Analyses

The amplified mtSSU fragments were ca. 600 bp in length for all taxa except *A. virosa*, for which it was approximately 2 kb in length, as visualized in agarose gels stained with ethidium bromide. We determined from nucleotide sequence alignment that the length difference of the PCR product of *A. virosa* was due to a single insert located approximately 200 bp upstream of primer MS2. In our data set, this insert was unique to *A. virosa* (i.e., was phylogenetically uninformative); it was therefore not sequenced entirely and excluded from the sequence alignment. Nucleotide sequence alignment of the mtSSU data was 544 positions in length, and 149 positions with ambiguous alignment were excluded from the analyses. Of the 395 included characters, 308 were constant, 34 variable characters were parsimony uninformative, and 53 were parsimony informative.

MP analyses yielded nine equally parsimonious trees of length 142 steps (CI = 0.704, RI = 0.798, RC = 0.562). Figure 1c depicts one token MP tree and shows that several branches collapsed in the strict consensus tree. The prominent feature of this tree is the presence of long branches and strong bootstrap supports for both

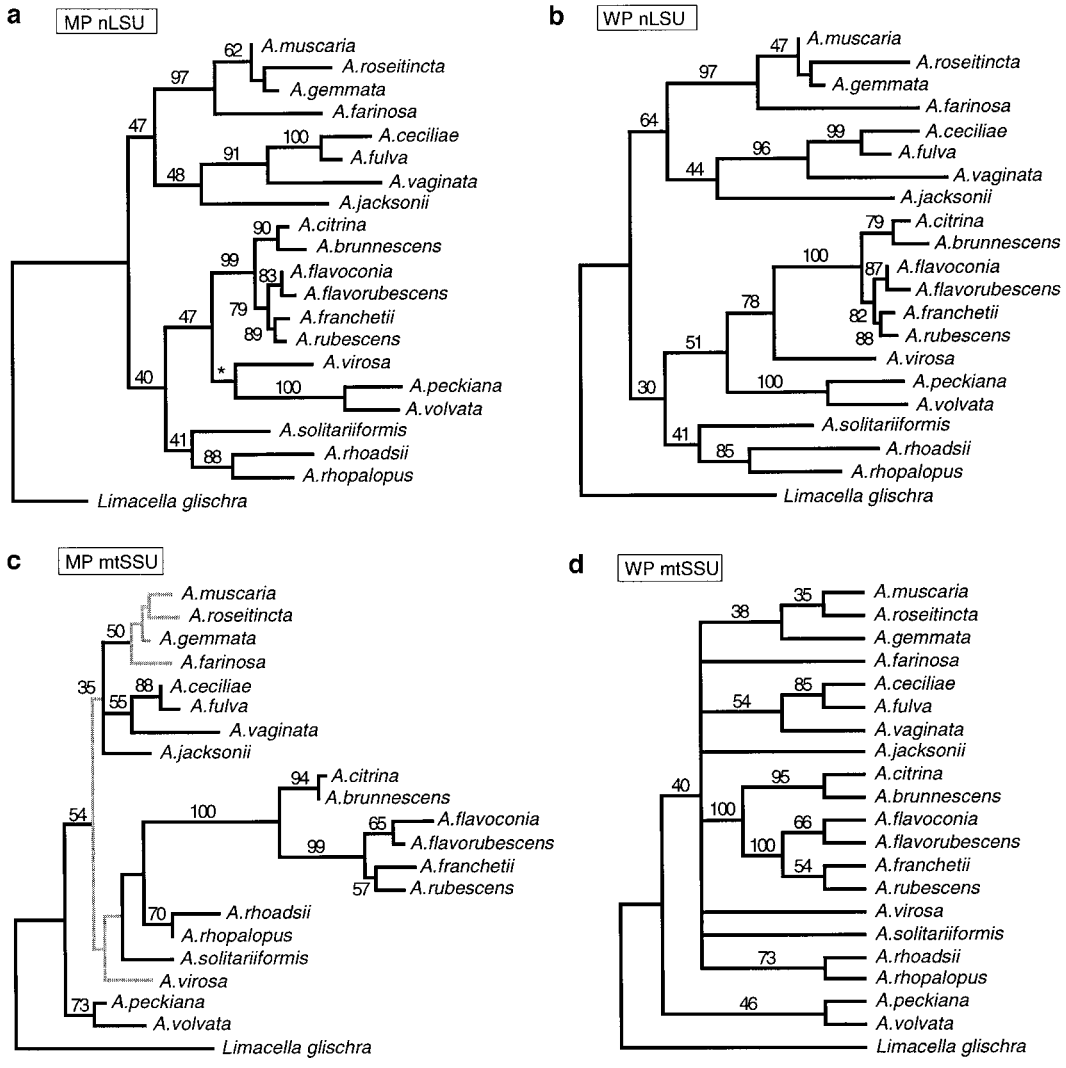

FIG. 1. Phylogenetic relationships in *Amanita* produced using equally weighted (MP) or weighted (WP) parsimony analyses from the nLSU-rDNA or mtSSU-rDNA data sets. Values above branches are bootstrap supports. (a) Single most-parsimonious tree produced in the MP analysis of the nLSU-rDNA data set; the star indicates a branch not present in the bootstrap tree. (b) Single most-parsimonious tree produced in the WP analysis of the nLSU-rDNA data set. (c) One of the 9 equally parsimonious trees produced in the MP analysis of the mtSSU-rDNA data set; branches drawn in grey collapse in the strict consensus tree. (d) Strict consensus of 17 equally parsimonious trees produced in the WP analysis of the mtSSU-rDNA data set.

subsection (100% BS) and series *Validae* (99% BS). Support for monophyly of subgenus *Amanita* (35% BS), section *Amanita* (50% BS), subsection *Vaginatae* (55% BS), and subsection *Amidellae* (73% BS) are weak to moderate. The basal position (54% BS) of *Amidellae* makes subgenus *Lepidella* paraphyletic. Section *Lepidella* was also paraphyletic.

The step matrix for WP analyses based on maximum-likelihood estimates of rates of nucleotide substitutions and LRT tests (see Table 4 and below) was as follows: the most common substitution types (CT and AG transitions, and AT transversions) were weighted "1," the rarest type (CG transversions) was weighted "3," and the intermediate type (AC and GT transversions) was weighted "2." The WP analyses yielded 17 trees of length 169 steps (CI = 0.663; RI = 0.769; RC = 0.510). Five of these trees were identical to trees produced in the MP analysis; the strict consensus tree of the 17 WP trees (Fig. 1d) was less resolved but similar to that of the MP analysis (Fig. 1c).

LRT tests indicated that a model with three substitution classes fits the data set: one substitution class for CT and AG transitions along with AT transversions, one class for AC and GT transversions, and one class for GC transversions (Table 4). Likelihood scores were significantly improved when the model also took into account the proportions of invariable sites (estimated to be 0.682), but did not significantly improve if rates for variable sites were allowed to vary following a gamma distribution (Table 4); therefore, the latter pa-

TABLE 2

Determination of the Best-Fit Evolutionary Model for the nLSU-rDNA Data Set Using Likelihood Ratio Tests

Evolution model[a]	Number of substitution types	Assumed bases frequencies	Among-site rate variation			Tree score − ln L	P^c
			Invariable sites	Rates of variable sites[b]	Number of rate categories		
JC	1	Equal	—	—	—	4395.40017	
F81	1	Estimated	—	—	—	4382.56982	<0.05
HKY	2	Estimated	—	—	—	4160.06987	<0.05
TN	3	Estimated	—	—	—	4138.52994	<0.05
GTR-4a	4	Estimated	—	—	—	4137.03491	n.s.
GTR-4b	4	Estimated	—	—	—	4137.06775	n.s.
GTR-5	5	Estimated	—	—	—	4136.28726	n.s.
TN	3	Estimated	0	Estimated	2	3935.52435	<0.05
TN	3	Estimated	Estimated	Equal	—	3901.94341	<0.05
TN	3	Estimated	Estimated	Estimated	2	3875.14821	<0.05
TN	3	Estimated	Estimated	Estimated	3	3870.29314	<0.05
TN	3	Estimated	Estimated	Estimated	4	3867.73737	<0.05
TN	3	Estimated	Estimated	Estimated	5	3866.21943	n.s.

Note. The arrow shows the best-fit model suggested from LRT tests.

[a] Listed in order of increasing complexity. JC, Jukes and Cantor (1969) model; F81, Felsenstein (1981) model; HKY, Hasegawa, Kishino, and Yano (1985) model; TN, Tamura and Nei (1993) model (two categories for transitions and one for transversions); GTR-4a, General-time-reversible (Yang, 1994) submodel with two categories for transitions and two for transversions as follows ([AT, AC, GT] and [GC]); GTR-4b, General-time-reversible (Yang, 1994) submodel with two categories for transitions and two for transversions as follows ([AT, AC, GC] and [GT]); GTR-5, General-time-reversible (Yang, 1994) submodel with two categories for transitions and three for transversions as follows ([AT, AC], [GC], and [GT]).

[b] Variable sites assumed to follow a gamma distribution with a gamma shape parameter.

[c] Compared with the best value previously calculated, likelihood was significantly improved ($P < 0.05$) or not (n.s.).

rameter was not included in the model. With these settings, an heuristic search with TBR branch swapping produced three trees of score 1352.027. These three trees score 142 steps under the MP criterion and represent a subset of the nine trees of identical score found in the MP analysis. One ML tree is shown in Fig. 2b.

Based on both the Templeton and the Kishino–Hasegawa tests, all MP, WP, and ML trees produced from the mtSSU-rDNA data set are not significantly different from each other (Table 3). The mtSSU-rDNA data set poorly resolves *Amanita* phylogeny, except that it strongly supports monophyly of the *Validae* and *Mappae* clades. Rate heterogeneity tests also indicate a significant acceleration of the mtSSU-rDNA in members of these two clades (Fig. 2b).

Comparison of Molecular Evolution of the nLSU-rDNA and the mtSSU-rDNA

In both the nLSU-rDNA and the mtSSU-rDNA, base frequencies were homogeneous across taxa (Table 5). Both genes are deficient in cytosine, but base composition was biased toward adenine (29.4%) and guanine (28.3%) in the nLSU-rDNA and toward adenine (34.1%) and thyamine (28.3%) in the mtSSU-rDNA, resulting in a higher overall AT content in the mitochondrial gene (62.4% vs 52.8%) (Table 5). Bias toward AT concentration in the mtSSU-rDNA was also observed in other mushroom groups (boletes: Bruns and Szaro, 1992; Ganodermataceae and Polyporaceae: J. M. Moncalvo, unpublished) and has been reported from other diverse organisms, such as Crustacea (*Daphnia:* 62.6–71%, Taylor *et al.*, 1996; *Lepidurus:* 66.7–70.6%, King and Hanner, 1998), treefrogs (59.4%; Richards and Moore, 1996), and paenungulates (56–59%; Lavergne *et al.*, 1996). It is also thought to be typical of mitochondrial genes in mammals (Brown *et al.*, 1982; Kocher *et al.*, 1989; Talbot and Shields, 1996). Bias toward AT concentration has been speculated to be associated with relaxation in selection (Sueoka, 1988, 1992; Hu and Thilly, 1994). That would agree with the view that mitochondrial genes (at least, mt-rDNA genes) have more limited functions than nuclear genes and are therefore under lower selection pressure.

In both genes, numbers of transitions and transversions increased almost linearly with increasing evolutionary distance (Fig. 3), indicating that there was no marked saturation in nucleotide substitutions in the aligned sequences (multiple hits). Maximum-likelihood estimates and LRT tests (Tables 2 and 4) indicated that the two molecules have two very different modes of evolution, although for both molecules three classes of substitution types fit the data best: the most frequent substitution in the nLSU-rDNA genes was CT, followed by AG, while AT and AG were nearly equally faster in the mtSSU-rDNA, followed by CT; all trans-

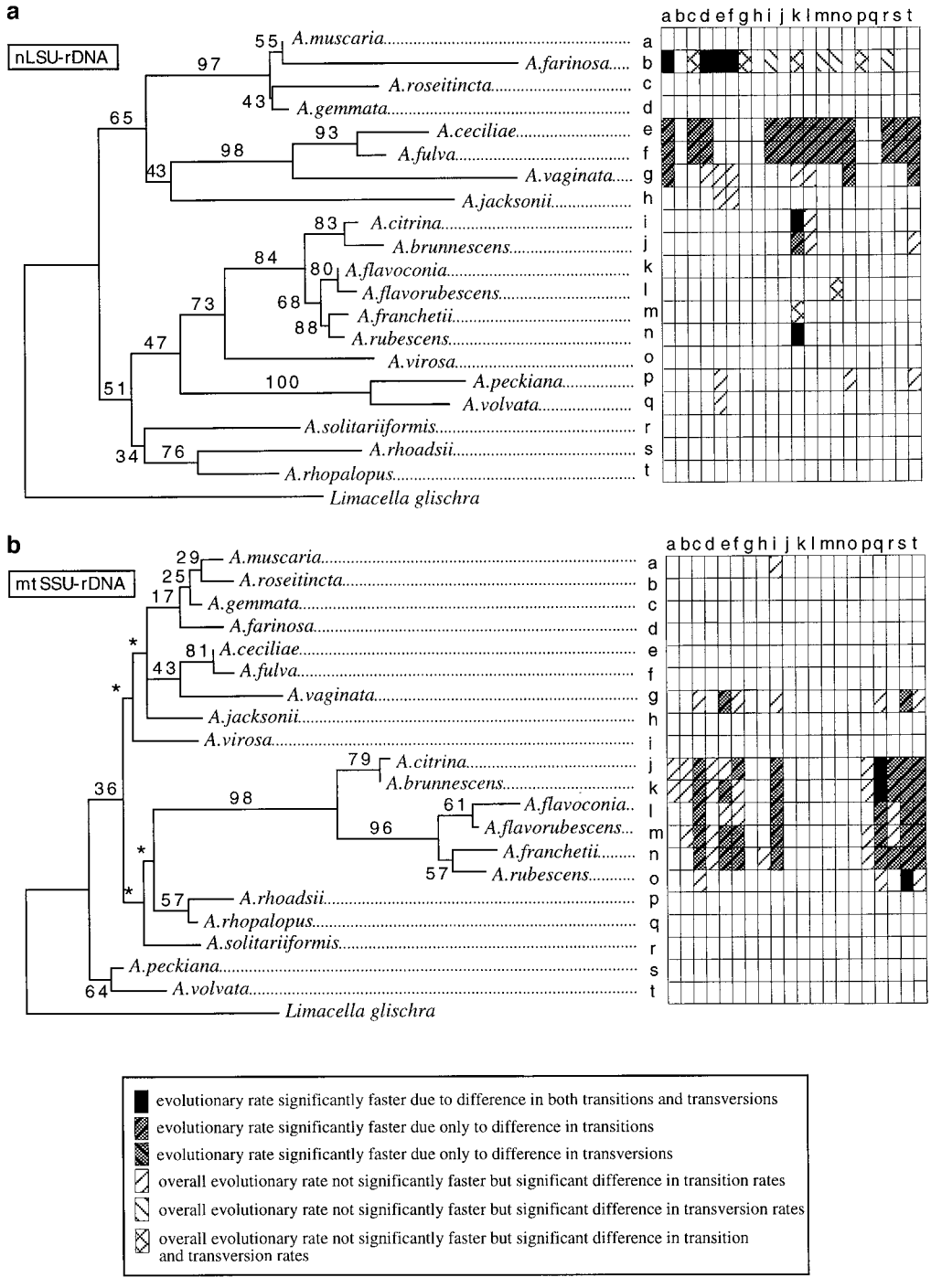

FIG. 2. Phylogenetic relationships in *Amanita* produced using maximum-likelihood and relative rate tests. Values above branches are bootstrap supports. (a) Single ML tree produced from the nLSU-rDNA data set. (b) One of the three equally likely trees produced from the mtSSU-rDNA data set.

versions were equally rare in the nLSU-rDNA genes, whereas GC transversions were rarest in the mtSSU-rDNA. There was a significant transition bias (Ti/Tv = 3.8) in the nLSU-rDNA data set, but not in the mtSSU-rDNA data set (Ti/Tv = 1.2), as a consequence of the high level of AT substitutions in that molecule (Fig. 3b). Transition biases have been commonly reported from nucleotide sequence matrices, including from mtSSU-rDNA data sets with high AT composition bias (e.g., Taylor *et al.*, 1996; King and Hanner, 1998; Richards and Moore, 1996; Lavergne *et al.*, 1996). It appears that the high level of AT substitutions observed

TABLE 3

Templeton and Kishino–Hasegawa Tests for Topological Congruence between Trees Produced from Separate Analyses of the nLSU-rDNA and mtSSU-rDNA Data Sets Using Equally Weighted Parsimony (MP), Weighted Parsimony (WP), and Maximum-Likelihood (ML)

		Tree scores and *P* values[a]					
Optimality criterion	Data set	MP-nLSU (1 tree)	WP-nLSU (1 tree)	ML-nLSU (1 tree)	MP-mtSSU (9 trees)	WP-mtSSU (17 trees)	ML-mtSSU (3 trees)
MP[b]	nLSU	554	555	559	572–582	572–599	575–578
			$P = 0.782$	$P = 0.297$	$P < 0.006$*	$P < 0.006$*	$P < 0.001$*
	mtSSU	147	148	149	142	142–145	142
		$P = 0.025$*	$P = 0.014$*	$P = 0.008$*		$P > 0.688$	$P = 1.000$
ML[c]	nLSU	3867.737	3861.213	3858.756	3882.042–3911.449	3882.340–3937.757	3888.288–3889.119
		$P = 0.142$	$P = 0.343$		$P < 0.049$*	$P < 0.039$*	$P < 0.005$*
	mtSSU	1363.593	1365.673	1369.088	1352.027–1355.264	1352.027–1359.003	1352.027
		$P = 0.074$	$P = 0.047$*	$P = 0.028$*	$P > 0.528$	$P > 0.458$	

[a] Significant difference at $P < 0.05$*.
[b] Templeton test.
[c] Kishino–Hasegawa test.

in the mtSSU-rDNA of *Amanita* is unusual, although it was also reported in the mtSSU-rDNA of boletes (Bruns and Szaro, 1992); it is not typical of higher fungi, since there is no such bias in the Ganodermataceae and Polyporaceae (J. M. Moncalvo, pers. observ.). Among-site rate variation in the nLSU-rDNA and the mtSSU-rDNA genes was also different; variable sites in the nLSU-rDNA follow a gamma distribution with a shape parameter $\Gamma = 0.6$ that fits in four rates categories as indicated from LRT tests (Table 2), whereas variable sites in the mtSSU-rDNA have a gamma shape parameter Γ equal to infinity that indicates equal rates among variable sites.

For both the nLSU-rDNA and the mtSSU-rDNA data, a molecular clock could be rejected (Table 6), indicating that both genes also exhibit among-taxa variation in rates of molecular evolution. Tests for rate heterogeneity among taxa (Fig. 2) indicated faster rates of molecular evolution for *A. farinosa* (subsection *Ovigerae*) and members of subsection *Vaginatae* (Fig. 2a) in the nLSU-rDNA gene. In contrast, rates of molecular evolution were faster in members of subsection *Validae* (series *Validae* and *Mappae*) in the mtSSU-rDNA gene (Fig. 2b). Further tests of the molecular clock in the mtSSU-rDNA data set revealed that rates are clockwise among both the faster and the slower evolving groups (see taxset 1 and 2 in Table 6, respectively), suggesting a bimodal rate with concerted speedup in all members of subsection *Validae*. In contrast, tests for the molecular clock in the nLSU-rDNA

TABLE 4

Determination of the Best-Fit Evolutionary Model for the mtSSU-rDNA Data Set Using Likelihood Ratio Tests[a]

			Among site rate variation				
Evolution model	Number of substitution types	Assumed bases frequencies	Invariable sites	Rates of variable sites	Number of rate categories	Tree scores − ln L	*P*
JC	1	Equal	—	—	—	1434.87970	
F81	1	Estimated	—	—	—	1407.78591	<0.05
HKY	2	Estimated	—	—	—	1400.78989	<0.05
GTR-2	2	Estimated	—	—	—	1390.66196	<0.05
GTR-3	3	Estimated	—	—	—	1388.65112	<0.05
GTR-3b	3	Estimated	—	—	—	1390.51956	n.s.
GTR-6	6	Estimated	—	—	—	1387.32588	n.s.
GTR-3	3	Estimated	Estimated	Equal	—	1352.46850	<0.05
GTR-3	3	Estimated	0	Estimated	4	1351.62378	n.s.
GTR-3	3	Estimated	Estimated	Estimated	4	1350.77657	n.s.

[a] Abbreviations and evolution models as in Table 2, except for: GTR-2, General-time-reversible (Yang, 1994) submodel with two categories ([CT, AG, AT] and [AC, GT, GC]); GTR-3, General-time-reversible (Yang, 1994) submodel with three categories ([CT, AG, AT], [AC, GT] and [GC]); GTR-3b, General-time-reversible (Yang, 1994) submodel with three categories ([AT], [AG, CT], and [AC, GT, GC]); GTR-6, General-time-reversible model (Yang, 1994) with six categories (one category for each substitution type).

TABLE 5

Maximum-Likelihood Estimates of Mean Base Frequencies with χ^2 Test of Homogeneity across Taxa

	Data sets	
	nLSU-rDNA	mtSSU-rDNA
Base Frequencies		
A	0.294	0.341
C	0.189	0.159
G	0.283	0.218
T	0.234	0.283
χ^2	1.000	1.000
GC	47.2%	37.6%
AT	52.8%	62.4%

data set indicated multimodal rates, since the evolutionary rates among the slowly evolving molecules were not clockwise (taxset 3 in Table 6).

Pairwise comparison of sequence divergences in the nLSU-rDNA and mtSSU-rDNA among *Amanita* taxa is plotted in Fig. 4, which clearly shows that a significant speedup in molecular rates of evolution in the mtSSU-rDNA has occurred among members of subsection *Validae*, as well as between members of that section and other *Amanita* taxa. This acceleration in rate of evolution of the mt-SSU rDNA in subsection *Validae* contrasts with the small amounts of molecular divergence observed among the other *Amanita* taxa, which resulted in lack of phylogenetic resolution among these taxa (Figs. 1c, 1d, and 2b). These results show that within a lineage one gene may undergo an evolutionary stasis in some taxa while accelerating in others; in addition, because molecular rates of evolution in the nLSU-rDNA for members of subsection *Validae* are slower than those in other taxa (Fig. 2a), molecular rates of evolution are not concerted within a genome.

Heterogeneity in rates and modes of evolution among taxa and between molecules have several important implications in phylogenetic reconstruction and time divergence estimates that we shall now examine.

Time Divergence Estimates

Several examples are known in which rates of molecular and morphological evolution are uncoupled (Bruna *et al.*, 1995; Harris *et al.*, 1998), for instance in

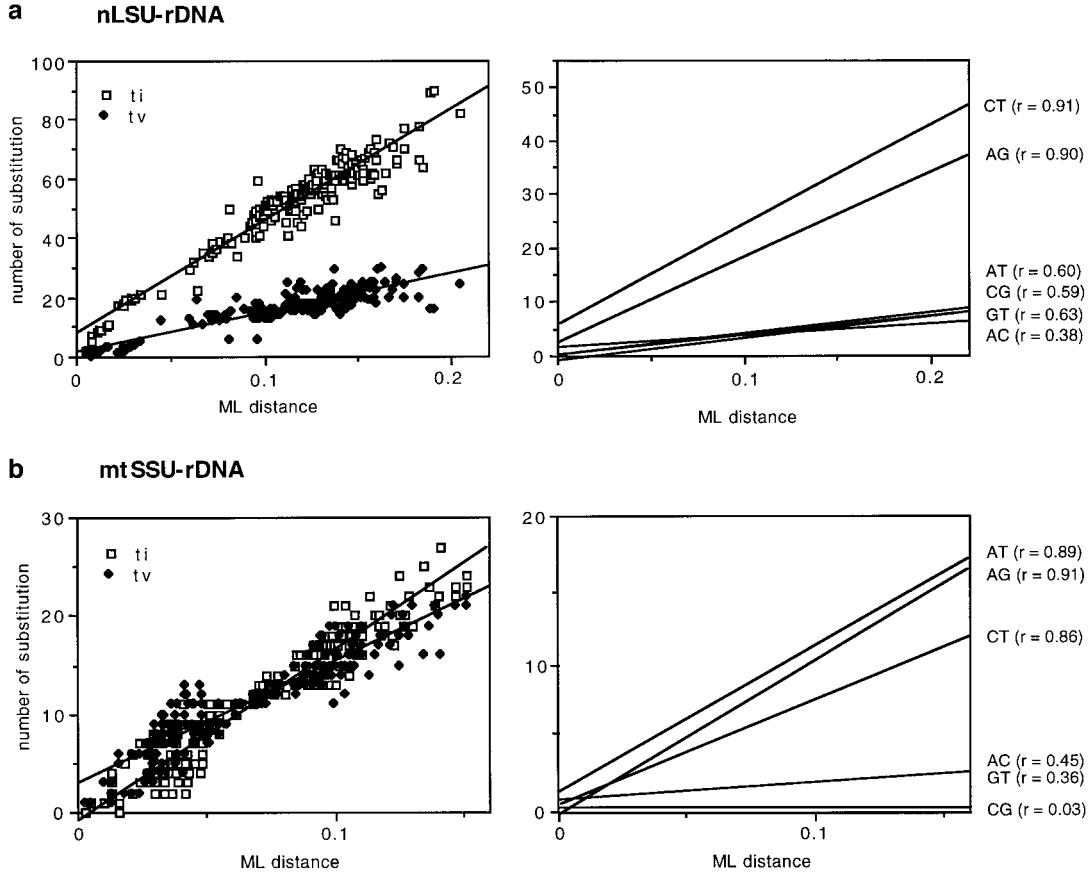

FIG. 3. Relationships between the number of nucleotide substitutions and the evolutionary distance in the nLSU-rDNA (a) and mtSSU-rDNA (b) genes.

TABLE 6

χ^2 Tests for Likelihood of Molecular Clock in the nLSU-rDNA and mtSSU-rDNA in *Amanita*

Data sets	Maximum likelihood scores (−ln L)		
	Molecular clock not enforced	Molecular clock enforced	P[a]
mtSSU			
All taxa	1352.02700	1374.18048	$P \ll 0.01$*
Taxset 1[b]	1090.63900	1099.11219	$P > 0.20$
Taxset 2[c]	867.48226	869.37923	$P \gg 0.20$
nLSU			
All taxa	3858.75677	3894.65641	$P \ll 0.01$*
Taxset 3[d]	3260.38495	3278.06189	$P < 0.01$*

[a] Molecular clock rejected at $P < 0.05$*.
[b] Members of Subsection *Validae* excluded.
[c] Members of Subsection *Validae* with *Limacella glischra* as outgroup.
[d] *A. farinosa* (Subsection *Ovigerae*) and members of Subsection *Vaginatae* excluded.

so-called "living fossils" of the genus *Lepidurus* (*Crustacea*), in which several cryptic species that are distinguished by high levels of molecular divergence are hidden behind a single morph (King and Hanner, 1998), or in the mushroom genus *Rhizopogon*, which has undergone accelerated morphological evolution to a false-truffle habit accompanied by little molecular divergence from morphologically well-distinct species (Bruns *et al.*, 1989). It is generally believed that morphological evolution is under stronger natural selection than molecular evolution and that various DNA sequences may evolve at constant rates over time and provide a molecular clock for dating past evolutionary events (Zuckerkandl and Pauling, 1965).

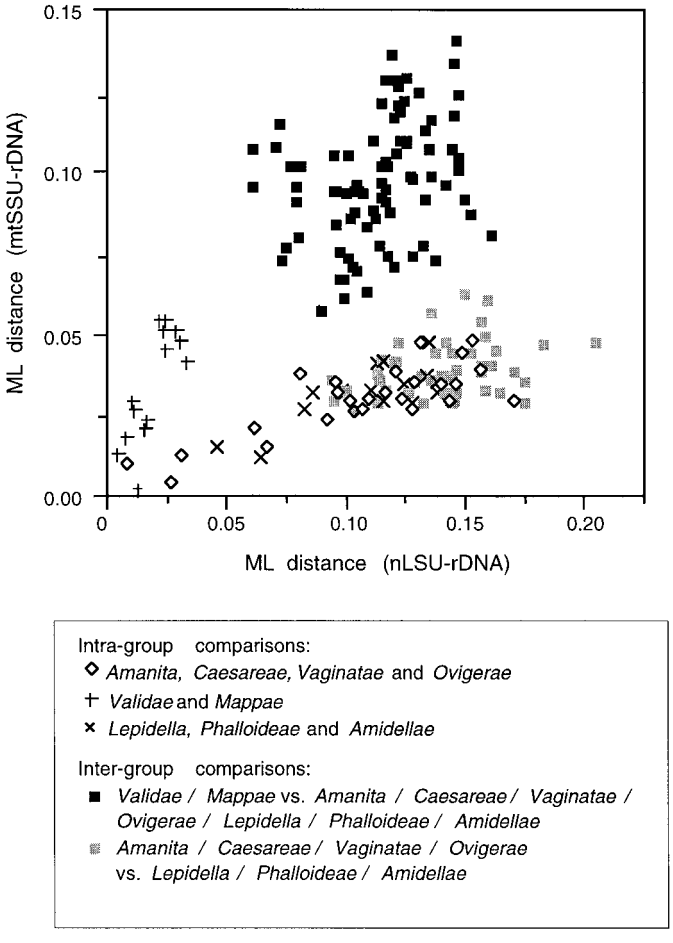

FIG. 4. Pairwise comparison of sequence divergence in the nLSU-rDNA and mtSSU-rDNA among *Amanita* species.

Calibration of molecular clocks in fungi has been hampered by lack of fossil records (there has been, however, at least one attempt of clock calibration based on the 18S rDNA by Berbee and Taylor (1993), which, taken together with fossils described from amber by Hibbett *et al.* (1997b), would permit an estimate for the origin of higher mushrooms between 60 and 220 mybp). Molecular clocks for the mtSSU-rDNA have been calibrated for animals (Brown *et al.*, 1982; Lynch and Jarrell, 1993) and subsequently used in several studies without further explicit testing of the clock. The molecular clock hypothesis is allied with the neutral theory of molecular evolution, but this may not hold if selective constraints and recombination were present (Ballard, 1998). Recombination of mitochondrial genes has been shown to occur in the mushroom genus *Armillaria* (Saville *et al.*, 1998) and might also occur in animals (Ballard, 1998).

This study provides evidence that rates of molecular evolution of both nLSU-rDNA and mtSSU-rDNA sequences can be markedly different, even among closely related taxa. Based on this observation, we would strongly recommend testing for a molecular clock and performing relative rate tests before estimating time divergence from molecular divergence. Rate heterogeneity in molecular divergence could explain discrepancies between estimated times of genetic separation based on the molecular clock and on vicariance events suggested from geological evidence, as reported, for instance, in Taylor *et al.* (1996).

Data Congruence and Combinability

Results of the ILD tests indicated that the two data sets are not significantly different from each other and therefore should be combined in parsimony analyses (*P* values were well above the incongruence threshold of 0.05 in both MP, $P = 0.79$, and WP, $P = 0.97$, tests). In contrast, tests for topological congruence indicated significant differences in tree topologies produced separately from nLSU-rDNA and mtSSU-rDNA sequences (Table 3). Topological incongruence between trees produced separately from data from different genes may result from (1) differences in their power of phylogenetic resolution, (2) discordance in rates or modes of evolution (Bull *et al.*, 1993), or (3) differences in their phylogenetic histories (Doyle, 1992). In this study, there is no evidence of difference in phylogenetic histories in the nLSU-rDNA and the mtSSU-rDNA genes in *Amanita:* branches in conflicts all have weak bootstrap support in the mtSSU phylogeny (Figs. 1 and 2). As suggested by de Queiroz (1993), bootstrap values for various clades can form a basis for the combination of data partitions. We attribute the topological incongruences observed in Figs. 1 and 2 to both differences in phylogenetic resolution (much lower in the mtSSU-rDNA) and discordance in modes and rates of evolution in the two genes (Figs. 2 and 3, Tables 2 and 4, and discussed above).

We conclude that the two data sets can be combined for parsimony analyses, because we have no evidence that the different genes have different histories. Moreover, combined data may amplify signal over noise (Kluge, 1989), and it has been repeatedly shown that increasing the number of characters in phylogenetic analyses increases phylogenetic accuracy (Huelsenbeck, 1995; Russo *et al.*, 1996; Poe and Swofford, 1999).

MP searches from the combined data sets yielded two equally parsimonious trees of length 701 steps (CI = 0.569; RI = 0.652; RC = 0.371) that differ from each other in the placement of *A. roseitincta* within subsection *Amanita.* One tree (Fig. 5a) was identical to the MP tree produced from the nLSU data set alone (Fig. 1a), with slightly higher bootstrap support for branches, except for monophyly of subgenus *Lepidella* and section *Phalloideae.* WP analyses of the combined data sets also yielded two trees (Length = 1186; CI = 0.5818; RI = 0.6593; RC = 0.3836) that differ from each other in the placement of *A. roseitincta;* one of these two trees is shown in Fig. 5b. WP trees (Fig. 5a) differ from MP trees (Fig. 5b) in the placement of *A. virosa* and in that subgenus *Lepidella* and section *Phalloideae* are paraphyletic in the WP analysis: subsection *Amidellae* is basal to the genus *Amanita,* and section *Lepidella* is basal to subgenus *Amanita,* but these basal relationships are not supported by bootstrapping (Fig. 5c).

Combining the nLSU-rDNA and mtSSU-rDNA data in ML analyses is problematic, because a single substitution model cannot accommodate the two data sets for the best-fit likelihood models of evolution of the two molecules are very different (see Tables 2 and 4), and PAUP* (Swofford, 1998), like other programs performing maximum-likelihood analyses (e.g., PHYLIPS: Felsenstein, 1993; FastDNAml: Olsen *et al.*, 1994), does not allow simultaneous use of multiple substitution models. However, to determine the sensitivity of tree topologies to various (and inadequate) ML models, we compared results from four searches using four different models: (1) a model using parameters determined from LRT tests to best fit the combined data set (BESTFIT model), (2) the model that best fit the nLSU-rDNA data set (nLSU model; Table 2), (3) the model that best fit the mtSSU-rDNA data set (mtSSU model; Table 4), and (4) the Jukes and Cantor (1969) model, which assumes equal base frequencies, one substitution type, and equal substitution rates among sites. All four searches yielded different trees that are, however, not significantly different from each other, based on the Kishino–Hasegawa test (data not shown). There were some topological differences between all trees indicating that, at least for our combined data set, tree reconstruction using ML is sensitive to the choice of model of sequence evolution. For instance, the tree produced using the Jukes–Cantor model was identical to the MP tree depicted in Fig. 5a and differed from the tree produced using the BESTFIT model (Fig. 5d) in the placement of *A. farinosa* and *A. virosa.* Results from the combined ML analyses are difficult to interpret, because it also remains unclear whether so-called best-fit models are always more able than simpler (or wrong) models to recover correct phylogenies, as shown by Yang (1997) and further discussed by Bruno and Halpern (1999). Difficulties in choosing an evolutionary model in the presence of heterogeneous data partitions might not be particular to multigene data sets, because a single molecule may also show different regions with marked differences in their rates and modes of evolution that cannot be accomodated into a single ML model. This calls into question the claimed superiority of maximum-likelihood over maximum-parsimony (see Edwards, 1995), since ML methods currently do not allow simultaneous use of multiple substitution models for combining heterogeneous data partitions.

Phylogenetic Implications

Many different trees have been produced in this study, using different data sets (separated or combined) and tree reconstruction methods (MP, WP, or ML). Results are summarized in Table 7. There was no conflict in tree topologies for statistically well-supported branches (BS $>$ 70%), with one exception (see below). Confidence values for some branches, but not all, were slightly higher when data sets were combined

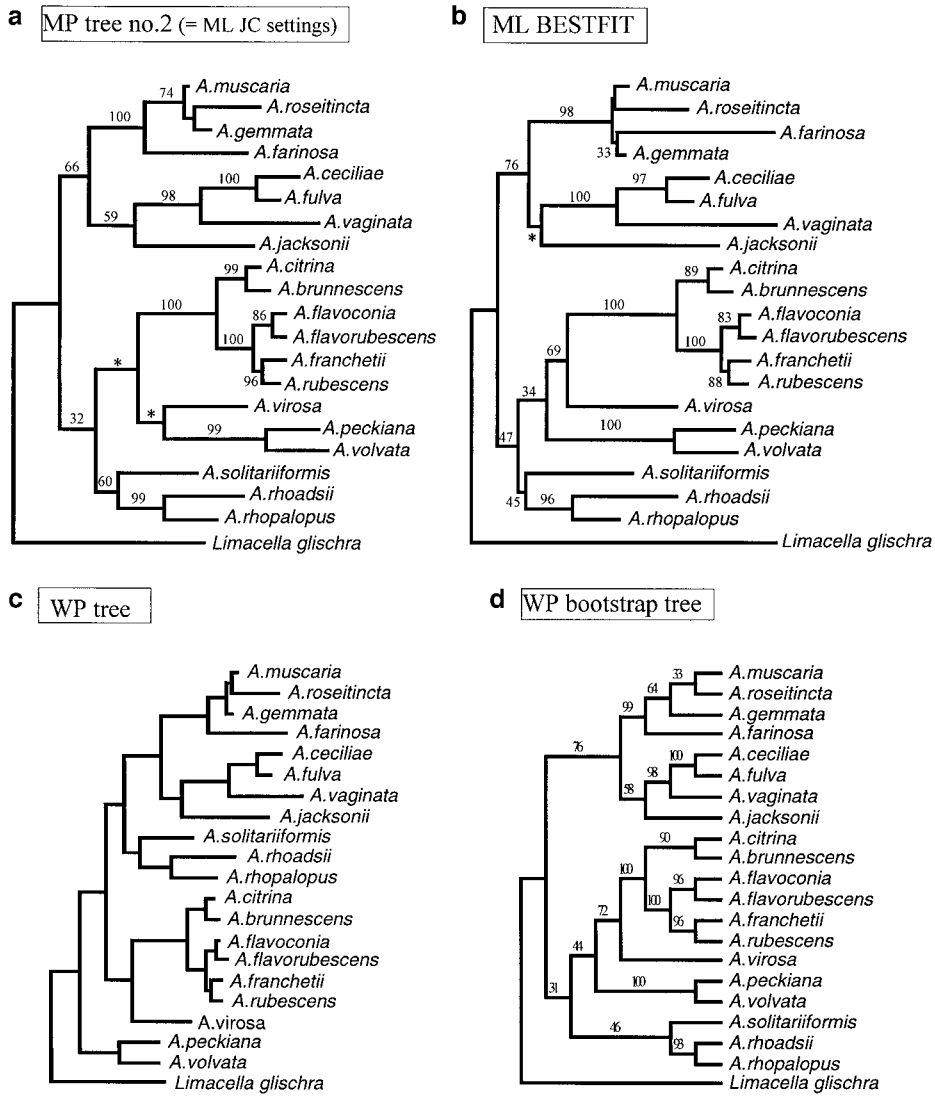

FIG. 5. Results of combined analyses of the nLSU-rDNA and mtSSU-rDNA data sets. Values above branches are bootstrap support. (a) One of the two trees produced using equally weighted parsimony; stars indicate branches absent from either the strict consensus or the bootstrap trees. (b) One of the two trees produced using weighted parsimony. (c) Bootstrap tree produced using weighted parsimony. (d) Tree produced using maximum-likelihood from the estimated "best-fit" model of the combined data set.

(Table 7). All trees (Figs. 1, 2, and 5) support monophyly of subgenus *Amanita*, section *Amanita*, section *Vaginatae*, subsection *Vaginatae*, subsection *Validae* and its series *Validae* and *Mappae*, and subsection *Amidellae*. Supports for monophyly of subgenus *Lepidella*, section *Lepidella*, and section *Phalloideae* are weak in the nLSU-rDNA analyses; in the combined analyses the support increases for section *Lepidella*, but decreases for subgenus *Lepidella* and section *Phalloideae* because monophyly of these two groups is generally not supported from analyses of mtSSU-rDNA sequence data. Depending on the analysis method, *A. virosa* (subsection *Phalloideae*) nested in various positions within subgenus *Lepidella* (Figs. 1, 2, and 5): in MP analyses of the nLSU-rDNA data set and in MP and ML-jc analyses of the combined data sets it was a sister taxon of subsection *Amidellae*, but with lack of bootstrap supports, and in the WP and ML analyses it was a sister taxon of subsection *Validae*, with bootstrap supports ranging from 69 to 78%. Because the latter analyses took into account heterogeneity in substitution rates in the sequence data, it could indeed be that subsection *Phalloideae* and *Validae* are sister groups.

There is one major conflict in our results concerning the monophyly of subsection *Amanita*. It is monophyletic in the MP and WP analyses of the nLSU-rDNA data set and of the combined data set, with low to moderately high bootstrap support (42–74%), and monophyletic in some ML analyses of the combined

TABLE 7

Comparative Bootstrap Support (in %) for Monophyly of Selected Groups in *Amanita*, Obtained from Separated and Combined Analyses of nLSU-rDNA and mtSSU-rDNA Sequence Data Using Equally Weighted (MP) Parsimony, Weighted Parsimony (WP), and Maximum-Likelihood (ML)[a]

Taxa	nLSU-rDNA			mtSSU-rDNA			Combined nLSU- and mtSSU-rDNA					
	MP	WP	ML	MP	WP	ML	MP	WP	ML	ML-nu	ML-mt	ML-jc
Sbg. *Amanita*	47	64	65	35	?[b]	0	66	76	76	+[c]	+	+
Sbg. *Lepidella*	40	30	51	No	No	No	32	31[d]	47	+	+	+
Sect. *Amanita*	97	97	97	50	?	17	100	99	98	+	+	+
Subsect. *Amanita*	62	47	No	?	38	25	74	64	No	+	+	+
Sect. *Vaginatae*	48	44	43	?	?	?	59	58	0	+	+	+
Subsect. *Vaginatae*	91	96	98	55	54	43	98	98	100	+	+	+
Sect. *Phalloideae*	47	51	47	?	No	No	0	44[d]	34	+	+	+
Subsect. *Validae*	99	100	84	100	100	98	100	100	100	+	+	+
Series *Validae*	79	82	68	99	100	96	100	100	100	+	+	+
Series *Mappae*	90	79	83	94	95	79	99	90	89	+	+	+
Subsect. *Amidellae*	100	100	100	73	46	64	99	100	100	+	+	+
Subsect. *Phalloideae* + *Amidellae*	0	No	No	No	No	No	0	No	No	No	No	+
Subsect. *Phalloideae* + *Validae*	No	78	73	No	?	No	No	72	69	+	+	No
Sect. *Lepidella*	41	41	34	No	?	No	60	46	45	+	No	+

[a] Maximum-likelihood used the best-fit model for the data being analyzed (ML), but in the combined analyses it also used the best-fit model for the nLSU-rDNA data set alone (ML-nu), for the mtSSU-rDNA data set alone (ML-mt), and the Jukes–Cantor model of sequence evolution (ML-jc) (see text).
[b] Unresolved position.
[c] Bootstrap support not calculated, but group present in the tree.
[d] Paraphyletic in the most-parsimonious trees but monophyletic in the bootstrap tree.

data set, but it is paraphyletic in the ML analyses of both the combined and the nLSU-rDNA analyses that used "best-fit" likelihood models (Table 7); in these analyses, *A. farinosa* (subsection *Ovigerae*) nests with either *A. muscaria* (55% BS, Fig. 2a) or *A. gemmata* (33% BS, Fig. 5d). Because we do not know the correct species phylogeny in *Amanita,* we cannot evaluate which of these analyses produced the most accurate phylogeny for *Amanita* and which were misleading. Theoretical and empirical studies using known phylogenies suggest greater effectiveness at estimating phylogenies when information about substitution bias is used to provide differential weightings for character transformations (Huelsenbeck, 1995); when rate heterogeneity is present in the data, it has been suggested that ML can better recover correct phylogenies (Gaut and Lewis, 1995; Cunningham *et al.,* 1998), whereas MP may recover a wrong topology with good bootstrap support (Cunningham *et al.,* 1998). In the light of these results, and because nucleotide composition and substitution biases, as well as substitution rate heterogeneity, are present in our data (Fig. 2), one should prefer the use of ML for the separate analyses of the nLSU-rDNA and mtSSU-rDNA; for the combined analysis, the WP analysis should be preferred, because the choice of a ML model to combine the two heterogeneous data sets was problematic and tree reconstruction was sensitive to the choice of the ML model (see above). Still, conflict remains: subsection *Amanita* is monophyletic in the WP analysis of the combined data (64% BS) and paraphyletic in the ML analysis of the nLSU-rDNA data.

CONCLUSIONS

Previous molecular phylogenies for *Amanita* were based on nLSU-rDNA data alone (Weiss *et al.,* 1998; Drehmel *et al.,* 1999) and were in good agreement with the morphological classification of Singer (1986). In this study, we showed that mtSSU-rDNA sequence data provide lower phylogenetic resolution than nLSU-rDNA sequence data and do not significantly conflict with nLSU-rDNA data. Combination of sequence data from these two genes did not significantly improve our understanding of *Amanita* phylogeny over that proposed in Drehmel *et al.* (1999) based on nLSU-rDNA data alone, although statistical support for most clades slightly improved in the combined analyses. It is generally admitted that increasing the number of characters in phylogenetic analyses increases phylogenetic accuracy. The fact that in this study the addition of mtSSU-rDNA data to nLSU-rDNA data did not significantly improve the phylogeny may be because the combined data sets have heterogeneous modes and rates of evolution, which cannot be accommodated simultaneously in complex models of evolution (e.g., ML) and for which simpler models (e.g., MP) may not be fully appropriate. In conclusion, whether or not com-

binability of multiple data sets is warranted, independent analyses should always be conducted to facilitate understanding differences between the various data sets and to best infer the multiple and dynamic relationships between molecular, morphological, and organismal evolution.

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