INVESTIGATION OF KALILO-LIKE PLASMIDS IN *NEUROSPORA* AND *GELASINOSPORA*

by

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Abstract

Mitochondrial DNA plasmids sharing great sequence homology with the senescence-inducing kalilo plasmid have been identified in natural isolates of *Neurospora crassa*, *Neurospora intermedia*, *Neurospora tetrasperma*, *Neurospora discreta* and a *Neurospora*-related genus *Gelasinospora*. Restriction enzyme analysis and sequence analysis have revealed that these plasmids are closely related by descent from a common ancestral plasmid. The phylogenetic tree constructed on the basis of the terminal inverted repeat sequences of the kalilo-like plasmids correlates well with the established taxonomy of their host fungi. The attempt to transfer kalilo-like plasmids to standard lab strains of *N. crassa* has had limited success. Only a few transferrants were obtained and they all proved to be unstable. Therefore, the comparison of plasmid function can not yet be made at the plasmid level with identical mitochondrial DNA and nuclear DNA background. However, the comparison of plasmid TIR sequences reveals some interesting features which may be relevant to the plasmid function. The failure of plasmid transfer indicates that horizontal transfer must be a rare event, though it may be evolutionarily significant as to contribute to the current distribution pattern of kalilo-related plasmids.

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This thesis is dedicated to my parents.

Chapter 1 Introduction

A general introduction to the genus *Neurospora*

Neurospora is a genus belonging to the class *Ascomycetes*, sub-class *Pyrenomycetes*. It has long been established as a model organism for eukaryotic genetics studies. It possesses a spectrum of nuclear, mitochondrial and plasmid genetic systems that are amenable to analyses by molecular biology methods. It is capable of heterokaryon formation, and mutants are easily obtained.

During the vegetative life cycle of *Neurospora*, there are haploid nuclei with only one set of seven chromosomes in each nucleus. The *Neurospora* genome is relatively small compared with other eukaryotes. It contains about 47 million nucleotide pairs; of these, 93% are unique sequences. The *Neurospora* mitochondrial genome is one of the best studied of all fungi. The mitochondrial DNA of *Neurospora crassa* (74-OR23-1A) has been cloned and the nucleotide sequence of 94% of its genome has been determined (For review, see Griffiths et al., 1995). Numerous mitochondrial plasmids have been found in natural isolates of *Neurospora*, which has resulted in a new area of genetic research.

The mating types and heterokaryon incompatibility in *Neurospora*

The life cycle of *Neurospora* allows both sexual and asexual reproduction. For sexual reproduction, some *Neurospora* species are heterothallic, such as *Neurospora crassa*, in which case crosses must involve direct interaction between strains of opposite mating types. Some species such as *Neurospora terricola* , are homothallic, or self-fertile. Others such as *Neurospora tetrasperma*, are pseudohomothallic. In *Neurospora tetrasperma*, individual ascospores are usually (but not always) heterokaryons that contain haploid nuclei of opposite mating types (Dodge,1927; Perkins, 1992).

The mating types of *Neurospora*, designated as *A* and *a*, are determined by codominant idiomorphs (Glass et al.,1988; reviewed by Metzenberg and Glass, 1990) located

on linkage group I (Perkins et al., 1982). The mating-type genes have a dual function: strains of opposite mating types are required for sexual reproduction, but only strains of the same mating type can form stable vegetative heterokaryons. The inability to make heterokaryons between specific strains is called heterokaryon incompatibility. It may be expressed as death or weak growth.

Besides difference in mating type, heterokaryon incompatibility can also be a result from allelic differences at heterokaryon incompatibility loci (*het* loci). Strains are compatible only when alleles at all *het*-loci are identical (Perkins, 1988). Studies in *N. crassa* suggest that the *het*-gene polymorphism is widespread in natural populations (Mylyk, 1976).

Fungal plasmids

Plasmids are small extragenomic DNA molecules that can reproduce inside living cells or organelles. They can replicate separately from the genome, or integrate into the genome and replicate as part of the genomic DNA. Plasmids were originally discovered in bacteria. They have also been found in eukaryotes including fungi and plants (reviewed by Meinhardt et al. 1990). Mitochondrial plasmids have been found in many different fungal cultures isolated directly from natural populations (reviewed by Griffiths, 1995). They are linear or circular DNA elements which may contain sequences coding for products that are involved in replication of the plasmids. The origin and function of these fungal plasmids are still unclear. In a few cases, the mitochondrial plasmids were found to be associated with a specific phenotype, such as the senescence-inducing plasmids kalilo and maranhar in *Neurospora*, but most fungal plasmids do not have any detectable effect on the phenotype of their host strains.

Fungal senescence

The phenomenon of fungal senescence has been best studied in *Podospora* and *Neurospora.* In the context of fungi, senescence may be defined as the progressive loss of growth potential culminating in death (Griffiths, 1992). It is different from the sporadic fungal organismal death regularly encountered in lab studies in that senescence has a more predictable and repeatable death pattern. Senescence can be measured by the growth of parallel fungal cultures in race tubes or in serial subcultures where symptoms are expressed and cultures die after a strain-specific distance or a strain-specific number of subcultures. The senescence symptoms include morphological abnormalities, such as hyphal tip swelling, and mitochondrial dysfunction, such as cytochrome abnormalities and rearranged mitochondrial DNAs. In this way, senescence resembles the phenotype of other mitochondrial mutants such as the *poky* and *stopper* mutants of *Neurospora.*

All natural isolates of *Podospora* species die (Esser et al., 1980). The senescence is concomitant with the appearance of new mtDNA-derived elements in mitochondria and rearrangement of mtDNA. Some nuclear and mitochondrial mutants have been found to prolong the lifespans of *Podospora* strains, such as the standard mitochondrial chloramphenicol-resistant marker as described by Belcour and Begel (1980) and the morphological double mutant *incoloris* and *vivax* (Tudzynski and Esser, 1979).

In *Neurospora*, senescence has only been found in natural field-isolated strains. The two best studied cases are the kalilo strains of *Neurospora intermedia*, and the maranhar strains of *Neurospora crassa.*

The kalilo plasmid: its genetic organization and relation to the onset of senescence phenotype in *Neurospora intermedia*

Approximately 30% of *N. intermedia* strains isolated from the Hawaiian island of Kauai show senescent phenotype (Griffiths and Bertrand,1984; Griffiths et al.1986; Debets et al., 1995). Molecular studies of these senescent strains led to the discovery of a 8.6 kb linear mitochondrial plasmid, which is named "kalilo", meaning "dying" in the Hawaiian language.

The kalilo plasmid resides in mitochondria either as a free plasmid or as a mitochondrial insertion sequence. The free plasmid has no detectable effect on the *N. intermedia* host. However, at some point, the kalilo plasmid may insert into mitochondrial DNA (mtDNA) and lead to mitochondrial dysfunction. The normal mtDNA is then progressively replaced by the insertion type. Therefore, it is reasonable to infer that senescence is a result of progressive loss of normal mitochondrial functions caused by plasmid insertion into mtDNA. Some kalilo-plasmid-containing strains were found to escape senescence by the function of nuclear suppressors (Griffiths st al., 1992; Yang and Griffiths, 1993). The suppressor may either inhibit the plasmid insertion or eliminate the plasmid to a barely detectable level.

The complete nucleotide sequence of the kalilo plasmid is now available (Chan et al., 1991). It turned out to be a 8643 bp linear DNA fragment with an organization typical of eukaryotic linear plasmids (Meinhardt et al., 1990; Griffiths et al., 1995). It has perfect terminal inverted repeats on both ends and two large open reading frames running in opposite directions towards the centre. There are also 120 kd terminal proteins covalently bound at its 5' ends. The overall structure of kalilo is shown in Fig. 1. The various features are described further:

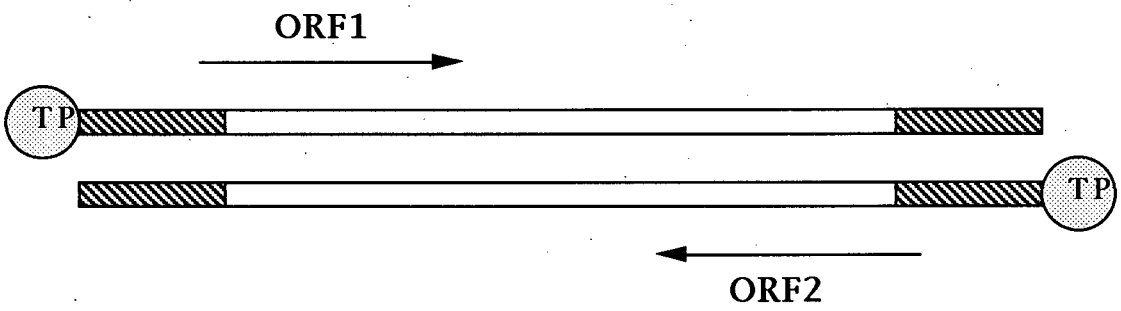

Fig. 1 The overall structure of kalilo plasmid

Terminal inverted repeats

Open reading frames (Arrows show the translational direction)

Terminal proteins

a. The open reading frames (ORFs):

The kalilo plasmid is known to be transcribed, though no translational product has been identified. Multiple transcripts of different sizes were found in all the developmental stages . Two transcripts of 4.4- and 4.8-Kb correspond to the two ORFs in size, position and polarity (Vickery and Griffiths, 1993). The two non-overlapping ORFs were proposed to be translated in the *Neurospora* mitochondrial genetic code (Nargang et al.,1984). ORF 1 codes for a putative protein 811 amino acids long that shows regional homology to bacteriophage T7 RNA polymerase, and ORF 2 codes for a putative protein 970 amino acids long that shares motifs of virus and bacteriophage DNA polymerases, and with putative proteins of ORFs of other eukaryotic plasmids (Chan et al., 1991).

b. The terminal proteins (TPs):

The 5' end of kalilo DNA bears a covalently bound 120 kd protein that protects the plasmid from digestion by 5' exonucleases (Vierula et al., 1990). Such a large protein is

unlikely to be encoded by one of the kalilo ORFs. The source of this protein remains unknown.

The function of the TP is also unknown, but it is believed to be at least partially responsible for the replication, as well as integration of the plasmid (Chan et al., 1991). The role of the terminal protein in replication and integration would be best addressed with *in vitro* assays using purified components or by an *in vivo* system that permits the generation and analysis of mutants of the multicopy plasmid. But neither of these options is available yet for the kalilo plasmid of *Neurospora*.

c. The terminal inverted repeats (TIRs):

The 1366 bp perfect TIRs of kalilo are the longest known to-date for a mitochondrial plasmid. Nucleotides 6 to 25 inwards from either end of the plasmid form short, imperfect palindromes (Chan et al. 1991). The TIRs also contain some very short ORFs, but none of them has been shown to be translated.

The ability to integrate into mtDNA and cause mitochondrial dysfunction is one of the unique properties of the kalilo plasmid. Almost full-length fragments insert into the mitochondrial genome by a mechanism that creates giant inverted repeats of mtDNA flanking the inserting sequence. A 5 bp match is found between the terminal sequence of kalilo plasmid and its insertion point in mtDNA. This 5 bp random sequence could be anywhere within the terminal 20 bp of the TIRs. Short segments of DNA (5-18 bp) distal to this 5 bp region are lost from both ends of the plasmid upon integration (Dasgupta et al., 1988)

Interestingly, the inserted form of maranhar, another linear senescence-inducing plasmid of *Neurospora*, is also flanked by very long inverted repeats of mtDNAs. But unlike kalilo, maranhar does not have terminal palindrome structure, and it integrates into the mtDNA without losing nucleotides from either terminus (Court et al., 1991). In addition, maranhar has no nucleotide sequence homology to either the host mtDNA or the kalilo DNA.

Comparison between these two *Neurospora* plasmids suggests that the integration of these plasmids occurs via a mechanism that may involve a panhandle structure formed by pairing of the nucleotides in the TIRs. The TIRs may also provide recognition sequences for the integration of the elements into mtDNA (Chan et al., 1991). The resolution of this problem might be achieved by the development of either an *in vitro* integration assay or an *in vivo* mutant system.

Kalilo-like plasmids found in other species of *Neurospora* and in *Gelasinospora*

Linear plasmids that show strong sequence homology to kalilo DNA have been found in natural isolates of *N. crassa, N. discreta* and *N. tetrasperma.* One kalilo-like plasmid was also found in *Gelasinospora.* The discovery of these new plasmids provides an opportunity to study the function and evolution of the kalilo plasmid.

Two of these kalilo-like plasmids had been studied intensively prior to the present work.

LA-kalilo plasmid

The LA-kalilo DNA was first identified in two Louisiana strains of *N. tetrasperma* (Marcinko-Kuehn et al., 1994). It showed strong nucleotide homology to kalilo DNA by Southern hybridization analysis. Besides, the LA-kalilo plasmid has a restriction map almost identical to that of kalilo DNA, only the termini were thought to be shorter by approximately 100 bp.

Many LA-kalilo-bearing strains senesced, but the presence of this plasmid does not guarantee senescence. The senescence phenotype is inconsistent and atypical when compared with that of kalilo-containing strains: the symptoms are slower to develop and parallel cultures often show differences in the expression or the time of death. Furthermore, LA-kalilo DNA does not insert into mtDNA.

Gel-kalilo plasmid

The Gel-kalilo DNA was found in a sample of *Gelasinospora* isolates from Louisiana soil. Sequence analysis of this plasmid shows remarkable similarity to kalilo DNA (Yuewang et al., 1996). Besides the identical genetic organization, the sequence similarity is 100% over large regions, and approximately 95% overall in the ORFs. The main differences are in the intergenic region and in the terminal inverted repeats. Both Gel-kalilo plasmid-containing and plasmid-free strains were grown in race tubes to investigate senescence. All the strains examined showed senescence. Therefore, it is impossible to correlate the possession of Gel-kalilo DNA with senescence.

Objectives of these studies

The work on the kalilo-like plasmids as described in the following chapters was performed in an attempt to answer the following questions.

1.What is the exact difference between kalilo and other kalilo-like plasmids? What is(are) the consequence(s) of this difference? Why do they have such different behavior in regards to insertion and senescence? Is it a difference of the plasmids, or a difference between their fungal hosts?

2. How did kalilo-like plasmids become distributed among *Neurospora* and *Gelasinospora* strains, by in situ evolution or horizontal transfer?

3. Is plasmid insertion the only determining factor of fungal senescence?

4. How do the terminal inverted repeats work during insertion?

Answers to these questions would help in the comprehension of the function and evolution of the kalilo plasmids, as well as the function of the TIR. Possession of perfect TIRs is a common feature of all eukaryotic plasmids (Meinhardt et al., 1990; Griffiths et al., 1995).

Chapter 2 Investigation of Kalilo-like Plasmids in Natural Isolates of *Neurospora*

Introduction

Several DNA elements sharing sequence homology with kalilo DNA have been found in the heterothallic species *N. intermedia*, *N. crassa* and *N. discreta*, the pseudohomothallic species *N. tetrasperma* and a homothallic species of a *Neurospora*-related genus *Gelasinospora*. The entire sequence of the kalilo-like plasmid in *Gelasinospora* (Gel-kalilo DNA) is available (Yuewang et al., 1996). The similarity of the LA-kalilo DNA in *N. tetrasperma* with the prototypic kalilo DNA has been well characterized by restriction enzyme digestion and Southern hybridization analysis (Marcinko-Kuehn et al., 1994). Restriction enzyme analysis (Fig. 2) suggests that the LA-kalilo plasmid is more similar to kalilo DNA than is Gel-kalilo DNA. However, the LA-kalilo plasmid is slightly shorter than kalilo DNA in the TIRs, so a sequencing analysis was necessary to identify the exact difference between kalilo and LA-kalilo DNA. The existence of other kalilo-homologous DNAs in *N. crassa*, *N. discreta* and *N. tetrasperma* was detected by dot-blots and Southern hybridization (Arganoza et al., 1994).

The discovery of kalilo-like DNA in *Neurospora* and *Gelasinospora* raised interest in studying senescence in these fungal strains and characterizing these kalilo-like DNA elements by gel electrophoresis, restriction enzyme analysis and sequencing analysis. This would enable a comparison to be made among the plasmid sequences which might help to understand the function and evolution of the kalilo plasmid.

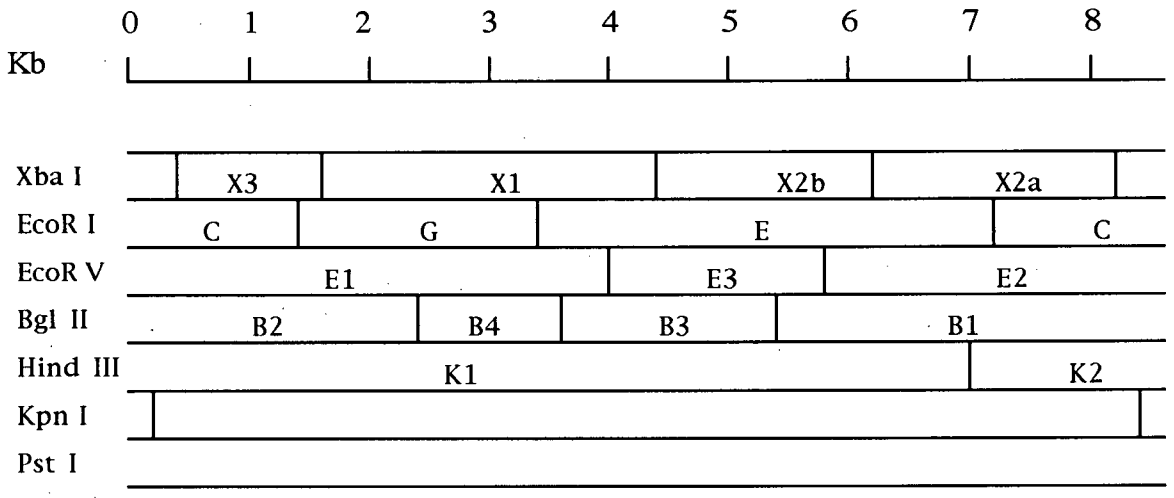

Fig. 2a

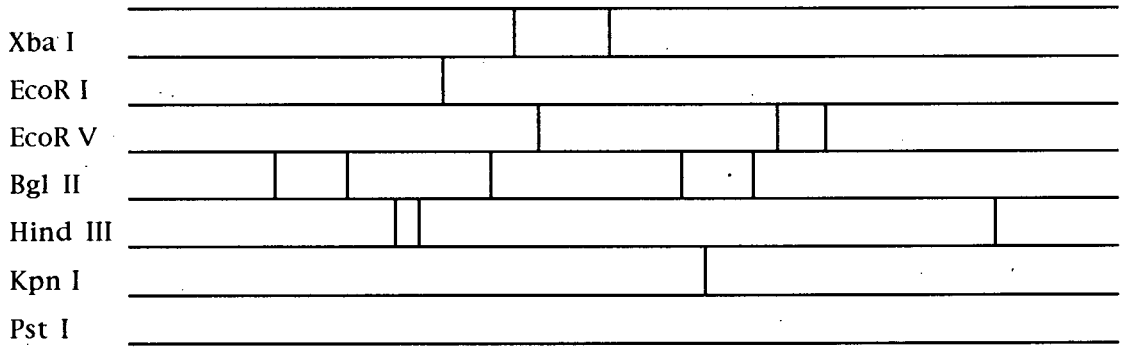

Fig. 2b

Fig. 2 Restriction maps of kalilo plasmid, LA-kalilo plasmid and Gel-kalilo plasmid. Restriction cutting sites are represented by vertical lines. Letters and numbers in Fig. 2a represent the codes used in subcloning.

a: Restriction map of kalilo DNA (Chan et al., 1991) and LA-kalilo DNA (Marcinko-Kuehn et al., 1994)
b: Restriction map of Gel-kalilo DNA (Yuewang et al., 1996)

Materials and Methods

Strains and culture conditions

The LA-kalilo-containing *Neurospora tetrasperma* strain (P4495) was collected and isolated from Louisiana soil samples by Dr. D. Jacobson. It was classified as *Neurospora tetrasperma* by the criteria established by Perkins et al. (1976). Other *Neurospora* strains were ordered from the Fungal Genetics Stock Center (FGSC). According to Arganoza et al. (1994), they all contain kalilo-related plasmids. Information on all the strains used in this work is listed in Table 1.

Table 1 *Neurospora* strains used for study of kalilo-related DNA plasmids

Fungus	Location	FGSC stock#	Mating type	Reference
N. crassa	Haiti	4709	a	
	Haiti	4710	A	
	Haiti	4711	A	
	Ivory Coast	4832	a	Fungal Genetics
N. discreta	Florida	5923	a	
	Papua New Guinea	6784	a	Newsletter
	Thailand	6790	a	
	Ivory Coast	6794	A	
N. intermedia	Hawaii	3718	A	No.41, 1994
	Hawaii	3721	a	
	Hawaii	3722	A	supplement
	Hawaii	5014	a	
N. tetrasperma	Moorea-Tahiti	6583	A+a	
	Moorea-Tahiti	6591	A+a	
	Louisiana	P4495	A+a	

All culturing and strain manipulation used standard techniques devised for *Neurospora*, as summarized by Davis and deSerres (1970).

Isolation of plasmid DNA

The plasmid DNA was co-purified with mitochondrial DNA using the small scale mtDNA method described by Myers (1988). Proteinase K digestion was necessary prior to phenol/chloroform extraction of proteins. For sequencing analysis, plasmid DNA needed to be further treated by *Pst I* and λ exonuclease. The restriction enzyme *Pst I* does not have any cutting site in all the kalilo-related DNA known so far (Yang and Griffiths, 1993), and the 5'-TPs protect the plasmids from being digested by λ exonuclease. Therefore, the plasmid DNA remained intact while the mtDNA was completely degraded by *Pst I* and λ exonuclease. Enzymes and small fragments of mtDNA were removed by phenol/chloroform extraction and ethanol/NH_4AC precipitation. Plasmid DNA purified this way can be used directly as a template for sequencing analysis.

Southern blot analysis:

The Southern hybridization methodology was similar to that described by Maniatis et al. (1982). The 8.2 kb *Kpn I* restriction fragment which encompassed most of the kalilo plasmid was cloned in the pUC18 vector and used as probe to detect kalilo-homologous sequences. The DNA probe was labelled with ^{32}P-dCTP using an oligolabelling kit available from Pharmacia.

Detection of plasmid insertion into mtDNA:

The mtDNA samples were digested by *Pst I*. Since *Pst I* does not have restriction site in the kalilo-like DNA, plasmid insertion can be detected by the presence of linear kalilo-homologous DNA fragments bigger than free plasmids (Yang and Griffiths, 1993). These fragments represent plasmid DNA flanked by mtDNA ending with *Pst I* sites.

DNA sequencing:

The DNA sequence was determined using an automated sequencing system (Applied Biosystems 377 Automated DNA Sequencer). Sequencing primers were synthesized oligonucleotides designed on the basis of the kalilo sequence (Chan et al., 1991) and sequencing data obtained in the present work. Plasmid DNA purified as described above was used as the template for sequencing. The first round of sequencing was primed by a primer close to the inner end of TIR. The DNA sequence of the rest of TIR was determined by primer walking, which was a series of unidirectional sequencing reactions in which the primer used in one reaction was designed on the basis of the outermost sequence of the previous reaction. The DNA sequence of the TIR was hence determined until additional sequence could not be obtained, and this was presumed to be the terminus of the plasmid (Yuewang et al., 1996).

Computer Analysis:

The DNA sequencing data were analyzed by computer programs including Assembly LIGN, MacVector, blastsearch (http://www.ncbi.nlm.nih.gov/Recipon/blast_search.shtml), PHYLIP (Joseph Felsenstein, Department of Genetics SK-50, University of Washington, Seattle, WA 98195) and the Wisconsin Sequence Analysis Package - Version 8 from Genetics Computer Group, Inc. (Devereux et al., 1984).

Results

Visualization of kalilo-like plasmids

Sixteen *Neurospora* isolates were proposed to contain kalilo-like plasmids by Arganoza et al. (1994). MtDNA of 14 of the 16 strains (Table 1) was extracted and run on 0.8% agarose gels. Plasmids were visualized as distinct bands after ethdium bromide staining (data not shown). The DNA was then transferred to nylon membrane and probed with the large kalilo *KpnI* fragment cloned in pUC18. The vector DNA does not have any homology with the fungal DNA, so anything hybridizing to the probe has homology with kalilo DNA.

As shown in Fig. 3, DNA plasmids showing strong homology to the kalilo probe were found in some but not all of the *Neurospora* strains being studied. Strains that do not have kalilo-homologous sequence either do not contain a kalilo-like plasmid, or they contain a low level of kalilo-like plasmid that was barely detectable by Southern hybridization. Interestingly, the kalilo-like plasmids in the two *N. discreta* strains are smaller than kalilo, while other kalilo-like plasmids in the *N. crassa* and *N. tetrasperma* strains are approximately of the same size as kalilo. Restriction analysis using *EcoRI* (Fig.4a) and *XbaI* showed that the smaller size of the *N. discreta* kalilo plasmid is due to its shorter terminal inverted repeats (a restriction map is shown in Fig. 4b). The existence of the TIR sequences in these kalilo-like plasmids was confirmed by digesting plasmid DNA samples with *Hind III*. *Hind III* cuts all the kalilo-like DNA only once and generates two fragments of different sizes. Southern hybridization was then performed using a cloned terminal kalilo fragment (C) (Fig. 2) as probe. Both *Hind III* fragments showed homology to the kalilo probe, confirming the existence of terminal repeat sequences on both ends (data not shown).

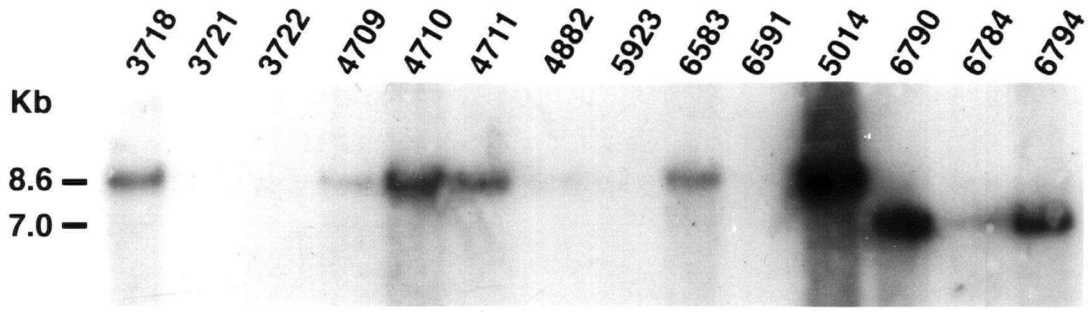

Fig. 3 Southern hybridization analysis of kalilo-like plasmids. Numbers cross the top are FGSC numbers of the *Neurospora* strains being studied. Numbers on the left side show the sizes of plasmid bands in Kb.

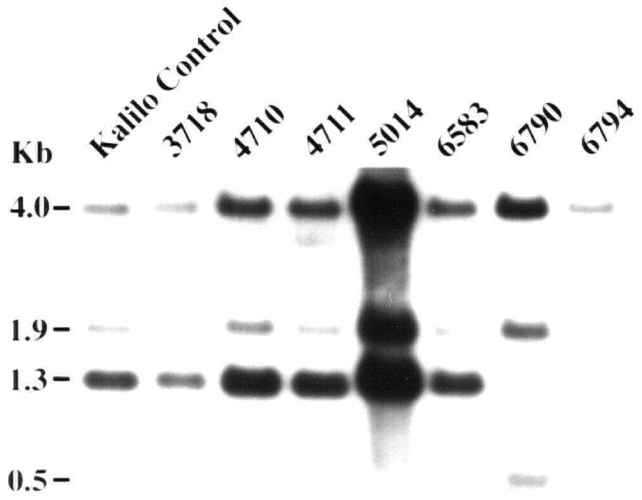

Fig. 4a

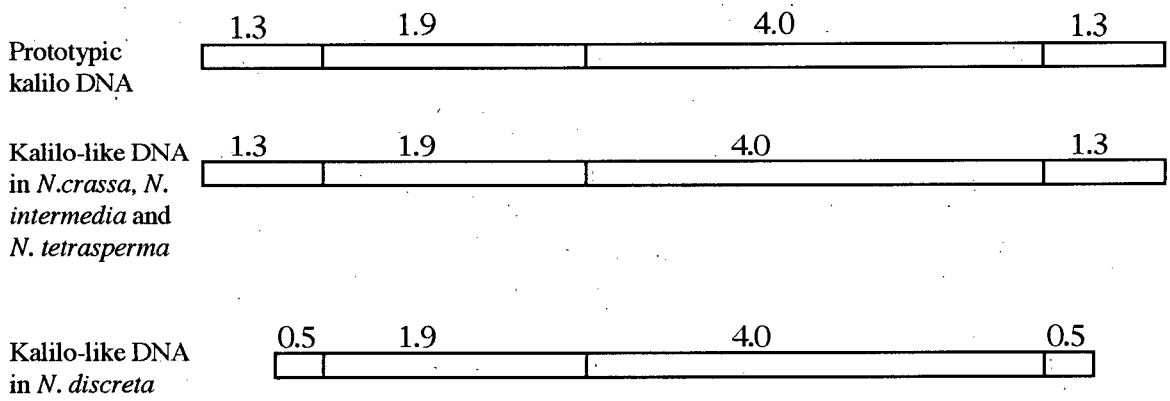

Fig. 4b

Fig. 4 Restriction analysis of kalilo-like plasmids by *EcoRI.*

a. Restriction fragments probed by a cloned kalilo *KpnI* fragment.

b. *EcoRI* restriction maps of kalilo-related plasmids. The horizontal bars represent DNA plasmids. The vertical lines represent *EcoRI* restriction cutting sites. The size of each fragment is shown in Kb above the corresponding rectangule.

Sequencing analysis of TIRs of kalilo-like plasmids and senescence studies of *Neurospora* strains containing kalilo-like plasmids

The TIRs of the kalilo-like plasmids were sequenced and the sequences were then aligned with the TIR sequence of the prototypic kalilo DNA (nucleotide sequence alignments are shown in Appendix I). Senescence was tested by both serial subculturing and race tube growth (de Groot, 1995). Onset of the senescent phenotype was characterized by slowing of hyphal growth in a race tube, or mycelial death in serial subcultures. This experiment lasted for 2 months. Strains did not show any senescent phenotype within 53-day race tube growth or 20 serial subcultures were classified as non-senescent. By looking at the sequence alignments and the senescence phenotype, the kalilo-like plasmids can be grouped into four major types.

Type I: Original kalilo

This is the prototypic kalilo sequence represented by the kalilo plasmid found in *N. intermedia* strains from the Hawaiian islands (Griffiths and Bertrand, 1984). Another version showing identical restriction map overall in the plasmid and 100% sequence identity in the TIRs was also found in a *N. tetrasperma* strain (6583) from Moorea-Tahiti. The kalilo plasmid in *N. intermedia* was also transferred to standard *N. crassa* strains with Oak Ridge background (Griffiths et al., 1990). All kalilo-containing strains show senescent phenotype accompanied by plasmid insertion into mtDNA (Bertrand et al., 1985; Griffiths et al., 1990; this study, data not shown).

Type II: LA-kalilo

The first example of this type is the kalilo-like plasmid found in two *N. tetrasperma* strains from Louisiana (Marcinko-Kuehn et al., 1994). A similar plasmid showing an identical restriction map overall in the plasmid and over 99% sequence similarity in the TIR was found in this study in a *N. crassa* strain (4711) from Haiti. The LA-kalilo-type plasmids shows the greatest similarity with type I kalilo DNA among all the kalilo-like plasmids. The only major difference lies in a 60 bp regions in the TIRs (Fig. 7). DNA alignment of this 60 bp in both kalilo and LA-kalilo is shown in Fig. 5.

Compared with kalilo DNA, the LA-kalilo plasmid has a 15 bp insertion and 50 bp deletion which result in a slightly shorter TIR as noticed by Marcinko-Kuehn et al.(1994). A blast search of the 15 bp insertion fragment did not reveal any significant matches in the gene data bank.

```
                    7860
TIR of kalilo DNA   GCACGTAGCA  AACTTATTGT  TATCCTTTCC  TCTTTCATTT
TIR of LA-kalilo DNA  ******GCTT  CTTACTATGT  A---------  ----------

                    7900
TIR of kalilo DNA   GCGTACAATT  ACGGTTTACA  TTATTATGAG
TIR of LA-kalilo DNA  ----------  ---*******  **---G****
```

Fig. 5 Comparison of the 60 bp region in TIRs of kalilo and LA-kalilo DNA

- Deletion in LA-kalilo DNA.
* Identical nucleotide between the two sequences.
Sequence underlined represent the 15 bp insertion.
Numbers shown above the sequences correspond to numbers in Fig. 6.

The senescent phenotype in LA-kalilo-containing strains is not so predictable as that in kalilo-containing stains. In the two LA-kalilo-containing *N. tetrasperma* strains, senescent symptoms are slower to develop and parallel cultures often show differences in the expression or the time of death (Marcinko-Kuehn et al., 1994). The senescent phenotype was not observed in the LA-kalilo-containing *N. crassa* strains (de Groot, unpublished result). Besides, LA-kalilo plasmids do not insert into mtDNA as shown by *Pst I* treatment (Marcinko-Kuehn, 1994; unpublished observations).

Type III: Short kalilo

The senescent phenotype was not observed in *N. discreta* strains carrying short kalilo DNA (de Groot, 1995). Compared with kalilo DNA, short kalilo plasmid has a big deletion in the TIRs. Based on the results of previous surveys of kalilo-like DNA in *Neurospora* and related fungal isolates (Table 2), the short kalilo DNA has so far only been detected in *N. discreta* strains.

Table 2 Previous survey of kalilo-like plasmids in *Neurospora* and related fungal isolates

Fungal isolates investigated	**Detection method**	**Results**	**Reference**
82 *N. intermedia* isolates collected from Hawaiian islands	Dot-blot	38% contain the senescence-inducing kalilo DNA	Debets et al., 1995
38 world-wide collection of *N. crassa* and *N. intermedia*	Southern hybridization	kalilo plasmid was only detected in *N. intermedia* strains from Hawaiian islands	Yang and Griffiths, 1993
39 *N. crassa* and 14 *N. tetrasperma* isolates collected from Louisiana	dot-blot and Southern hybridization	Only two *N. tetrasperma* strains contain LA-kalilo DNA	Marcinko-Kuehn et al., 1994
225 worldwide collection of *Neurospora* and related fungal isolates	dot-blot Southern hybridization	kalilo-homologous plasmids were found in 16 *Neurospora* strains	Arganoza et al., 1994
16 kalilo-containing strains proposed by Arganoza et al., 1994	Southern hybridization	Two *N. discreta* strains contain short kalilo DNA, while some other strains contain kalilo or LA-kalilo DNA.	This paper

Type IV: Gel-kalilo

The kalilo-like plasmid found in two *Gelasinospora* strains from Louisiana (Yuewang et al., 1996) is the only member in this group. It shows the biggest divergence from the original kalilo plasmid. Its full DNA sequence has been published and all *Gelasinospora* strains were shown to be senescent, whether they contain the plasmid or not (Yuewang et al., 1996). Restriction enzyme analysis using *Pst I* did not show any sign of plasmid insertion into mitochondrial genome (data not shown). In this case, senescence must be caused by mechanisms other than plasmid integration.

The results of the plasmid and senescence studies in kalilo-like DNA-containing fungal strains are summarized in Table 3. A comparison of the four types of TIRs is shown in Fig. 6.

Table 3 Summary of species distribution and senescence of kalilo-like plasmids

Type of kalilo	**Occurrence**	**Senescence observed**	**References**
kalilo	*N. intermedia* (Hawaii) *N. tetrasperma* (Moorea-Tahiti) *N. crassa* (Oak Ridge)[a]	Yes Yes Yes	Debets et al., 1995 This paper Griffiths et al., 1990
LA-kalilo	*N. tetrasperma* (Louisiana) *N. crassa* (Haiti)	Not consistent No	Marcinko-Kuehn et al., 1994 This paper
Short kalilo	*N. discreta* (Thailand) *N. discreta* (Ivory Coast)[b]	No	This paper
Gel-kalilo	*Gelasinospora* (Louisiana)	All *Gelasinospora* isolates show senescent phenotype.	Yuewang et al., 1996

a: The kalilo plasmids was transmissed to *N. crassa* from *N. intermedia* by hyphal contact.

b: Nucleotide sequence for short kalilo in this strain is not available yet.

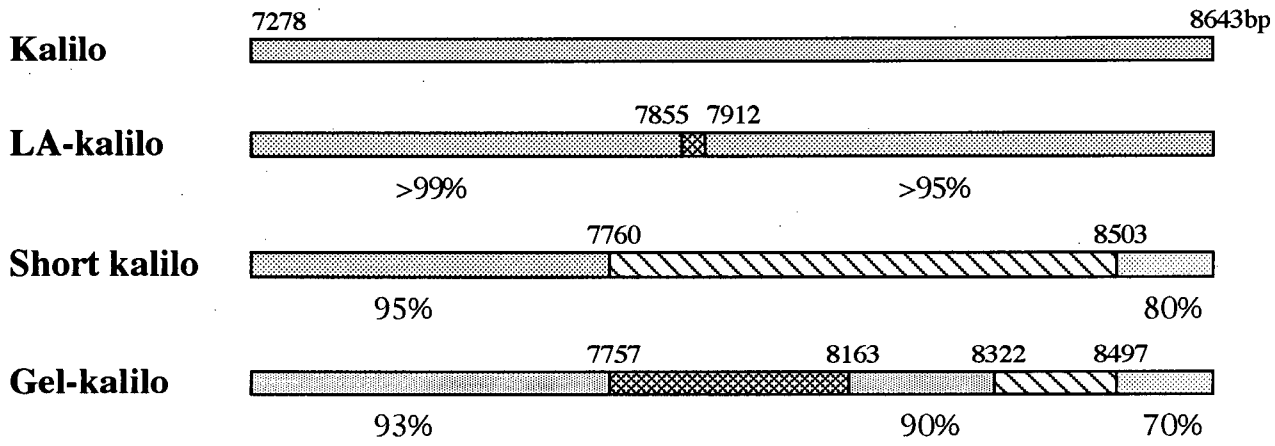

Fig. 6 Comparison of the TIRs of kalilo-like plasmids. The figure shows only one of the perfect TIRs of each plasmid. Numbers above the bars represent locations of fragments relative to kalilo DNA. Percentages below the bars represent degree of sequence homology to kalilo DNA.

Kalilo sequence

Deletion

Deletion + Insertion

Sample sequence analysis of ORFs of kalilo-like plasmids

Approximately 2 kb sequences of the ORFs of LA-kalilo DNA from *N. tetrasperma* strain P4495 were analyzed. The regions sequenced are shown by locations of the primers in Fig. 7. Nucleotide sequence alignments of these regions for kalilo DNA, LA-kalilo DNA and Gel-kalilo DNA are shown in Appendix II. The amino acid sequence alignments of the Gel-kalilo ORFs to the prototypic kalilo ORFs have been published (Yuewang et al., 1996), but the amino acid sequence alignments of the LA-kalilo ORFs to the kalilo ORFs are not yet available due to the lack of entire sequence of LA-kalilo DNA. At the nucleotide sequence level, LA-kalilo DNA show more than 99% similarity with kalilo DNA in the sampled ORF regions while Gel-kalilo DNA shows approximately 95% similarity with kalilo DNA overall in the ORFs (Yuewang et al., 1996).

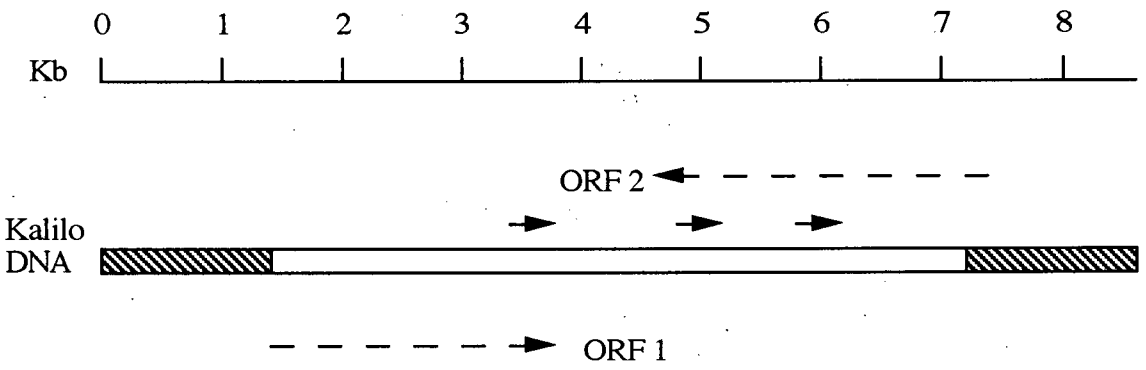

Fig. 7 Sequencing sample regions of the ORFs of the LA-kalilo plasmid

Sequencing primers

TIRs

Discussion

Several kalilo-like plasmids have been identified in a world-wide collection of strains of *Neurospora* and a related genus *Gelasinospora*. Discovery of these kalilo-like plasmids raised our interest in the evolution of these plasmids and their host strains. Studies on the kalilo plasmid family could also provide useful information on the structure and function of TIRs, which are common features of linear eukaryotic plasmids (Meinhardt et al., 1990; Griffiths, 1995).

The kalilo-like plasmids found so far are all linear DNA elements with identical terminal inverted repeats on both ends. Their resistance to λ exonuclease suggested the presence of terminal proteins (TPs) covalently bound to the 5'-ends (Vierula et al., 1990), which is a common structure of eukaryotic linear plasmids (Meinhardt et al., 1990; Griffiths, 1995). In addition, all of the kalilo-like plasmids show strong cross-hybridization with kalilo DNA at the DNA level. The LA-kalilo plasmids have a restriction map almost identical to that of kalilo DNA. Sample sequences in ORFs of LA-kalilo show more than 99% identity to kalilo ORFs. The Gel-kalilo DNA has a very different restriction map to kalilo, but its

sequence shows remarkable similarity. When compared with kalilo plasmid, the ORF1 sequence of Gel-kalilo is 95% similar at the DNA level and 91% similar at the amino acid level; the ORF2 sequence shows 93% similarity to kalilo DNA excluding length mutations (Yuewang et al., 1996). The short-kalilo plasmids have a restriction map identical to kalilo DNA in the ORF regions. These features make it reasonable to conclude that these kalilo-like plasmids are related by descent from a common ancestral plasmid. An unrooted phylogenetic tree of the kalilo-like plasmids was then constructed based on the sequences of their TIRs. The TIR sequences of the prototypic kalilo plasmid (Chan et al., 1991), the LA-kalilo plasmid in *N. tetrasperma* strain P4495, the short kalilo plasmid in *N. discreta* strain 6790 and the Gel-kalilo plasmid (Yuewang et al., 1996) were aligned by the multi-sequence alignment programs as described in the methods. This alignment (as shown in Appendix IIIa) shows neither the big deletion regions in the middle of the TIRs of short kalilo and Gel-kalilo plasmids, nor the sequence similarity in the temini of all kalilo-related plasmids. Therefore, the TIR sequences were re-aligned manually (alignment as shown in Appendix IIIb, the alignment of Gel-kalilo DNA to kalilo DNA was taken from Yuewang et al., 1996). This hand-made alignment matched up all corresponding regions including the terminal ends (as in Fig. 6) and the big deletions in Gel-kalilo DNA and short kalilo DNA were shown as big gaps in the middle of the TIRs. However, when both sequence alignments were subject to phylogenetic analyses by the PHYLIP programs (including a parsimony tree generated on the basis of bootstrap resampled data, and a UPGMA tree based on DNA distance matrix) and the Wisconsin Sequence Analysis programs, phylogenetic trees with identical branching pattern were obtained, though the branch lengths varied from tree to tree. A phylogenetic tree with identical branching pattern was also obtained when only the inner 500 bp of the TIRs were analyzed. The branching order information obtained from all the trees as described above was summarized in the unrooted phylogenetic tree shown in Fig. 8. The plasmid distribution pattern is superimposed to it in Fig. 9 to view the evolution better.

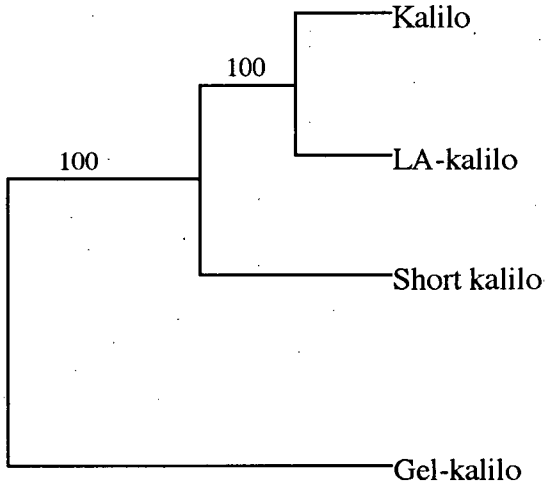

Fig. 8 An unrooted phylogenetic tree for kalilo-related plasmids based on their TIR sequences. The TIR sequence of kalilo DNA was taken from Chan et al. (1991). The TIR sequence of Gel-kalilo DNA was taken from Yuewang et al. (1996). TIR sequences of LA-kalilo and short kalilo DNAs were obtained as described in this paper. In the tree shown above, the branching order is informative but the branch length is not. Numbers above lines show the percentage of bootstraped data sets supporting the branching pattern.

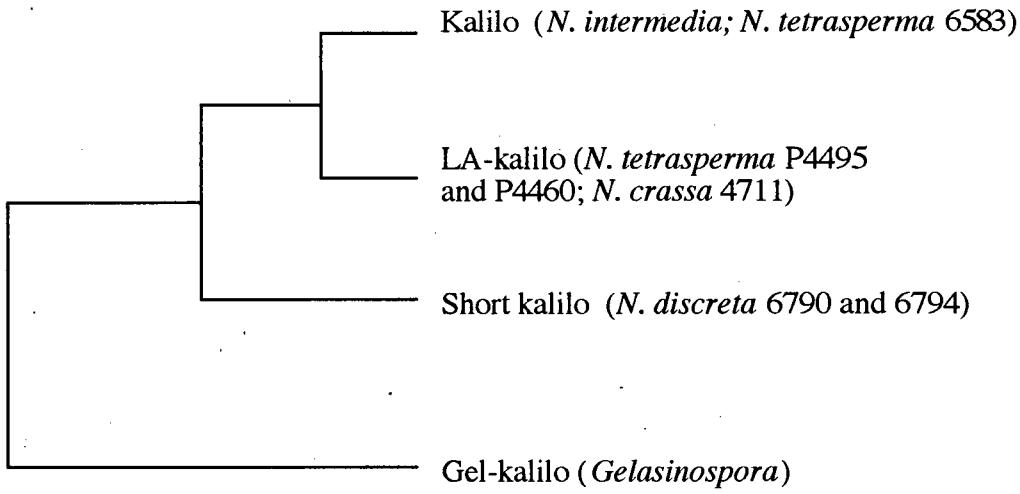

Fig. 9 An unrooted phylogenetic tree for kalilo-like plasmids superimposed with fungal distribution of the plasmids. The branching order is imformative but the branch lengths do not reflect the actual distance between strains

This phylogeny of the kalilo-related plasmids coincides with the established taxonomy of the genera *Neurospora* and *Gelasinospora* based on morphological characters (Wehmeyer, 1975) in that all *Neurospora* species are placed on a different branch away from *Gelasinospora*. The same taxonomy is also supported by other molecular phylogenetic studies of Taylor et al. (1990) using rDNA internal spacer. The intergeneric nucleotide substitution was found to be 2-3% while the interspecific rate was 0.5%.

This phylogeny of the kalilo-related plasmids also coincides to the phylogenetic relationship of *Neurospora* species established by Randall and Metzenberg (1995) using DNA sequences of mating type genes (*mt-A1* genes) in that *N. crassa*, *N. tetrasperma* and *N. intermedia* are more closely related to each other than to *N. discreta* . However, the tree based on TIR sequences of kalilo-like plasmids suggests a different placement of *N. tetrasperma* in relation to *N. crassa* and *N. intermedia* from that proposed by Randall and Metzenberg (1995). Their tree based on mt-A1 genes suggested that *N. intermedia* and *N. tetrasperma* are closely related and *N. tetrasperma* may be descended from *N. intermedia* or be one of its recent ancestors. However, our tree based on kalilo-like plasmids showed a close affinity of *N. tetrasperma* to both *N. crassa* and *N. intermedia*. *N. tetrasperma* may have evolved through two different routes from *N. crassa* and *N. intermedia*. Therefore, *N. tetrasperma* may not be truly monophyletic as proposed by Taylor and Navtig (1989) based on mitochondrial DNA RFLP analysis of some *N. tetrasperma* strains (not including those strains from Louisiana and Moorea-Tahiti used in this work). Substantial genetic differences within a subgroup of *N. tetrasperma* strains from Louisiana were observed in the phylogenetic studies by Merino et al. (1996) using nuclear DNA RFLP analysis and this may also suggest different ancestral origins of *N. tetrasperma* strains.

The good correlation between the phylogeny of the kalilo-like plasmids with that of their host fungi is a strong evidence that the kalilo-like plasmids may have evolved before the divergence of *Neurospora*/*Gelasinospora* genera and *Neurospora* species. The current distribution pattern of kalilo-related plasmids was formed by long-time *in situ* evolution of the

plasmids. Plasmids were lost in some strains by chance or by suppressor mutations (Griffiths et al., 1992). The differences between kalilo-related plasmids reflect differences between their genetic background. The more similar two plasmids are to each other, the more similar their genetic backgrounds should be, and the closer their host strains are related. Therefore, the kalilo plasmid family, and maybe also other mitochondrial plasmid families (Navtig et al., 1984), could be used as markers for phylogenic relationship between different fungal strains. However, the plasmid distribution pattern may also be explained by horizontal transfer. When we look at the distribution pattern of kalilo and LA-kalilo plasmids, kalilo type plasmids were found in natural isolates of *N. intermedia* from Hawaii and *N. tetrasperma* from Moorea-Tahiti; while LA-kalilo type plasmids were found in *N. tetrasperma* from Louisiana and *N. crassa* from Haiti. Hawaii and Moorea-Tahiti, and Louisiana and Haiti are both geographically close pairs of locations that may allow transfer of plasmids through direct contact of different strains. The feasibility of both *in situ* evolution and horizontal transfer has been discussed at length by Taylor et al.(1985, 1986) , Navtig et al. (1984) and Yuewang et al. (1996). It is still difficult to determine which model accounts for the distribution of kalilo-like plasmids, though both possibilities might be ture.

Comparison of the TIRs of kalilo, LA-kalilo , short kalilo and Gel-kalilo plasmids reveals some interesting features.

The inner 500 bp of TIR

This region contains the translational start site of the ORFs (Chan et al., 1991). It is highly conserved among the kalilo-like plasmids. This region may contain important sequences or structures for the translation and function of the ORFs, which have not yet been studied.

The terminal 140 bp

This is a less conserved region. It has been proposed that transcription of the kalilo plasmid is initiated at 101 nucleotide from the termini and elongates towards the center (Vickery and Griffiths, 1993). A further sequence comparison was made in a 52 bp region flanking the transcription initiation site (alignment is shown in Fig. 10). The sequence identity is shown to be approximately 90% among all the kalilo-related plasmids in this region. The sequence upstream of the proposed initiation site is AT-rich while the sequence downstream forms a short imperfect palindrome which is part of a bigger palindrome sequence as noticed by Chan et al. (1991). Since not much information is yet known about the promoter sequences in kalilo plasmid, this 52 bp region could be a very good place to start searching. The terminal 20 bp region in the termini of kalilo plasmid was proposed to be important in the integration of the plasmid into mitochondrial genome (Chan et al., 1991). In fact, kalilo and LA-kalilo have an almost identical terminal sequence, but they show a substantial difference in insertion behavior. This suggests that the terminal 20 bp may be necessary but not sufficient for integration. Other factors may be involved. An example is the nuclear suppressors which were found to inhibit the senescence-inducing ability of kalilo plasmid in some *N. intermedia* strains. The suppressors may be functioning by inhibiting plasmid insertion or by eliminating the plasmid DNA to an almost undetectable level (Griffiths et al., 1992; Yang and Griffiths, 1993). Such suppressors may also exist in other fungal strains. Besides, plasmid insertion may not be the only reason leading to senescence as in the case of *Gelasinospora* .
Fungal senescence is a far more complicated process than had been thought. It may involve interaction between the mtDNA and the plasmid DNA and it may also involve certain factors from the nuclei. A good way to study the senescence mechanism is to transfer the kalilo-like plasmids into one *Neurospora* strain and compare their function in a new genetic background. This attempt will be discussed in detail in Chapter 3.

	8557	8543		
kalilo/LA-kalilo	TTTTCATTTT	TATACCACAC	CCTAATGGGG	AGATAAACGT
Short kalilo	******-***	**C*******	**********	**********
Gel-kalilo	******A***	*********T	**********	***G*T***A

	8527	8506
kalilo/LA-kalilo	CTTTATCGCC	CC
Short kalilo	**********	**
Gel-kalilo	******T***	**

Fig.10 Sequence alignment of a 52 bp region flanking the proposed transcription initiation site in kalilo-related plasmids. The C at position 8543 is the transcription initiation site proposed by Vickery and Griffiths (1993). Sequence from nucleotides 8506 to 8531 forms a short imperfect palindrome.

* Identical nucleotide as in kalilo DNA
\- Deletion compared with kalilo DNA

The 740 bp region in the middle of TIRs.

This region has the most divergence among the kalilo-like plasmids. This suggests that the region may be functionally unimportant. Differences in this region include insertions, deletions and several point mutations. A blast search of the insertion sequences in LA-kalilo and Gel-kalilo did not reveal any significant matches in the gene data bank. The origin of these unique sequences remains mysterious. A possibility is that these sequences were from mtDNA through recombination between plasmid and mtDNA during insertion. But there is no

evidence that these sequences show homology with *N. crassa* mtDNA (Dr. Rick Collins, personal communication). Interestingly, a 200 bp kalilo sequence, which is not present in short-kalilo, was amplified by PCR from mtDNA sample of *N. discreta* strain 6790 (unpublished observations). This may suggest the interaction between mtDNA and plasmid DNA, which may be a source of variation for both molecules.

Chapter 3 Transfer of Kalilo-related Plasmids to Standard Lab Strains of *Neurospora crassa*

Introduction

One of the characteristic properties of kalilo DNA is its ability to insert into the mtDNA and cause progressive loss of normal mitochondrial functions leading to fungal senescence. The mechanism of insertion and senescence is of great interest. As discussed in Chapter 1 and Chapter 2, the terminal inverted repeats flanking the kalilo plasmid may play an important role in the integration events. Since all the kalilo-like plasmids known so far differ from the original kalilo DNA mainly in the TIR region, they serve as useful natural mutants to study the function and mechanism of the TIRs.

The kalilo-related plasmids were found in four different *Neurospora* species and two *Gelasinospora* strains from around the world, and they have different effects in their hosts, i.e. some insert and cause senescence while others do not. Fungal senescence is a complicated process which may involve interaction between nuclear DNA, mtDNA and plasmid DNA. These features make it hard to study the function by making comparisons only on the plasmid level. Ideally, if all the kalilo-related plasmids could be transferred into the same *Neurospora* species, comparison could be made in the same genetic context. *Neurospora crassa* is the best studied *Neurospora* species in terms of physiology, cytology and genetics. Many modern molecular biology techniques are applicable to this well characterized organism. The sequence of its mtDNA is available. In addition, the original kalilo DNA was transmitted successfully from *N. intermedia* to standard lab strain of *N. crassa* with an Oak Ridge background by hyphal contact (Griffiths et al., 1990). Its function as an "inducer" of senescence was hence confirmed in both of these *Neurospora* species.

In this chapter, the attempts to transfer kalilo-like plasmids, particularly the LA-kalilo plasmid, to standard lab strains of *N. crassa* will be discussed in detail. Although the plasmid transfer project has had only limited success so far, some interesting phenomena were observed during the experiments. These may provide insight into plasmid stability in different genetic backgrounds and into horizontal transfer of mitochondrial plasmids between different *Neurospora* species.

Materials and methods

Fungal strains

The transfer of kalilo-like plasmids was attempted using the LA-kalilo plasmid, which shows the greatest similarity to the prototypic kalilo DNA. The LA-kalilo plasmid-containing *N. tetrasperma* strain P4495 (A+a) was used as plasmid donor strain, and several *N. crassa* lab strains with different auxophic markers were used as plasmid recipients (Table 4). After the discovery of the LA-kalilo plasmid in a natural isolate of *N. crassa* from Haiti (4711), the transfer was also attempted intraspecifically in *N. crassa.*

Table 4 *N. crassa* lab strains used as recipients in the plasmid transfer experiments

Transfer methods	***N. crassa* recipient strains used**	**Genotype**[5]
Hyphal contact	24-3[1]	*a, ad-3A, al-2, his-3, nic-2, pan-2*
	I-32-14[2]	*a, ad-3B, al-2, leu-3, arg-1, tol, cDE*
	I-33-2	*a, ad-3B, al-2, leu-3, arg-1, tol, cDE*
	I-34-6	*A, ad-3A, nic-2, al-2, tol*
Spheroplast fusion	704	*a, his-1*
Cross and heterokaryon formation	2-17-825[3]	*A, ad-3A*
	12-21-17	*A, ad-3B, al-2, cot-1, pan-2*
	1423[4]	*A, al-2, pan-1, CDE*
	1424	*A, al-2, pan-1, CdE*
	1425	*A, al-2, pan-1, cDE*
	1426	*A,.al-2, pan-1, cdE*

1. 24-3 is derived from a cross between *N. crassa* lab strains 74A-Y112-M15, *ad-3A* and 74-OR21-1a, *his-3, nic-2, al-2,pan-2* (Griffiths et al., 1974).
2. I-32-14, I-33-2 and I-34-6 are stocks derived by A.M. deLange.
3. 2-17-825 is a nitrous acid-induced *ad-3A* mutant arising from a base-pair substitution (Malling and deSerres, 1968).
4. 1423, 1424, 1425 and 1426 are FGSC stocks.

Methods for plasmid transfer:

The plasmid transfer has been attempted using different methods as described below.

1. Hyphal contact.

Transfer of a linear mitochondrial plasmid was observed between distantly related fungi upon hyphal contact (Kempken, 1995). The plasmid-containing *Ascobolus immersus* strain 2/I was grown together with the plasmid-free *Podospora anserina* strain s- in petri dishes. An incompatible reaction between the two strains was observed as a clear cut zone at

the hyphal contact region. *P. anserina* mycelia were removed at different timepoints for DNA analysis. The mitochondrial plasmid was found transferred from *A. immersus* to *P. anserina* independent of mitochondrial DNA and nuclear DNA, though its stability in the new host was low.

The transfer of kalilo plasmid from *N. intermedia* to *N. crassa* was accomplished in a similar way (Griffiths et al., 1990). Nonsenescent auxotrophs of *N. crassa* were grown together with a kalilo-containing auxotrophic *N. intermedia* strain (2360 *his*). No true heterokaryons were formed, but the strains grew together and the mycelia intertwined. The kalilo plasmid was later found transferred to the *N. crassa* strain independent of mtDNA and nuclear DNA.

A procedure similar to the ones described above was adapted to transfer LA-kalilo DNA from *N. tetrasperma* to *N. crassa*. The plasmid-containing *N. tetrasperma* strain P4495 was grown together with *N. crassa* auxotrophs (Table 4) on both slants and petri dishes on supplemented medium. Since the *N. crassa* strains showed more vigorous growth than the *N. tetrasperma* strain on supplemented medium, the *N. tetrasperma* strain was inoculated 12-24 hours prior to the inoculation of the *N. crassa* strains to synchronize the growth. Yang and Myers (unpublished result) also minimized the growth of auxotrophic *N. crassa* strains by decreasing the amount of supplements to the medium. *N. crassa* strains were picked out by testing the auxotrophic markers and existence of kalilo DNA was tested by dot-blot probing.

2. Spheroplast fusion.

The mycelial cell wall was removed by Novozyme treatment. Mycelial cells were used because the *N. tetrasperma* strain made very few conidia. The cell wall-free spheroplasts of plasmid-containing *N. tetrasperma* (P4495) and plasmid-free *N. crassa* (704, *his,a*)were mixed in 40% PEG4000, 0.01M $CaCl_2$ and 0.05 Tris-glycine, PH 7.6 and incubated at 30 °C to induce cell fusion (Dr. H. Bertrand, personal communication). It was hoped that the LA-kalilo plasmid could be transferred through this direct cell contact as in other cases of

mitochondrial plasmids, such as the transfer of pClB4 plasmid between *Claviceps purpurea* strains (Gessner-Ulrich and Tudzynski, 1994). Protoplasts of the *C. purpurea* plasmid donor strain were mixed with protoplasts of the *C. purpurea* recipient strain at a ratio of 10:1 in 30% PEG 6000, 0.75 mM $CaCl_2$. 0.05 mM glycine, PH 7.5. After 10 minutes incubation at 28 °C, the protoplasts were diluted and plated on appropriate regenerating medium. Plasmid transfer was then detected by mitochondrial DNA analysis. Five transferrants were recovered in 300 colonies tested.

3. Crossing and heterokaryon formation

Since LA-kalilo was also found in a *N. crassa* strain from Haiti (4711), the transfer experiment has been tried intraspecifically. The LA-kalilo-containing wild type *N. crassa* strain was used as a maternal parent in a cross to an auxotrophic *N. crassa* strain of Oak Ridge background (2-17-825 *ad-3A, a*). The plasmid should hence be passed to all ascospore progeny. The ascospore progeny with the auxotrophic marker could then be used to make forced heterokaryons with *N. crassa* lab strains with different auxotrophic markers (Table 4). The plasmid was expected to be transferred through heterokaryon formation or transient hyphal fusion.

Dot-blot hybridization:

Total DNA was extracted by grinding mycelia harvested from overnight liquid culture with acid-washed sand. Cells and mitochondria were lysed with LETS buffer (0.1 M LiCl, 10mM EDTA, 10mM Tris-HCl and 0.5% SDS). Sand and cell wall debris were removed by centrifugation at 12,000rpm for 5 minutes. The supernatants containing fungal DNA were then transferred to nylon membrane using a dotblot apparatus from BioRad. ^{32}P-dCTP labelled kalilo DNA 8.2 Kb *Kpn I* fragment cloned in pUC 18 vector was used to probe the filter.

Results

The attempt to transfer LA-kalilo plasmid to lab strains of *N. crassa* has had limited success so far. Some *N. crassa* transferrants have been obtained, but they all proved to be unstable.

Hyphal contact:

When the wild-type plasmid donor strain was grown together with auxotrophic *N. crassa* strains on supplemented medium, the *N. crassa* strains always grew much faster and better than the *N. tetrasperma* strain and they soon dominated the culture. This may be due to faster hyphal growth or better conidiation of *N. crassa* strains. The excess amount of *N. crassa* conidia also made the dot-blot screening for plasmid transferrants laborious work. About 2000 *N. crassa* colonies were isolated and tested by dot-blot hybridization. However, no plasmid transferrant was detected. It is possible that the *N. tetrasperma* strain died out before any hyphal interaction could take place to allow the plasmid transfer.

Spheroplast fusion:

Over 300 *N. crassa* regenerants were tested by dot-blotting. Six possible LA-kalilo plasmid transferants were identified by a positive signal on dotblot hybridization (data not shown). When these potential transferants were subcultured and subject to mtDNA analysis, no plasmid DNA was detected even by Southern hybridization (data not shown).

Cross and heterokaryon formation:

An auxotrophic marker *ad-3A* was introduced into the LA-kalilo-plasmid-containing strain by crossing the wild type *N. crassa* strain 4711 to an adenine-requiring lab strain 2-17-825 (a, *ad-3A*). The plasmid-containing strain was used as maternal parent. Six ascospore progeny with the *ad-3A* marker and the appropriate mating type were selected and used to make forced heterokaryons with a *N. crassa* lab strain containing *ad-3B* and *cot* markers (Table 4). No heterokaryon was formed though limited hyphal growth of the *N. crassa* strain was observed in some tubes. This limited growth could be due to either back mutation of the

auxotrophic markers, or cross-feeding between different strains. The growing *N. crassa* mycelia were subcultured and subject to dot-blot hybridization analysis, but no plasmid transfer was detected.

Plasmid transfer was also attempted using Gel-kalilo DNA (X. Yang and G.A. Kuldau, unpublished results). Both methods of hyphal contact and spheroplast fusion were tried and proved to be unsuccessful.

Discussion

The transfer of mitochondrial plasmids independent of mtDNA and nuclear DNA has been reported both intraspecifically (Debets, et al., 1994; Gessner Ulrich and Tudzynski, 1994; Collins and Saville, 1990) and interspecifically (Griffiths et al., 1990) in filamentous fungi. Recent work by Kempken (1995) showed that the mitochondrial plasmid could also be transferred between distantly related fungi, such as the discomycete *Ascoblolus immersus* and the pyrenomycete *Podospora anserina*, though the plasmid had low stability in the new host strain. These studies suggest that mitochondrial plasmids can be transferred independent of mitochondrial and nuclear DNA upon rare and unstable hyphal interaction. Therefore it is conceivable that horizontal transfer may contribute to the current distribution pattern of linear plasmids in fungi.

However, in the present work, the attempt to transfer LA-kalilo plasmid from *N. tetrasperma* to standard lab strains of *N. crassa* with Oak Ridge background proved to be unsuccessful. Three different methods were used. These were: hyphal contact, spheroplast fusion, and heterokaryon formation following introduction of a suitable forcing marker. Altogether 2300 *N. crassa* colonies were screened and only 6 possible plasmid transferants were picked up by dot-blot probing. But when these possible transferrants were subcultured and subjected to mtDNA analysis, no plasmid DNA could be detected by Southern hybridization. If this observation is not due to an artifact of the dot-blot procedure, it must reflect the extremely unstable nature of LA-kalilo plasmid in the new host. Similar findings

was also reported by Kempken (1995). In his work of transferring a mitochondrial plasmid between two distantly related fungi, the plasmid had a considerably lower copy number in the new host fungus, and the amount was soon reduced to a level that was detectable only by PCR amplification. The low stability of transferred plasmids suggests that maintenance of a plasmid in a certain fungal host may require compatibility between mitochondrial DNA, nuclear DNA and plasmid. It is possible that maintenance of the kalilo plasmid requires certain products from the mitochondrial DNA, such as the replication machinery since there is yet no evidence that the kalilo plasmid is using its own DNA polymerase for replication. Plasmid incompatibility due to failure in replication has long been noticed. In bacteria, when two plasmids are both present in a cell and their replication is subject to a common regulation, either one will be lost (Broda, 1979).

Plasmid incompatibility with mtDNA was observed in yeast (Gunge and Yamane, 1984). The linear killer plasmids pGKL1 and pGKL2 from *Kluyveromyces lactis* were stable when transferred to a [*rho*^{0}] petite mutant of *Saccharomyces cerevisiae* lacking mitochondrial DNA. But they became unstable and were lost when mitochondria of a wild type strain ([*rho*$^+$]) of *S. cerevisiae* was introduced into the same cell. Incompatibility between plasmids from *K. lactis* and mitochondrial DNA from *S. cerevisiae* was a reasonable explanation for this phenomenon. It was proposed that a replication advantage of mitochondrial DNA over pGKL plasmids may account for this incompatibility between mtDNA and foreign plasmid.

Incompatibility may also occur between plasmid and nuclear genes. One example is the plasmid suppressors found in the nuclei of *Neurospora* that may eliminate plasmids to a barely detectable level. The molecular mechanism underlying this process is not clear yet (Griffiths et al., 1992; Yang and Griffiths, 1993). Other examples are the nuclear morphological mutants which have life-prolonging effect in *Podospora*, such as the double mutants *incoloris vivax* (*i viv*) and *grisea vivax* (*gr viv*). Small amounts or even no senescence-inducing mitochondrial DNA elements could be detected in these strains (Tudzynski and Esser, 1979; Tudzynski et al., 1980).

In addition to plasmid incompatibility with the mitochondrial DNA and nuclear DNA, another possible reason for the failure in obtaining LA-kalilo plasmid transferrants in the present work is the wide-spread heterokaryon incompatibility between different fungal strains. Reduced horizontal transfer efficiency was noticed in previous work on the transfer of kalilo plasmid between incompatible *N. intermedia* strains and between incompatible *N. crassa* strains (Debets et al., 1994). The same phenomenon was also observed in the horizontal transfer of a virus-like double-stranded RNA between incompatible strains of the chestnut blight ascomycete *Cryphomectria parasitica* (Anagnostakis, 1982). Heterokaryon incompatibility is an ubiquitous phenomenon in filamentous fungi. Studies in *N. crassa* showed that the heterokaryon genes are highly polymorphic in the natural populations (Mylyk, 1976). It was proposed that heterokaryon incompatibility may provide an efficient way to inhibit the spread of harmful cytoplasmic elements among populations (Caten, 1972; Begueret et al, 1994).

In summary, due to the failure in transferring kalilo-like plasmids into standard lab strains of *N. crassa*, comparison of plasmid function can not yet be made only at the plasmid level. The mechanisms of plasmid insertion and senescence remain open questions. Stable plasmid horizontal transfer must be a rare event as shown by previous and present work. The transfer efficiency can be greatly reduced by the widespread vegetative incompatibility between different fungal strains. Transferred plasmids may also be eliminated from the new host due to its incompatibility with the mitochondrial genome or nuclear genome. Hence, although horizontal transfer between incompatible strains and different species remains a distinct possibility, the present results suggest that this is not a common occurrence even under conditions designed to maximize transfer. Therefore, it is unconvincing to involve horizontal transfer as an explanation of present distribution pattern of kalilo-related plasmids in nature.

Summary

Four types of kalilo-related plasmids have been identified in world-wide collected isolates of *Neurospora* and *Gelasinospora* (Table 3). The phylogeny of these kalilo-related plasmids coincides roughly to the established taxonomy of their host strains based on morphological and molecular studies. This is a strong evidence for the *in situ* evolution of kalilo-related plasmids with their fungal hosts.

Transfer of kalilo-like plasmids to *Neurospora crassa* standard lab strains has had limited success in the present work. Only six unstable transferrants were selected by dot-bloting hybridization in a sample size of 2300 colonies. The plasmid was lost during subculture. The failure to obtain stable plasmid transferrants may be due to plasmid incompatibility with mitochondrial genome or nuclear genome. The widespread heterokaryon incompatibility in filamentous fungi could also inhibit the efficient horizontal transfer of mitochondrial plasmids.

Based on these observations, it may be concluded that the distribution pattern of kalilo-related plasmid was formed by long-time *in situ* evolution starting before the divergence of *Neurospora/Gelasinospora* genera and the divergence of *Neurospora* species. Plasmids were then lost from some strains by chance or by suppressor mutations (Griffiths et al., 1992; Yang and Griffiths, 1993). However, horizontal transfer may contribute to the distribution of plasmids in a few cases, especially plasmid distribution in closely related species (such as kalilo plasmid and LA-kalilo plasmid in *N. intermedia, N. crassa* and *N. tetrasperma*) and in a population of one single species (such as the distribution of kalilo plasmid among the Hawaiian islands population of *N. intermedia*).

Due to the failure in transferring kalilo-like plasmids into standard lab strains of *N. crassa*, comparison of plasmid function can not yet be made at the plasmid level. The mechanisms of plasmid insertion and senescence remain open questions. So far only the type I kalilo plasmids showed consistent correlation with the onset of insertion-induced senescent

phenotype. The correlation between senescence and other kalilo-like plasmids is not clear. Senescence may be caused by reasons other than plasmid insertion as observed in the cases of LA-kalilo-containing *N. tetrasperma* (Marceko-Kuehn et al., 1994) and Gel-kalilo-containing *Gelasinospora* (Yuewang et al., 1996). Senescence is a complicated process. Beside the plamid, certain genes in mtDNA and nuclear DNA may also be involved.

Bibliography

Anagnostakis S.L., 1982. *Biological control of chestnut blight.* Science 215:466-471

Arganoza M.T., Min J., Hu Z. and Akins R.A., 1994. *Distribution of seven homology groups of mitochondrial plasmids in Neurospora: evidence for widespread mobility between species in nature.* Curr. Genet. 26:62-73

Begueret J., Beatrice T. and Corinne C., 1994. *Vegetative incompatibility in filamentous fungi: het genes begin to talk.* Trends in Genetics 10(12):441-446

Belcour L. and Begel O., 1980. *Life-span and senescence in Podospora anserina.* J. Gen. Microbiol. 119:505-515

Bertrand H., Chan B. and Griffiths A.J.F., 1985. *Insertion of a foreign nucleotide sequence into mitochondrial DNA causes senescence in Neurospora intermedia.* Cell 41:877-884

Broda P., 1979. Plasmids. pp47-76. W.H.Freeman and Company Ltd.

Caten C.E., 1972. *Vegetative incompatibility and cytoplasmic infection in fungi.* J. Gen. Microbiol. 72:221-229

Chan B.S., Court D.A., Vierula P.J. and Bertrand H.,1991. *The Kalilo linear senescence-inducing plasmid of Neurospora is an invertron and encodes DNA and RNA polymerases.* Curr. Genet. 20:225-237

Collins R.A. and Saville B.J., 1990. *Independent transfer of mitochondrial chromosomes and plasmids during unstable vegetative fusion in Neurospora.* Nature 345:177-179

Court D.A., Griffiths A.J.F., Kraus S.R., Russell P.J. and Bertrand H., 1991. *A new senescence-inducing mitochondrial linear plasmid in field-isolated Neurospora crassa strains from India.* Curr. Genet. 19:129-137

Dasgupta J., Chan B.S., Keith M.A. and Bertrand H., 1988. *Kalilo insertion sequences from the senescent strains of Neurospora intermedia are flanked by long inverted repeats of mitochondrial DNA.* Genome 30 (Suppl.1):318

Davis R.H., deSerres F.J., 1970. *Genetic and microbiological techniques for Neurospora crassa.* Meth. Enzymol. 17A:79-143

Debets F., Yang X. and Griffiths A.J.F., 1994. *Vegetative incompatibility in Neurospora: its effect on horizontal transfer of mitochondrial plasmids and senescence in natural populations.* Curr. Genet. 26:113-119

Debets F., Yang X. and Griffiths A.J.F., 1995. *The dynamics of mitochondrial plasmids in a Hawaiian population of N. intermedia.* Curr. Genet. 29:44-49

Devereux J., Haeberli P. and Smithies O., 1984. *A comprehensive set of sequence analysis programs for the VAX.* Nuc. Acids Res. 12(1):387-395

Dodge B.Q., 1927. *Nuclear phenomena associated with heterothallism and homothallism in the ascomycetes Neurospora.* J. Agric. Res. 35:289-305

Esser K., Tudzynski P., Stahl U.and Kuck U.,1980. *A model to explain senescence in the filamentous fungus Podospora anserina by the action of plasmid-like DNA.* Mol. Gen. Genet. 178:213-216

Gessner-Ulrich K. and Tudzynski P., 1994. *Studies on function and mobility of mitochondrial plasmids from Claviceps purpurea.* Mycol. Res. 98(5):511-515

Glass N.L., Vollmer S.J., Staben C., Grotelueschen J., Metzenberg R.L. and Yanofsky C., 1988. *DNAs of the two mating-type alleles of Neurospora crassa are highly dissimilar.* Science 241:570-573

Griffiths A.J.F., 1992. *Fungal senescence.* Annu. Rev. Genet. 26:351-372

Griffiths A.J.F., 1995. *Natural plasmids of filamentous fungi.* Microbiol. Rev. 59(4):673-685

Griffiths A.J.F., and Bertrand H, 1984. *Unstable cytoplasms in Hawaiian strains of Neurospora intermedia.* Curr. Genet. 8:387-398

Griffiths A.J.F., Collins R.A. and Nargang F.E., 1995. Mitochondrial genetics of Neurospora. The Mycota II, genetics and biotechnology. pp 93-105 Springer-Verlag Berlin Heidelberg.

Griffiths A.J.F., deLange A.M. and Jung .J.H., 1974. *Identification of a comples chromosome rearrangement in Neurospora crassa.* Can. J. Genet. Cytol. 16:805-822

Griffiths A.J.F., Kraus S.R., Barton R., Court D.A., Myers C.J. and Bertrand H., 1990. *Heterokaryotic transmission of senescence plasmid DNA in Neurospora.* Curr. Genet. 17:139-145

Griffiths A.J.F., Kraus S.R., Bertrand H., 1986. *Expression of senescence in Neurospora intermedia.* Can. J. Genet. Cytol. 28:459-467

Griffiths A.J.F., Yang X., Barton R. and Myers C., 1992. *Suppression of cytoplasmic senescence in Neurospora.* Curr. Genet. 21:479-484

Gunge N. and Yamane C., 1984. *Incompatibility of linear DNA killer plasmids pGKL1 and pGKL2 from Kluyyveromyces lactic with mitochondrial DNA from Saccharomyces cerevisiae.* J. of Bacteriol. 159(2):533-539

Jacobson D.J., 1991. *Control of mating type heterokaryon incompatibility by the tol gene in Neurospora crassa and N. tetrasperma.* Genome 35:347-353

Kempken F., 1995. *Horizontal transfer of a mitochondrial plasmid.* Mol. Gen. Genet. 248:89-94

Malling H.V. and deSerres F.J., 1968. *Correlation between base-pair transition and complementation patterns in nitrous acid-induced ad-3B mutants of Neurospora crassa.* Mutation Res. 5:359-371

Maniatis T., Fritsch E.F., Sambrook J., 1982. *Molecular cloning: a laboratory manual.* Cold Spring harbor Laboratory, Cold Spring Harbor, New York

Marcinko-Kuehn M., Yang X., Debets F., Jacobson D.J. and Griffiths A.J.F., 1994. *A Kalilo-like linear plasmid in Lousisiana field isolates of the pseudohomothallic fungus Neurospora tetrasperma.* Curr. Genet. 26:336-343

Meinhardt F, Kempken, Kamper J. and Esser K., 1990. *Linear plasmids among eukaryotes:fundamentals and application.* Curr. Genet. 17:89-95

Merino S.T., Nelson M.A., Jacobson D.J. and Natvig D.O., 1996. *Pseudohomothallism and evolution of the mating-type chromosome in Neurospora tetrasperma.* Genetics 143:789-799

Metzenberg R.L. and Glass N.L., 1990. *Mating type and mating strategies in Neurospora.* BioEssays 12:53-59

Myers C.J., 1988. *Transmission of Kalilo DNA in senescent strains of Neurospora intermedia.* Ph.D. thesis, the University of British Columbia, Canada

Mylyk O.M., 1976. *Heteromorphism for heterokaryon incompatibility genes in natural populations of Neurospora crassa.* Genetics 83:275-284

Nargang F.E., Bell J.B., Stohl L.L. and Lambowitz A.M., 1984. T*he DNA sequence and genetic organization of a Neurospora mitochondrial plasmid suggest a relationship to i ntrons and mobile elements.* Cell 38:441-453

Navtig D.O., May G. and Taylor J.W., 1984. *Distribution and evolutionary significance of mitochondrial plasmids in Neurospora spp.* J. Bacteriol. 159(1):288-293

Perkins D.D.,1988. *Main features of vegetative incompatibility in Neurospora.* Fungal Genetics Newsletter 35:44-46

Perkins D.D., 1992. *Neurospora: the organism behind the molecular revolution.* Genetics 130:687-701

Perkins D.D., Radford A, Newmeyer D and Bjorkman M , 1982. *Chromosomal loci of Neurospora crassa.* Fungal Genetics Newsletter 33: 15

Perkins D.D., Turner B.C. and Barry E.G., 1976. *Strains of Neurospora collected from nature.* Evolution 30:281-313

Randall T.A. and Metzenberg R.L., 1995. *Species-specific and mating type-specific DNA regions adjacent to mating type idiomorphs in the genus Neurospora.* Genetics141:119-136

Taylor J.W., Bruns T.D., White T.J., 1990. *Unusual rate of molecular evolution in the ribosomal DNA intermal transcribed spacer region of Sordariaceae?* Mycological Society of America Newsletter 41:40-41

Taylor J.W. and Natvig D.O., 1989. *Mitochondrial DNA and evolution of heterothallic and pseudohomothallic Neurospora species.* Mycol. Res. 93(3):257-272

Taylor J.W., Smolich B.D. and May G., 1985. *An evolutionary comparison of homologous mitochondrial plasmids DNAs from three Neurospora species.* Mol. Gen.Genet. 201:161-167

Taylor J.W., Smolich B.D. and May G., 1986. *Evolution of mitochondrial DNA in Neurospora crassa.* Evolution 40:716-739

Tudzynski P. and Esser K., 1979. *Chromosomal and extrachromosomal control of senescence in the ascomycete Podospora anserina.* Mol. Gen. Genet. 173:71-84

Tudzybnski P., Stahl U., Esser K., 1980. *Transformation to senescence with plasmid-like DNA in the ascomycete Podospora anserina.* Curr. Genet. 2:181-184

Vierular P.J., Cheng C.K., Court D.A., Humphrey R.W., Thomas D.Y. and Bertrand H., 1990. *The kalilo senescence plasmid of Neurospora intermidia has covalently-linked 5' terminal proteins.* Curr. Genet. 17:195-201

Vickery D.B. and Griffiths A.J.F., 1993. *Transcription of the kalilo linear senescence plasmid from Neurospora intermedia.* Plasmid 29:180-192

Wehneyer L.E., 1975. *The Pyrenomycetous fungi.* pp 127-132, The New York Botanical Garden.

Yang X. and Griffiths A.J.F., 1993. *Plasmid suppressors active in the sexual cycle of Neurospora intermedia.* Genetics 135:993-1002

Yuewang W., Yang X. and Griffiths A.J.F., 1996. *Structure of a Gelasinospora linear plasmid closely related to the Kalilo plasmid of Neurospora intermedia.* Curr. Genet. 29:150-158

Appendix I

Sequence alignments of the TIR of kalilo plasmid (sequence data taken from Chan et al., 1991) with the TIRs of other kalilo-like plasmids.

a. the TIR of LA-kalilo plasmid in *N. tetrasperma* strain P4495.
b. the TIR of kalilo-like plasmid in *N. crassa* strain 4711
c. the TIR of kalilo-like plasmid in *N. tetrasperma* strain 6583
d. the TIR of kalilo-like plasmid in *N. discreta* strain 6790.

* Identical nucleotide as in kalilo plasmid
\- Deletion
. Nucleotide sequence not determined in the present work
Numbers shown above the sequences correspond to numbers in Fig. 6.

Sequence alignment of a and b with TIR of kalilo:

```
                                                                    7327
Kalilo   GGTTGCGTGC GAATTCCTCA TATTAATGCG ATAATTGAAA AAAGAAAATT
a        ********** ********** ********** ***_****** **********
b        .......... .......... .......... .......... ..........

                                                                    7377
Kalilo   TGCTCATGAT AAAAATATTT AATATAAATT ATGTTCTTTT ACGTCGTAGC
a        ********** ********** ********** ********** **********
b        .......... .......... .......... .......... ..........

                                                                    7427
Kalilo   CTTTCTATAC ACTCCATTTA GTTTTGGAAA TAAGGCACCC TTTCCTCTAA
a        ********** ********** ********** ********** **********
b        .......... .......... .......... .....***** **********

                                                                    7477
Kalilo   GATAATGGGG GCGGATAAAA CATAAAAGCC ACTTTCCCCC AAGCGGGCGT
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    7527
Kalilo   AGATCCCATA CCTCACGGTA TGGATAATTC GGCATTTCAG TAGACGACTT
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    7577
Kalilo   TTTTGTTTAA GATTTTTTTG GGAAAACCCA AAAGTGATAA ACAACTAGCC
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    7627
Kalilo   AGATTCTCCC CAATTATGGC CGTGCCTTAT ACTTTTTATC ATATACTAGG
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    7677
Kalilo   GACTTATATA ATAAAATTCT AGTATAGTTT TGTCACCAAT ATTTTAAGAA
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********
```

```
                                                                    7727
Kalilo   TCAGTCTACC CTCTACAATT GAATAGATTG TTATATGAAA AATAAAATTT
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    7777
Kalilo   CTTATAAGTC TATTTATTTC TTTTTTATTT TTGGTTTCTA CCCTTTTATT
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    7827
Kalilo   AAATTGGATT CTTTCGGGTC CCATTCATAA TCAACCTACA GGTTATGGTC
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    7875
Kalilo   TTTCACCACT TCCCCGCTCA CTGCATTGAC CGCACGTAGC AAACTTA-T-
a        *C******** ********** ********** TA*****-** -TT****C*A
b        *C******** ********** ********** TA*****-** -TT****C*A

                                                                    7924
Kalilo   TGTTATCCTT TCCTCTTTCA TTTGCGTACA ATTACGGTTT ACATTATTA-
a        ***A------ ---------- ---------- ------**** *****----G
b        ***A------ ---------- ---------- ------**** *****----G

                                                                    7974
Kalilo   TGAGTAGATT TCAGCTCCCT TCACGCCACT AATTGCTATT GGGGTCATCT
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    8023
Kalilo   ACGGACCT-C TTCTCCCCCT TTCACCATAC AGGTACTTCG TTTTGCTTTT
a        **A*****C* ********** ********** ********** **********
b        **A*****C* ********** ********** ********** **********

                                                                    8073
Kalilo   CCCTGTAGAT TACTTGCACT TTAGGTTCTC TTACTCAGTC TGCGGTACTT
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    8123
Kalilo   CTTAGACAAT ACTGTACTCA CAGGCCTTTC TTCGCTGCTC AGTTCTAAAG
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    8173
Kalilo   TTAGTCCTGG TTGATTTATT TACTGTTACT TTTGGTTTCC TTTTTAATAA
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    8223
Kalilo   AAATTGGGAT TTTTTCTATT TATCAAGTCG AGGGTTCGTT CTGGTTCATT
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********
```

```
                                                                    8273
Kalilo   ACTATCGATC ACCGTACTCT GTTTTCTGGA TATTCTAAGC CTCAGTTATT
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    8323
Kalilo   GGCCTTCAGA TTCCCCAGCC CTGCGCCTTC AATAGTGGGA TGTTCTAGAA
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    8373
Kalilo   TAAATCTTCC AGTTCGCTTA AATTCACTCA TGGACATTAC ATTGAATACT
a        ********** ********** ********** ********** **********
b        ********** ********** ********** *********A **********

                                                                    8423
Kalilo   TTCCCTTAGC AACCGTGTGT CTCTTAGACA ATACCACACA CGTACACCGG
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    8473
Kalilo   TACCCAGATT TAATAATGCT ACCCACTACC CTGATTGTAT CCCTGGAATC
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    8523
Kalilo   ATTACTCAAG GTGGGATATT CTACCCCACC ATGGGGCGAT AAAGACGTTT
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    8573
Kalilo   ATCTCCCCAT TAGGGTGTGG TATAAAAATG AAAAAATCAA AAAGAGAGAG
a        ********** ********** ********** ********** **********
b        ********** ********** ********** ********** **********

                                                                    8623
Kalilo   TACTAGAAAT GATAAAAAGA TCACAAAGGG TTAAAATAGG AACAAAAGGG
a        ********** ********** ******C*** ********** **********
b        ********** ********** ******T*** ******A*** **********

                                    8643
Kalilo   GTCAGTGGTG CCCCTTACAC
a        ********** **********
b        **GC****** **TA******
```

Sequence alignment of c with TIR of kalilo plasmid:

```
                                                                    7327
Kalilo   GGTTGCGTGC GAATTCCTCA TATTAATGCG ATAATTGAAA AAAGAAAATT
c        .......... .......... .......... .......... ..........

                                                                    7377
Kalilo   TGCTCATGAT AAAAATATTT AATATAAATT ATGTTCTTTT ACGTCGTAGC
c        .......... .......... .......... .......... ..........

                                                                    7427
Kalilo   CTTTCTATAC ACTCCATTTA GTTTTGGAAA TAAGGCACCC TTTCCTCTAA
c        .......... .......... .......... .......... ..........

                                                                    7477
Kalilo   GATAATGGGG GCGGATAAAA CATAAAAGCC ACTTTCCCCC AAGCGGGCGT
c        ********** ********** ********** ********** **********

                                                                    7527
Kalilo   AGATCCCATA CCTCACGGTA TGGATAATTC GGCATTTCAG TAGACGACTT
c        ********** ********** ********** ********** **********

                                                                    7577
Kalilo   TTTTGTTTAA GATTTTTTTG GGAAAACCCA AAAGTGATAA ACAACTAGCC
c        ********** ********** ********** ********** **********

                                                                    7627
Kalilo   AGATTCTCCC CAATTATGGC CGTGCCTTAT ACTTTTTATC ATATACTAGG
c        ********** ********** ********** ********** **********

                                                                    7677
Kalilo   GACTTATATA ATAAAATTCT AGTATAGTTT TGTCACCAAT ATTTTAAGAA
c        ********** ********** ********** ********** **********

                                                                    7727
Kalilo   TCAGTCTACC CTCTACAATT GAATAGATTG TTATATGAAA AATAAAATTT
c        ********** ********** ********** ********** **********

                                                                    7777
Kalilo   CTTATAAGTC TATTTATTTC TTTTTTATTT TTGGTTTCTA CCCTTTTATT
c        ********** ********** ********** ********** **********

                                                                    7827
Kalilo   AAATTGGATT CTTTCGGGTC CCATTCATAA TCAACCTACA GGTTATGGTC
c        ********** ********** ********** ********** **********

                                                                    7877
Kalilo   TTTCACCACT TCCCCGCTCA CTGCATTGAC CGCACGTAGC AAACTTATTG
c        ********** ********** ********** ********** **********

                                                                    7927
Kalilo   TTATCCTTTC CTCTTTCATT TGCGTACAAT TACGGTTTAC ATTATTATGA
c        ********** ********** ********** ********** **********

                                                                    7977
Kalilo   GTAGATTTCA GCTCCCTTCA CGCCACTAAT TGCTATTGGG GTCATCTACG
c        ********** ********** ********** ********** **********
```

```
                                                                    8027
Kalilo  GACCTCTTCT CCCCCTTTCA CCATACAGGT ACTTCGTTTT GCTTTTCCCT
c       ********** ********** ********** ********** **********

                                                                    8077
Kalilo  GTAGATTACT TGCACTTTAG GTTCTCTTAC TCAGTCTGCG GTACTTCTTA
c       ********** ********** ********** ********** **********

                                                                    8127
Kalilo  GACAATACTG TACTCACAGG CCTTTCTTCG CTGCTCAGTT CTAAAGTTAG
c       ********** ********** ********** ********** **********

                                                                    8177
Kalilo  TCCTGGTTGA TTTATTTACT GTTACTTTTG GTTTCCTTTT TAATAAAAAT
c       ********** ********** ********** ********** **********

                                                                    8227
Kalilo  TGGGATTTTT TCTATTTATC AAGTCGAGGG TTCGTTCTGG TTCATTACTA
c       ********** ********** ********** ********** **********

                                                                    8277
Kalilo  TCGATCACCG TACTCTGTTT TCTGGATATT CTAAGCCTCA GTTATTGGCC
c       ********** ********** ********** ********** **********

                                                                    8327
Kalilo  TTCAGATTCC CCAGCCCTGC GCCTTCAATA GTGGGATGTT CTAGAATAAA
c       ********** ********** ********** ********** **********

                                                                    8377
Kalilo  TCTTCCAGTT CGCTTAAATT CACTCATGGA CATTACATTG AATACTTTCC
c       ********** ********** ********** ********** **********

                                                                    8427
Kalilo  CTTAGCAACC GTGTGTCTCT TAGACAATAC CACACACGTA CACCGGTACC
c       ********** ********** ********** ********** **********

                                                                    8477
Kalilo  CAGATTTAAT AATGCTACCC ACTACCCTGA TTGTATCCCT GGAATCATTA
c       ********** ********** ********** ********** **********

                                                                    8527
Kalilo  CTCAAGGTGG GATATTCTAC CCCACCATGG GGCGATAAAG ACGTTTATCT
c       ********** ********** ********** ********** **********

                                                                    8577
Kalilo  CCCCATTAGG GTGTGGTATA AAAATGAAAA AATCAAAAAG AGAGAGTACT
c       ********** ********** ********** ********** **********

                                                                    8627
Kalilo  AGAAATGATA AAAAGATCAC AAAGGGTTAA AATAGGAACA AAAGGGGTCA
c       ********** ********** ********** ********** **********

                                    8643
Kalilo  GTGGTGCCCC TTACAC
c       ********** ******
```

Sequence alignment of d with TIR of kalilo plasmid:

```
                                                                    7327
Kalilo   GGTTGCGTGC GAATTCCTCA TATTAATGCG ATAATTGAAA AAAGAAAATT
d        .......... .......... .......... .......... ..........

                                                                    7377
Kalilo   TGCTCATGAT AAAAATATTT AATATAAATT ATGTTCTTTT ACGTCGTAGC
d        .......... .......... .......... .......... **********

                                                                    7427
Kalilo   CTTTCTATAC ACTCCATTTA GTTTTGGAAA TAAGGCACCC TTTCCTCTAA
d        ********** ********** ********** ********** **********

                                                                    7475
Kalilo   GATAAT-GGG GGCGGATAAA ACATAAAAGC CACTTT-CCC CCAAGCGGGC
d        ******G*** ********** ********** ******C*** **********

                                                                    7525
Kalilo   GTAGATCCCA TACCTCACGG TATGGATAAT TCGGCATTTC AGTAGACGAC
d        ********** ********** ********** ********** **********

                                                                    7572
Kalilo   --TTTTTTGT TTAAGATTTT TTTGGG-AAA ACCCAAAAGT GATAAACAAC
d        TT******** ***__**A** ******A**** *G******** **********

                                                                    7622
Kalilo   TAGCCAGATT CTCCCCAATT ATGGCCGTGC CTTATACTTT TTATCATATA
d        ********** ******_*** ********** ********** **********

                                                                    7672
Kalilo   CTAGGGACTT ATATAATAAA ATTCTAGTAT AGTTTTGTCA CCAATATTTT
d        ********** ********** **G******* ********** **********

                                                                    7721
Kalilo   AAGAATCAGT CTACCCTCTA CAATTG-AAT AGATTGTTAT ATGAAAAATA
d        ********** ********** ********** ********** *****____*

                                                                    7769
Kalilo   AAATTTC-TT ATAAGTCTAT TTATTTCTTT TT-TATTTTT GGTTTCTACC
d        ***_*****A ********** **_******* **A*T***__ ----------

                                                                    7819
Kalilo   CTTTTATTAA ATTGGATTCT TTCGGGTCCC ATTCATAATC AACCTACAGG
d        ---------- ---------- ---------- ---------- ----------

                                                                    7869
Kalilo   TTATGGTCTT TCACCACTTC CCCGCTCACT GCATTGACCG CACGTAGCAA
d        ---------- ---------- ---------- ---------- ----------

                                                                    7919
Kalilo   ACTTATTGTT ATCCTTTCCT CTTTCATTTG CGTACAATTA CGGTTTACAT
d        ---------- ---------- ---------- ---------- ----------

                                                                    7969
Kalilo   TATTATGAGT AGATTTCAGC TCCCTTCACG CCACTAATTG CTATTGGGGT
d        ---------- ---------- ---------- ---------- ----------
```

```
                                                                    8019
Kalilo  CATCTACGGA CCTCTTCTCC CCCTTTCACC ATACAGGTAC TTCGTTTTGC
d       ---------- ---------- ---------- ---------- ----------

                                                                    8069
Kalilo  TTTTCCCTGT AGATTACTTG CACTTTAGGT TCTCTTACTC AGTCTGCGGT
d       ---------- ---------- ---------- ---------- ----------

                                                                    8119
Kalilo  ACTTCTTAGA CAATACTGTA CTCACAGGCC TTTCTTCGCT GCTCAGTTCT
d       ---------- ---------- ---------- ---------- ----------

                                                                    8169
Kalilo  AAAGTTAGTC CTGGTTGATT TATTTACTGT TACTTTTGGT TTCCTTTTTA
d       ---------- ---------- ---------- ---------- ----------

                                                                    8219
Kalilo  ATAAAAATTG GGATTTTTTC TATTTATCAA GTCGAGGGTT CGTTCTGGTT
d       ---------- ---------- ---------- ---------- ----------

                                                                    8269
Kalilo  CATTACTATC GATCACCGTA CTCTGTTTTC TGGATATTCT AAGCCTCAGT
d       ---------- ---------- ---------- ---------- ----------

                                                                    8319
Kalilo  TATTGGCCTT CAGATTCCCC AGCCCTGCGC CTTCAATAGT GGGATGTTCT
d       ---------- ---------- ---------- ---------- ----------

                                                                    8369
Kalilo  AGAATAAATC TTCCAGTTCG CTTAAATTCA CTCATGGACA TTACATTGAA
d       ---------- ---------- ---------- ---------- ----------

                                                                    8419
Kalilo  TACTTTCCCT TAGCAACCGT GTGTCTCTTA GACAATACCA CACACGTACA
d       ---------- ---------- ---------- ---------- ----------

                                                                    8469
Kalilo  CCGGTACCCA GATTTAATAA TGCTACCCAC TACCCTGATT GTATCCCTGG
d       ---------- ---------- ---------- ---------- ----------

                                                                    8519
Kalilo  AATCATTACT CAAGGTGGGA TATTCTACCC CACCATGGGG CGATAAAGAC
d       ---------- ---------- ---------- -T***-**** **********

                                                                    8568
Kalilo  GTTTATCTCC CCATTAGGGT GTGGTATAAA AATGAAAAAA -TCAAAAAGA
d       ********** ********** *****G*-** ********** G*******TC

                                                                    8616
Kalilo  GAGAGTACTA GAAATGATAA AAAGATCACA AAGGGTTA-A AAT-AGGAAC
d       ***G****** ********** ***A*AA**T *T***-**C* ***A*TT**A

                                                    8643
Kalilo  AAAAGGGGTC AGTGGTGCCC CTTACAC
d       ****--**** *-*******- -*-----
```

Appendix II

DNA sequence alignment of kalilo DNA, Gel-kalilo DNA and LA-kalilo DNA in the ORFs. The kalilo sequence was taken from Chan et al. 1991. The Gel-kalilo sequence was taken from Yuewang et al., 1996. The sample sequences of LA-kalilo DNA were obtained as described in Chapter 2.

a: DNA sequence alignment of ORF1
b: DNA sequence alignment of ORF2

* Identical nucleotide as in kalilo plasmid
\- Deletion
. Nucleotide sequence not determined in the present work
Numbers shown above the sequences correspond to numbers in Fig. 6.

Appendix IIa

```
                                                                      4950
Kalilo       ACTAATCATA TATTTCCCCA TATCTCTTAT ATCTTCAACG TTCAATTC--
Gel-kalilo   ********** *****T**** ********** ********** **********
LA-kalilo    .......... .......... .......... .......... .....***AT

                                                                      4999
Kalilo       -CCCCATTTG AGGAAGGACT GGTAAAGGTA AATATTCATA TCTTTTGTTA
Gel-kalilo   ********** ********** *****T**** ****A***** ****GG*G**
LA-kalilo    T********** ********_* ********** ********** **********

                                                                      5049
Kalilo       CCTTTTATTT TAACAAAATC CTCACCATTT TTATTAACAA TCTCTATTTT
Gel-kalilo   ********** ********** T********* ********** **********
LA-kalilo    ********** ********** ********** ********** **********

                                                                      5099
Kalilo       ATAATCTTTT AAATTTTCCC TGAATTTATA ATCAATTTTA TTTAAAAAAT
Gel-kalilo   ********** ********** ********G* ********** C*********
LA-kalilo    ********** ********** ********** ********** **********

                                                                      5149
Kalilo       CATTTTTAGA GTATAATAAA ATAAAACACT CTCTAACTTG TTCAGCCAAT
Gel-kalilo   ********** ********** ********** ********** **********
LA-kalil     ********** ********** ********** ********** **********

                                                                      5199
Kalilo       TTATACATAT CATTAGGATG AGTACCAAAA CACTCATGTA TAGGTAAGAT
Gel-kalilo   ***A****** ********** T**T****** **A******* *T*****A**
LA-kalilo    ********** ********** ********** **A******* **********

                                                                      5249
Kalilo       ATAAGAATCT CAACTATCAA TTATCATTGT TAAGTGTGAT GCATCTAATG
Gel-kalilo   ********** ********** ***A*****G ********C* **********
LA-kalilo    ********** ********** ********** ********** **********

                                                                      5299
Kalilo       AATGAATAAT ATTAGGGATG ATTGCTTGGA CCTCTCTTCT ACTATCTTTT
Gel-kalilo   *******T** *********A **G***G*** *T******** *********A
LA-kalilo    ********** ********** ********** ********** **********
```

```
                                                                    5349
Kalilo          TCATTAACTC AAGATCTTAA TACTGCTGTT CTATTTTTCC CTAGGAAATT
Gel-kalilo      ***C****** ********** ********** ********** **********
LA-kalilo       ********** ********** ********** ********** **********

                                                                    5399
Kalilo          TATAGTTAAT TTTTTAACCT TAGATAGATT ATATCTTTGT GTTAACTCTA
Gel-kalilo      ********** ****G***T* ******A*** ********** **********
LA-kalilo       ********** ********** ********** ********** **********

                                                                    5449
Kalilo          AACCATCGGG TGTAGATCAT GATAAAGGAA TATCTAACTT TAAATAGATT
Gel-kalilo      *******A** ******A**G ****TC**** ******G*** C*****T***
LA-kalilo       ********** ********** ********** ********** **********

                                                                    5499
Kalilo          TTAGCTAAGC GAGTAAGATA GTCATAAATA GAGTGAAGTT TAGGGAATTG
Gel-kalilo      ****T***** ********** C********* ********** **********
LA-kalilo       ********** ********** ********** ********** **********

                                                                    5549
Kalilo          ATTGAATATA TTATCATTAA TAATACTAGC CAGAGTTTCA ACTTCAAAGG
Gel-kalilo      ********** ********** ********** T********* **********
LA-kalilo       ********** ********** ********** ********** **********

                                                                    5599
Kalilo          TGTCTAGGAC AACAAAGTCA CCATTTTTAG TAGGAACTAA ATAATCATAA
Gel-kalilo      ********** ******T*T* ********** ********** **********
LA-kalilo       ********** ********** ********** ********** **********

                                                                    5649
Kalilo          ACTTTTATTT CTTTCCCCTT TGAAATAACT ATTTTCTCTA CTTTTTCCAA
Gel-kalilo      ********** ********** ********T* ********** **********
LA-kalilo       ********** ********** .......... .......... ..........

                                                                    5699
Kalilo          TTTACTTTTT AACTGTTCTG TAATACCATA TGTAGTAACA TTATAACTTT
Gel-kalilo      ******C*** **T******* ********** ********** **********
LA-kalilo       .......... .......... .......... .......... ..........

                                                                    5749
Kalilo          TAGTCATAAT AACTTTTTTA AGTAAACTTC TATTAAGAGA GATATCCGAA
Gel-kalilo      ********** ********** ********** ********** **********
LA-kalilo       .......... .......... .......... .......... ..........

                                                                    5799
Kalilo          AATTTAAGAT TTTTAAATTT TTTTTCAGCA GATTCGTTAA TAGCTTTATT
Gel-kalilo      *******C** ****T***** ********** ********** **********
LA-kalilo       .......... .......... .......... .......... ..........

                                                                    5849
Kalilo          TATTGCAGGA ATAAGTTGAG AATAGAAATC GTTTACACTC TCACCACTAT
Gel-kalilo      *********T ********T* ********** ********** **********
LA-kalilo       .......... .......... .......... .......... ..........
```

```
                                                                    5899
Kalilo        TAATAAGATT AACATATTTT CCCAACTCAA GGTCAAGAAG CATTGCGGCA
Gel-kalilo    ********** C********* ********** *A******** **********
LA-kalilo     .......... .......... .......... .......... .........*

                                                                    5948
Kalilo        AAATGTTGAA CACCACTACA TGTTGCATCA AGG-AAAATA GGATTAAAAA
Gel-kalilo    ********** ********** ********** ********** **********
LA-kalilo     ********-* ******G*** ********** ***A****** **********

                                                                    5998
Kalilo        CTGGGTAATC AGGATTTTCT TTTAATTTCC TCATAGTTAA ACAGAAAGCT
Gel-kalilo    ******G*** ********** ********** G********* **********
LA-kalilo     ********** ********** ********** ********** **********

                                                                    6048
Kalilo        GCAAATAATG TTGGAGATTC AGCTTTTAAT ATAAACTCCT TATCCATAGC
Gel-kalilo    ********** ********** ********** *****A**** **********
LA-kalilo     ********** ********** ********** ********** **********

                                                                    6098
Kalilo        TATTATATTA TCTAAATTTT CAACCACTCA GTTGAATCTA TCTTGAAATG
Gel-kalilo    ********** ********** ********** ********** **********
LA-kalilo     ********** ********** ********** ********** **********

                                                                    6148
Kalilo        ATTTTTTACT AAATTTACCA CCATCATTAT AAATATTAGC CCCATATACA
Gel-kalilo    ********** ********** **T*****G* ********** **********
LA-kalilo     ********** ********** ********** ********** **********

                                                                    6198
Kalilo        TAGAAAAAGA ATAAACCCTT CTCATCTAAT TTCTTACCTT CAAATAAATT
Gel-kalilo    ********** *********C T********* ****G***** **********
LA-kalilo     ********** ********** ********** ********** **********

                                                                    6248
Kalilo        TATTAAAGCT AAAGATAATT CAGATCCTTG ATAATCAAGA TAGAAAGATT
Gel-kalilo    ********** ********** ********** ***GC***** **********
LA-kalilo     ********** ********** ********** ********** **********

                                                                    6298
Kalilo        GAGTGTAAAT ACGCCCTCTT CAATCTATAT TAACATTTAA ATAAAAGGGA
Gel-kalilo    ********** ********** ********** ********** **********
LA-kalilo     ********** ********** ********** ********** **********

                                                                    6348
Kalilo        GTATTCAAAT AAGTTCTAGC AATATCTATA GTAATCATAT TATTAATATA
Gel-kalilo    ********** *****T**** ********A* ********** **********
LA-kalilo     ********** ********** ********** ********** **********

                                                                    6398
Kalilo        AGTATCTTTT TTAGTATTTT TATAAAAATC TAGAATGAAA GACCCCTCAT
Gel-kalilo    *********C *****C**** ********** **T******* *****T****
LA-kalilo     ********** ********** ********** ********** *****-****
```

```
                                                                    6448
Kalilo          TTTGAAGATA AGATAATAAA TCACCATTAA TTTTAAATTT GAAAGCATTT
Gel-kalilo      ********** *****G**** ***G****** ********** **********
LA-kalilo       ********** ********** ********** ********** **********

                                                                    6498
Kalilo          AATTTATTAA TTACATTATA CAACTTATCA GTTATTGCTA TTTTATGTCT
Gel-kalilo      ********** ********** ********** C********* *******A**
LA-kalilo       ********** ********** *_******** *****G**** _*********

                                                                    6548
Kalilo          ATGATGAAAT GATCCAACCA CTAAAGATTC ATCTTGAGTA TCAGTTAAAT
Gel-kalilo      ********** ********** *******A** G***GC*T** ***T******
LA-kalilo       ********** ********** ********** C********* **********

                                                                    6598
Kalilo          TTATAAGATT TCCTCCCCTT ATACCATGTC CTCATGGTAA TGGTGGACAT
Gel-kalilo      ******C*C* ********** ********** A****AT*** **********
LA-kalilo       ********** ********** ***....... .......... ..........

                                                                    6648
Kalilo          CCATTGGAAG AGCTTTTGGA GATATAACTA AACTTTCCAT AATTTCATTT
Gel-kalilo      ********** ********** ********** ********** ********A*
LA-kalilo       .......... .......... .......... .......... ..........

                                                                    6698
Kalilo          TCATATTCCT TATTTATTTT TAAAGAATAA ATTTCATTTT CAAACACCCT
Gel-kalilo      ********T* ********** ********** ********** **********
LA-kalilo       .......... .......... .......... .......... ..........

                                                                    6748
Kalilo          CTCAAAAATA GGATTAAGGG GTGATGTGAA GGTATTAATA AAAATATCAC
Gel-kalilo      ******T*** ********A* *******A** ********** **********
LA-kalilo       .......... .......... .......... .......... ..........

                                                                    6798
Kalilo          CCAATTTTAC AAAAAACCCT ACATCAAATT TATTATAGTT TGAGAAATCA
Gel-kalilo      ********** ********** *A******** *******C*C G*G******T
LA-kalilo       .......... .......... .......... .......... ..........

                                                                    6848
Kalilo          TCAAAGCTCA TAAACTCATC TTTATAGTAT TCTGTGTAAA TATGTCTAGT
Gel-kalilo      ********** ****A****T *******G** ***C****** **********
LA-kalilo       .......... .......... .......... .......... ..........

                                                                    6898
Kalilo          AATACTATAA CCAATTCTAT AGTCTAAATT TGTTAAAGAT GATTTACTAT
Gel-kalilo      ********** ********** ********** **G******* **********
LA-kalilo       .......... .......... .......... .......... ..........

                                                                    6948
Kalilo          AACTAGCTAA TAAGTATGAT CATGCGATAA AAATAAATTT CTCATTCACA
Gel-kalilo      ********** ********** ********** ********** T*********
LA-kalilo       .......... .......... .......... .......... ..........
```

```
                                                                    6998
Kalilo        ATATAATCTT TTAGTGATTT AGATATTTTA TTAAGATTAC CATTACTTTT
Gel-kalilo    ********** *********A ********** ********** **********
LA-kalilo     .......... .......... .......... .......... ..........

                                                                    7048
Kalilo        ATAAAGCTTT AGAAGATCAA ATGCTCTATC ATGTATTAAC CTGTGTTTGG
Gel-kalilo    ********** ********** ********C* *********T **C*******
LA-kalilo     .......... .......... .......... .......... ..........

                                                                    7098
Kalilo        TATACATAGT TGATGTAATA TCAGATTTAA CATCAGTTGA AGGTGTAGCT
Gel-kalilo    C********* ****T***** ********T* *****A**** **********
LA-kalilo     .......... .......... .......... .......... ..........

                                                                    7148
Kalilo        AGCTTCAATC ATTCCCTCTC AATAATCTCT TGAGCCTTAT GTAGATCCTT
Gel-kalilo    *A******** *****G**** G********* **G******* AA********
LA-kalilo     .......... .......... .......... .......... ..........

                                                                    7198
Kalilo        TTTATGCTCA GAAAGAATTT TTGAAACATT ATTTAGGAAT AATGATAATT
Gel-kalilo    ********** ********C* ******T*** *G******** **********
LA-kalilo     .......... .......... .......... .......... ..........

                                                                    7248
Kalilo        TAGGGTCGCG TTTCTTTTCA GAAGAAAAGT TACGAATATT TTTAAACTCC
Gel-kalilo    ****C**AGA ***T****** *T******** ********** ***T*TTG**
LA-kalilo     .......... .......... .......... .......... ..........

                                                                    7298
Kalilo        GAGTAATTAA ACTTAATAAA ACGGAGGCTG GTTGCGTGCG AATTCCTCAT
Gel-kalilo    ********** ********** ********** ********AA **********
LA-kalilo     .......... .......... .......... .......... ..........

                                                                    7334
Kalilo        ATTAATGCGA TAATTGAAAA AAGAAAATTT GCTCAT---- ----------
Gel-kalilo    ********** ********** ********** *A****AATA AAATTTTTAA
LA-kalilo     .......... .......... .......... .......... ..........

                                                                7334
Kalilo        ---------- ---------- ---------- ---------- ------
Gel-kalilo    ATAAATATTC TGTTCTTTTA CGTCGTAGCC TTTCTATACA CTCCAT
LA-kalilo     .......... .......... .......... .......... ......
```

Appendix IIb

```
                                                                                                1309
Kalilo      ---------- ---------- ---------- ---------- ---------- ----------
Gel-kalilo  ATGGAGTGTA TAGAAAGGCT ACGACGTAAA AGAACAGAAT ATTTATTTAA AAATTTTATT
LA-kalilo   .......... .......... .......... .......... .......... ..........

                                                                                                1369
Kalilo      ATGAGCAAAT TTTCTTTTTT CAATTATCGC ATTAATATGA GGAATTCGCA CGCAACCTTG
Gel-kalilo  ****T***** ********** ********** ********** ******TTCA **********
LA-kalilo   .......... .......... .......... .......... .......... ..........

                                                                                                1429
Kalilo      GGAAGCCTCC CTTGTTTTAT TAAGTTTAAT TATTTATCTT CCTCTTCACA CTCAAAATTT
Gel-kalilo  *C******** **A******* ********** ********** ******T*A* ******T***
LA-kalilo   .......... .......... .......... .......... .......... ..........

                                                                                                1489
Kalilo      TATAGTACTT CAACTGATTC ACCCATGGGA CATATTGTAT CAGCTAGTAA ATCTTCATGA
Gel-kalilo  ********** ********** ********** ********** ********** **********
LA-kalilo   .......... .......... .......... .......... .......... ..........

                                                                                                1549
Kalilo      TATAATTATT ATTCACATAA AAATTATGAT CCTTTAACAA GAGAATCATT TCATGATGAA
Gel-kalilo  ********** T****GTG** ********** ***A**T**C CTTTT***** AG*AA*****
LA-kalilo   .......... .......... .......... .......... .......... ..........

                                                                                                1609
Kalilo      CTAAGAGCCT TTATTTCATT ATATAAAAAA CAATTCAAAG AAGAAAAGTT TTTTTTTATG
Gel-kalilo  ********** ********** ********** ********** ********** ****A*****
LA-kalilo   .......... .......... .......... .......... .......... ..........

                                                                                                1669
Kalilo      CTAAGAGGCT TTATTTCATT ATATAAAAAA CAATTCAAAG AAGAAAAGTT TTTTTTTATG
Gel-kalilo  ********** ********** ********** *T******** ********** **********
LA-kalilo   .......... .......... .......... .......... .......... ..........

                                                                                                1727
Kalilo      ACTGACCCAC TAGAAGTTTT ACGTCTTCTA GAGGCTATTA ATTCAATTTA TATG--CATA
Gel-kalilo  ******T**T *T***A**** *AA****A** *CC***T*** *********_ *_*******-
LA-kalilo   .......... .......... .......... .......... .......... ..........

                                                                                                1787
Kalilo      CACATACAGG GATTAGTGAG GCTGTTTCAG AACCATTTGA GTATGAGCTC TTTAGTTTAG
Gel-kalilo  *-***CC--- -T**TC**** ********** *******G** *A*******T ***TC****C
LA-kalilo   .......... .......... .......... .......... .......... ..........

                                                                                                1847
Kalilo      GAGATGGTTT ACCAAAAGGA AATATTATTT TCACTTTTAA ACCTACAAGT AATCCTAGTA
Gel-kalilo  CTA******* ********** ********** ********** ********A* *******AAG
LA-kalilo   .......... .......... .......... .......... .......... ..........

                                                                                                1907
Kalilo      TTAAAACAAA ATATGAAGCA CATAAATCAA ATATTAAAAG AAATAAAAAT ATTAATTTAT
Gel-kalilo  ********** *******AA* *T**TG**** *GGA*****T ********** GC********
LA-kalilo   .......... .......... .......... .......... .......... ..........
```

```
                                                                    1967
Kalilo      CTAAGAAAAA TCCTCTTAAC AAATTTAAGT ATAATGGTTA TACTATACCA AATACCATGG
Gel-kalilo ********** ********** **G******* ********** ********** **A*******
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2027
Kalilo      ATCTTTCACA ATGACCCAAC ATTCATTTTA TAAATGATGG TAAAAATGCT GTGTCTTTGA
Gel-kalilo ********** ********** *******A** *T******C* G********* **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2087
Kalilo      ATAATATTAT TAAATCAGGG GTTGATAATA TGACCCTTTC TTTCTTTATC ACTATTAACA
Gel-kalilo ********** ********** AA******** ********** ********** *******C**
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2147
Kalilo      AAAAATATAA TGAAATTACT GTTTTATTGA ATAATACACC TATTTTCAAG ATTAAAGATG
Gel-kalilo **TTT*T*** *******C** ********** ********** ********** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2207
Kalilo      AAAAAATAAT GTCTGAAGAT GATTTATCTT CTTTTAAAAG AACAATTACA GAAAATGAAC
Gel-kalilo ---******** *GA*A***** ********** ********** ********** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2267
Kalilo      AGGATAAAGT TTATGTTTTT GAAAATGGAG AAATGGTTTT CTTCAGTGAG AATGTTAAAA
Gel-kalilo ********** ********** ********TA ********** *****C**** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2327
Kalilo      CTAGTTTTAT AAAGAAAATA ACTCGTCAAG ACTTAATTAA TTTTGAAAAT CCTAAAATTA
Gel-kalilo *G*C****** ***A****** *A******** ********** ********** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2387
Kalilo      TAACCTTAGA TCTTGAAACT AGAAGTGTTC CAATACATCC TATAAAAGAA GGAAAGGATG
Gel-kalilo ********** ********** ********** ********** ********** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2447
Kalilo      GGAAGGAAGG AAAAGTTGAC TCAATTATGT TTCCTATACT TATGTCAGTA TACAATGGAA
Gel-kalilo ********AA *******A** *****G**** ********G* *******A** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2507
Kalilo      AATTTGTTAA ATCTTTTCTT TTTAGTCAAT CAGCATGAGA GACTGAAATG ATGAACGCAT
Gel-kalilo ********** ********** ********** ********** ********** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2567
Kalilo      TTAAATCTAT TATGTTGAGA AAATATGATG GTTACAAAGT TTATACTCAT AATTTCTCAT
Gel-kalilo ********** ********** ********** ********** ******A*** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........
```

```
                                                                    2627
Kalilo      ATTTTGATGG AATTTTCATC ATTGACATCC TTTCAAGACT CGGTGAGGTA AAACCATTTA
Gel-kalilo ********** ********** ********** ********** ********** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2687
Kalilo      TGAGAAATGG TAAAATTTTA AAACTAACTT TTAATTTTAC ACTACCAAAC TCCAAAAGAA
Gel-kalilo ********** ********** ********** ********** ********** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2747
Kalilo      AATATACACT TTATTTTATG GATTCTCTGC TTATTTTACC AGATTCTTTG GATAAATTAT
Gel-kalilo ********** *C*A****** ********** ********** ********** **A*******
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2807
Kalilo      CAAATTTTTT TAACAATAAA GTTAAGAAAT TGTTTTTTCC CCATTCATTC CTAGATGATA
Gel-kalilo **TTA***** ********** ****T****A GC******** ********** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2867
Kalilo      ATACTATTCC CATAAATTAT GTTGGAAAAT GCCCTGATTA TAAGTATTTT CCAAAGGCTT
Gel-kalilo ***G****T* ********** ********** ********** ********** *****A****
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2927
Kalilo      ATACTGAAGA TTTCACAATT GAACAATATC AAGAATATGC TAATAAATTT AAAAATAATA
Gel-kalilo G****A**** ********** ***A****** ********** CGG******* **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    2987
Kalilo      ATTGAGATTT AAAAAAAGAA TTAATTAAAT ATTGTGAAAT TGATACTATA GCACTTTATC
Gel-kalilo ********** ********** ********** ********** ********** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    3047
Kalilo      AAGTTTTAGT AAGTTTTCAA AGAAAAATTT ATGATAAATT TATGATAGAT TGTACTAAAT
Gel-kalilo ********** **A******G ********** ********** ********** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    3107
Kalilo      ATCCAACAAT TCCTTCATTA GCATTTGCTA TTTTTAGAAA AAAATATCTT GTTGAAGATA
Gel-kalilo ********** ********** ********** ********** ***T****** *****T****
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    3167
Kalilo      TGATTCCCAA TATTAAATCT AAACTTCATA ATATTATTAA ACTTAGTTAT TTTGGTGGAA
Gel-kalilo ********** ********** ********** ********** **G******* **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    3227
Kalilo      TCTGTGAGCT ATATAAACCA TTCGGAGTTA ATATCAAATC ATATGATGTT AATTCACTTT
Gel-kalilo ********** ********** *CT******* ********** ********** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........
```

```
                                                                    3287
Kalilo      ACCCATTCGC CATGAAATAC TTCAAAATGC CATCTGGAAT TCCTAAATAT GTTAAAGGAA
Gel-kalilo *********G ********T* ****C****G *T******** ********** *********G
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    3344
Kalilo      CGTTACAAAA CATTGTAAGA TTCACA---G ATTCTATTTG TGAAGTCCCA TTTGGATTTT
Gel-kalilo **A**G**** T***AG**A* ****T***** ******C*GA ***GA***** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    3404
Kalilo      ATAATGTAAA GGTAAAAACC CCTTTAAATT TAGATAAACC TTTTCTTCCT ACAAGACTTA
Gel-kalilo ********** *******G** ********** ********** ********** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    3464
Kalilo      ATACACCAGC CGGGACAAGA ACAGCTTTCC CACTTGGTCA ATGAGAAGGT TGATATTTCT
Gel-kalilo *****G**** ********** ***AT***** ********** ********** **********
LA-kalilo  .......... .......... ***A****** *********T ********** **********

                                                                    3524
Kalilo      CTGAGGAAAT ATTGAATGCT ATGAAGCATG GTTATGAATT TGAATTTATA GAGGGTTATT
Gel-kalilo ********** ********** ******T*** ********** ********C* *****G*T**
LA-kalilo  ********** ********** ********** ********** ********** **********

                                                                    3584
Kalilo      TATTTGAAGA ATCATCTATG TTTGATGAAT ATATAGATCT TTTATACAAT ATTAAAAAAA
Gel-kalilo ********A* *****A**** *****G***A ********** ********** **********
LA-kalilo  ********** *****A**** ********** ********** ********** **********

                                                                    3644
Kalilo      ATTCTCCTAA GGAGTCTCCC TGATACTATA TTTCTAAACT CCTTATGAAC TCTTTATATG
Gel-kalilo ********** ********** ********** ********** ********** **********
LA-kalilo  ********** ********** ********** ********** ********** **********

                                                                    3704
Kalilo      GTCGATTTGG ATTAAATCCC GAGGGGGAGG AAATCTTTAT TACTAGTGAA GAAGAGGGAG
Gel-kalilo **A******* ********** ********** *****A**** ********** **********
LA-kalilo  ********** ********** ********** ********** ********** **********

                                                                    3764
Kalilo      ATGCAATTAT AGCAACAAAG GAGTATGTAA CAATTACACC TTTATCAAGC GGTAATGTTT
Gel-kalilo **AA*C**** **A*****T* ********** ********** *****T***T **********
LA-kalilo  ***A****** ********** ********** ********** ********** **********

                                                                    3824
Kalilo      TAATTTCAGC TAAATTACCT GAAGAAGCAT TTGGAGATAT GAATATATCA GTACCCATTT
Gel-kalilo **********T ********** ********** ****G***** ********** **********
LA-kalilo  ********** ********** ********** ********** ********** **********

                                                                    3884
Kalilo      CATCAGCTAT TGCTGCATAC TCTCGAATAC ATATGTCACA TTTTTTAACT AAATATTCAA
Gel-kalilo ********** ********** ***A****** ********** ********A* **********
LA-kalilo  ********** ********** ********** ********** ********** **********
```

```
                                                                    3944
Kalilo      ATAACATTTA TTATATTGAT ACTGACGGTA TTAAAGTTGA TATCGATCTT GATAAAGATG
Gel-kalilo ********** ********** ********** ********** ********** **********
LA-kalilo  ********** ********** ********** ********** ********** **********

                                                                    4004
Kalilo      AAGTTGATTC AAAAGAGTTA GGAAAAATGA AATATGAATA TGTCTTTGAA GAATACACTA
Gel-kalilo ********** T********* **G******* ********** ******G*** *******GC*
LA-kalilo  ********** ********** ********** ********** ********** **********

                                                                    4064
Kalilo      GTTTAGGACC TAAAGTTTAC GGTGGATTAT TGTATGATAA AAAAGGTAAG TTAACAGAAT
Gel-kalilo ********** *********T ********** ********** ********** **********
LA-kalilo  ********** ********** ********** ********** ********** **********

                                                                    4124
Kalilo      TAGTAAAACT TAGAGGTTAT AGCTCAAAAC TCCCTTATAA TAAGTTAAAA GAGGGATTGG
Gel-kalilo ********** ********** ********** ********** ***A****** **********
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                                    4184
Kalilo      TAAAAGACCA TACGTTAGAA TTGACTCAGA AAAAATGAAA AAGAAAATTA TCAGAGTCTA
Gel-kalilo *******T** **T*A***** **A******* ********** ********** *****A****
LA-kalilo  .......... .......... .......... .......... .......... ..........

                                                          4219
Kalilo      CTGTTTACTT GAAGAACAAC CATTTACTGT TTCTG
Gel-kalilo **A******* ********** ********** *****
LA-kalilo  .......... .......... .......... .....
```

Appendix III

Alignment of part of the TIR sequences of the prototypic kalilo DNA from *N. intermedia*, the LA-kalilo DNA from the *N. tetrasperma* strain P4495, the short kalilo DNA from the *N. discreta* strain 6790 and the Gel-kalilo DNA from *Gelasinospora*. The sequences of kalilo DNA and Gel-kalilo DNA were taken from Chan et al. 1991 and Yuewang et al., 1996, respectively.
a:The alignment was made by using the multi-sequence alignment program from the Baylor College of Medicine on internet.
b:The alignment made manually. Sequence homology of all corresponding regions are shown as in Fig. 6.
* Identical nucleotide as in Kalilo plasmid
- Deletion
Numbers shown above the sequences correspond to numbers in Fig. 6.

Appendix IIIa

```
                                                                    7461
Kalilo          CACCCTTTCC TCTAAGATAA T-GGGGGCGG ATAAAACATA AAAGCCACTT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ********** ********** *G******** ********** **********
Gel-kalilo      ********** ********** ********** ********G* **********

                                                                    7510
Kalilo          T-CCCCCAAG CGGGCGTAGA TCCCATACCT CACGGTATGG ATAATTCGGC
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    *C******** ********** ********** ********** **********
Gel-kalilo      *C******** ********** ********** *****C**** **********

                                                                    7559
Kalilo          ATTTCAGTAG ACGACTTTTT TGTTTAAGAT TTTTTTGGG- AAAACCCAAA
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ********** ********** *T*G*TTA** A********A ****G*****
Gel-kalilo      ********** *********C ********** ****___**G ****A**CG*

                                                                    7609
Kalilo          AGTGATAAAC AACTAGCCAG ATTCTCCCCA ATTATGGCCG TGCCTTATAC
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ********** ********** *********_ ********** **********
Gel-kalilo      ********** ********** ********** T*ATG*C*GT AA********

                                                                    7658
Kalilo          -TTTTTATCA TATACTAGGG ACTTATATAA TAAAATTCTA GTATAGTTTT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ********** ********** ********** ******G*** **********
Gel-kalilo      A*C******* ********** ********** ******A*** *********A

                                                                    7707
Kalilo          GTCACCAATA TTTTAAGAAT CAGTCTACCC TCTACAATTG -AATAGATTG
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ********** ********** ********** ********** A*********
Gel-kalilo      ********** ********** ********** ********** **********

                                                                    7757
Kalilo          TTATATGAAA AATAAAATTT CTTATAAGTC TATTTATTTC TTTTTTATTT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    *********_ ___****** C A*A******* *****T**CT ****A*T***
Gel-kalilo      *********_ *******A** ********** *****T***G *****AT*G*
```

```
                                                                    7802
Kalilo          T-----TGGT TTCTACCCTT TTATTAAATT GGATTCTTTC GGGTCCCATT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    *CCAGGG*CG A*AA*GA*G* ****CTCCCC ATTAGGG*GT **TGTAA*A*
Gel-kalilo      *TTTTA*TT* **A*TTTT** A*T**TT*** TCC**T***T A*T*ATA**C

                                                                    7852
Kalilo          CATAATCAAC CTACAGGTTA TGGTCTTTCA CCACTTCCCC GCTCACTGCA
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    G*A**AAGT* AA*A*TCGAG G*TA**AGA* ATGA*AAAAA AAAA***ATG
Gel-kalilo      TCC*CC**CT A*T**----- ---------- ----**G**T *AATCG*AGT

                                                                    7902
Kalilo          TTGACCGCAC GTAGCAAACT TATTGTTATC CTTTCCTCTT TCATTTGCGT
LA-kalilo       ********** **-----G** *C**AC***- ---------- ----------
Short kalilo    GGT**AAATA A*TAA***AA GG*CA*GG*G *C*------- ----------
Gel-kalilo      **C*GT*TTA *GGCAT**TA *C*CC***-- ---------- ----------

                                                                    7952
Kalilo          ACAATTACGG TTTACATTAT TATGAGTAGA TTTCAGCTCC CTTCACGCCA
LA-kalilo       ---------* *AGTTTAC** ********** C********* **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      ---------- ------AG** *G*TT*G*** GAG*C*AG*T *GATCGTA*C

                                                                    8001
Kalilo          CTAATTGCTA TTGGGGTCAT CTACGGACCT -CTTCTCCCC CTTTCACCAT
LA-kalilo       ********** ********** ********** C********* **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      *A****CTCT **C**A*T*A T**TTCTAGC A**AGAA*TA AGGGATAG*A

                                                                    8051
Kalilo          ACAGGTACTT CGTTTTGCTT TTCCCTGTAG ATTACTTGCA CTTTAGGTTC
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      TA*CTAGT*C **GAC*TT*G *GA*T*TGG* G**T*AGTGT TAGGTCA*AG

                                                                    8101
Kalilo          TCTTACTCAG TCTGCGGTAC TTCTTAGACA ATACTGTACT CACAGGCCTT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      CA*AT*CT*A AGATT*T*TG GAGAG**C*G *GCTC*AT*A T**CCAAA**

                                                                    8151
Kalilo          TCTTCGCTGC TCAGTTCTAA AGTTAGTCCT GGTTGATTTA TTTACTGTTA
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      CTC*TT*G*A *T*A*G**T* *--*GA*T** AACCCCA*AC CCC*A**G*G

                                                                    8201
Kalilo          CTTTTGGTTT CCTTTTTAAT AAAAATTGGG ATTTTTTCTA TTTATCAAGT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      GGA*ATTC*A *T****A*TA *********T *********G **********
```

```
                                                                    8250
Kalilo          CGAGGGTTCG TTCT-GGTTC ATTACTATCG ATCACCGTAC TCTGTTTTCT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      ********** ****G***** ********T* ********** *********G

                                                                    8300
Kalilo          GGATATTCTA AGCCTCAGTT ATTGGCCTTC AGATTCCCCA GCCCTGCGCC
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      ********** ********** ********** *********C AG**CT***T

                                                                    8346
Kalilo          TTCAATAGTG GGATGTTCTA GAATA----A ATCTTCCAGT TCGCTTAAAT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      **G***GG** ********** CCTC*CCAGG GGTGATA*A* A**TA*CTC*

                                                                    8396
Kalilo          TCACTCATGG ACATTACATT GAATACTTTC CCTTAGCAAC CGTGTGTCTC
LA-kalilo       ********** ******A*** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      C*C*ATTA** *TG*GGT**A A*TATGAAAA ***G*AA**A A*A**A*TAA

                                                                    8446
Kalilo          TTAGACAATA CCACACACGT ACACCGGTAC CCAGATTTAA TAATGCTACC
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      AA*A*GG*** AA*A*TTAAA *GGCT*A**A AT*ACAA*** C**G*GGGAG

                                                                    8496
Kalilo          CACTACCCTG ATTGTATCCC TGGAATCATT ACTCAAGGTG GGATATTCTA
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      TGG**T**CT TACAACA--- ---------- ---------- ----------

                                                                    8546
Kalilo          CCCCACCATG GGGCGATAAA GACGTTTATC TCCCCATTAG GGTGTGGTAT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      ---------- ---------- ---------- ---------- ----------

                                                                    8596
Kalilo          AAAAATGAAA AAATCAAAAA GAGAGAGTAC TAGAAATGAT AAAAAGATCA
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      ---------- ---------- ---------- ---------- ----------

                                                                    8643
Kalilo          CAAAGGGTTA AAATAGGAAC AAAAGGGGTC AGTGGTGCCC CTTACAC
LA-kalilo       ********** ********** *********G C*********T A*******
Short kalilo    ---------- ---------- ---------- ---------- -------
Gel-kalilo      ---------- ---------- ---------- ---------- -------
```

Appendix IIIb

```
                                                                    7461
Kalilo          CACCCTTTCC TCTAAGATAA T-GGGGGCGG ATAAAACATA AAAGCCACTT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ********** ********** *G******** ********** **********
Gel-kalilo      ********** ********** ********** ********** **********

                                                                    7510
Kalilo          T-CCCCCAAG CGGGCGTAGA TCCCATACCT CACGGTATGG ATAATTCGGC
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    *C******** ********** ********** ********** **********
Gel-kalilo      *T******** ********** *****A**** *****C**** **********

                                                                    7559
Kalilo          ATTTCAGTAG ACGACTTTTT TGTTTAAGAT TTTTTTGGG- AAAACCCAAA
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ********** ********** *T*G*TTA** A********A ****G*****
Gel Kalilo      ********** ********** ********** ****--***A ****-**CG*

                                                                    7609
Kalilo          AGTGATAAAC AACTAGCCAG ATTCTCCCCA ATTATGGCCG TGCCTTATAC
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ********** ********** *********- ********** **********
Gel-kalilo      ********** ********** ********** T*ATG*C*GT AA********

                                                                    7658
Kalilo          -TTTTTATCA TATACTAGGG ACTTATATAA TAAAATTCTA GTATAGTTTT
LA-kalilo       ********** ********** ********** ********** **********
short-Kalilo    ********** ********** ********** ******G*** **********
Gel-kalilo      A*C******* ********** ********** ******A*** **********

                                                                    7707
Kalilo          GTCACCAATA TTTTAAGAAT CAGTCTACCC TCTACAATTG -AATAGATTG
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ********** ********** ********** ********** A*********
Gel-kalilo      ********** ********** ***C****** ********** **********

                                                                    7757
Kalilo          TTATATGAAA AATAAAATTT CTTATAAGTC TATTTATTTC TTTTTTATTT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    *********- ---****** C A*A******* *****T**CT ****A*T***
Gel-kalilo      *********- *******A** ********** *****T***G *****AT*G*

                                                                    7806
Kalilo          TTGGTTTCTA CCCTTTTATT AAATTGGATT CTTTCGGGTC CCATTCATA-
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    *--------- ---------- ---------- ---------- ----------
Gel-kalilo      *--T***A*T TTT*A**T** TT***TTT*A T**C*TTT*T TT*G*T***A

                                                                    7852
Kalilo          -ATCAACCTA CAGGTTATGG TC--TTTCAC CACTTCCCCG CTCACTGCA-
LA-kalilo       ********** ********** *****C**** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      TC**C***C* *TAT*C**T* C*TGAA**-G T*G**T*AGT G*T*GG***T
```

```
                                                                    7900
Kalilo          TTGACCGCAC GTAGCAAACT TATTGTTATC CTTTCCTCTT TCATTTG--C
LA-kalilo       *****TA*** **-----G** *C**AC***- ---------- ----------
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      AAT*TCT*CT TA**ATTGT* *GGA*AG*G* *-GAGC**GA **G*ACCCAA

                                                                    7948
Kalilo          GTACAATTAC GGTTTACATT AT--TATGAG TAGATTTCAG CTCCCTTCAC
LA-kalilo       ***------- -********* -----G**** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      A*T*TC**T* **A***-*** **TC**GC*C ****AC*A** --GGA*AG*A

                                                                    7997
Kalilo          GCCACTAATT GC-TATTGGG GTCATCTACG GACCTCTTCT CCCCCTTTCA
LA-kalilo       ********** ********** *********A ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      TAACTAGT*C CGGAC**TT* TGAC*T*GG* *TTTCAG*G* TAGGTCA*-*

                                                                    8044
Kalilo          CCATACAGGT AC---TTCGT TTTGCTTTTC CCTGTAGATT ACTTGCACTT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      G****TCC-* *AAGA**-** **G*AGAGAG **GAGCTCGA T*A*A*C*AA

                                                                    8093
Kalilo          TAGGTTCTCT TACTCAGTC- TGCGGTACTT CTTAGACAAT ACTGTACTCA
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      -*--****** *T*GG*T*AA ***-T**A-* GA*TCT-**C C*CA***C*C

                                                                    8143
Kalilo          CAGGCCTTTC TTCGCTGCTC AGTTCTAAAG TTAGTCCTGG TTGATTTATT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      A*T*GTGGGA *ATT**A--- ---------- ---------- ----------

                                                                    8192
Kalilo          TACTGTTACT TTTGGTTTCC TTTTTAATAA AA-ATTGGGA TTTTTTCTAT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      ---------- ---------* ********** **A*****T* ********GC

                                                                    8241
Kalilo          TTATCAAGTC GAGGGTTCGT TCT-GGTTCA TTACTATCGA TCACCGTACT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      *********- ********** ***G****** *******T** **********

                                                                    8290
Kalilo          CTGTTTTCTG GATATTCTAA GCCTCAGTTA TTGGCCTTCA GATTCCCC-A
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      ********G* ********** ********** ********** ********C*
```

```
                                                                    8340
Kalilo          GCCCTGCGCC TTCAATAGTG GGATGTTCTA GAATAAATCT TCCAGTTCGC
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      *****-***T **G***G*** ********** ---------- ----------

                                                                    8390
Kalilo          TTAAATTCAC TCATGGACAT TACATTGAAT ACTTTCCCTT AGCAACCGTG
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      ---------- ---------- ---------- ---------- ----------

                                                                    8440
Kalilo          TGTCTCTTAG ACAATACCAC ACACGTACAC CGGTACCCAG ATTTAATAAT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      ---------- ---------- ---------- ---------- ----------

                                                                    8490
Kalilo          GCTACCCACT ACCCTGATTG TATCCCTGGA ATCATTACTC AAGGTGGGAT
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ---------- ---------- ---------- ---------- ----------
Gel-kalilo      ---------- ---------- ---------- ---------- ----------

                                                                    8540
Kalilo          ATTCTACCCC ACCATGGGGC GATAAAGACG TTTATCTCCC CATTAGGGTG
LA-kalilo       ********** ********** ********** *****T**** **********
Short kalilo    ---------- -***-***** ********** ********** **********
Gel-kalilo      ------**T* ****G***-T ******T*** *A*C****** *******A**

                                                                    8590
Kalilo          TGGTATAAAA ATGAAAAAAT CAAAAAGAGA GAGTACTAGA AATGATAAAA
LA-kalilo       ********** ********** ********** ********** **********
Short kalilo    ****G***** TGA*****G* ******TC** *G******** **********
Gel-kalilo      ********** *******CC* G*****A*** *TA*TAA*A* **G*******

                                                                    8640
Kalilo          AGATCACAAA GGGTTAAAAT AGGAACAAAA GGGGTCAGTG GTGCCCCTTA
LA-kalilo       *********T *********A ********** *****GC*** *****TA***
Short kalilo    *A*AA**T*T ****AC**** *ATT*A**** A**T--CA** *****T----
Gel-kalilo      *AT*A*A*GG CT*A****TA *CA*TA*C** ****GAGTG* TAT***-***

                    8643
Kalilo          CA-C-
LA-kalilo       *****
Short kalilo    --*-*
Gel-kalilo      **A*A
```