Evolution of Mushroom Mitochondrial DNA: *Suillus* and Related Genera

Thomas D. Bruns* and Jeffrey D. Palmer

Department of Biology, University of Michigan, Ann Arbor, Michigan, 48109-1048, USA

Summary. Mapping studies were performed with 18 cloned probes on mitochondrial DNA (mtDNA) from 15 species of *Suillus* and four species from three related genera of fleshy pore mushrooms (Boletaceae). Within *Suillus,* mtDNAs vary in size from 36 to 121 kb, differ in gene order by only one major rearrangement, and have diverged in nucleotide sequence within the large subunit ribosomal RNA gene region by up to 2.9%. Three additional gene orders exist in related genera. Two of the three can be transformed into the predominant *Suillus* order by either one or two rearrangements. The fourth requires two to three rearrangements to be converted to any of the others. The minimum estimates of nucleotide divergence within the large subunit ribosomal RNA gene region vary from 8.3% to 11% in comparisons between *Suillus* and these related species. Trees based on restriction-site and size differences within the mitochondrial ribosomal RNA genes were consistent with the hypothesized sequence of genome rearrangements and provide suggestive evidence for a major expansion of the mitochondrial genome within *Suillus.* Structural and sequence changes in mtDNA provided information about phylogenetic relationships within the Boletaceae.

Key words: Southern blots — Restriction maps — Ribosomal RNA genes — Introns — Phylogenetic trees — Boletaceae

Introduction

Mitochondrial DNA (mtDNA) is attractive for systematic studies in fungi because it is a small, easily isolated, well-studied molecule. However, a major obstacle to using mtDNA for phylogenetic studies is the high frequency and large size of length mutations (Sanders et al. 1977; Taylor et al. 1986; Bruns et al. 1988). Alignment of independently constructed restriction-site maps is thus difficult, as is recognition of site similarities (Bruns et al. 1988). Moreover, the number of length mutations prevents one from assuming that loss and gain of individual restriction sites are independent events in many regions of the mitochondrial genome. Here we circumvent the problem of frequent length mutations by using large-scale restriction mapping only to study rearrangements, and by intensively mapping small regions whose size is relatively conserved.

Previous work on mtDNA of *Suillus* focused on intra- and interspecific size variation and documented the gene order of five species (Bruns et al. 1988). We now extend knowledge of evolutionary changes in the molecule by using 18 cloned fragments of *Suillus* mtDNA to construct more detailed restriction maps for 14 additional species and by constructing trees relating the 19 species studied.

Materials and Methods

Cultures and DNA Extraction. Fungal cultures used are listed in Table 1. These were maintained and grown in large batches as described (Bruns et al. 1988). Total DNA was either extracted from fresh mycelia (Bruns et al. 1988) or from lyophilized samples (Murray and Thompson 1980). MtDNA was purified as previously described (Bruns et al. 1988).

Offprint requests to: T.D. Bruns
* *Current address:* Department of Botany, University of California, Berkeley, California 94720, USA

Table 1. Fungal cultures studied

Species	Isolate[a]	Location	Abbreviation[b]
Suillus americanus	TDB-588	Southern Michigan, USA	AMER
S. cavipes	TDB-646	Southern Michigan, USA	CAV
S. grevillei	TDB-570	Southern Michigan, USA	GREV
S. grisellus	TDB-574	Southern Michigan, USA	GRIS
S. luteus	TDB-571	Southern Michigan, USA	LUT
S. ochraceoroseus	SAR-84-137	Washington, USA	OCH
S. placidus	TDB-725	Southern Michigan, USA	PLAC
S. sinuspaulianus	DAOM 66995	Quebec, Canada	SIN
S. spectabilis	TDB-641	Southern Michigan, USA	SPEC
S. spraguei (pictus)	TDB-638	Northern Michigan, USA	SPRA
S. subalutaceus	ACAD 15288	Nova Scotia, Canada	SUB
S. tridentinus	HB-347	West Germany	TRI
S. tomentosus	TDB-661	Colorado, USA	TOM
S. variegatus	HB-325	West Germany	VAR
S. viscidus	TDB-576	Southern Michigan, USA	VIS
Gyrodon lividus	HB-346	West Germany	GL
G. merulioides	TDB-532	Southern Michigan, USA	GM
Paragyrodon sphaerosporus	TDB-420	Western Minnesota, USA	PS
Xerocomus chrysenteron	TDB-635	Northern Michigan, USA	XC

[a] TDB cultures were isolated and maintained as described by Bruns et al. (1988). Voucher collections of the basidiocarps are deposited at the University of Michigan Herbarium (MICH). Other cultures are from the following sources: SAR-84-137: Steven Rehner, University of Washington, Seattle; ACAD 15288 (F2): Kenneth A. Harrison, Acadia University, Nova Scotia; HB-347, HB-325, HB-346: Helmut Besl, University of Regensburg, Regensburg, West Germany; DAOM 66995 (B109): Biosystematics Research Institute, Ottawa, Ontario, Canada (see Pantidou and Groves 1966)

[b] Used in Figs. 3 and 8

Cloning. HindIII fragments from *Suillus luteus* mtDNA were shotgun-cloned from a total digest by standard methods (Maniatis et al. 1982). Selected fragments from *Suillus sinuspaulianus* were isolated and cloned in SeaPlaque low melting point agarose (FMC) gels. Recombinant plasmids were screened for inserts of the same size as genomic fragments. Southern-blot hybridizations were used to confirm the identity of each clone and to identify those that contained portions of the eight mitochondrial genes surveyed. The 18 clones used in this study are summarized in Table 2.

Southern-Blot Hybridizations. Restriction fragments were separated by gel electrophoresis and blotted as described previously (Bruns et al. 1988), with the exception that blotting was bidirectional and 4% NuSieve (FMC) agarose was used to resolve fragments of 75–1500 bp. Radioactive *Suillus* clones were hybridized for 12–30 h at 60°C in 5× saline sodium citrate (SSC; 1× SSC = 0.15 M NaCl, 0.15 M sodium citrate, pH 7.0), 2× Denhardt's, 100 μg/ml sheared salmon sperm DNA. Following hybridization, the filters were washed for one to several hours in 2× SSC at 60°C. We found the lower stringency necessary to obtain adequate signals when several of the *Suillus* clones were hybridized to mtDNAs of related genera. These conditions were employed routinely, because no problems were encountered with background or extensive cross-hybridization due to dispersed repeats. Autoradiograms were exposed for 1–7 days at −80°C with intensifying screens. Bound probes were stripped from the filters with 0.4 N NaOH at 50°C or with boiling 0.1 × SSC prior to reuse of the filters.

Map Construction. Restriction-site maps were deduced primarily from Southern-blot hybridization data as illustrated in Fig. 1. Sites for the following 13 restriction enzymes were mapped in this manner: AvaI, AvaII, BamHI, BglII, BstEII, ClaI, CfoI, EcoRI, HaeIII, HindIII, PstI, PvuII, XhoI.

Phylogenetic Analysis. PAUP version 2.4 (Swofford 1985) was used for parsimony analysis. The branch and bounds algorithm was employed to find the shortest trees. The Mulpars option and global branch swapping were used to find trees that required one additional step. The contree algorithm was used to produce strict consensus trees as defined by Rohlf (1982).

Results

Size Variation of MtDNAs

It was previously known that size of mtDNA varies over threefold (36–121 kb) within *Suillus* and exhibits a nearly continuous distribution (Bruns et al. 1988). The present study shows that virtually every region of the mitochondrial genome appears to be size variable when all 15 species are compared (Fig. 3). Exceptions are limited to small regions such as those that hybridize to clones 4 and 11 (Fig. 3). The former is adjacent to the 5' end of the large ribosomal RNA (LrRNA) gene and the latter contains the 3' end of the small ribosomal RNA (SrRNA) gene. The existence of other small size-conserved regions is obscured at this level of comparison.

The region that includes cytochrome oxidase subunit I (COI) (clones 1, 2, and 16) is extremely variable in size. Previously, we have noted intraspecific size variation in this region and speculated that it was caused by variation in number of introns within COI (Bruns et al. 1988). We now have evidence that at least one intron exists within one of most size-variable parts of the region (Fig. 4).

Rearrangements and Duplications

Suillus luteus is the only species of *Suillus* among the 15 studied that has a different fragment order. A single transposition of the SrRNA gene region (i.e., regions hybridizing to clones 10 and 11) can account for the difference. This rearrangement was noted by Bruns et al. (1988), but without the mapped position of the region that hybridizes to clone 9 it was unclear whether the transposition involved the ATP synthase subunit 9 (ATP9) gene (probe 8) or the SrRNA gene (Fig. 3). Additional rearrangements are evident in comparisons involving mtDNAs of three related genera (Fig. 5). The four different orders shown in this figure can be interconverted by postulating four rearrangements (Fig. 5). These results clearly demonstrate that large-scale rearrangements are rare events in these genomes.

Small-scale rearrangements could exist that have gone undetected by our methods. We frequently observed faint hybridization of probes to nonadjacent restriction fragments, but because of difficulties associated with mapping these weak signals we have generally ignored them. Two cases that we studied involve duplication (clones 13 and 16, Fig. 2; clone 6, Figs. 3 and 4), and the latter may also involve transposition.

Structural Maps of the Ribosomal Gene Regions

Initial mapping results revealed clusters of conserved sites in all species. Two of the larger clusters were in the regions of the LrRNA and SrRNA genes (Fig. 6). We examined these two regions in more detail by employing Nusieve agarose gels with single and double digests of the enzymes AluI, Sau3A, MboII, and RsaI to produce fine-structure restriction-site maps of the two regions (Fig. 7). Alignment of maps was difficult in regions bordering introns of the LrRNA gene in *Suillus* and in highly divergent regions in comparisons between *Suillus* and related genera. In all cases, ambiguous site alignments were entered as missing data for the tree analysis (see below). This sacrifices some resolution, but causes no other difficulties. In the calculation of sequence divergence, however, regions containing these ambiguous site alignments were excluded. This biases the results toward an underestimation of sequence differences between the most divergent taxa, because estimates are based only on the most conserved regions of these genes.

Sequence Divergence

We estimated nucleotide divergence among these taxa using restriction-site differences within the LrRNA gene by methods of Nei and Li (1979). Eight species of *Suillus* that we call group 1 were found

Table 2. MtDNA fragments used for hybridization mapping

Num-ber[a]	Fragment (kb)	Vector[b]	Genes included[c]
1	1.3 HindIII	pUC12	Intron(s) of COI
2	3.0 HindIII	pUC12	Portion of COI and ?
3	5.5 HindIII	pUC12	ATP6 and ?
4	1.3 ClaI/PvuII	BS M13+	?
5	3.6 HindIII	pUC12	Portion of LrRNA and introns
6	1.3 HindIII	pUC12	Intron of LrRNA
7	2.4 HindIII	pUC19	COII and ?
8	0.085 and 0.065 Alu	—	ATP9
9	0.6 ClaI/PstI	pIC20H	?
10	0.9 SstI/HindIII	BS M13+	SrRNA 5' end and ?
11	0.6 HindIII	pUC12	SrRNA 3' end
12	4.8 PstI/ClaI	pIC20H	?
13	3.5 ClaI	pIC20H	COB and ?
14	2.3 ClaI/BamHI	pIC20H	COIII and ?
15	5.6 BamHI/SstI	pUC19	?
16	5.9 SstI/BamHI	pUC19	COI and ?
A	7.4 ClaI/XhoI	BS M13+	LrRNA, COII, and ?
B	6.3 PstI/PstI	pIC20H	SrRNA and ?

Clones 1–3, 5, 6, and 11 were derived from *S. luteus* TDB-571. Clone 8 was isolated from petite mtDNA of *Saccharomyces cerevisiae* DS400/A3 (Macino and Tzagoloff 1979). All others were derived from *S. sinuspaulianus* DAOM 66995. All recombinant plasmids were transformed in *E. coli* strain JM83

[a] Used in Figs. 2, 3, 5, 6, and 7

[b] Unusual vectors used: pIC20H (Marsh et al. 1984); BS M13+ (BlueScript KS M13 phagmids, Stratagene cloning systems, San Diego)

[c] Gene content was determined by Southern-blot hybridization of heterologous gene probes as described by Shumard et al. (1986) with modifications of Bruns et al. (1988). Introns of the LrRNA gene were determined by fine-structure mapping (see text). ? indicates undefined gene content

to have less than 1% divergence within the region. The largest divergence within the genus is 2.9%, between group 1 and *S. luteus*. The smallest divergence between *Suillus* and the related genera is over twofold larger (8.3%), and the largest (11%) is nearly four times greater (Table 3). Furthermore, these divergence estimates between *Suillus* and the related genera probably underestimate total divergence because they are based only on the most conserved portions of the LrRNA region.

Phylogenetic Analyses

The initial phylogenetic analysis used as characters 41 restriction sites, three mini-inserts from both rRNA genes, and the two introns from the LrRNA gene (Table 4). Invariant and cladistically uninformative restriction sites and sites within the introns of the LrRNA genes were excluded from the analysis, and care was taken to ensure that four-base
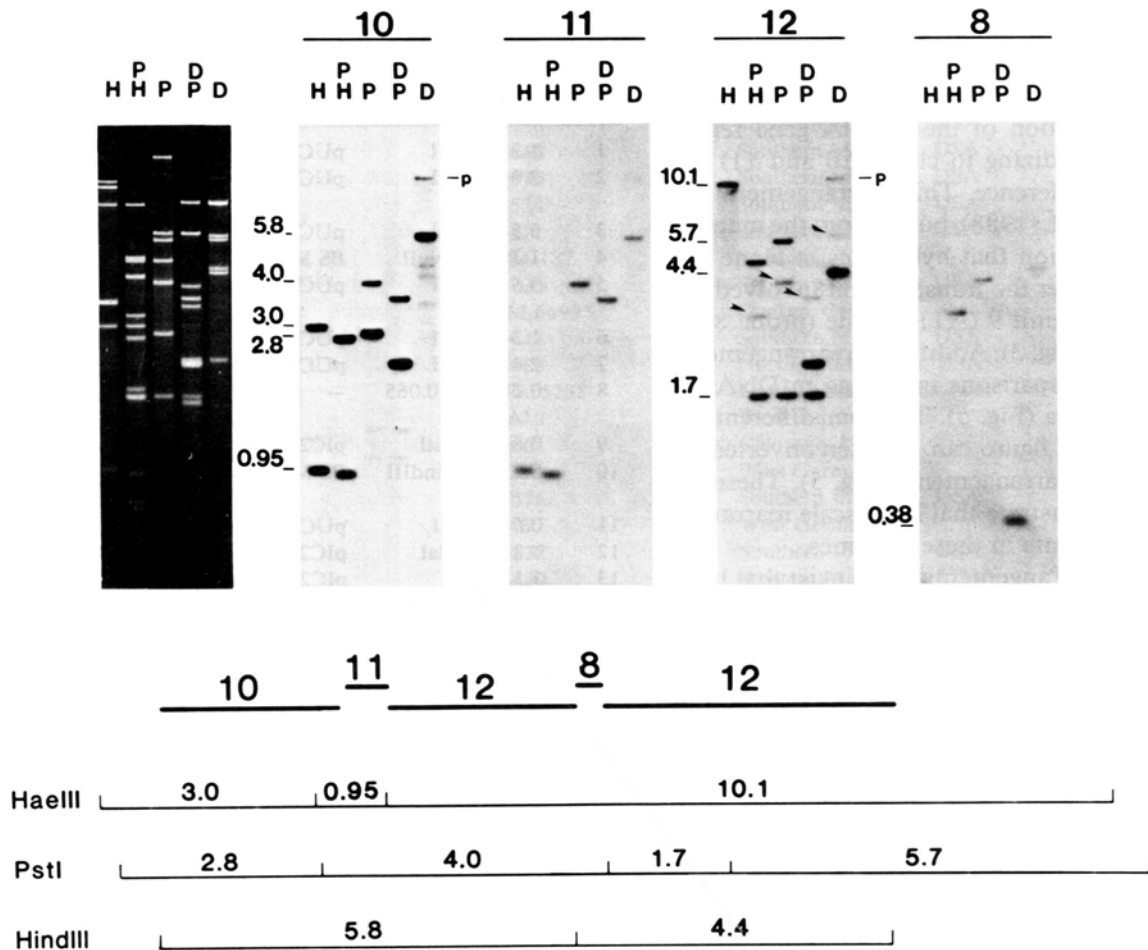
Fig. 1. Hybridization mapping of a portion of the mtDNA from *Paragyrodon*. Restriction fragments produced by single and double digestion with HaeIII (H), PstI (P), and HindIII (D) were separated in a 0.8% agarose gel, blotted onto a reusable nylon filter, and hybridized in four separate experiments with radiolabeled probes. Probe numbers (8, 10–12) indicate their predominant order within *Suillus*. The positions of these fragments in *Paragyrodon* have changed relative to *Suillus* by the transposition of the ATP9 gene (probe 8). Note the low intensity of hybridization to part of clone 12 (arrows). This region is barely detectable by hybridization in *Gyrodon merulioides* and may be undetectable or absent from *Xerocomus chrysenteron* (see text). Weak hybridization of probe 8 to the 10.1-kb HaeIII fragment can be seen on a longer exposure (not shown). The top HindIII band in hybridizations of probes 10 and 12 is a partial (p).

recognition subsets of six-base sequences were not counted twice (e.g., HindIII and AluI). A total of 54 equally parsimonious trees requiring 61 steps exist if these characters are polarized by outgroup analysis. The strict consensus tree of these 54 trees (not shown) contains little resolution of relationships within the genus but demonstrates that *Suillus* sensu lato is monophyletic with respect to the four other taxa. A minimum of 21 character-state changes, over a third of all changes in the entire tree, separates *Suillus* from the outgroups (Fig. 8, tree A).

To refine the analysis within *Suillus* we examined the character states of the hypothetical ancestor of *Suillus* as constructed from the initial outgroup-rooting algorithm and found three of the character-state assignments to be questionable. All three involve restriction sites in the SrRNA gene (characters 31, 32, and 34) that would be assumed to be absent

in the ancestor of *Suillus* by outgroup rooting. However, the presence of these sites in most *Suillus* species (11 of 15, 13 of 15, and 13 of 15) can be used to infer instead that the sites were primitively present in the genus (i.e., by ingroup criteria).

To examine the effect of the character-state polarizations of these sites we constructed a hypothetical ancestor (HTA) and used it in an ancestor-rooted analysis. The characters discussed above were entered as missing data in the HTA to allow either polarity. All other characters that could be unambiguously polarized by the outgroups were, and those that were ambiguous with respect to outgroups were also coded as missing data (Table 4). The parsimony analysis was then repeated using ancestor rooting. A total of 15 equally parsimonious trees were found. These 15 trees described only two trees that differ in structure. The remaining 13 differ in their arbi-

Suillus sinuspaulianus

COI ATP6 LrRNA COII   ATP9   SrRNA   COB COIII

S. luteus clones   1 2   3   5 6   11

S. sinus clones   16   4   7   9   10   12   13   14   15

Aval
Xhol
Avall
Bglll
BamHI
BstEll
Clal
Sstl
EcoRl
Hindlll
Cfol
Haelll
Pstl
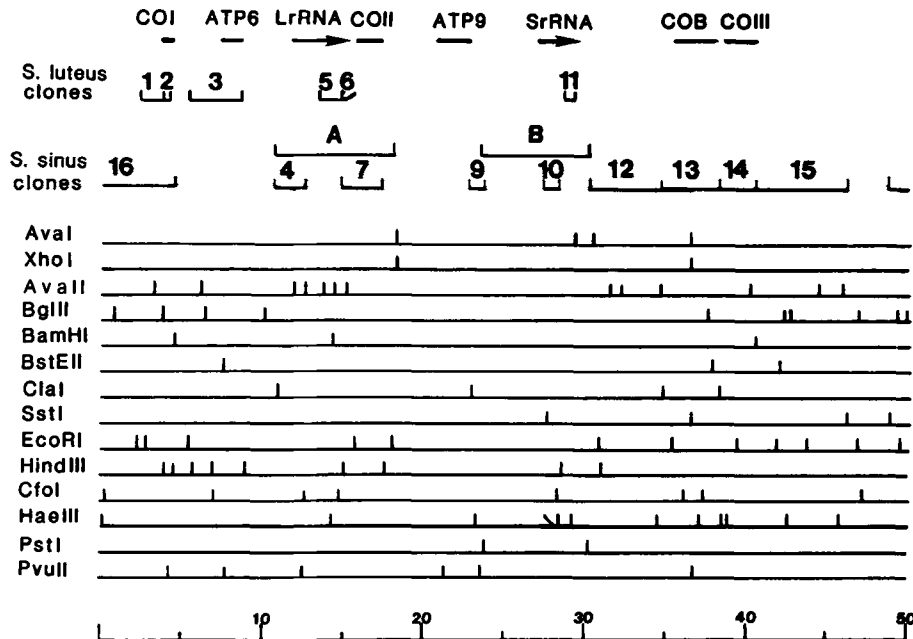Pvull

10   20   30   40   50

Fig. 2. Positions of mitochondrial genes and *Suillus* clones within the mitochondrial genome of *S. sinuspaulianus*. Hybridization limits of clones from *Suillus luteus* and *S. sinuspaulianus* (S. sinus) are given below the genes. Cross-hybridization of clones 13 and 16 occurs but is not illustrated above. The probes employed are those described in Table 2. Gene locations were determined by hybridization of gel-isolated fragments from *Saccharomyces cerevisiae* as described by Bruns et al. 1988). Similar maps were constructed for each species. However, in many species regions that contained little or no detectable hybridization to the cloned probes were encountered. When these areas were extensive, some sites could not be mapped, but large restriction fragments that spanned these regions allowed portions of the molecule that did hybridize to be unambiguously ordered. Gene abbreviations: COI, COII, COIII = cytochrome oxidase subunits 1, 2, and 3; ATP6, ATP9 = ATP synthase subunits 6 and 9; LrRNA, SrRNA = large and small subunit ribosomal RNA; COB = cytochrome oxidase b apoprotein.

Table 3. Nucleotide sequence divergence within the LrRNA gene region

	Suillus group 1[a]	*Gyrodon merulioides*	*Gyrodon lividus*	*Paragyrodon sphaerosporus*
Suillus group I	0.32–0.85			
S. luteus	2.4–2.9			
S. spraguei	1.9–2.4			
Gyrodon merulioides	8.8–9.2			
G. lividus	8.7–9.2	2.2		
Paragyrodon sphaerosporus	8.3–8.9	3.1	0.5	
Xerocomus chrysenteron	11–11.4	4.9	3.2	2.7

Values are based upon difference among 21–39 restriction sites within the LrRNA gene region (Fig. 7) and are the averages of values calculated from restriction enzymes with different recognition sequence sizes weighted by the reciprocals of their variances as suggested by Nei and Li (1979). Estimates were calculated using equations 8 and 9 of Nei and Li (1979); these produced identical values to two significant figures. Ranges given are minimum and maximum values calculated from the eight members of group 1. The comparisons given show the largest divergence within *Suillus* (group 1 versus *S. luteus* and *S. spraguei*) and the smallest between *Suillus* and the related genera (group 1 *Suillus* vs three other genera). Discrepancies in sizes of the range are caused by differences in the size of the regions (and therefore number of restriction sites) compared

[a] *Suillus* group 1 includes the following species: *S. cavipes, S. variegatus, S. tomentosus, S. ochraceoroseus, S. spectabilis, S. sinuspaulianus, S. subalutaceus, S. americanus*

trary resolution of trichotomies. A strict consensus tree of the 15 is shown (Fig. 8, tree A). All 15 trees postulate the presence in the HTA of the three sites in question.

Although tree A (Fig. 8) is the most parsimonious, over 100 trees exist that require only one ad-

ditional step. Statistically many of these less parsimonious trees may be equivalent descriptions of these data (cf. Templeton 1983a,b). Therefore, we examined 100 of the trees requiring one additional step to find which features among them were most consistent. Branching differences within the trees
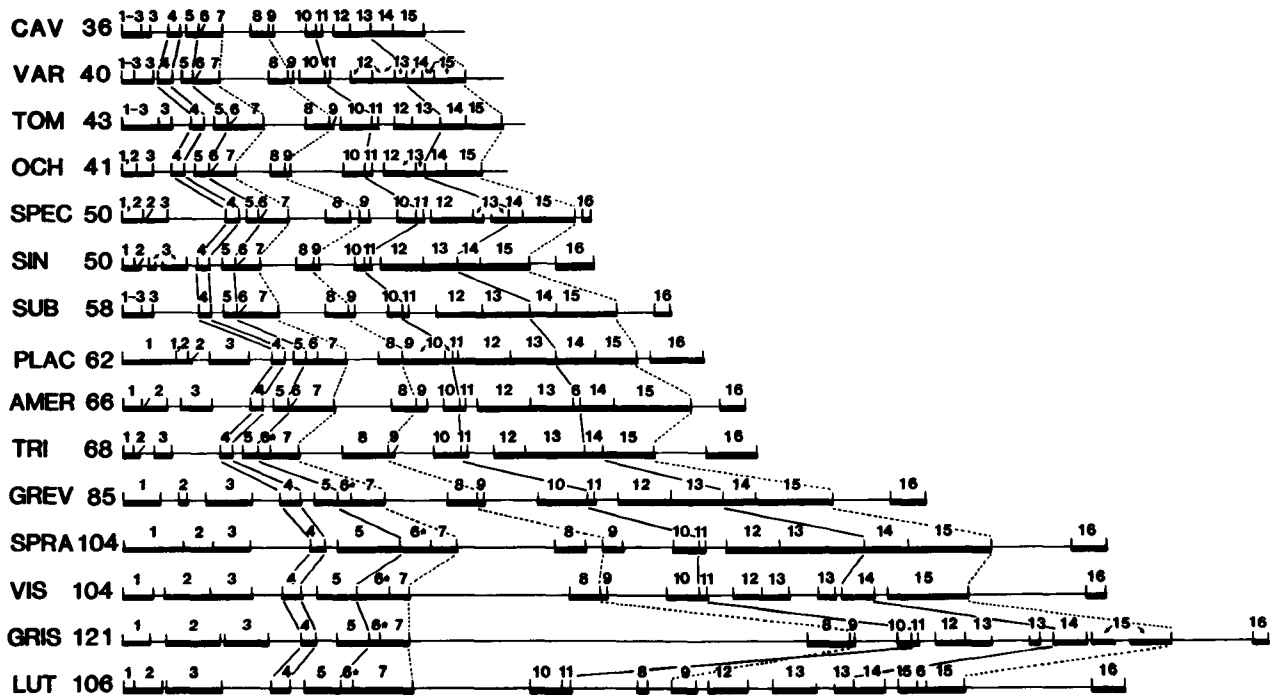
354

Fig. 3. Fragment order and size variation in the mtDNAs of 15 *Suillus* species. The circular mtDNAs are shown linearized in approximate alignment according to the positions of hybridization of the 16 indicated probes. Probe 16 overlaps and includes sequences that hybridize to probes 1 and 2; its position is shown only for regions to which it hybridizes uniquely. Genome sizes are given in kilobase pairs after the species abbreviation. The limits of the regions to which the the probes hybridize are defined by sites of 13 restriction enzymes given in the text. Solid lines connect restriction sites that border the same hybridization region and are shared by all *Suillus* species. Dotted lines connect hybridization borders that are defined by one of several closely spaced restriction sites. Clone 6 hybridizes to two nonadjacent regions in two genomes (*S. luteus*, *S. americanus*). Strong hybridizations of this clone are indicated with stars, other regions hybridize only weakly to the clone. Note that the mapped limits of many hybridizing regions differ, and distances between regions vary dramatically. Order, however, differs only in *S. luteus* and only by a single transposition of regions 10 and 11. Species abbreviations are given in Table 1.

examined were limited to different placements of *S. placidus* or the *S. subalutaceus–S. americanus* branch. The most common alternatives to that shown in tree A place *S. placidus* on a common branch with *S. ochraceoroseus,* or on the opposite side of the *S. subalutaceus–S. americanus* branch. The position of the root within *Suillus* was found to be the most common difference between the trees.

We then added two morphological characters to the analysis to aid in the identification of the root. The two characters added, spore color and clamp connections, were chosen because their primitive states can be inferred fairly confidently from the outgroups and because they are the most significant defining characters of *Fuscoboletinus* and *Boletinus* (treated here as members of *Suillus* sensu lato). Tree B (Fig. 8) shows the most parsimonious solution to these data. This tree requires one additional loss of a restriction site compared to tree A but explains the presence of clamp connections in basidiocarps and spore color without postulating convergence or reversal, whereas tree A must postulate reversal of both of these features in *S. cavipes.*

Although fragment order changes were not included in the tree analysis, all but one are congruent

with it (Fig. 8, tree A). Four of the five orders can be explained by the single rearrangements postulated above. The *S. luteus* order is derived from the predominant *Suillus* order by transposition of the SrRNA gene region (Fig. 3, probes 10 and 11). Fragment orders B, C, and D (Fig. 5) can be explained by a single transposition of ATP9 (probe 8), followed by a single rearrangement of regions hybridizing to clones 12 and 13. The fragment order of *Xerocomus,* however, is derived from order C according to these trees, rather than from order B as depicted in Fig. 5. Therefore, at least one additional step appears to be necessary to explain this rearrangement. This step could involve the reversal of the ATP9 (probe 8) transposition, along with the two inversions suggested in Fig. 5. Alternatively, two inversions can interconvert orders A and C if one postulates that a small region homologous to clone 12 is absent or undetectable by hybridization in order A (Fig. 5, legend). This latter explanation is supported by the observation that this region hybridizes rather weakly to clone 12 in *Paragyrodon* (Fig. 1) and *G. lividus* and is barely detectable in *G. merulioides* (Fig. 5).

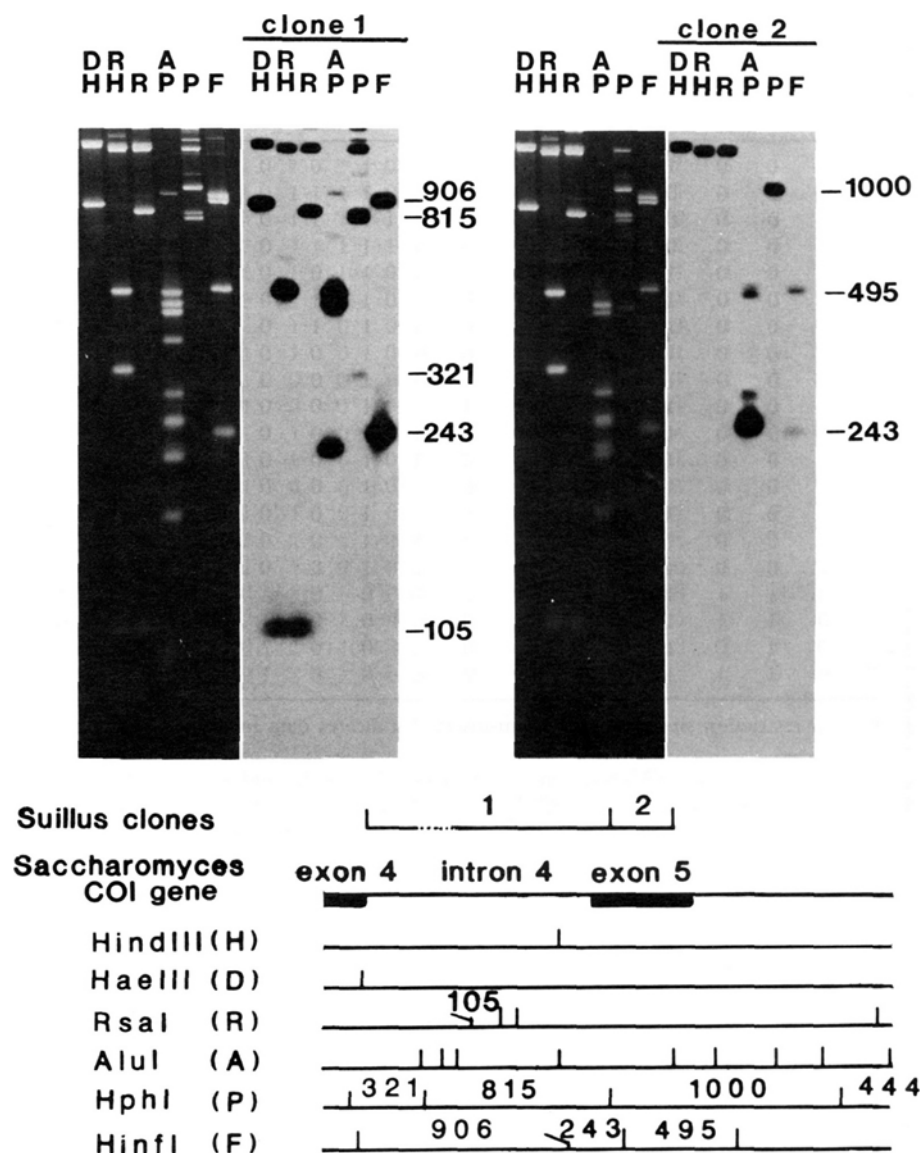Both trees (Fig. 8) show a striking pattern with

Fig. 4. Indirect evidence for an intron in the COI gene of *Suillus luteus*. *Suillus luteus* clones 1 and 2 were hybridized to filter-bound mtDNA restriction fragments of *Saccharomyces cerevisiae* petite mutant DS6/A407. A linearized restriction-site map of the petite mitochondrial genome is shown, based on the sequence data of Bonitz et al. (1980). Note that clone 1 hybridizes to the 105-bp RsaI restriction fragment. This fragment, which is internal to the *Saccharomyces* intron, contains the second half of the region encoding the conserved LAGLI–DADG decapeptide motif (Waring et al. 1982; Hensgens et al. 1983) that is characteristic of the open reading frames of maturase genes in fungal group-one introns. Clone 2 hybridizes to adjacent exon 5 sequences of the *Saccharomyces* gene. Faint bands, particularly in the HpaI and HphI/AluI lanes, are due to partial digestion. The lower of the two bands around 800 bp in the HphI lanes are partials containing the 321- and 444-bp fragments. Restriction enzyme abbreviations used in the upper figure are shown in the lower figure.

respect to mitochondrial genome size. Species that are grouped together tend to have similar-sized genomes, and all species with mtDNAs larger than 80 kb are identified as a single monophyletic group. The species in this clade also contain both LrRNA introns, but the two introns together account for less than 6 kb of the size increase. A larger portion of the size differences occurs between regions hybridizing to probe fragments 1–4, 7 and 8, and 15 and 16 (Fig. 3).

Convergence and reversal were rather high in relation to the low estimates of nucleotide divergence. In both trees, 6 of the 22 informative characters change twice within the *Suillus* portion of the trees. Among the restriction-site changes, one convergent gain (character 12) and one loss–gain (character 31) are postulated by both trees. All other double changes involve convergent loss, which is a highly probable event (Templeton 1983a). Character 27, a mini-insert in the LrRNA gene, undergoes three changes in both trees, and character 38, a restriction site in the SrRNA gene, is gained three times in tree A and lost four times in tree C. Mapping errors are possible with respect to the latter site because it occurs at the border of the size-conserved zone, so unlike internal sites its presence or absence can only be inferred by one fragment. Elimination of the site from the analysis results in 81 equally parsimonious trees with HTA-rooting. The position of the root and the *S. subalutaceus–S. americanus* branch and resolution of trichotomies are the only differences among 100 of these trees. They agree with trees A or B in all other respects.

Our use of single isolates for most species may add uncertainty to the phylogenetic analysis. Is it reasonable to assume that variation of these characters within a species is minor compared to variation between species? For restriction sites this as-

Table 4. Characters for tree analysis

Taxa	Character numbers[a]																			
					5						10					15					20
HTA	0	0	1	1	1	0	0	1	0	1	0	0	1	1	0	0	1	1	1	1
S. luteus	1	1	?	1	1	0	0	0	0	?	0	0	1	1	1	0	?	0	0	?
S. grisellus	1	1	?	1	1	0	0	0	0	?	0	0	1	1	1	0	?	1	1	?
S. viscidus	1	1	?	1	1	0	0	0	0	?	0	0	1	1	1	0	?	1	1	?
S. tridentinus	0	1	1	1	1	0	0	1	0	1	0	0	1	1	0	0	1	1	1	1
S. grevillei	1	1	0	1	1	0	0	1	0	1	?	0	1	1	1	0	?	1	1	1
S. spraguei	1	1	0	1	1	0	0	1	0	1	0	0	1	1	1	0	?	0	0	?
S. placidus	0	1	1	1	1	0	0	1	0	1	0	0	1	1	0	0	1	1	1	1
S. subalutaceus	0	0	1	1	1	0	0	1	0	1	0	0	1	1	0	0	1	1	1	1
S. americanus	0	0	1	1	1	0	0	1	0	1	0	1	1	1	0	0	1	1	1	1
S. cavipes	0	0	1	1	1	0	0	1	0	1	0	1	1	1	0	0	1	1	1	1
S. sinuspaulianus	0	0	1	1	1	0	0	1	0	1	0	0	1	1	0	0	1	1	1	1
S. ochraceoroseus	0	0	1	1	1	0	0	1	0	1	0	0	1	1	0	0	1	1	1	1
S. spectabilis	0	0	1	1	1	0	0	1	0	1	0	0	1	1	0	0	1	1	1	1
S. variegatus	0	0	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1
S. tomentosus	0	0	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1
X. chrysenteron	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	1	1	?
P. sphaerosporus	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	1	0	1	1	0
G. merulioides	0	0	1	1	0	1	0	1	1	0	0	0	0	0	0	?	0	1	1	0
G. lividus	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	1	0	1	1	0

1 indicates the presence and 0 the absence of a restriction site, intron, or mini-insert. ? indicates data missing due to mapping ambiguities

[a] Introns A and B = characters 1 and 2. Restriction sites in the LrRNA region = characters 3–26, 29, 30, and 46. Restriction sites in the SrRNA region = characters 31–36 and 38–45. Mini-inserts in the LrRNA region = characters 27 and 28. Mini-insert in the SrRNA region = character 37. MboII sites are not mapped for most species in the LrRNA region and so were excluded from the analysis
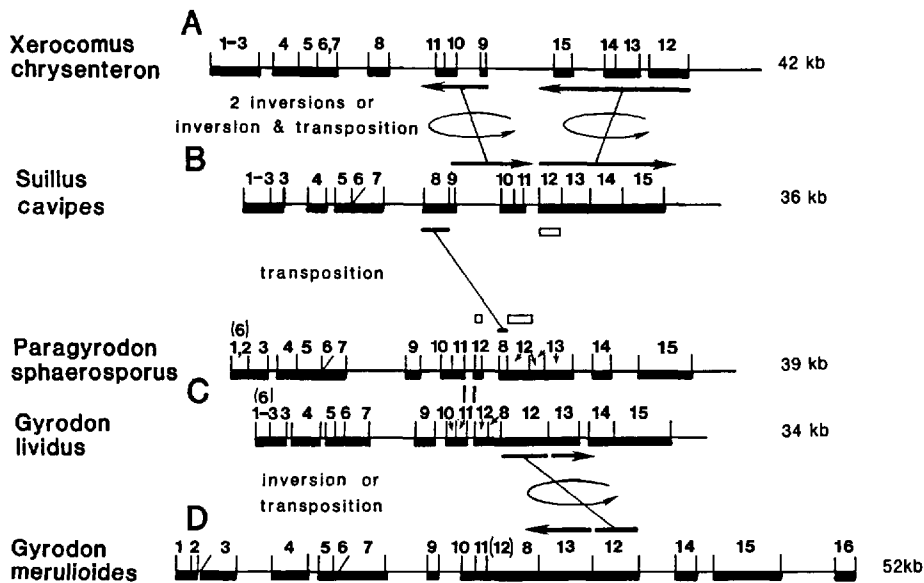

Fig. 5. MtDNA rearrangements among related genera of the Boletaceae. The hybridization limits of 16 probes are shown in the linearized maps of the circular mtDNAs of *Suillus cavipes* and four species from related genera. Probe 16 hybridizes to the same regions as probes 1 and 2 in all genomes but is shown only where it hybridized uniquely to additional fragments (e.g., map D). Four different orders exist in these taxa with respect to these probes. The orders can be interconverted by postulating the four rearrangements shown. Alternatively, if the small region hybridizing to the portion of clone 12 located between regions 11 and 8 in order C is assumed to be absent or undetectable in order A, then orders A and C can be interconverted directly by inversions of two regions in order C: 9-10-11-(12)-8 and 12-13-14-15. Weak hybridizations of clones 6 and 12 are indicated by parentheses. The former clone contains part of an intron of the LrRNA gene from *S. luteus*.

Table 4. Extended

			Character numbers																						
			25					30					35					40					45		
1	?	1	0	1	?	?	0	1	1	?	?	0	?	1	1	0	1	1	?	0	1	1	?	0	0
?	?	?	?	1	1	1	0	1	1	1	1	0	1	1	1	1	0	1	0	?	1	1	0	0	?
?	1	?	?	?	1	?	1	1	1	0	1	0	0	1	1	0	0	1	1	0	1	1	0	0	?
?	1	1	?	?	1	1	1	1	1	0	1	0	1	1	1	0	0	1	1	0	1	1	0	0	?
1	1	1	0	1	1	1	0	1	1	0	1	0	1	1	1	0	1	1	0	0	1	1	0	0	?
?	1	1	0	?	1	0	0	1	1	0	1	?	1	1	1	0	0	1	0	1	1	1	0	0	?
?	?	1	0	1	1	0	0	1	1	1	1	0	1	1	1	1	0	1	?	1	1	1	0	?	?
1	0	1	0	1	1	0	0	1	1	1	0	0	1	1	1	0	0	1	0	0	1	1	0	0	?
1	0	1	0	1	0	0	0	1	1	1	1	0	1	1	1	0	1	1	0	0	1	1	0	0	0
1	0	1	0	1	0	0	0	1	1	1	1	0	1	1	1	0	1	1	0	0	1	1	0	0	0
1	0	1	0	1	1	0	0	1	1	1	1	0	1	1	1	0	0	1	0	0	1	1	1	0	0
1	0	1	0	1	1	0	0	1	1	1	1	0	1	1	1	0	1	1	0	0	1	1	1	0	1
1	0	1	?	1	0	0	0	1	1	1	0	0	1	1	1	0	0	1	0	0	?	1	1	0	1
1	0	1	0	1	1	0	0	1	1	1	1	0	1	1	1	0	0	1	0	0	1	1	1	0	1
1	0	1	0	1	1	0	0	1	1	1	1	0	1	1	1	0	1	1	0	0	1	1	0	0	0
1	0	1	0	1	1	0	0	1	1	1	1	0	0	1	1	0	1	1	0	0	1	1	0	0	0
?	?	?	0	0	?	?	0	0	0	0	0	1	0	0	0	0	1	0	0	0	?	?	?	?	0
0	?	0	0	0	?	?	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	?	1	0
0	?	?	1	0	?	?	0	0	0	0	0	1	0	?	?	0	1	1	1	0	?	?	?	0	0
0	?	0	1	0	?	?	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	?	1	0

sumption appears to be well founded because studies of intraspecific variation of mtDNA in other fungi as well as in *Suillus* have shown that site differences within a species are rare throughout the size-conserved portion of the mitochondrial genome (Sanders et al. 1976; Taylor et al. 1986; Bruns et al. 1988). Furthermore, the low level of divergence present even among species that are morphologically dis-
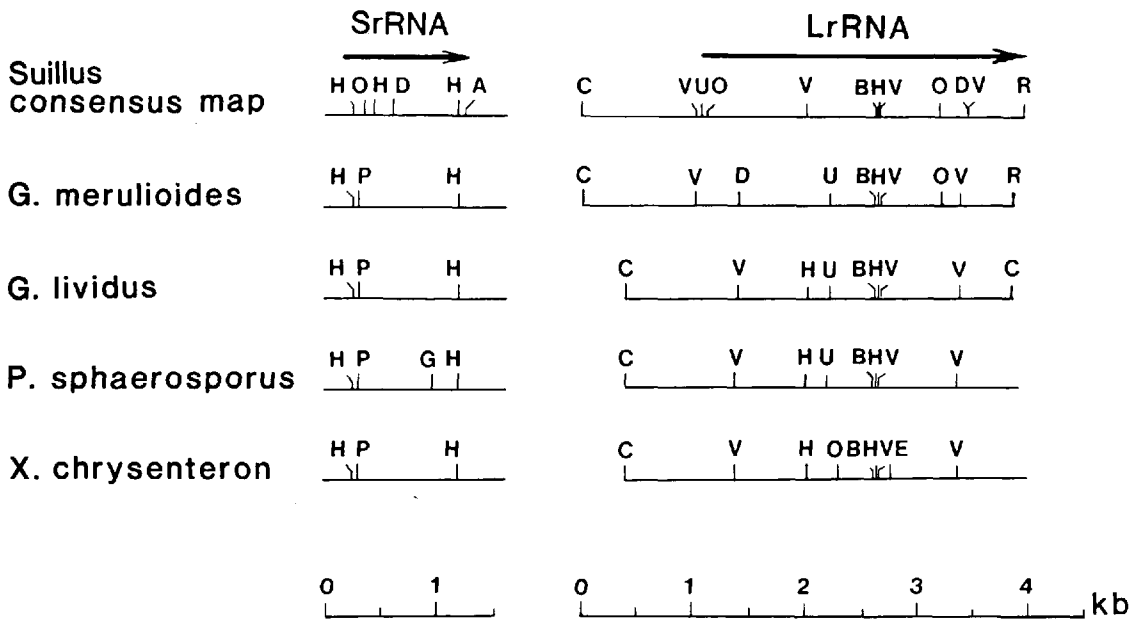

SrRNA →   **LrRNA** →

Suillus consensus map
SrRNA: H OH D   H A
LrRNA: C   VUO   V   BHV   O DV   R

G. merulioides
SrRNA: H P   H
LrRNA: C   V D   U BHV   OV   R

G. lividus
SrRNA: H P   H
LrRNA: C   V   HU BHV   V   C

P. sphaerosporus
SrRNA: H P   G H
LrRNA: C   V   HU BHV   V

X. chrysenteron
SrRNA: H P   H
LrRNA: C   V   H OBHVE   V

Scale: 0 — 1   0 — 1 — 2 — 3 — 4 kb

Fig. 6. Clusters of similar restriction sites in the mitochondrial ribosomal RNA gene regions. The *Suillus* consensus maps contain restriction sites shared by most of the 15 species studied. The following variation exists in these regions: The AvaI (A) and HindIII (D) sites of the SrRNA region are absent in two and four species, respectively, and additional BglII (G) and HindIII (D) sites are each found in one species. Within the LrRNA region the BamHI (B) site and one or two of the AvaII (V) sites are absent from several species. Species that lack any of these sites also have one or two introns within the gene. An additional HaeIII site in the 5′ portion of the region is shared by six species. An additional ClaI, BglII, and CfoI site are each found in one or two species. Restriction site abbreviations: AvaI (A), BamHII (B), ClaI (C), HindIII (D), BstEII (E), BglII (G), HaeIII (H), CfoI (O), PstI (P), EcoRI (R), PvuII (U), AvaII (V).
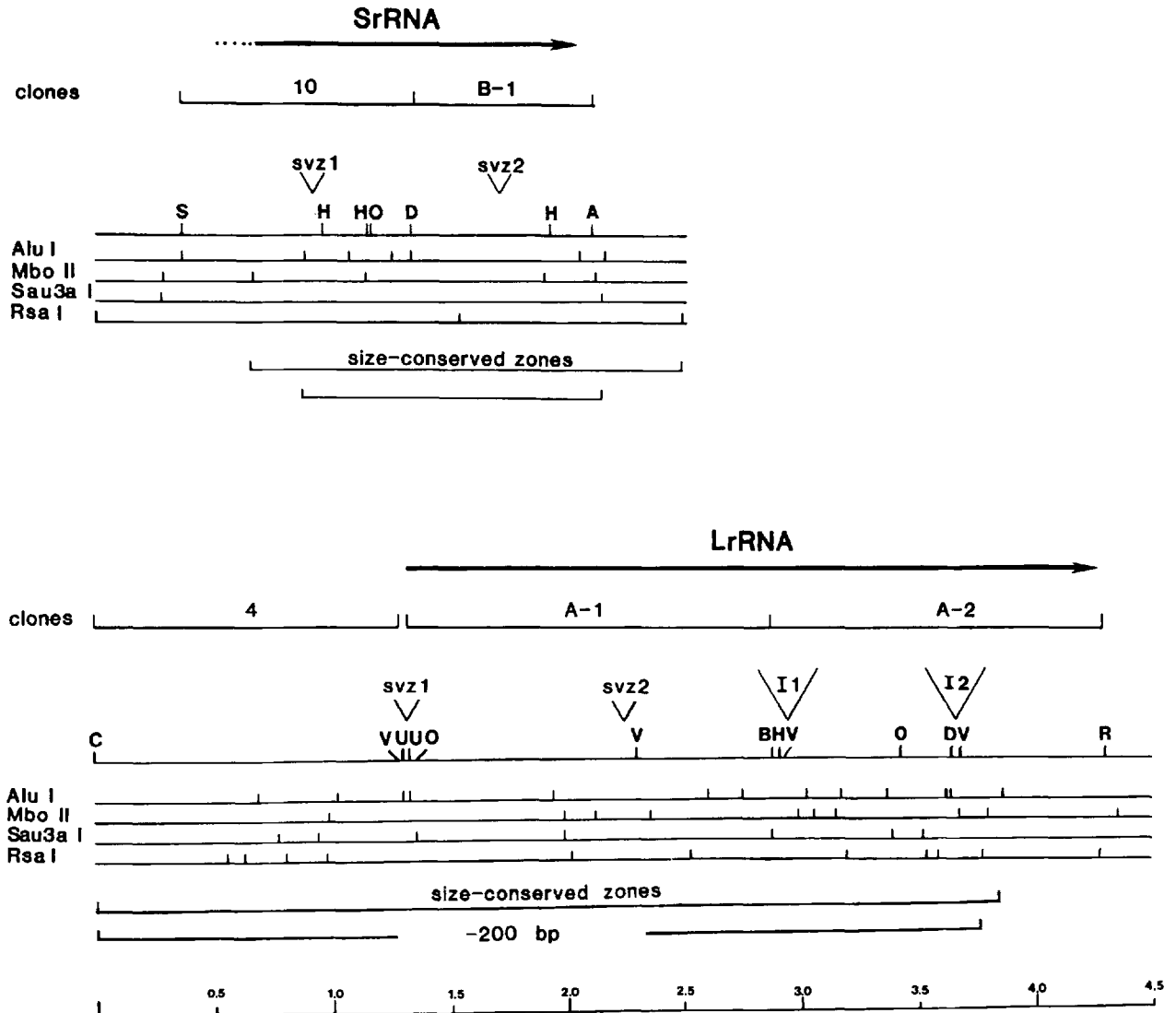
Fig. 7. Fine structure restriction-site maps of the mitochondrial small and large subunit ribosomal RNA genes of *S. sinuspaulianus*. The approximate limits of the two genes and their orientations (arrow points 3') were determined by hybridization of heterologous gene probes from *Saccharomyces cerevisiae*. The dotted 5' end of the arrow indicates weak hybridization of the 15S probe in this region. Homologous clones used as probes to construct the maps are illustrated below the genes. The cross-hybridizing regions in other species were also mapped with these probes. The areas that are relatively size-conserved among the 15 species of *Suillus* (top size-conserved zone) and within the four species outside the genus relative to *Suillus* (bottom size-conserved zone) are indicated below each map. The locations of two large introns (I-1 and I-2) present in the LrRNA gene of several *Suillus* species are shown. Four regions that exhibit size variations of 10–30 bp within the *Suillus* genes are indicated as size-variable zones (SVZ). Three of the four size-variable zones had two discrete size classes in the species of *Suillus* examined, whereas SV2 in the SrRNA gene exhibited nearly continuous size variation. Restriction site abbreviations: AvaI (A), BamHI (B), ClaI (C), HindIII (D), BstEII (E), HaeIII (H), CfoI (O), EcoRI (R), PvuII (U), AvaII (V).

tinct and that have mtDNAs of vastly different sizes also suggests that intraspecific variation is minor.

Intraspecific variation in intron content of mitochondrial genes is, however, a common phenomenon in fungi (Collins and Lambowitz 1983; Jacquier and Dujon 1983). Nevertheless, no size variation was found in the LrRNA gene region of *Neurospora crassa*, which contains one intron (Taylor et al. 1986). In *Suillus* both LrRNA introns occur in the two isolates of *S. viscidus* and three of *S. luteus* examined, but we have examined only single strains of other species. In the phylogenetic analysis both introns are consistent characters (Fig. 8). When they are removed from the analysis, the number of equally parsimonious trees increases significantly, but none of the 200 trees examined postulated convergent gain of intron 1. Hence, in *Suillus* this intron appears to be a reliable phylogenetic trait. Without
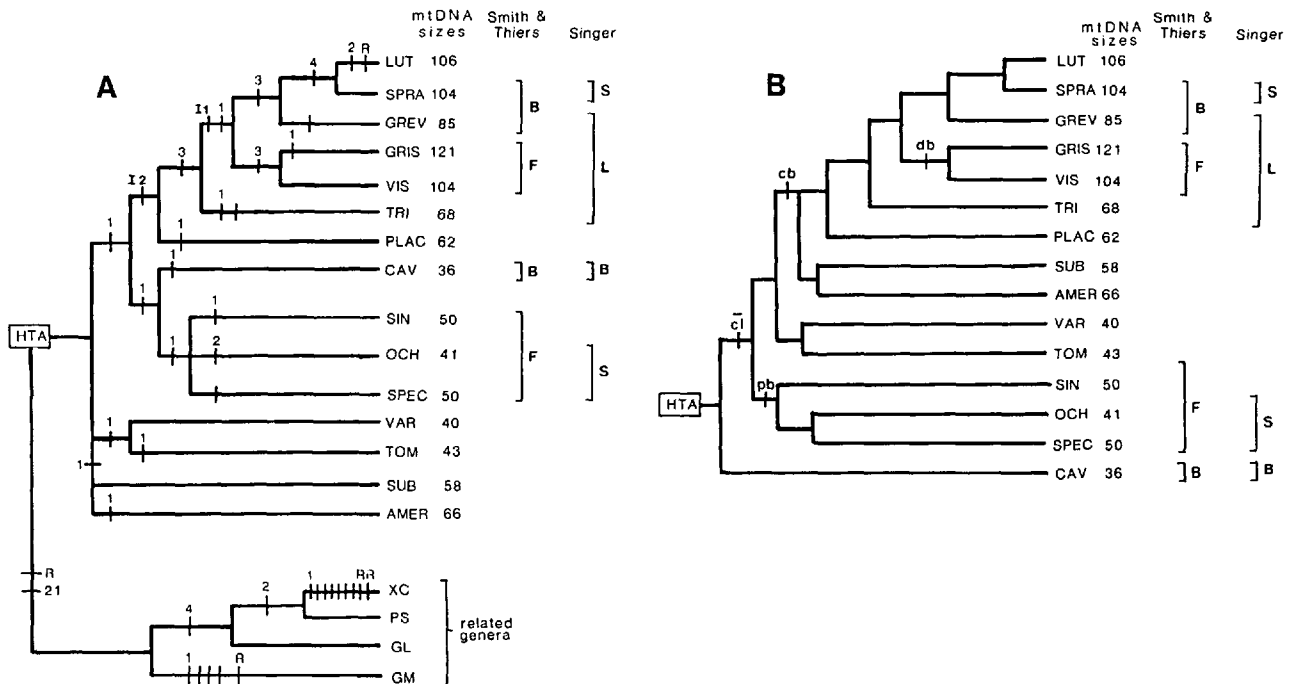
Fig. 8. Phylogenetic analyses of restriction-site and size differences within the mitochondrial ribosomal RNA gene regions. The cladistic analyses were based upon the characters described in Table 4. Relationships depicted within *Suillus* result from ancestor-rooted analyses using a hypothetical ancestor (HTA) described in the text. Sizes of the mitochondrial genomes were not included in the analysis, but are given in kilobase pairs after the species abbreviations. Supraspecific taxa discussed in the text are indicated by brackets to the right. Abbreviations are: Smith and Thiers = *Suillus* section *Boletinus* (B), *Fuscoboletinus* (F); Singer = *Suillus* sect. *Solidipes* (S), *Suillus* section *Larigni* (L), *Boletinus* (B). Species abbreviations are given in Table 1. **A** Cladistic analysis based only on molecular characters. The *Suillus* portion of the tree shows the strict consensus of 15 equally parsimonious trees requiring 32 steps. The character-state changes are shown with numbered bars. Unnumbered bars indicate additional changes, unique to a single species, that were not included in the analysis. The gain of the two introns in the large subunit ribosomal RNA gene are shown (I1 and I2). The branch containing the related genera is attached at the root of the *Suillus* tree as identified by ancestor rooting. Relationships shown among the related genera and distance to *Suillus* are those found with the initial outgroup-rooted analysis. Five rearrangements (R) of the mitochondrial genome postulated in Figs. 3 and 5 can be added directly to this tree, and account for the distribution of the five fragment orders found if the alternative pathway between the orders of *Xerocomus* (XC) and *Paragyrodon* (PS) is assumed (Fig. 5). **B** Most parsimonious tree found after addition of two morphological characters. Distributions of the four major spore colors, olivaceous brown (ob), purplish brown (pb), cinnamon brown (cb), and dull brown (db), and the presence of clamp connections (cl) within basidiocarps can be explained as single unique changes in this cladogram. This tree is also the most parsimonious if one assumes that purplish brown and dull brown are identical colors, as proponents of *Fuscoboletinus* have argued (Pomerleau and Smith 1962), but it then postulates convergent gain of that color. An additional loss of restriction site 38 is the only difference in a molecular character in this tree compared to tree A. The distribution of the latter restriction site is highly convergent in both trees (see text). Spore color was coded as an unordered multistate character. The hypothetical ancestor of *Suillus* is assumed to have clamp connections in the basidiocarp and an olivaceous brown spore color because both character states occur in species of *Gyrodon* and *Paragyrodon*. The minus sign above cl indicates loss of the character state.

intron 2 in the analysis, the position of *S. placidus* is variable, and, as a result, a convergent gain of this intron is frequently postulated.

Discussion

Molecular Evolution of the Mitochondrial Genome

The nearly continuous array of mtDNA sizes within *Suillus* and the roughly uniform distribution of size changes throughout most regions of the molecule (Fig. 3) are consistent with the theory of Bruns et al. (1988) that large interspecific differences result from the accretion of numerous smaller length mutations. This model would predict that closely related species should have similar-sized mtDNAs. Our phylogenetic analyses are consistent with this prediction (Fig. 8a and b).

MtDNA in many *Suillus* species is much larger than that found in species of other genera studied (36–121 vs 35–52). This observation suggests that either the largest mtDNAs in *Suillus* have expanded, or the small molecules are the products of convergent loss of sequences. The phylogenetic analyses are most consistent with the former hypothesis, because all species of *Suillus* with mtDNAs larger than

80 kb are represented as a monophyletic group (Fig. 8a and b). Expansion of the regions hybridizing to clones 1 and 2 and expansion between the regions hybridizing to probe fragments 7 and 8 and 15 and 16 appear to account for much of the increase (Fig. 3). Inclusion of the introns from the LrRNA in the phylogenetic analysis accounts for only a minor portion of the size increase, but does have the indirect effect of strengthening recognition of the clade as a monophyletic lineage.

Restriction-site differences within the LrRNA region reveal a low rate of nucleotide divergence relative to size change in the genome. Within group 1, mtDNAs vary in size almost twofold (36–66 kb), whereas nucleotide divergence in the LrRNA region is less than 1%. This low level of divergence within group 1 appears to be caused by retention of a primitively shared set of restriction sites (Fig. 8). The greatest divergence within *Suillus* occurs within a single lineage (the *S. tridentinus–S. luteus* clade, Fig. 8), in which the genome size has risen substantially. These results suggest unequal rates of mtDNA sequence divergence within *Suillus*.

Small size differences are common within the regions of both rRNA genes in *Suillus*. These are comparable in size to the 40- and 60-bp mini-inserts found within the mitochondrial rRNA genes of *Saccharomyces cerevisiae* (Dujon 1980; Sor and Fukuhara 1982). Sequences of the latter suggest a close relationship to the G–C clusters that are present as multiple dispersed copies in the yeast mitochondrial genome (Dujon 1980; Sor and Fukuhara 1982). Small length mutations have also been mapped in the nuclear 28S rRNA gene of anurans (Hillis and Davis 1987).

The occurrence of two large introns in the mitochondrial LrRNA gene of several *Suillus* species is noteworthy. Many fungi have a single intron near the 3' end of the gene (Heckman and RajBhandary 1979; Lazarus et al. 1980; Jacquier and Dujon 1983), but two introns have been reported previously only in *Podospora anserina* (Wright and Cummings 1983). Our phylogenetic analyses suggest that the largest and most 5' of the *Suillus* introns has been acquired uniquely.

Large-scale rearrangements of the mitochondrial genome are clearly the least frequent molecular evolutionary events in these taxa. We have made this point elsewhere with respect to the order of eight mitochondrial genes in five species of *Suillus* (Bruns et al. 1988). We can now expand this conclusion to 16 hybridizing regions of the molecule in 15 species of *Suillus* and 4 species of related genera. The low rate of rearrangement is especially surprising given the large and variable sizes of the genomes. This situation contrasts with the high frequency of rear-

rangements in plant mtDNA (Palmer and Herbon 1986).

The five fragment orders that exist among the fungi studied are potentially interconvertible by five rearrangements. These include two inversions, two transpositions, and one additional inversion or transposition (Figs. 3 and 5). All of the rearrangements include or border the probe 12 region. This correlation may be coincidental, but if not it implies that this region is more prone to both inversions and transpositions than other parts of the molecule.

Phylogenetic Inferences

The mtDNAs of *Suillus* sensu lato are clearly monophyletic with respect to the outgroups chosen. This conclusion relates directly to a controversy concerning the relationship of *Paragyrodon* to *Suillus*. Smith and Thiers (1971) considered *Paragyrodon* to belong in *Suillus*. Watling (1969) maintained the monotypic genus *Paragyrodon* but transferred it to the subfamily Suilloideae. Our results strongly support the classification of Singer (1986), who places the species in the subfamily Gyrodontoideae with *G. lividus* and *G. merulioides*. The monophylesis of *Suillus* (inclusive of *Fuscoboletinus* and *Boletinus*) also agrees with Singer's (1986) concept of the Suilloideae, but conflicts with the classification of Pegler and Young (1981), who place *Suillus* and *Boletinus* in different families.

The relatively low nucleotide divergence (ca. 3%) between *Xerocomus* and either *Paragyrodon* or *G. lividus* demonstrates that it is related to the other members of the Boletaceae studied. This conclusion agrees with most previous taxonomic treatments of the family, but does conflict with Heim's (1971) theory of a polyphyletic origin of the Boletaceae.

Fuscoboletinus was identified as polyphyletic in all of our trees. This result stems from the significant divergence of *S. (Fuscoboletinus) viscidus* and *S. (Fuscoboletinus) grisellus* from the three other species that are placed in *Fuscoboletinus* (Fig. 8b). These two species groups of *Fuscoboletinus* share no uniquely derived molecular characters and are separated by a minimum of seven character-state changes in both the outgroup and ancestor-rooted trees (Fig. 8a and b). Five of the seven characters are completely consistent within all of these trees, but one would be forced to postulate convergence or reversal for all five characters in any tree in which *Fuscoboletinus* is depicted as monophyletic. Furthermore, the four species of section *Larigni* appear to be related by a common ancestor. The grouping of these species into a common lineage is significant because the section is pivotal to the view of Singer

and others that *Fuscoboletinus* is polyphyletic (Singer et al. 1963; Singer 1986).

Our data provide little resolution of relationships within group 1 (Fig. 8), primarily because of the low level (<1%) of nucleotide sequence divergence between species in the group (Table 3). The inclusion of species from both *Boletinus* and *Fuscoboletinus* in this group, however, underlines the close relationship of these genera to *Suillus* sensu stricto and suggests that the utility of recognizing the former two genera is questionable.

Suillus luteus and *S. spraguei* were consistently identified as a monophyletic group in all our analyses. This result finds no support in any existing classification and remains an anomaly in need of further study.

Further resolution of the phylogenetic relationships within these fungi will clearly require additional data. Mapping of more restriction sites is one possibility, but recent advances in direct sequencing with the polymerase chain reaction may provide a better alternative for rapid acquisition of phylogenetically informative molecular data (Saiki et al. 1988).

References

Bonitz SG, Coruzzi G, Thalenfeld BE, Tzagoloff A, Macino G (1980) Assembly of the mitochondrial membrane system: structure and nucleotide sequence of the gene coding for subunit 1 of yeast cytochrome oxidase. J Biol Chem 255:11927–11941

Bruns TD, Palmer JD, Shumard DS, Grossman GI, Hudspeth MES (1988) Mitochondrial DNAs of *Suillus*: three-fold size change in molecules that share a common gene order. Curr Genet 13:49–56

Collins, RA, Lambowitz AM (1983) Structural variations and optional intron in the mitochondrial DNA of *Neurospora* species isolated from nature. Plasmid 9:53–70

Dujon B (1980) Sequence of the intron and flanking exons of the mitochondrial 21S rRNA gene of yeast strains having different alleles at the omega and rib-1 loci. Cell 20:185–187

Heckman JE, RajBhandary UL (1979) Organization of tRNA and rRNA genes in *N. crassa* mitochondria: intervening sequence in the large rRNA gene and strand distribution of the RNA genes. Cell 17:583–595

Heim R (1971) The interrelationships between the Agaricales and the Gastromycetes. In: Petersen RH (ed) Evolution in the higher Basidiomycetes. University of Tennessee Press, Knoxville, pp 505–534

Hensgens LAM, Bonen L, de Haan M, van der Horst G, Grivell LA (1983) Two intron sequences in yeast mitochondrial COX1 gene: homology among URF-containing introns and strain-dependent variation in flanking exons. Cell 32:379–389

Hillis DM, Davis SK (1987) Evolution of the 28S ribosomal RNA gene in anurans: regions of variability and their phylogenetic implications. Mol Biol Evol 4:117–125

Jacquier A, Dujon B (1983) The intron of the mitochondrial 21s rRNA gene: distribution in different yeast species and sequence comparison between *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. Mol Gen Genet 192:487–499

Lazarus CM, Lunsdorf H, Han U, Stepien PP, Kuntzel H (1980) Physical map of *Aspergillus nidulans* mitochondrial gene coding for ribosomal RNA: an intervening sequence in the large rRNA cistron. Mol Gen Genet 177:389–397

Macino G, Tzagoloff A (1979) Assembly of the mitochondrial membrane system: the DNA sequence of a mitochondrial ATPase gene in *Saccharomyces cerevisiae*. J Biol Chem 254:4617–4623

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor NY

Marsh JL, Erfle M, Wykes EJ (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene 32:481–485

Murray MC, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

Nei M, Li W (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

Palmer JD, Herbon LA (1986) Tricircular mitochondrial genomes of *Brassica* and *Raphanus*: reversal of repeat configurations by inversion. Nucleic Acids Res 14:9744–9764

Pantidou ME, Groves JW (1966) Cultural studies of Boletaceae: some species of *Suillus* and *Fuscoboletinus*. Can J Bot 44:1371–1392

Pegler DN, Young TWK (1981) A natural arrangement of the Boletales with reference to spore morphology. Trans Br Mycol Soc 76:103–146

Pomerleau R, Smith AH (1962) *Fuscoboletinus*, a new genus in the Boletaceae. Brittonia 14:156–172

Rohlf FJ (1982) Consensus indices for comparing classifications. Math Biosci 59:131–144

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Highuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Sanders JPM, Heyting C, Verbeet MP, Meijlink CPW, Borst P (1977) The organization of genes in yeast mitochondrial DNA. III. Comparison of the physical maps of the mitochondrial DNAs from three wild-type *Saccharomyces* strains. Mol Gen Genet 157:239–261

Singer R (1986) The Agaricales in modern taxonomy, ed 4. Koeltz Scientific Books, Koenrgstein

Singer R, Snell WH, Dick E (1963) The genus *Fusoboletinus*. Mycologia 55:352–357

Smith AH, Thiers HD (1971) The Boletaceae of Michigan. University of Michigan Press, Ann Arbor

Sor F, Fukuhara H (1982) Nature of an inserted sequence in the mitochondrial gene coding for the 15S ribosomal RNA of yeast. Nucleic Acids Res 10:1625–1633

Swofford DL (1985) PAUP—phylogenetic analysis using parsimony version 2.4. User's manual. University of Illinois, Urbana

Taylor JW, Smolich BD, May G (1986) Evolution and mitochondrial DNA in *Neurospora crassa*. Evolution 40:716–739

Templeton AR (1983a) Phylogenetic inferences from restriction endonuclease cleavage site maps with particular reference to evolution of humans and apes. Evolution 37:221–224

Templeton AR (1983b) Convergent evolution and nonparametric inferences from restiction data and DNA sequences. In: Weir BS (ed) Statistical analysis of DNA sequence data. Marcel Dekker, New York, pp 151–180

Watling R (1969) The genus *Paragyrodon*. Notes R Bot Gard Edinb 29:67–73

Waring RB, Davies RW, Scazzacchio C, Brown TA (1982) Internal structure of a mitochondrial intron of *Aspergillus nidulans*. Proc Natl Acad Sci USA 79:6332–6336

Wright RM, Cummings DJ (1983) Mitochondrial DNA from *Podospora anserina* IV. The large ribosomal RNA gene contains two long intervening sequences. Curr Genet 7:151–157