Phylogenetic Relationships Among Ascomycetes: Evidence from an RNA Polymerase II Subunit

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In an effort to establish a suitable alternative to the widely used 18S rRNA system for molecular systematics of fungi, we examined the nuclear gene RPB2, encoding the second largest subunit of RNA polymerase II. Because RPB2 is a single-copy gene of large size with a modest rate of evolutionary change, it provides good phylogenetic resolution of Ascomycota. While the RPB2 and 18S rDNA phylogenies were highly congruent, the RPB2 phylogeny did result in much higher bootstrap support for all the deeper branches within the orders and for several branches between orders of the Ascomycota. There are several strongly supported phylogenetic conclusions. The Ascomycota is composed of three major lineages: Archiascomycetes, Saccharomycetales, and Euascomycetes. Within the Euascomycetes, plectomycetes, and pyrenomycetes are monophyletic groups, and the Pleosporales and Dothideales are distinct sister groups within the Loculoascomycetes. We confirm the placement of Neoleotia within the Archiascomycetes, suggesting that fruiting body formation and forcible discharge of ascospores were characters gained early in the evolution of the Ascomycota. These findings show that a slowly evolving protein-coding gene such as RPB2 is useful for diagnosing phylogenetic relationships among fungi.

Introduction

The Ascomycota are a large and important group of fungi, distinguished from other fungi by a saclike ascus containing haploid ascospores (Alexopoulos, Mims, and Blackwell 1996). The Ascomycota encompass over 32,000 species, amounting to 40% of all described fungi (Hawksworth 1991; Honegger 1991; Hawksworth et al. 1995), and its members form symbiotic, parasitic, and saprobic associations with both animals and plants, as well as lichen associations with green algae and cyanobacteria (Alexopoulos, Mims, and Blackwell 1996). Ascomycetes present a challenge to taxonomists because few morphological characters appear to be phylogenetically informative. As a result, conflicting classification schemes have been proposed for the higher categories of Ascomycota (Barr 1983, 1987, 1990; Eriksson and Hawksworth 1993; Alexopoulos, Mims, and Blackwell 1996).

In this study, we examine the relationships between ascmycete groupings proposed by Alexopoulos, Mims, and Blackwell (1996) that are based on a synthesis of the 18S rDNA data and morphological characters (fig. 1). Three major groups of the Ascomycota were proposed on the basis of 18S rDNA studies (Berbee and Taylor 1992a; Kurtzman 1993; Nishida and Sugiyama 1993). The basal group is the Archiascomycetes, fungi with highly variable morphological and biochemical characters. Evidence for this clade rests primarily on shared rDNA sequences, but strong support for monophyletic Archiascomycetes is lacking (Nishida and Sugiyama 1993, 1994). The remaining ascomycetes comprise two sister groups strongly supported by rDNA analyses, the Saccharomycetales and Euascomycetes. Members of the Saccharomycetales differ from Euascomycetes both by the presence of a yeast phase and by the absence of both ascogenous hyphae and fruiting body formation (Alexopoulos, Mims, and Blackwell 1996). Euascomycetes are both the most complex in morphology and the most diverse group in the Ascomycota. The major characters used to delineate the principal lineages of Euascomycetes are the morphology of the fruiting body (ascocarp) and the structure of the ascus (Nannfeldt 1932; Luttrell 1955; Alexopoulos, Mims, and Blackwell 1996). A number of monophyletic lineages have been identified based on 18S rDNA data, but the relationships among the groups within the Euascomycetes are not resolved completely (fig. 1; Berbee and Taylor 1995; Gargas and Taylor 1995; Spathafora 1995; Berbee 1996).

To achieve a more complete view of the evolutionary relationships between groups of the Ascomycota, it will be necessary to broaden the base of molecular characters used. Protein-coding genes are promising in this regard because of their high content of functional information. It is likely that protein sequences will come to play an increasingly important role in phylogenetic studies of eukaryotes. The single-copy nuclear gene sequences that encode the two major subunits of each nuclear RNA polymerase are advantageous for this purpose because of their large size and easy accessibility by PCR amplification (James, Whelen, and Hall 1991; Stiller and Hall 1997; Denton, McConaughy, and Hall 1998). The largest number of extant sequences for comparison are those for nuclear DNA-dependent RNA polymerase II, the enzyme that transcribes pre-mRNA. Its two largest subunits have proven to be useful for broad-scale evolutionary studies of a variety of eukaryotic organisms (Iwabe et al. 1991; Sidow and Thomas 1993; Klenk et al. 1995; Stiller and Hall 1997; Denton, McConaughy, and Hall 1998). In yeast, the 140-kDa second largest subunit is encoded by RPB2 and participates extensively in catalyzing elongation (Thuriaux and Sentenac 1992). Twelve highly conserved regions with >85% identity in amino acid sequences were iden-
RPB2 sequences from fungi, plants, and animals (James, Whelen, and Hall 1991; Denton, McConaughy, and Hall 1998). PCR priming within these highly conserved regions allows recovery of the RPB2 genes from many different organisms for phylogenetic comparison.

The objectives of this study are to develop RPB2 as a new gene system for fungal molecular systematics, and with it to establish phylogenetic relationships among different orders of Ascomycota. Sequences of their RPB2 genes and proteins can provide an independent genetic data set with which to examine the evolution of morphological characters. The major question investigated is whether RPB2 analyses support the evolutionary conclusions of previous studies based on 18S rDNA sequences.

Materials and Methods

Specimens Examined

Twenty-eight fungal specimens, representing 1 basidiomycete and 27 ascomycetes, were used in this study. The sources of fungal strains and GenBank accession numbers for RPB2 gene sequences are listed in table 1. The classification of taxa is based on that of Alexopoulos, Mims, and Blackwell (1996).

DNA Extraction, PCR Amplification, Cloning, and Sequencing

Fungal cultures were grown on media recommended in the culture catalogs of the American Type Culture Collection and Centraalbureau voor Schimmelcultures. Genomic DNA was recovered from fresh fungal cultures using a cetyltrimethylammonium bromide (CTAB) extraction method (Rogers and Bendich 1994). The set of general oligonucleotide primers for amplifying regions 3–11 of RPB2 genes (fig. 2) was designed on the basis of conserved RPB2 sequences in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, Arabidopsis thaliana (GenBank accession number Z19120), *Homo sapiens* (GenBank accession number X63563), and *Drosophila melanogaster* (GenBank accession number M29646). Overlapping fragments of RPB2 were amplified by the polymerase chain reaction (PCR) with *Taq* DNA polymerase following the manufacturer’s recommendations (Life Technologies, Inc., Gaithersburg, Md.). The PCR conditions included: (1) hot start with 95°C for 5 min; (2) 30 cycles of 1 min at 95°C, 2 min at 55°C (or 50°C), an increase of 1°C/s to 72°C, and 2 min at 72°C; and (3) a 10-min incubation at 72°C. DNA fragments from regions 3–5 and 3–7 (fig. 2) were amplified with a 50°C annealing temperature. Regions 5–7, 6–7, 6–11a, and 7–11b (fig. 2) were amplified with a 55°C annealing temperature. PCR-amplified RPB2 fragments from genomic DNA were cloned using the pCR2.1 plasmid vector (Invitrogen Inc., Carlsbad, Calif.). A number of clones for each RPB2 fragment were subjected to automatic sequencing (ABI PRISM Dye Terminator Cycle Sequencing and ABI PRISM Sequencer model 377, Perkin-Elmer). Fungal-specific primers were then designed based on the RPB2 sequences recovered from 10 ascomycetes (fig. 2). These primers were used to amplify and directly sequence overlapping regions of RPB2 from 16 additional fungi.

Sequence Alignment and Phylogenetic Analyses

The predicted amino acid sequences of RPB2 between regions 3 and 11 of 26 fungi were aligned to RPB2 sequences of *S. cerevisiae* and *S. pombe* using CLUSTAL V (Higgins, Bleasby, and Fuchs 1992), with subsequent manual adjustment. The resultant alignment consisted of 915 aligned amino acid positions including gaps. The percentage of identity at each of 915 aligned positions from 28 fungi was calculated and plotted to show the rate heterogeneity of amino acid replacements among sites. Regions that could not be aligned reliably were removed, leaving a total of 873 amino acid positions for phylogenetic analyses. The basidiomycete *Agaricus bisporus* was used as an outgroup to evaluate the phylogenetic relationships within Ascomycota. Phylogenetic analyses were carried out using protein parsimony, distance, and protein maximum-likelihood algorithms. All parsimony analyses were conducted using PAUP, version 3.1.1 (Swofford 1993), with both equal-weights parsimony and a weighted step matrix based on the JTT matrix (Felsenstein 1981; Jones, Taylor, and Thornton 1992). The heuristic search using the random-addition-of-taxon option was performed with 100 replicates to increase the chance of finding all of the most parsimonious trees. A distance matrix was constructed with the PAM matrix using Prodist of PHYLIP, version 3.6 (Felsenstein 1993), and a distance-based tree was produced by the neighbor-joining algorithm. To evaluate support for particular nodes, 500 parsimony and neighbor-joining bootstrap replicates were performed. Protein maximum-likelihood analyses were conducted using both PAML, version 1.1 (Yang 1995) and PUZZLE, version 4.0 (Strimmer and Haeseler 1997). Both programs incorporate the JTT matrix for weighting amino acid substitutions and gamma-distributed rates to allow for rate heterogeneity among sites. The gamma distribution parameter α was estimated to be 0.33 for our data set. Eight rate categories were used in the analyses. In order to test alternative phylogenetic hypotheses, analyses were conducted using PAML to construct the most like-
ly trees under the constrained conditions, and the resulting trees were evaluated together with the most parsimonious tree and the neighbor-joining tree in PUZZLE using the KHT paired-site tests (Templeton 1983; Kishino and Hasegawa 1989).

**Results**

**Sequence Conservation and Variation in Fungal RPB2 Sequences**

A total of 915 amino acid positions, spanning from motif 3 to motif 11 of RPB2, were sequenced for 25 ascomycetes and 1 basidiomycete (table 1 and fig. 2). Considerable rate heterogeneity of amino acid replacements among sites was observed (fig. 3), with the extent of variation within conserved sequence motifs being less than 15% (fig. 3a). The most conserved regions were the first and last 350 amino acids, averaging 78% and 88% identity, respectively, across taxa (fig. 3a). The central tract of 215 residues was the most variable, having an average of 58% identity (fig. 3a).

In order to assess and localize the sequence divergence within selected groups of ascomycetes, the regional variability of RPB2 protein sequences was plotted
(fig. 3). The variation of RPB2 protein sequences within the taxa sampled from six different ascomycete orders are shown in fig. 3b-e. For the closely related fungi (fig. 3f), the percentage of variation between sequence motifs 6 and 7 approaches 20% in places and averages 8%. Thus, this central region can be used to investigate phylogenetic relationships between closely related taxa and will be particularly useful for analyses that deal with large numbers of such taxa.

Only 7 out of the twenty-eight fungal RPB2 sequences have introns within the regions we sequenced (fig. 4). At least 2 of these introns appear to have been present in the ancestral fungal RPB2 gene based on the homologous intron positions in Arabidopsis (fig. 4). This suggests that introns may have been gained and lost frequently over the course of fungal evolution and, therefore, may not be reliable as phylogenetic characters.

Phylogenetic Relationships Between Major Lineages Within Ascomycota

Across the taxa studied, nucleotides at third codon positions are substantially saturated (data not shown). Accordingly, we used the translated protein sequences rather than DNA sequences for our phylogenetic analyses.

The single most parsimonious tree from weighted analysis was identical in topology to the tree obtained by maximum-likelihood analyses (fig. 5A). The distance tree (neighbor-joining) was similar in topology to the most parsimonious tree except for the placement of the Chaetothyriales (fig. 5B). With the basidiomycete A. bisporus as an outgroup (fig. 5), there are three major lineages within the Ascomycota: Archiascomycetes, Saccharomycetales, and Euascomycetes. Of these, Archiascomycetes, represented here by S. pombe and N. vitellina, comprise the most basal group. The node separating Archiascomycetes from the Saccharomycetales and Euascomycetes has 78% bootstrap support in parsimony and 99% in distance analysis (fig. 5). The Saccharomycetales and the Euascomycetes, each of which is monophyletic, are sister groups with strong support (fig. 5).

Relationships Between Different Orders Within Euascomycetes

Among Euascomycetes, six well-supported clades are apparent (clades A–F in fig. 5). Members of the Pleosporales, Dothideales, and Melanommatales of loculoascomycetes clustered together with bootstrap values of 94% and 98% (clade A in fig. 5). In this clade, Aureobasidium pullulans and Dothidea insculpta of the Dothideales grouped together with a 100% bootstrap value, and Mycosphaeria citrullina of the Dothideales and Sporormiella minima of the Melanommatales clumped within members of the Pleosporales with bootstrap values of 79% and 97% (fig. 5). Other very well supported monophyletic groups included the members in Chaetothyriales (clade B), plectomycetes including the Eurotiales and Onygenales (clade C), pyrenomycetes including the Sordariales and Microascales (clade D), and the members in Leotiales (clade E) (fig. 5).

Because all six clades (A–F) of Euascomycetes had significant statistical support (fig. 5), a phylogenetic analysis with each of these clades constrained was performed in order to deduce their interrelationship. Ex-
Fig. 3.—The variability of RPB2 amino acid sequences. The percentage of identity was calculated at each amino acid position, then averaged over contiguous blocks of five amino acids; % variability = 100 − % identity. The horizontal axis shows the amino-acid-encoding parts of RPB2, the vertical axis on the left side shows the percentage of identity, and the vertical axis on the right side shows the percentage of variability. a, The variability of RPB2 within basidiomycetes and ascomycetes. b, The variability of RPB2 within Sordariales and Microascales. c, The variability of RPB2 within Pleosporales. d, The variability of RPB2 within Eurotiales and Onygenales. e, The variability of RPB2 within sampled taxa of Leotiales. f, The variability of RPB2 between Capronia and Exophiala.

As a test of significance of the relationships between groups of Euascomycetes, KHT paired-site tests were conducted to test alternative phylogenetic hypoth-
Fig. 4.—Introns in fungal RPB2 genes. Positions within RPB2 are marked by arrows, with the flanking amino acids given by one-letter designations and residue positions in coordinates based on the Saccharomyces cerevisiae RPB2 gene (GenBank accession number M15693). Phase 0 introns are between two amino acids, phase 1 introns are indicated by (1) where the intron is inserted between the first and second codon positions, and phase 2 introns are indicated by (2) where the intron is inserted between the second and third codon positions. The species in which introns were found are listed below the intron positions.

Fig. 5.—Phylogenetic trees based on the RPB2 amino acid sequences. A, The single most parsimonious tree obtained with weighted protein parsimony using heuristic searches with 100 replicates and the random-addition-of-taxon option (PAUP, version 3.1.1). Agaricus bisporus was used as the outgroup. The bootstrap values were determined from 500 replications. The major clades within the Euascomycetes are indicated by the circled letters A–E. B, The neighbor-joining tree constructed using the Neighbor program of PHYLIP, version 3.5c. Distances were estimated using the Dayhoff PAM matrix to weight amino acid substitutions (Prodist, PHYLIP, version 3.5c). The bootstrap values were determined from 500 replications.
topology with the distance tree (table 2 and fig. 5A), is not significantly less likely than the most parsimonious tree (table 2 and fig. 5A).

Discussion

In general, the RPB2 phylogeny of the Ascomycota (fig. 5) is congruent with the previous rDNA analyses, in that both genes support three major lineages of Ascomycota: Archiascomycetes, Saccharomycetales and the Euascomycetes (Kurtzman 1993; Nishida and Sugiyama 1994; Hasse et al. 1995; Spatafora 1995; Berbee 1996). Their sister group relationship is indicative of an independent origin for the Saccharomycetales distinct from that of all present day Euascomycetes. Their positions in the RPB2 phylogeny are consistent with morphology in that most Euascomycetes have the ability to form a fruiting body and are structurally more complex than either Archiascomycetes and Saccharomycetales.

Table 2
Kishino-Hasegawa Paired-Site Tests

<table>
<thead>
<tr>
<th>Trees Tested</th>
<th>Steps of Parsimony</th>
<th>In Likelihood</th>
<th>SD</th>
<th>P</th>
<th>Rejected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Most parsimonious tree (fig. 5A)</td>
<td>34,808</td>
<td>−16,340.74</td>
<td>8.33</td>
<td>0.2420</td>
<td>Best</td>
</tr>
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<td>Neighbor-joining tree (fig. 5B) (Monophyletic loculoascomycetes)</td>
<td>34,810</td>
<td>−16,350.49</td>
<td>8.33</td>
<td>0.2420</td>
<td>No</td>
</tr>
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<td>Monophyletic discomycetes = ((Dothi, Pleo, Chaeto), Plecto, Pyreno, Disco)</td>
<td>34,940</td>
<td>−16,388.02</td>
<td>18.51</td>
<td>0.0108</td>
<td>Yes</td>
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<td>Monophyletic ununiculate Euascomycetes = ((plecto, pyreno, disco), other Euroascomycetes)</td>
<td>34,861</td>
<td>−16,367.70</td>
<td>12.42</td>
<td>0.0300</td>
<td>Yes</td>
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<td>Plectomycetes as basal group of filamentous ascomycetes = ((plecto, (other filamentous ascomycetes)))</td>
<td>34,992</td>
<td>−16,381.82</td>
<td>14.73</td>
<td>0.0052</td>
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<tr>
<td>Pyrenomycetes as basal group of filamentous ascomycetes = ((pyreno, (other filamentous ascomycetes)))</td>
<td>34,988</td>
<td>−16,414.28</td>
<td>18.34</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
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</table>

NOTE.—ln likelihood = natural log likelihood; P = the confidence limit for rejection of the alternative tree topology.

1 The most parsimonious tree (same topology as the most likely tree), the neighbor-joining tree, and the most likely trees under the constrained conditions. Dothi = Dothideales; Pleo = Pleosporales; Chaeto = Chaetothyriales; plecto = plectomycetes; pyreno = pyrenomycetes; disco = discomycetes.

2 Tree length for each tree topology is determined by weighted protein parsimony using PAUP version 3.1.1.

3 Best = the tree with the highest likelihood; Yes = rejected by maximum likelihood (P < 0.05); No = not rejected by maximum likelihood.

Monophyletic Classes in Euascomycetes

The classes Plectomycetes and Pyrenomycetes generally have not been accepted as taxonomic categories; however, phylogenetic analyses based both on RPB2 and rDNA data show each of these classes to be monophyletic (Berbee and Taylor 1992; Spatafora and Blackwell 1993; 1994; Spatafora 1995). Certain characters are useful in distinguishing between Plectomycetes and Pyrenomycetes. In general, Plectomycetes have a completely closed fruiting body and scattered ascospores while the majority of Pyrenomycetes have a flask-shaped fruiting body and unitunicate ascospores with forcibly discharged ascospores.

Pleosporales and Dothideales of the Loculoascomycetes

Loculoascomycetes was proposed by Nannfeldt (1932) and Luttrel (1955) to include taxa with bitunicate ascospores. The individual orders of Loculoascomycetes are distinguished mainly on the basis
of centrum development and the position of asci in the fruiting body (Barr 1987).

In the RPB2 phylogeny, Melanomrnatales clusters within the Pleosporales with bootstrap values of 79% and 97% (fig. 5), which indicates that there is no significant phylogenetic difference at the ordinal level between cellular (wide and septate) and trabeculate (thin and nonseptate) pseudoparaphyses (downward growing, sterile hyphae). On the other hand, the presence of pseudoparaphyses is a unique character with which to delineate the Pleosporales complex.

In RPB2 trees, members of the Dothideales group together with 100% bootstrap support except for Mycosphaerella citrullina which may have been misclassified. These taxa are distinct and separate from the Pleosporales clade in the RPB2 trees (fig. 5) suggesting that the absence of pseudoparaphyses is a character that typifies Dothideales.

The Pleosporales and Dothideales clades group together with 94% or 98% bootstrap values in RPB2 trees (clade F in fig. 5), indicating that they are sister groups with a common ancestor. No conclusion was reached from rDNA analyses as to the relationship between Pleosporales and Dothideales (Berbee 1996; LoBuglio, Berbee, and Taylor 1996; Winka, Ericksson, and Bang 1998).

The Position of Chaetothyriales

The position of one order of loculoascomycetes was not defined clearly in the RPB2 tree. Although three representatives of the Chaetothyriales grouped together with 100% bootstrap support in all analyses based on their RPB2 sequences (fig. 5), the phylogenetic placement of the order as whole is ambiguous. They branch either as the sister group to the plectomycetes (fig. 5A) or with the Pleosporales + Dothideales clade (clade F, fig. 5B); however, neither placement has strong statistical support. Parsimony analysis requires only two additional steps for the tree in figure 5B beyond that for the tree in figure 5A, and the KHT tests (table 2) shows no significant difference in likelihood between these two branching positions. The inconclusive placement of the Chaetothyriales based upon RPB2 sequence data mirrors the disagreement on this point from other types of analyses.

The affinity of Chaetothyriales to loculoascomycetes indicated in figure 5B is strongly supported by the presence of ascostromata, bitunicate asci, apical pseudoparaphyses, and multiseptate ascospores. Such a large number of derived morphological features in combination is not plausibly explained by convergence. A recent study based upon 18S rDNA shows that the Chaetothyriales grouped either with the plectomycetes with no support in parsimony and distance analyses, or with other bitunicate loculoascomycetes in likelihood analysis (Winka, Ericksson, and Bang 1998). Determination of the phylogenetic position of the Chaetothyriales with confidence awaits further investigation. At this point, however, there is no convincing evidence either in RPB2- or rDNA-based phylogenies against the close relationship between Chaetothyriales and other loculoascomycetes that is suggested by shared morphological features.

Paraphyletic discomycetes

Discomycetes, a group characterized by an open fruiting body, is paraphyletic in RPB2 phylogenies (fig. 5). Discomycete taxa with operculate asci (Pezizales) appear to be basal to all other euascomycetes, while those with inoperculate asci (Leotiales) are a sister group to the pyrenomycetes. Although this topology has only weak bootstrap support (fig. 5), the same relationships are shown in rDNA phylogenies (Gargas 1992; Saenz, Taylor, and Gargas 1994; Gargas and Taylor 1995; Berbee 1996; Lobuglio, Berbee, and Taylor 1996). Moreover, the sister relationship between pyrenomycetes and Leotiales is supported by the shared morphological characters of inoperculate unitunicate asci and paraphyses.

A tree forcing a monophyletic grouping of the members of Pezizales and Leotiales is rejected by the KHT test (table 2), reinforcing the paraphyly of discomycetes. In addition, the monophyly of unitunicate Euascomycetes is rejected in the KHT test (table 2). Because the Pezizales is basal to the Euascomycetes, loculoascomycetes appear to have evolved from within the unitunicate Euascomycetes (fig. 5 and table 2).

Conclusions

Overall, RPB2 analyses confirmed many of the relationships previously proposed in rDNA trees. Very well supported classes and orders are apparent in the RPB2 phylogeny; however, the relationships among these groups are not established unequivocally. In addition, the relatively short branches in the RPB2 tree that lead to these classes and orders probably reflect a rapid and ancient radiation of Euascomycetes (fig. 5). It would be of great interest to know just when this radiation occurred, and what kinds of bottlenecks (environmental factors, population size, and/or gene fitness) drove the process. While the taxa used in this study represent all major recognized groups of Ascomycota, they encompass only a small part of the diversity of this, the largest group of fungi. The excellent resolution achieved and the ready accessibility of RPB2 gene sequences using these conserved PCR primers provides a basis for extending the phylogeny of Ascomycota outwards from the core relationships described here.

Acknowledgments

We are grateful to Mary Berbee, Sara Landvik, Joseph Ammirati, and John Stiller for their valuable comments on the manuscript. We thank Mary Berbee, Ellie Duffield, Linda Kohn, Klete Kurtzman, Karen La Fe, Karry O’Donnell, Joey Spatafora, and Wendy Untereiner for providing the DNA samples and fungal cultures, John Stiller for assistance on primer design, and Joseph Felsenstein for advice on phylogenetic analyses.
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RODNEY L. HONEYCUTT, reviewing editor

Accepted August 30, 1999