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Ribosomal DNA and Resolution of Branching Order among the Ascomycota: How Many Nucleotides Are Enough?

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Molecular phylogenies for the fungi in the Ascomycota rely heavily on 18S rRNA gene sequences but this gene alone does not answer all questions about relationships. Particularly problematical are the relationships among the first ascomycetes to diverge, the Archiascomycetes, and the branching order among the basal filamentous ascomycetes, the Euascomycetes. Would more data resolve branching order? We used the jackknife and bootstrapping resampling approach that constitutes the "pattern of resolved nodes" method to address the relationship between number of variable sites in a DNA sequence alignment and support for taxonomic clusters. We graphed the effect of increasing sizes of subsamples of the 18S rRNA gene sequences on bootstrap support for nodes in the Ascomycota tree. Nodes responded differently to increasing data. Some nodes, those uniting the filamentous ascomycetes for example, would still have been well supported with only two thirds of the 18S rRNA gene. Other nodes, like the one uniting the Archiascomycetes as a monophyletic group, would require about double the number of variable sites available in the 18S gene for 95% neighbor-joining bootstrap support. Of the several groups emerging at the base of the filamentous ascomycetes, the Pezizales receive the most support as the first to diverge. Our analysis suggests that we would also need almost three times as much sequence data as that provided by the 18S gene to confirm the basal position for the Pezizales and more than seven times as much data to resolve the next group to diverge. If more data from other genes show the same pattern, the lack of resolution for the filamentous ascomycetes may indicate rapid radiation within this clade.

INTRODUCTION

Molecular phylogenetic studies of the Ascomycota up to the present have relied heavily on 18S ribosomal RNA gene sequences. The gene sequences consistently show that the Euascomycetes form a monophyletic group consisting of filamentous ascomycetes with fruiting bodies. The Euascomycetes includes some of the most familiar and economically important ascomycetes—*Penicillium*, *Peziza*, *Neurospora*, and so on. This cluster of taxa includes the most developmentally complex species in the Ascomycota. A well-resolved phylogeny for the branching order at the base of this group would provide a basis for understanding the pattern of evolution of morphological complexity. However, this area in the phylogeny is far from well resolved. The operculate cup fungi of the Pezizales appear at the base of the filamentous ascomycetes in some studies (Gargas *et al.*, 1995; Landvik *et al.*, 1996), whereas *Penicillium* and allied genera, or *Neurospora* along with other pyrenomycetes and loculoascomycetes, are basal in others (Berbee and Taylor, 1993; Spatafora, 1995).

Resolving the branching order at the base of the Ascomycota would contribute to revealing the characters of the very first ascomycetes. One of the surprises from rRNA-based molecular systematics was finding a morphologically heterogeneous cluster of species, the Archiascomycetes, at the base of the Ascomycota (Berbee and Taylor, 1992, 1993; Nishida and Sugiyama, 1993, 1994; Gargas *et al.*, 1995; Landvik, 1996; Sjamsuridzal *et al.*, 1997; Sugiyama, 1998). The Archiascomycetes vary from *Neolecta*, a filamentous genus making complex fruiting bodies, to *Taphrina*, a genus of obligate plant parasites with alternating hyphal and yeast stages, to *Pneumocystis carinii*, a human lung parasite, to *Schizosaccharomyces*, a genus of saprophytic fission yeasts. Based on morphology, no one would have proposed grouping these fungi together against the other ascomycetes. Based on rRNA sequence data, the Archiascomycetes appear monophyletic in some studies (Berbee and Taylor, 1992, 1993; Nishida and Sugiyama, 1993; Sjamsuridzal *et al.*, 1997; Sugiyama, 1998) and paraphyletic in others (Gargas *et al.*, 1995; Landvik *et al.*, 1996).

More data from other genes may provide more reso-

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lution for Archiascomycete and Euascomycete nodes. However, before we begin another expensive and time-consuming round of sequencing, we wanted to estimate whether more data would be likely to solve the problems. For some questions, perhaps the 1750 or so base pairs of sequence data in the 18S rRNA gene are not quite enough but adding an equal amount of similar sequence data for the same taxa may provide the resolution. On the other hand, if the lack of resolution of branching order was due to very rapid radiation of the lineages, then perhaps no amount of data would provide answers.

Lecointre *et al.* (1994) and Philippe *et al.* (1994) proposed a resampling approach for estimating how much more data would be needed for a statistically supported answer to a phylogenetic question. They initially resampled from an aligned sequence data set without replacement to generate jackknife subsets of various lengths. They then analyzed each jackknife data subset using neighbor-joining bootstrapping. For nodes that represented well-supported evolutionary events, increasing the amount of sequence data in the jackknife samples also increased the average neighbor-joining bootstrap support for the node. Lecointre *et al.* (1994) suggested that the bootstrap support "pattern for resolved nodes" or PRN, increases with increasing data following a curve of the formula

$$f(x) = k(1 - e^{-b(x - x')}) \quad (1)$$

In this equation, $f(x)$ is the bootstrap percentage for a node that resulted from 1000 neighbor-joining replicates of a jackknife sample, where x is the number of nucleotides that were sampled in the jackknife replicate; k is the asymptote of the curve, equal to 100% for a fully resolved node reaching 100% bootstrap support; b is a shape parameter determining how quickly the curve arrives at 100%, and x' is the x intercept for the curve. Using nonlinear regression, values for x', k, and b can be estimated by fitting the resampling results to the equation for the curve. If k is assumed to be 100%, then the equation above can be rearranged and estimates of b can be used to predict x, the number of nucleotides that would be needed to reach a specified bootstrap level (Lecointre *et al.*, 1994; Philippe *et al.*, 1994; Steiner and Müller, 1996). If the specified bootstrap level is 95%, for example,

$$x = x' - (1/b)\ln(1 - (95\%/100\%)) \quad (2)$$

Like the divergence order of the filamentous ascomycetes, the divergence order of the first multicellular animals has been a difficult problem in molecular phylogenetics. Philippe *et al.* (1994) estimated that the coelomate protostomes (annelids, arthropods, etc.) could be established as a monophyletic group with 99% neighbor-joining bootstrap support with about twice the number of variable nucleotides available from 18S rRNA gene sequences. To establish the deuterostomes (echinoderms + chordates) as a monophyletic group would, they estimated, require more than three times the amount of data available from the 18S genes. They introduced the stimulating (but controversial; see Wray *et al.*, 1996; Morris, 1997; Ayala *et al.*, 1998) argument that the lack of metazoan resolution reflected rapid radiation. Indicating general interest in the "rapid radiation" problem, their approach was reviewed in *Nature* (News and Views) (Gee, 1995).

In this paper, we apply the theory of the pattern of resolved nodes to two phylogenetically difficult problems for the Ascomycota. First, would more data support the monophyly of the Archiascomycetes and second, can the branching order of the first filamentous ascomycetes ever be resolved?

MATERIALS AND METHODS

This study is based on a data set including 34 complete or nearly complete 18S rRNA gene sequences from GenBank (Table 1). Five of the sequences are outgroup sequences representing the three classes of the Basidiomycota. The remaining sequences are from ascomycetes, including a broad range of Archiascomycetes and Euascomycetes. The number of taxa in the data set was a compromise between our desire to include as many lineages as possible and the PRN programs' high computer processor time and disk storage requirements. We aligned the sequences manually. All sites are included in the analysis and we ignored gaps. For comparison with the PRN results, we used PAUP* version 4.0b2a (Swofford, 1999) to perform 500 parsimony bootstrap replicates using the heuristic search with tree bisection and reconnection options. Using PAUP, we also performed 2000 neighbor-joining bootstrap replicates with a Kimura two-parameter correction for multiple hits and a transition/transversion rate of two.

We used the PRN programs to perform 2000 jackknife replicates of the 707 variable sites in the 18S data set. Each of the first 200 replicates included 25 sites randomly sampled from the original 707 sites; the second 200 replicates each included 50 sites; the third 200 replicates included 75 sites; the fourth included 100 sites and so on, increasing by increments of 50 sites for a total of 10 different sequence lengths up to a maximum of 400 sites. The 400-site maximum represented about 60% of the total sites. Increasing the number of sites further would have had the undesirable effect of also increasing the chance of sampling the same sites in all of the replicates. The PRN program cluster then performed neighbor-joining bootstrap replicates using distances calculated with a Kimura two-parameter correction and transition/transversion ratio of two. For

TABLE 1

Sequences Included in Phylogenetic Analysis

Taxon	Accession No.
Ascomycota	
Ascobolus lineolatus	L37533
Capronia masonni	X79318
Ceramothyrium linnaeae	AF022715
Debaryomyces hansenii	X62649
Dipodascopsis uninucleata	U00969
Dothidea hippophaës	U42475
Eurotium rubrum	U00970
Gyromitra esculenta	U42648
Helvella lacunosa	U42654
Herpotrichia diffusa	U42484
Hypomyces chrysospermus	M89993
Lecanora dispersa	L37535
Neolecta irregularis	Z47721
Neolecta vitellina	Z27393
Neurospora crassa	M11033
Pleospora herbarum	U05201
Pneumocystis carinii	L27658
Protomyces inouyei	D11377
Protomyces macrosporus	D85143
Rhytisma salicinum	U53370
Saccharomyces cerevisiae	V01335
Saitoëlla complicata	D12530
Schizosaccharomyces japonicus	Z32848
Schizosaccharomyces pombe	X54866
Sclerotinia sclerotiorum	X69850
Spathularia flavida	Z30239
Taphrina deformans	U00971
Urnula hiemalis	Z49754
Xylaria carpophila	Z49785
Basidiomycota	
Boletus satanas	M94337
Leucosporidium scottii	X53499
Sporobolomyces roseus	X60181
Tilletia caries	U00972
Udeniomyces puniceus	D31658

the 2000 new data subsets, the program made and analyzed 2,000,000 bootstrap replicates. The PRN programs, running on a modest Silicon Graphic IRIX R4000 computer with 32 MB of RAM, required 12 days for this analysis.

Different nodes appeared in the different replicates and particularly when the number of sites sampled was small, most of the nodes appearing in the bootstrapping were found very infrequently. Nodes that appeared in fewer than 1% of the bootstrap replicates and nodes appearing in fewer than 1000 of the jackknifed data sets were dropped from further analysis. This left 92 nodes for examination.

We selected nodes for further study that were related to the monophyly of the Archiascomycetes and the branching order of the basal filamentous ascomycetes. For selected nodes, we fit the predicted curve to a scatter plot of bootstrap percentages for each length of sites sampled using SPSS for Windows Advanced Statistics Release 6.0. The nonlinear regression relied on an iterative approach (unlike the exact approach that is possible in linear regression) to optimize the values of x', k, and b by minimizing the sum of squares of the residuals. We used the default Levenberg–Marquardt estimation.

How many nucleotides would be necessary to arrive at 95% bootstrap support for a given node, assuming that the node could be fully resolved? In mathematical terms, finding out required assuming that $k = 100\%$ and estimating the values for b. Using the estimate of b, we could then estimate the corresponding number of nucleotides needed for the 95% bootstrap support level.

RESULTS

The 92 PRN nodes included all the 25 resolved nodes present in a neighbor-joining bootstrap consensus tree (Fig. 1). As expected, both bootstrap analysis and PRN analysis strongly support such phylogenetic features as (1) the separation of the Ascomycota from the Basidiomycota, (2) the monophyly of the filamentous ascomycetes (Euascomycetes), (3) the monophyly of the true yeasts (Hemiascomycetes), and (4) the monophyly of the pyrenomycete clade with *Xylaria* and *Neurospora* (Fig. 1, Table 2). According to the PRN estimate, the pyrenomycete clade would still receive 95% bootstrap support with only 235 of the variable 707 sites in the data set (Fig. 2A, Table 2). The monophyly of the Hemiascomycetes would be supported at the 95% level with only 424 of the 707 sites (Fig. 2B, Table 2). Although we have not presented the graphs for the other groups receiving 100% bootstrap support in the neighbor-joining analysis, they show similar patterns of clear increase in bootstrap support with increasing data.

The node at the base of the Archiascomycetes received 90% bootstrap support from neighbor-joining and 59% bootstrap support from parsimony (Fig. 1). Depending on the particular jackknife sampling, the bootstrap support for this node varied widely, from 5 to 94% (Figs. 3A and 3B). Using Eq. (2) above, we predicted that about 700 additional variable sites per species, or an additional 1700 or so base pairs of data per species, with a level of variability similar to the 18S sequences, would be required to give this node 95% bootstrap support.

The PRN analysis offers little encouragement for resolving the divergence order at the base of the Archiascomycetes. Of the 92 nodes retained from PRN analysis, 4 placed different taxa at the base of the Archiascomycetes. No single arrangement received strong support, although the highest level of average PRN bootstrap support for any node was for placing *Pneumocystis* at the base (Table 2). Assuming that this phylogenetic position was correct, we calculated that for 95% bootstrap support, about 5800 more variable sites per species would be needed (Table 2).

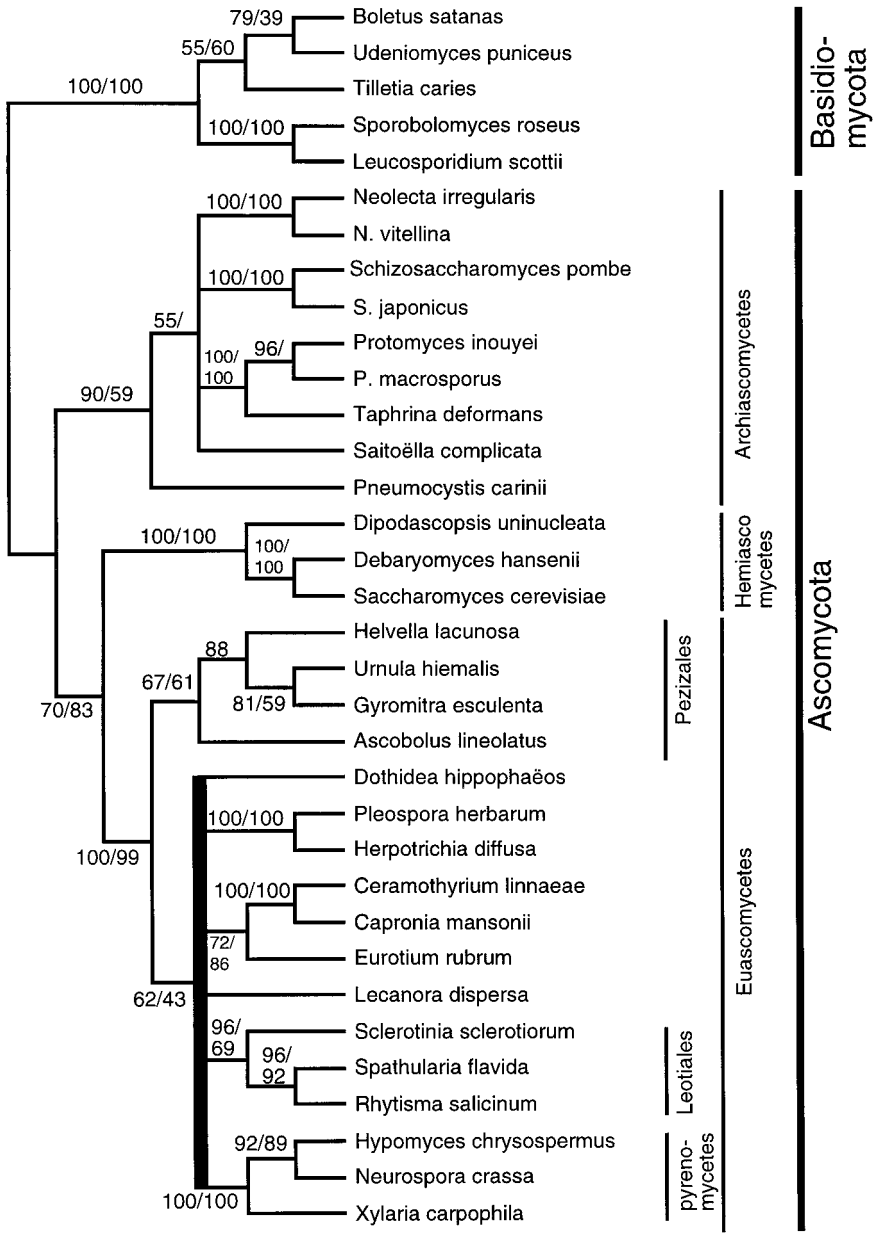


FIG. 1. Neighbor-joining consensus tree from 2000 bootstrap replicates performed with PAUP*. The first number of each pair is the neighbor-joining bootstrap percentage; the second is the parsimony bootstrap percentage from 500 searches. When parsimony and neighbor-joining consensus trees were in conflict, only the neighbor-joining percentage is given. The unresolved polytomy at the base of the Euascomycetes is in bold.

Of the 92 nodes retained from PRN analysis, 5 involved rearrangements at the base of the Euascomycetes. The highest level of average PRN bootstrap support was captured by a node separating the cup fungi in the Pezizales from the other fungi with fruiting bodies. This node that places the Pezizales in the basal position received 62% neighbor-joining and 43% parsimony bootstrap support (Fig. 1). Would more data produce 95% bootstrap support for this node? The PRN analysis shows that increasing the size of the subsampled data set increased the average bootstrap support for this node (Figs. 3C and 3D). Assuming that the maximum bootstrap support with infinite data would be 100%, about 2000 more variable sites would be required for 95% bootstrap support. That translates into a requirement for about 4900 more base pairs of sequence data that show a level of variability similar to the 18S sequences (Table 2). Apart from the possible basal divergence of the Pezizales, none of the potential early Euascomycetes divergences received support. For

TABLE 2

Estimates of Additional Data Required for 95% Bootstrap Support for Specified Nodes[a]

Taxa united in node	*b*	*x′*	Required no. of variable sites for 95% bootstrap support: Average	95% Confidence interval	Required no. of nucleotides for 95% support	How many more nucleotides/ species?
Pyrenomycetes	0.013	11.48	235	225–246	582	
Euascomycetes	0.0073	34.97	448	435–461	1108	
Hemiascomycetes	0.0073	11.77	424	408–442	1051	
Archiascomycetes	0.0022	33.8	1408	1359–1467	3485	1735
Pezizales as basal euascomycetes	0.0011	44.17	2672	2541–2818	6614	4864
Pezizales + Leotiales + Rhytisma basal	0.0005	56.9	6171	5818–6569	15274	13524
Leotiales + Rhytisma	0.0004	44.5	7826	7298–8436	19370	17620
Pneumocystis basal in Archiascomycetes	0.001	14.4	3044	2868–3250	7535	5785

[a] Estimates of *b* and *x′* are used in calculating the number of nucleotides needed for 95% bootstrap support using Eq. (2).

example, a node representing the hypothesis that another cluster of cup fungi, including the genera *Rhytisma* and *Sclerotinia*, diverged after the Pezizales received only 25% neighbor-joining and 26% parsimony bootstrap support. The average PRN bootstrap support increased with increasing data (Figs. 3E and 3F) but with considerable scatter. Assuming that the node could be supported at the 100% level with infinite data, about 5500 more variable sites, more than seven times as much data as provided by the 18S genes, would be required to achieve 95% bootstrap support (Table 2).

DISCUSSION

Bootstrapping predicts whether other, similar data would resolve the same nodes in a tree. The PRN model goes a step further and predicts how many more nucleotides would be required for a specified level of bootstrap support for a node, assuming that the additional

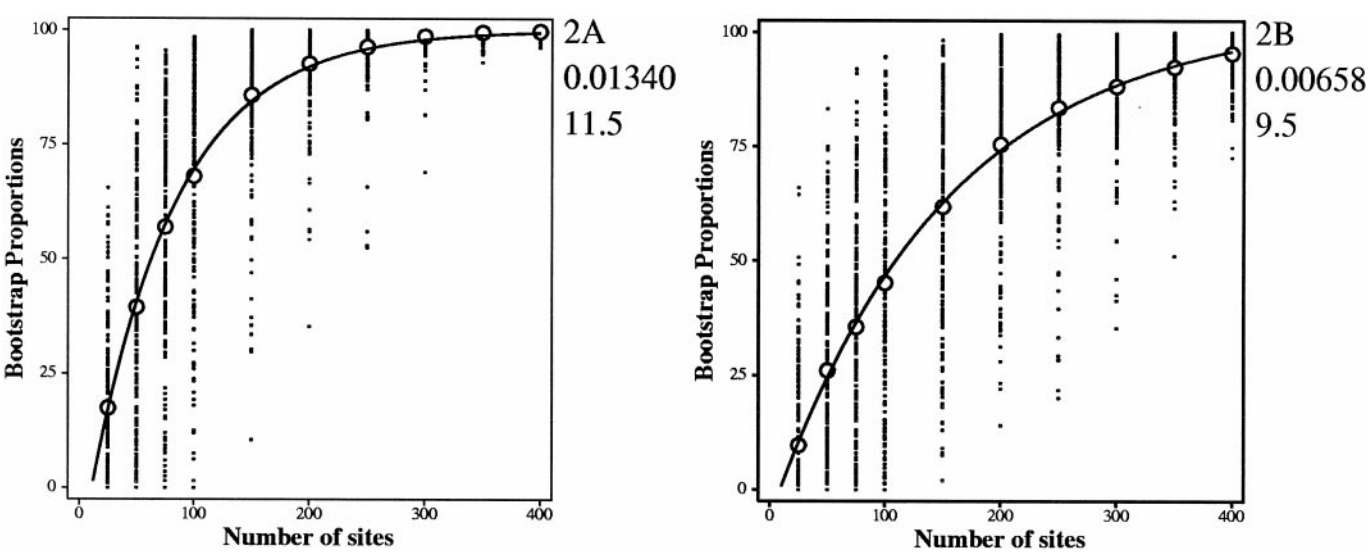
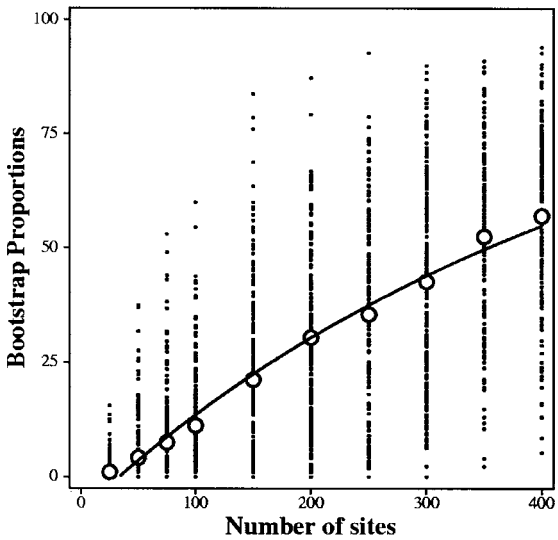
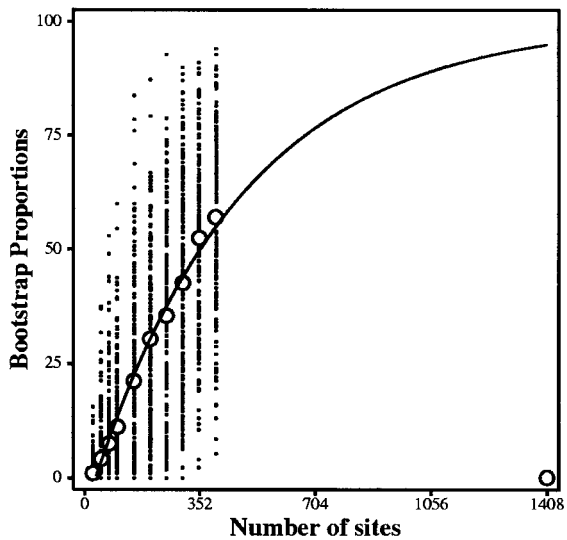


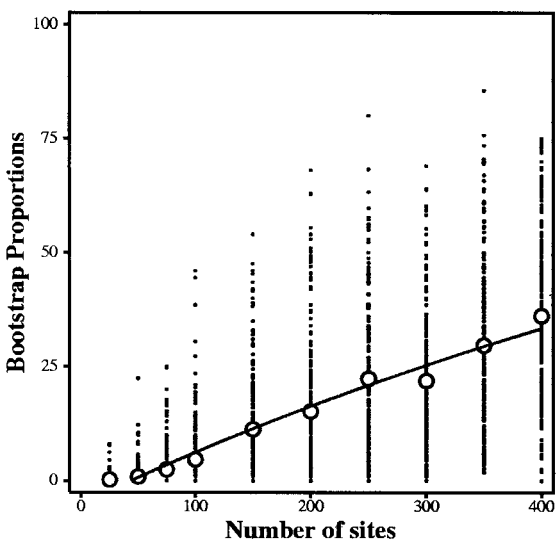
FIG. 2. Scattergrams of the effect of increasing the number of sites in jackknife samples on bootstrap support for selected nodes. (A) The pattern of support for the well-resolved node uniting pyrenomycetes including *Neurospora* and *Xylaria*. (B) The node uniting the true yeasts in the Hemiascomycetes. Each point represents the neighbor-joining bootstrap percentage or $f(x)$ for the node from one jackknife subsample. The open circle is the mean bootstrap support for the 200 jackknife data subsets of the specified length. The values of the shape parameter *b* and the *x* axis intercept *x′* are given below the figure numbers. The curve is fitted based on Eq. (1): $f(x) = k(1 - e^{-b(x-x')})$.



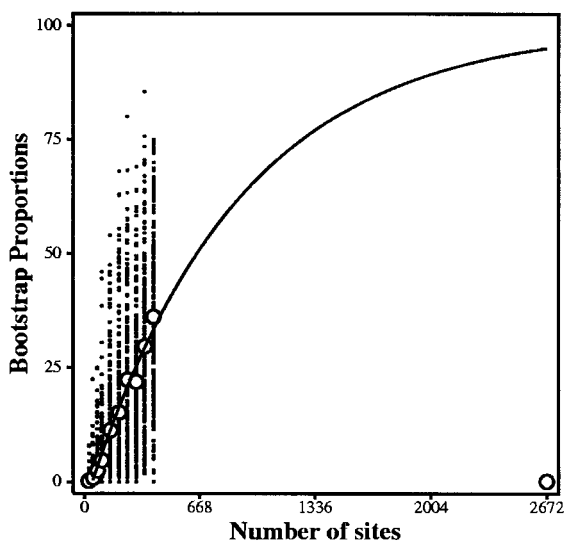
3A
0.00217
33.8



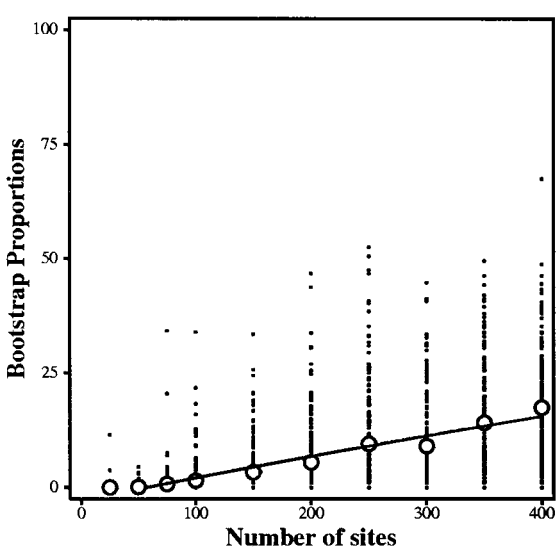
3B
0.00217
33.8



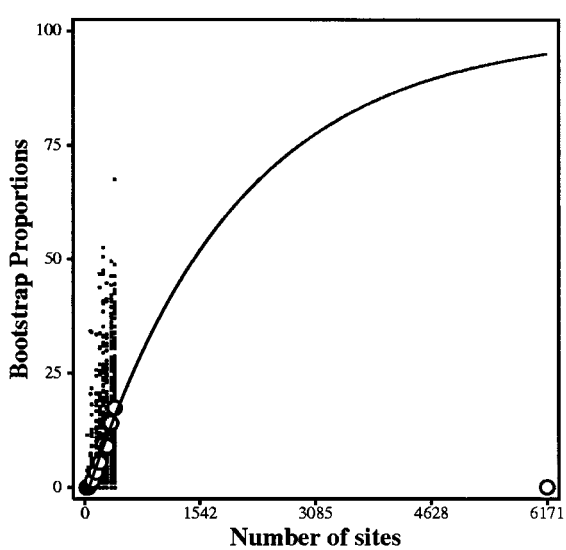
3C
0.00114
44.2



3D
0.00114
44.2



3E
0.00049
56.9



3F
0.00049
56.9

nucleotides follow the same evolutionary patterns. However, characteristics specific to a particular gene, or a particular data set, including heterogeneity in substitution rates among taxa and taxon sampling, may determine whether specific nodes can be resolved. Our analysis offers specific predictions. We hope our predictions will be challenged by new data sets with more taxa and other genes.

Among unsolved phylogenetic problems, establishing the monophyly of the Archiascomycetes with statistical support may be relatively tractable. Support for the node uniting the Archiascomycetes is still in the steeply ascending phase, offering hope that more data will provide even clearer evidence for monophyly. Even for this node, however, the PRN analysis suggests that 95% bootstrap support will require almost as much additional data as is now represented by the 18S sequence alignment. Encouragingly, none of the conflicting nodes, placing the Archiascomycetes as a paraphyletic group, received strong support or responded to increasing data with strongly increasing bootstrap percentages.

Establishing the branching order of the first filamentous ascomycetes may be more difficult. Of the possible basal lineages, the 18S data provide most support for the Pezizales as the basal Euascomycetes. However, substantial sequencing effort would be required for clear support of the Pezizales in the basal position. We estimate that the approximately 4200 bp of sequence data from the combined 18S and 28S rRNA gene sequences would still fall short of providing 95% neighbor-joining bootstrap support for a basal position of the Pezizales among the filamentous ascomycetes. The 18S gene provides no information on the divergence order for the next lineages of the Euascomycetes. Indicating the lack of hope for resolution of branching order here, the support for the best of the possible nodes for this group of fungi increases only slightly with increasing data (Figs. 3C and 3D). If other genes reveal a similar pattern, this will offer a pessimistic prognosis for reconstructing branching orders among the Euascomycetes.

If the lack of phylogenetic resolution indicates rapid radiation of taxa, no amount of data will resolve the branching order. For multicellular animals, after calibration of the rate of accumulation of mutations, Philippe *et al.* (1994) suggested that the 18S rRNA gene resolves only branching events that occurred more than 40 Ma apart and that a radiation must have occurred over a shorter time to account for the lack of resolution of branching order. Assuming that the 18S rRNA genes have evolved faster in fungi than in animals (Doolittle *et al.*, 1996) or at similar rates in animals and fungi (Berbee and Taylor, 1993), the filamentous ascomycete radiation may also have occurred quickly.

If molecular phylogenetics cannot untangle the pattern of relationships among the first filamentous ascomycetes, then perhaps alternative questions could be addressed. Possibly, examining the fossil record will offer clues to whether, where, and when rapid radiation took place. As Taylor and Berbee (1993, 2000) have pointed out, most of the lineages of the filamentous ascomycetes produce diverse and abundant conidia. These conidia are tough structures that often survive as dispersed fossils capable of surviving treatment with strong acids that dissolve away surrounding rock. Showing that a fossil record for small fruiting bodies of filamentous ascomycetes may be found in very old, well-preserved plant leaves and stems, Taylor *et al.* (1999) described a possible pyrenomycete from the Lower Devonian. Lichens have been reported from the fossil record and a subset of these might be good indicators for the presence of Euascomycetes. Euascomycetes would probably be the fungal partners in fossilized lichens representing terrestrial associations between fungi with septate hyphae and algae, where the hyphae were in close proximity to the algae, and where the whole thallus grows attached to or appressed to a substrate. Finding evidence of rapid increase in numbers and diversity of fossilized conidia, fruiting bodies, and lichens should contribute to reconstructing early evolution among these fungi.

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FIG. 3. Pattern of response to increased sample size, for nodes that do not receive 95% bootstrap support with existing data. The curve is fitted based on Eq. (2), assuming that infinite data would give 100% bootstrap support: $x = x' - (1/b)\ln(1 - (95\%/100\%))$. The values of the shape parameter b and the x axis intercept x are given below the figure numbers. Scatterplots for nodes (A) and (B) show the pattern for the Archiascomycetes. The curve estimated from resampling existing data is shown in node (A) and the curve is extrapolated to show the amount of data necessary for 95% bootstrap support in (B). Based on this extrapolation, 1408 variable sites would be necessary for 95% bootstrap support for this node. Nodes (C) and (D) represent the assumption that the Pezizales were the first Euascomycetes to diverge. Again, the curve from existing data in (D) is extrapolated, assuming that the node would eventually receive 100% bootstrap support. The plots for nodes (E) and (F) represent the assumption that the clade with *Sclerotinia* and *Rhytisma* was the second Euascomycetes lineage, after the Pezizales, to diverge. The extrapolation in node (F) shows that much additional data would be required to generate strong bootstrap support for this node.

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