

Achlya mitochondrial DNA: gene localization and analysis of inverted repeats

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Summary. Mitochondrial DNA from four strains of the oomycete *Achlya* has been compared and nine gene loci mapped, including that of the ribosomal protein gene, *var1*. Examination of the restriction enzyme site maps showed the presence of four insertions relative to a map common to all four strains. All the insertions were found in close proximity to genic regions. The four strains also contained the inverted repeat first observed in *A. ambisexualis* (Hudspeth et al. 1983), allowing an examination by analysis of retained restriction sites of the evolutionary stability of repeated DNA sequences relative to single copy sequences. Although the inverted repeat is significantly more stable than single copy sequences, more detailed analysis indicates that this stability is limited to the portion encoding the ribosomal RNA genes. Thus, the apparent evolutionary stability of the repeat does not appear to derive from the inverted repeat structure per se.

Key words: *Achlya* – Mitochondrial DNA – Inverted repeat – Gene localization

Introduction

Present knowledge of the organization and expression of the mitochondrial genomes of the fungi and their allies is primarily derived from the ascomycetes. Studies of these organisms have demonstrated a five-fold variation in genome size (c.f. Clark-Walker et al. 1981a vs. Garber and Yoder 1984), a diversity in mitochondrially encoded genes c.f. Macino and Tzagoloff 1979 vs. Sebald et al. 1979), highly variable gene orders (Dujon 1983), and differences in the genetic code (c.f. Browning and RajBhandary 1982 vs. Bonitz et al. 1980; Hudspeth et al. 1982). In non-asco-

mycete classes, however, information is limited to the diversity of genome sizes in basidiomycetes (e.g. Specht et al. 1983; Hintz et al. 1985) and restriction endonuclease maps for the myxomycete *Physarum* (Kawano et al. 1982) and the oomycetes *Achlya* and *Phytophthora* (Hudspeth et al. 1983; Boyd et al. 1984; Klimczak and Prell 1984).

Our previous studies of *Achlya* mtDNA revealed the presence of a large inverted repeat in the 50 kb circular genome. Although typical of chloroplast DNAs (Palmer 1985), large inverted repeats have been found in the mtDNA of only *Achlya* and three other mitochondrial genomes – the yeast *Kloeckera africana* (Clark-Walker et al. 1981b), the sporozoan *Isospora gondii* (Borst et al. 1984), and the linear genome of the ciliate *Tetrahymena pyriformis* (Goldbach et al. 1978). While both ribosomal cistrons are completely encoded in chloroplast and *Achlya* repeats, only the L-rRNA is in the *Tetrahymena* repeat, and only a portion of the L-rRNA is in the *Kloeckera* repeat.

Intramolecular recombination between inverted repeats in circular eukaryotic DNAs has been detected in the *Saccharomyces cerevisiae* 2 μ m circle (Guerineau et al. 1976), chloroplast genomes (Palmer 1983; Aldrich et al. 1985), and the *Achlya* mitochondrial genome (Hudspeth et al. 1983; Boyd et al. 1984). Palmer and Thomson (1982) have shown that chloroplast genomes undergo less DNA sequence rearrangement throughout their genomes when an inverted repeat is present, and that the rate of sequence divergence of the repeat itself is less than that of non-repeated sequences (Palmer et al. 1983).

We have extended our studies of *Achlya* mtDNA to include four strains representing two species. We report here the localization of four region-specific insertions, seven additional gene loci, and an examination of the stability of repeat DNA sequences relative to single copy sequences.

Methods and materials

Strains. *A. ambisexualis* 734 and *A. heterosexualis* 1–4 and 8–6 were obtained from the Alma Barksdale Collection (New York Botanical Garden, Bronx, NY, USA). The *S. cerevisiae* petite strains encoding ribosomal cistrons were the gifts of P. S. Perlman (Ohio State University, Columbus, OH, USA). Petites containing genic sequences of COB, COI, COIII, ATPase 6, and ATPase 9 were the gifts of A. Tzagoloff (Columbia University, New York, NY, USA). COII sequences in a bacterial plasmid were the gift of T. Fox (Cornell University, Ithaca, NY, USA).

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Abbreviations. ATPase 6, 9: genes for ATPase subunits 6 and 9; COI, II, III: genes for cytochrome oxidase subunits 1, 2, and 3; COB: gene for apocytochrome b; L-, S-RNA: genes for the mitochondrial large and small ribosomal RNAs; mtDNA: mitochondrial DNA; *var1*: gene for the *S. cerevisiae* mitochondrially encoded ribosomal protein; m.u.: map units; bp: base pairs; kb: kilobase pairs

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Table 1. *Saccharomyces cerevisiae* gene probes

Probe	Strain	Source	Sequence reference
COI	DS6/407	<i>Hinf</i> / <i>TagI</i> : base 130 to 410 (exon 5)	Bonitz et al. (1980)
COII	Clone ^a	<i>Hinf</i> : base 79 to 430	Coruzzi and Tzagoloff (1979)
COIII	DS/40	<i>Hinf</i> / <i>PvuII</i> : base 341 to 707	Thalenfeld and Tzagoloff (1980)
COB	DS400/A12	<i>Hinf</i> : base 55 to 533 (exon 1, short form)	Nobrega and Tzagoloff (1980)
ATPase 6	DS14	<i>EcoRI</i> / <i>MboI</i> : base 40 to 696	Macino and Tzagoloff (1980)
ATPase 9	DS400/A3	<i>AluI</i> : base 20 to 178	Macino and Tzagoloff (1979) Hensgens et al. (1979)
21SrDNA	A12-48 ^b	<i>AluI</i> : base 698-1867, 1965-2658, and 2709-2975	Sor and Fukuhara (1983)
15SrDNA	BD11	<i>AluI</i> / <i>BamHI</i> : base 154-720	Sor and Fukuhara (1980) Li et al. (1982)
Var 1	A17-10	<i>HhaI</i> / <i>HaeIII</i> : base 199 to 1217	Hudspeth et al. (1982)

All probes contain only genic or exon sequences with the exception of 30 3' flanking *var1* nucleotides. ATPase 6 and 9 probes consist of two fragments and the large rDNA of four fragments

^a 2.4 kb *HaeIII* fragment from wild type *S. cerevisiae* tailed into *PstI* of pBR 322

^b ω^* - strain

Isolation of DNAs. *Achlya* mycelia grown at room temperature in PYG (Griffin et al. 1974) were harvested in late logarithmic phase from moderately aerated 16 l carboys by filtration through a single layer of Miracloth (Calbiochem, La Jolla, CA, USA). Following a 2 l water rinse the mycelia were soaked in 10 vol (wet weight) of 100 mM EDTA (pH 7.5) at 4° C for 1 h, then collected by filtration through Miracloth, with the excess liquid removed by blotting with absorbent towels. The mycelial mat was ground to a fine powder in liquid nitrogen in 5-10 g aliquots. The frozen powder was resuspended with stirring in 1.0 M sorbitol-0.1 M EDTA-0.1 M Tris (pH 7.5), allowed to thaw at 4° C, and mtDNA isolated as previously described (Hudspeth et al. 1983). DNAs used for probing Southern blots were isolated as previously described from yeast strains (Hudspeth et al. 1980) and a bacterial plasmid (Maniatis et al. 1982).

Restriction enzyme digestion and gel electrophoresis. Restriction endonucleases were obtained from International Biotechnologies, Inc., New Haven, CT, USA, New England Biolabs, Beverly, MA, USA, Boehringer Mannheim, FRG, or Bethesda Research Laboratories, Gaithersburg, MD, USA. Digestions were carried out in buffers recommended by the manufacturers. Electrophoresis and photography of 0.8% or 0.6% agarose and 3.5% polyacrylamide slab gels were as previously described (Moore et al. 1977; Hudspeth et al. 1983).

Transfer hybridizations. Transfer of restriction fragments from 0.8% agarose gels to nitrocellulose filters (Schleicher and Schuell, Keene, NH, USA) and hybridization were carried out as described by Southern (1975) with the modifications previously noted (Hudspeth et al. 1983). The gene probes (Table 1) were electroeluted from 3.5% polyacrylamide gels and nick-translated (Rigby et al. 1977) for 1 h at 11° C. Hybridizations were at 40° C for the *var1* probe and at 50° C for all others.

Results

Restriction maps

Physical maps of four *Achlya* mitochondrial genomes were constructed by restriction endonuclease analysis. Initial analyses of the enzyme digests indicated a variation in individual strains and species both in genome size and in the number and location of restriction sites. To establish the colinearity of the *Achlya* strains used here and in a prior study (Hudspeth et al. 1983), we derived a minimal composite map for these four heterothallic strains. This map was constructed both by retaining only the 41 restriction sites held in common by all four strains, and by selecting the minimum strain distance between any two common sites (as determined below). Figure 1 compares this common map with the four individual maps, which specify only the 22 additional site variations.

We have used the combination of restriction maps to further refine the minimal and maximal lengths of the inverted repeat, small single copy, and large single copy regions of the *Achlya* genome previously established (Hudspeth et al. 1983). Our new estimates of the extent of these three genomic regions are based upon the presence of restriction sites in other strains that are more proximal to the repeat boundaries than those previously identified in E-87. These sites are the repeat *BstEII* sites adjacent to the large single copy region in 1-4 and 8-6; the small single copy *Bg/III* site adjacent to the repeat in 8-6; and the large single copy *PstI* site adjacent to the repeat in 734 and 8-6. The repeat *Bg/III* site adjacent to the small single copy region in E-87 is common to all four *Achlya* strains and remains the most proximal to that single copy boundary.

We assume these site differences to result from a single nucleotide substitution rather than from a repeat length expansion, based on the following considerations. For the variable *BstEII* site, we are unable to detect any strain fragment length variation in either the 6.0 or the 3.5 kb *Bg/III* fragments that cross the large single copy repeat boundaries. The non-repeated *PstI* site lies in the non-variable 3.5 kb *Bg/III* fragment. Similarly, for the 8-6 small single copy *Bg/III* site, the 1.0 kb *HpaI* fragment crossing the small single copy boundary is invariant in strains 734, 1-4, and 8-6. Calculations using these new boundary sites, in conjunction with the elimination of fragment length variation due to insertions (see below), allow us to describe more accurately the basic *Achlya* mitochondrial genome. The 49.1 kb common circle contains two inverted repeats of 9.94-11.24 kb, separated by a small single copy region of 6.23-8.31 kb and a large single copy region of 20.48-21.01 kb. As discussed below, the common genome may be expanded up to at least 51.8 kb by optional insertions in each of the three genomic regions.

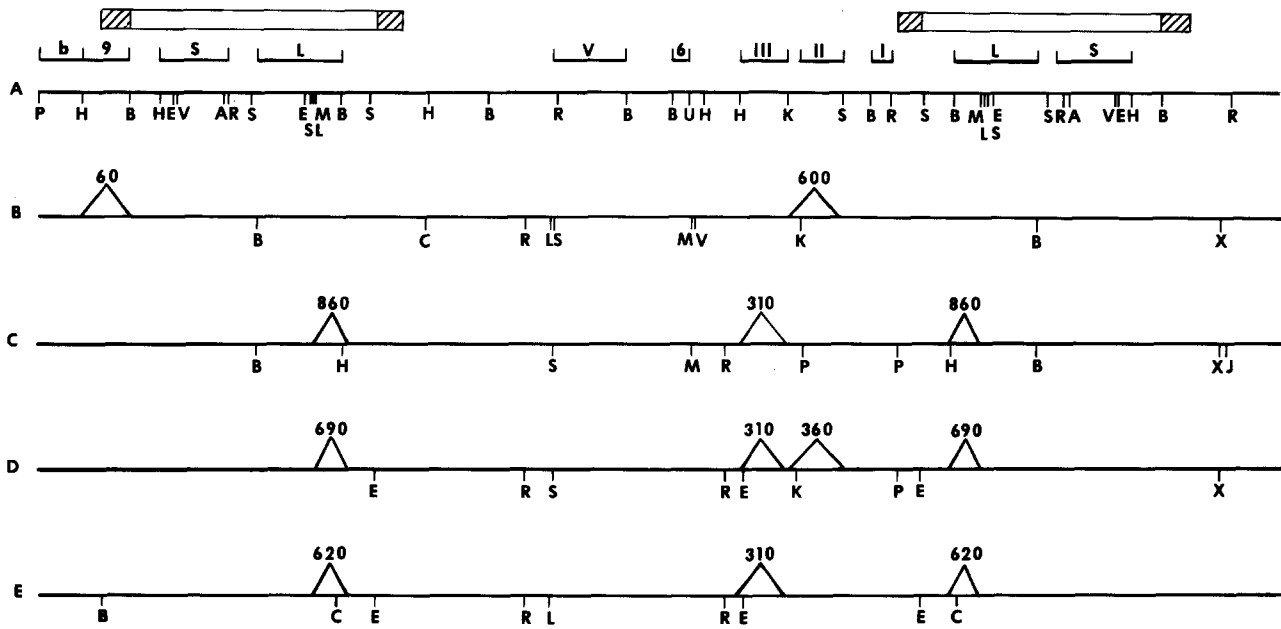


Fig. 1 A-E. Common and individual restriction site maps of *Achlya* strains. **A** Summary restriction site map showing only sites common to all four strains. Strains E-87 **B**, 734 **C**, 1-4 **D**, and 8-6 **E** contain the restriction sites shown plus those of the common map **A**. Triangles in **B-E** indicate the location and size (in bp) of the inserts discussed in the text. The fragments which hybridized to gene probes (see Table 1) are indicated above the summary map. The minimum (*open bars*) and maximum (*hatched ends*) lengths of the inverted repeats are shown above the genes. Enzyme abbreviations: A, *SstII* (*SacII*); B, *BglIII*; C, *Clal*; E, *BstEII*; H, *HpaI*; J, *BglI*; K, *KpnI*; L, *SalI*; M, *BamHI*; P, *PstI*; R, *EcoRI*; S, *SstI* (*SacI*); U, *PvuI*; V, *PvuII*; X, *XhoI*

Insertions and deletions

To date the only detailed studies localizing restriction fragment length polymorphisms in fungal mitochondrial genomes are of the ascomycetes *Saccharomyces* (Sanders et al. 1977), *Aspergillus* (Earl et al. 1981), and *Neurospora* (Mannella et al. 1979). We have analyzed the four *Achlya* genomes presented here by systematic side-by-side comparisons of DNA fragment lengths generated by restriction enzyme sites common to all four strains. The use of common sites allowed us to examine the genomes so that the largest comparison made was limited to approximately 5 kb fragments. Analyses (repeated at least twice for any set of fragments) using 0.8% and 0.6% agarose or 3.5% polyacrylamide gels allowed us to detect differences as small as 60 bp. While we assumed in our analysis the minimum number of insertion or deletion events that best describes DNA fragment length variations, we cannot, of course, exclude the possibility that fragment length variations resulted instead from a larger number of smaller insertions or deletions.

We found only four regions of insertion or deletion events in the genomes. These events were found both in the repeat and in the two single copy regions. Figure 2 illustrates the major fragment length polymorphism revealed by *SstI* digestion, which is localized to the inverted repeat in the 3' region (see below) of the L-rRNA cistron. Further restriction analyses of the inverted repeat localized the fragment length polymorphism to the region between the *BamHI* and the *BglIII* sites, extending from E-87 map coordinates 21.9 to 24.1 and from 73.6 to 75.8 (Hudspeth et al. 1983). These data indicate that the fragment variations relative to E-87 are due to insertions of 0.62 kb (8-6), 0.69 kb (1-4), and 0.86 kb (734).

Comparison of restriction fragment lengths derived solely from the small single copy region reveals a difference

only in strain E-87. The DNA sequence length between the E-87 *HpaI* (3.4 m.u.) and *BglIII* (7.2 m.u.) sites is 1.91 kb, but only 1.85 kb in the other three strains. Assumption of a single 0.06 kb insertion in E-87 in this region restores the common restriction enzyme fragment length colinearity throughout the entire small single copy region.

Insertion or deletion events occurring in the large non-repeated region appear to be more complex. The only detectable events were located between the E-87 *HpaI* (56.3 m.u.) and *SstI* (64.5 m.u.) sites, which encompass two *KpnI* sites in E-87. Distances between these *HpaI* and *SstI* sites are estimated to be 3.06, 3.055, 2.85, and 2.85 kb in E-87, 1-4, 734, and 8-6, respectively. However, the comparative distance from *HpaI* to the first *KpnI* site in E-87 is 0.31 kb less than in the other genomes. The distances from the single or second (when present) *KpnI* site to the *SstI* site are 1.96 kb in E-87, 1.92 kb in 1-4, and 1.91 kb in 734 and 8-6. In addition, when two *KpnI* sites are present, the distance between them also varies, being 0.55 kb in E-87 and 0.35 kb in 1-4. We interpret these data to indicate two independent events. The first is a deletion of 0.31 kb in the *HpaI-KpnI* region of E-87 relative to the other strains, and the second is a variable insertion of either 0.6 kb (E-87) or 0.36 kb (1-4), between the first *KpnI* site and extending past the second. In the absence of other common sites in the *HpaI-SstI* region, however, we cannot exclude the possibility of a single insertion or deletion event bridging the common *KpnI* site.

Relative stability of genomic regions

We have analyzed the relative susceptibility to single nucleotide changes of inverted repeat and non-repeated sequences in the mitochondrial genomes of the four *Achlya* strains using equations (Nei and Li 1979; Brown et al. 1979)

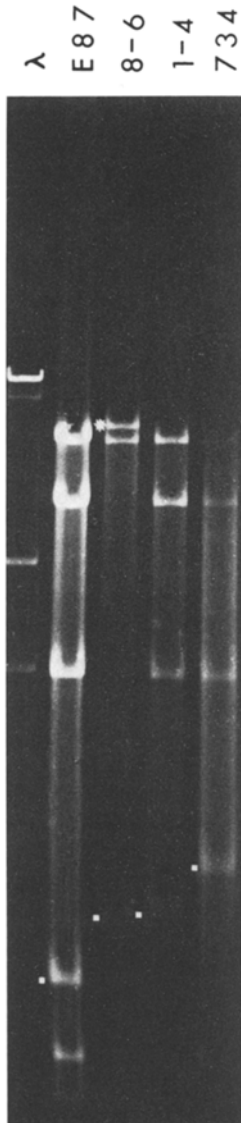


Fig. 2. Repetitive inserts. EthBr-stained gel of *Sst*I digests of mtDNA from four *Achlya* strains. The white squares denote the fragments that map to the same area of the genome yet differ in size (Fig. 1). Other inserts found in the larger *Sst*I fragments are unresolvable under these conditions. The 8-6 fragment (*) differs in size due to a deleted site. λ denotes bacteriophage lambda DNA digested with *Hind*III

that relate the number of shared restriction sites to percent sequence divergence. Our analysis is limited to the gain or loss of sites whose recognition sequence requires six specific nucleotides. We have retained the enzymes *Bst*EII and *Bgl*I in our analysis since the additional one and five nucleotides in their respective recognition sites are completely degenerate. However, we have excluded from analysis all variant restriction sites that are associated with a known insertion event. These are the repeat insertion *Cla*I of strain 8-6, the first large single copy insertion *Bst*EII of 1-4 and 8-6, and the second *Kpn*I large single copy insertion of E-87 and 1-4 (Fig. 1). In addition, we have also excluded the previously reported *Xor*II site at E-87 coordinate 68.6 (Hudspeth et al. 1983) as we are unable to confirm its presence with the isoschizomer *Pvu*I. Thus, our analysis is based upon a total of 59 restriction sites, 41 of which are common to all four strains.

The estimated sequence divergence of these *Achlya* mtDNAs is presented in Table 2. The known intramolecular recombination between repeat elements (Guerineau et al. 1976; Hudspeth et al. 1983; Boyd et al. 1984) has led to the suggestion that the symmetrical nature of nucleotide changes in repeats is due to a copy-choice correction (Ko-

Table 2. Analysis of sequence divergence

Region	Sites compared	Sites shared	Percent sequence divergence
Total genome	59	41	6.1
Total genome less one repeat	43	28	7.2
Total repeat one copy	16	13	3.5
Repeat rDNA one copy	11	10	1.6
Repeat non-rDNA one copy	5	3	8.5
Total single copy	27	15	9.8
Large single copy	21	12	9.3
Small single copy	6	3	11.6
Non-rDNA less one repeat	32	18	9.6
Non-rDNA total genome	48	31	7.3

Shared sites/compared sites = S; Number of nucleotides recognized = N; % sequence divergence = $-100 (\ln S)/N$ (Brown 1979; Nei and Li 1979)

lodner and Tewari 1979; Myers et al. 1982) and is thus independent of sequence divergence. Therefore, we have based our divergence values on those derived from the total genome minus one repeat.

The most striking feature of the divergence analysis is the apparent conservation of repeated sequence DNA relative to single copy DNA. A two to three-fold difference is present in all pairwise combinations except that of 734 with 1-4. To test the significance of these differences we compared the number of variable sites from the two genomic regions of all strains with the number of invariant sites. We scored a variable site change as a single data event when it was absent in at least one of the four genomes. In this manner we were in effect analyzing the overall susceptibility of the genomes to nucleotide substitution rather than the divergence between individual strains. The results indicate an overall divergence of 7.2 percent, with a 2.8 fold difference between repeat (3.5%) and single copy (9.8%) sequences.

This approach also allowed us to address the question of whether the repeat conservation is due to intramolecular recombination events or to the conservative nature of the ribosomal cistrons. We thus compared repeat rDNA (as determined below) with repeat non-rDNA sequences. Although our comparisons are based upon only 11 and 5 restriction sites, respectively, the repeat rDNA value of 1.6% divergence and the repeat non-rDNA value of 8.5% divergence strongly argue that the repeat sequence conservation is due to the encoding of rRNA sequences.

Gene loci

To determine the relationship between coding regions and the observed genome variability, we established the locations of nine potential *Achlya* mitochondrial genes. These sequences were detected by Southern analysis using as probes the isolated *S. cerevisiae* mtDNA fragments described in Table 1. We attempted to minimize the possibility of cross-hybridization of non-genic regions by radio-labeling *S. cerevisiae* sequences containing only coding regions. The sole exception was the probe for *var1*, which includes 30 nucleotides of 3' flanking sequence. Although the degree of homology of different *S. cerevisiae* probes appeared to vary, based on hybridization signal intensity, our hybridizations were done at 50° C to provide a criterion low enough

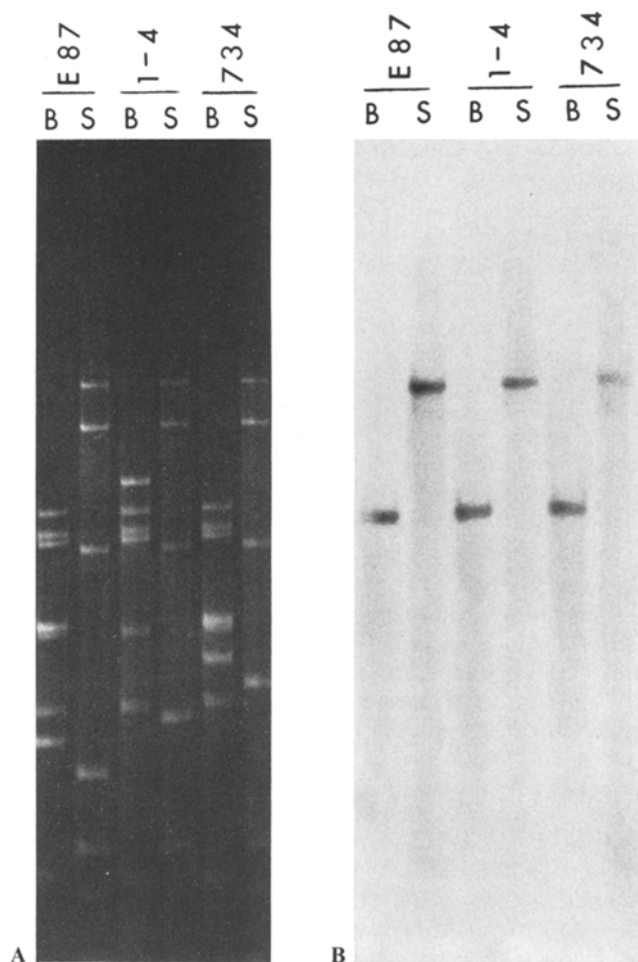


Fig. 3 A, B. Transfer hybridization of mtDNA to localize the COIII gene. **A** EthBr-stained gel of *Bgl*III and *Sst*I digests of three *Achlya* strains. **B** Autoradiograph of these DNAs transferred to nitrocellulose and hybridized with yeast mtDNA sequences (Table 1)

to allow hybridization of eight of the nine probes. The stringency at this criterion resulted in hybridization only to specific contiguous *Achlya* DNA regions. Due to the high A-T content of the *var1* probe (Hudspeth et al. 1982), those hybridizations were performed at 40° C, a criterion required for homologous hybridization of the *var1* gene (Zassenhaus et al. 1983). The results of the hybridization of the COIII gene is shown in Fig. 3, and the localization of all the gene probes in Fig. 1.

The gene mapping results for the nine loci assayed show that only the ribosomal cistrons are encoded within the repeat unit. By using specific 5' and 3' regions of the yeast L-rRNA probe, we have localized all the homologous *Achlya* regions to a repeat *Bgl*III fragment with the 5' sequences proximal to the S-rRNA locus. The 3' sequences extend into the *Bam*HI-*Bgl*III portion of this fragment, which is also the region of the repeat insertion/deletion event. The S-rRNA probe hybridized to sequences between the *Hpa*I and *Eco*RI restriction sites near the small single copy region. By assuming reasonable length and sequence conservation with *S. cerevisiae* S-rRNA, and the same polarity as the L-rRNA, the maximum limits for the 5' and 3' termini are 154 bp preceding the *Hpa*I site and 966 bp past the *Eco*RI site. *Achlya* sequences homologous to the seven *S. cerevisiae* protein encoding probes are found for COI,

II, and III, ATPase 6, and *var1* in the large single copy region, and for ATPase 9 and COB in the small single copy sequences.

Examination of insertion/deletion regions showed, surprisingly, that all four regions of variable sequence length were in close proximity to potential genic regions. For the three single copy coding regions, the insertions or deletions need not be within the proximal coding sequences, since the smallest restriction fragment containing these regions of size variation is larger than the entire *S. cerevisiae* coding sequence from which the probe was derived. Furthermore, there has been no prior demonstration in other fungi of optional introns being associated with any of these loci. For instance, in the large single copy DNA the COIII gene maps to the same *Hpa*I-*Kpn*I restriction fragment that contains the 310 bp insert in 734, 1-4, and 8-6. However, the minimal size of this fragment (2.0 kb in E-87) is far greater than either the size of the gene probe (367 bp) or the size of the entire *S. cerevisiae* coding sequence (810 bp). Similar arguments also apply to the COII and ATPase 9 regions.

The large rRNA cistron has been mapped to fragments adjacent to, and within, the *Bam*HI-*Bgl*III fragment containing the inverted repeat strain-specific insertion. Although it is tempting to speculate that this insertion is analogous to optional L-rRNA introns described in other fungi (Dujon 1980; Netzker et al. 1982; Burke and RajBhandary 1982), we have not determined the polarity of the hybridization in the insert-containing fragment and cannot ascertain whether the insertion sequences interrupt the L-rDNA sequence or are merely located at its 3' terminus.

Both COB and COI loci in *S. cerevisiae* contain combinations of multiple introns that can extend the gene loci to over 10 kb (Grivell et al. 1980, 1982). The probes used in locating homologous *Achlya* genes were exon regions of less than 0.5 kb. Although both *Achlya* genes could theoretically extend through proximal insertion regions, too little of the potential genic region has been analyzed thus far to evaluate this possibility.

Finally, we note that the dual orientation of the *Achlya* mitochondrial genome results in two distinct gene orders. One of these is that illustrated in Fig. 1. The second order results from an inversion of either single copy region through a recombination event between the repeats.

Discussion

Our detailed restriction analysis of four closely related heterothallic *Achlya* strains has enabled us to establish a common physical map of their mitochondrial genomes. Reference to this map has provided us with a minimal framework to reflect changes in both restriction endonuclease sites and DNA sequence organization resulting from insertions. The insertions (Fig. 1) not only account for the size variation of the strains, but suggest that insertion events are relatively region-specific. Indeed, though there are seven insertions of different sizes, they occur only in four reasonably well-defined regions of the genome. Previous comparisons of mtDNA physical maps from the ascomycetes *Saccharomyces* (e.g. Sanders et al. 1977), *Aspergillus* (Earl et al. 1980), and *Neurospora* (Mannella et al. 1979) have shown similar region-specific insertions. In many instances these insertions are associated with genic regions, either as introns interrupting coding sequences (e.g. Bonitz et al. 1980; No-

brega and Tzagoloff 1980), or flanking coding regions (Hudspeth et al. 1984). In *Achlya* we note that all of the insertions also correlate with genic regions, but we cannot yet define their exact relationship to coding sequences.

Our restriction analyses have allowed us to assess the relative rates of sequence divergence between inverted repeat and single copy sequences. Whether our analysis is based upon pairwise comparisons or the comparison of total site variations involving all four heterothallic strains, the data clearly indicate the relative conservation of inverted repeat sequences. Furthermore, the relative conservation of inverted repeat sequences was still retained when we carried out pairwise comparisons of our four strains with the more distantly related homothallic *A. klebsiana* (Boyd et al. 1984).

The relative stability of the repeat regions may result from two fundamentally different mechanisms. One is a consequence of the inverted repeat per se, and would result from conversion of a mutation arising in a repeat arm through intramolecular recombination. The other is a consequence of the particular sequences in the repeat arms, and would result from genetic selection. We favor the latter mechanism for two reasons. First, consideration of the effect of intramolecular recombination versus intermolecular recombination on neutral new mutations indicates that the former can affect the rate of fixation, but not, in fact, the probability of fixation over evolutionary time. Second, our data suggest (Table 2, lines 4 vs. 5) that the conservation is limited to the portion of the repeat encoding the rRNAs. However, the paucity of sites in the non-rDNA repeat limits the strength of this conclusion. The same interpretation was also reached in a study of legume chloroplast genomes (Palmer et al. 1983), although those data were also deficient in non-rDNA restriction sites in the repeat. These interpretations are in accord with the well-known relative stability of rDNA, which for mtDNA has been observed in apes and humans (Brown et al. 1982; Cann et al. 1984). However, it is likely that any coding region would appear stable relative to the genome as a whole, suggesting, as our data indicate, that the non-rDNA region of the repeat does not have any other significant coding function.

Analysis of our sequence divergence data at the interspecies level does not conform to the designated speciation for *A. ambisexualis* and *A. heterosexualis*. The interspecies comparisons of strain 734 and 1–4 indicate a lesser degree of divergence than the intraspecies comparison of 734 and E-87. This appears to be a reflection of the close affinity of these species [only three of over 50 *Achlya* species are heterothallic (Dick 1973)], rather than the inappropriateness of the analysis, since a single site change in each of the pairings restores the species relationships. Furthermore, pairwise comparisons with the homothallic *A. klebsiana* demonstrate significant divergence. From a systematics viewpoint, then, sequence divergence data in the genus *Achlya* may be useful in distinguishing between two or more closely allied groups and/or between diverse species, but not for differentiating between closely related species.

The detection of *Achlya* gene loci is based upon heterologous hybridizations using *S. cerevisiae* genic regions of known DNA sequence. These genic probes are devoid of introns and flanking sequence, with the sole exception of the 30 nucleotides at the 3' end of *var1*. The reliability of this approach has been well documented by subsequent DNA sequence analysis in several fungi (e.g. Macino and

Tzagoloff 1980) and other lower eukaryotes (de la Cruz et al. 1984). We note only two instances of the inappropriate localization of mitochondrial genes by this technique: A *Zea mays* COII probe hybridized to *Trypanosoma brucei* sequence (Johnson et al. 1982 vs. Benne et al. 1983), and an *S. cerevisiae* COB probe used in *Podospira anserina* (Wright et al. 1982 vs. Jamet-Viorny et al. 1984). The gene map presented here in one of its two orientations represents the first for a non-ascomycetous filamentous fungus.

Although we have established regions of mitochondrial homology between *Achlya* and *S. cerevisiae* coding sequences, we have not shown that these sequences are either transcriptionally or translationally active. This consideration is most important in terms of the ATPase 9 and *var1* loci. With the exception of *S. cerevisiae* and some other yeasts (Clark-Walker and Sriprakash 1983), all animal and fungal systems examined encode the functional mitochondrial ATPase subunit 9 in the nuclear genome. In *Neurospora* and *Aspergillus* (Van den Boogaart et al. 1982; Brown et al. 1984), however, in spite of the functional gene's being nuclear, undisturbed ATPase 9 sequences are present in the mitochondrial genomes and are only minimally, if at all, transcribed (de Vries et al. 1983). Mitochondrial ribosomal proteins also appear to be nuclearly encoded with the exceptions of S-5 in *Neurospora* and *Aspergillus* (La Polla and Lambowitz 1981) and *var1* in *S. cerevisiae* and other yeasts (Terpstra et al. 1979; Clark-Walker and Sriprakash 1983). To date the only mitochondrial homologies to either ATPase 9 or to *var1* sequences reported outside of the ascomycetes have been seen in the basidiomycete genus *Coprinus* (Weber et al. 1986).

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