PHYLOGENETIC ANALYSIS OF *SCLEROSPORA GRAMINICOLA* USING INTERNAL TRANSCRIBED SPACER REGION-2

A Thesis

by

APARNA VISWANATHAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2003

Major Subject: Plant Pathology

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Approved as to style and content by:

Clint Magill (Chair of Committee)	Heather Wilkinson (Member)
Spencer Johnston (Member)	Dennis C. Gross (Head of Department)

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ABSTRACT

Phylogenetic Analysis of *Sclerospora graminicola* Using
Internal Transcribed Spacer Region -2. (August 2003)
Aparna Viswanathan, B.Sc.; M.Sc., Gujarat Agricultural University
Chair of Advisory Committee: Dr. Clint Magill

The internal transcribed spacer region (ITS) from fourteen samples of *Sclerospora graminicola* was amplified using a nested PCR. These regions of the rDNA were amplified by a combination of conserved primers (ITS 1 and 6 and ITS 3 and 4). These products were cloned into a puc-4 vector and transformed into *E.coli* competent cells. The inserts from transformants were isolated, then sequenced by the Gene Technologies Lab, Texas A& M University. The sequences produced were and aligned with the ITS2 region of other known Oomycete fungi(*Peronospora, Phytophthora* and *Pythium*) using CLUSTALX.

Phylogenetic relationship among sequences from Indian and African samples was examined using a consensus bootstrap parsimony tree. All the samples from India, Mali, Nigeria and Niger, with the exception of Niger 4, formed a monophyletic group with the Oomycetes (*Peronospora, Phytophthora* and *Pythium*). The three samples Niger 4, BFaso 2 and BFaso 17 from Burkina Faso shared a close relationship with the outgroup *Cladosporium herbareum*.

DEDICATION

This thesis is dedicated to my beloved parents V.G. Viswanathan and Uma Viswanathan and my brother Arun who have supported me all the way since the beginning of my studies. They have been a great source of motivation and inspiration. It has been the greatest blessing being your daughter and your sister.

This thesis is also dedicated to my loving in-laws P.S. Lakshmanan and Radha Lakshmanan who have loved me, believed in my capabilities and encouraged me to keep going.

I also dedicate this thesis to my fiancé Sreenivas who has instilled in me the drive and determination to follow my dreams and pursue my goals, loves me unconditionally and is my pillar of strength.

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During this entire journey of my four years at A&M many friends and colleagues have come and gone, leaving a lasting impression. Every passing day when things didn't go the way I wanted , which as researchers we all go through, the task seemed to be getting tougher. There were moments of joy, celebration, sorrow, despair, frustrations and finally today all the effort has taken the form of this thesis.

I would like to express my heartfelt gratitude to Dr.Clint Magill, who never gave up on me even when self-doubt crept into me. No matter what time of day, I always had the freedom to go knock on his door and say, " I would like to discuss something" and was always welcomed with a smile. He always made sure whatever the problem, I could discuss it with him. His enthusiasm, encouragement and interest in my work kept me going.

I thank my committee members, Dr. Heather Wilkinson and Dr. Spencer Johnston, for their cooperation and advice in guiding me through my research and the completion of this thesis. Special thanks to Dr. Wilkinson for her untiring efforts in helping me with my results and guiding me at every step. I would also like to thank them for caring and taking an interest in me as a person, not just as a student.

This journey would never have begun if it were not for the implicit faith that my loving parents have bestowed in my capabilites and encouraged me to pursue my dreams. Through the highs and lows of each day, even with miles existing between us, they were constantly there to support, encourage and love me. My brother, Arun , has been one of my best friends and a constant moral support, listening to my stories and making me laugh and cheered me up when all seemed to be falling apart. Though it has only been a couple of months since I got engaged, my in-laws have been a constant source of love and encouragement to me. Every call and every mail I received from them was filled with warmest wishes and prayers for my success. Thank you so much, it means a lot to me and kept me going.

What would a life be without the love and happiness that friends bring into our lives. We all came from different walks of life and then moved on to become the most fun group I ever had. My room-mate Pals has been a family to me, all those days when she offered me strength, gave me her shoulder to cry on, made me smile and laugh, cooked lovely meals for me and took over my share of household chores so I could give all my attention to my work during the course of my research and thesis writing. A special thanks to my friends: Sandy, Vinu, Sunny, Srini, Hari, Karthik Swami, Nitin, Mamtha, Deepa, Rashmi, Chai, Anitha, Kavi, Reshma, Siddy, Abhi , Ajit and Jaydeep. In the past couple of months I missed their birthdays and didn't return calls, yet they always made sure I was never lonely by constantly sending me cheerful mails and cards. Thank you so much guys and girls, you know I am always at a loss of words when it comes to expressing it all to you.

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Last, but not the least, I want to thank my wonderful fiancé, Sreenivas, for supporting me, loving me and being there for me throughout these trying moments. His constant encouragement and faith in me made even the toughest of tasks look doable. No matter what day or time he was there to give me strength to keep going, patiently let me cry my heart out, listen to all my frustrations and made sure I was ready to face any challenge at any given moment. His soothing words smoothened the roughest of paths and his confidence in me made me grow stronger and helped me accomplish what I could never have achieved alone. Through the mountaintops and craters of this journey he walked by my side. Thank you so much Jaanu, for being there for me always and for loving me so much and for believing in me.

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CHAPTER I
INTRODUCTION

Pearl millet originated in Africa and was subsequently introduced into India. Although the crop is quite hardy, it still suffers from various biotic stresses. One of the major biotic factors that reduces yield is the downy mildew disease caused by *Sclerospora graminicola.* It appears that the pathogen develops a host-specific population(s), and that this population(s) survives as long as the particular host is grown but dies out when the host is withdrawn (10).

Sclerospora graminicola is an obligate biotropic oomycete that reproduces asexually to produce motile zoospores and sexually through the production of soil-borne oospores. It is heterothallic and the existence of variable pathotypes is known. *Sclerospora graminicola* is highly variable; and because of the inherent variability in the pathogen resistance in pearl millet to downy mildew has been short lived (1).

Downy mildew disease has been very serious and widespread throughout the pearl millet growing regions of Asia and Africa. Pearl millet hybrids are more popular in India than anywhere else. With the beginning of hybrid cultivation in India during the late 1960s, downy mildew became more visible on hybrids than on local open-pollinated cultivars. Since that time, there have been several epidemics and a number of new hybrids have been introduced and several subsequently have gone out of production due to increased levels of susceptibility to the disease. During 1971-72, downy mildew appeared in an epidemic form on a highly popular hybrid HB 3 grown in several states. With the introduction of more genetic diversity into the pearl millet hybrids grown in India, vulnerability to downy mildew has been reduced considerably from what it was a few years ago. Resistance breakdown has been less of a problem in Africa where land races are cultivated almost exclusively except for some areas where heterogeneous varieties are also used (Thakur, Personal Communication).

This thesis follows the style of Phytopathology.

Ball (1) reported that, cultivars of pearl millet were challenged by isolates of downy mildew collected from various locations in West Africa and India in order to ascertain whether variability in cultivar response was genetically or environmentally determined.

The most important conclusion was that variation is determined by the host and pathogen genotypes (1). The results suggested that the potential exists for the entire pathogen population to alter dramatically from one growth season to the next. The apparent stability that exists in West African landraces, for example, the Gambia, could be the result of an equilibrium between constantly changing genomes of hosts and pathogen, with the hosts acting as natural multilines. With the pathogen population capable of such rapid change, i.e. change from one season to the next, there would be great risks of resistance breakdown if homogenous stands of hybrid varieties were grown. The most important conclusion was that variation is determined by the host and pathogen genotypes (1).

West African isolates of the pathogen were generally more pathogenic than Indian isolates. However, there were also substantial differences between two isolates collected from different host cultivars at the same location in Upper Volta. One of the primary objectives of the ICRISAT breeding program is to improve and stabilize yields through new improved cultivars. Many local cultivars of pearl millet in West Africa produce yields lower than 616 kg/ha. Resistance to downy mildew is one of the major selection criteria and promising cultivars are initially tested in downy mildew nurseries at ICRISAT, Hyderabad or other locations in India. The cultivars that exhibit sufficiently low levels of downy mildew are incorporated into international multi-location trials. In the multi location trials certain cultivars have shown distinct differences. In particular, F1 hybrid varieties developed in India tended to be highly susceptible in West Africa, particularly in Nigeria. Unless cultivars that are introduced can provide a broad basis of resistance, rapid selection for successful pathotypes may negate the yield and quality advantages promised by the improved cultivars (1).

Host resistance has been the most important strategy for the control of this disease. In order to develop breeding strategies for cultivars with stable and durable host resistance to downy mildew it is important to generate information about the pathogen populations. To, date pearl millet downy mildew can be identified after visual symptoms are noticed. However, if a DNA probe or PCR primers could be developed that are specific for *S. graminicola*, tissues from the leaves or stored seeds could be tested for the presence of the pathogen prior to symptom development. Currently, the simplest procedure is to characterize them at the molecular level. Since there is a lack of knowledge about the relationships existing within *S .graminicola* isolates from India and Africa, this study used molecular systematics to determine a phylogeny for the pathogen.

This investigation had the specific objective of developing a phylogeny of the pathogen *S .graminicola* based on comparisons of DNA sequences for a ribosomal internal transcribed spacer and to use the resulting phylogenetic tree to establish a relationship between the various isolates from India and four African countries.

Internal Transcribed Spacer 2 (ITS2) of the ribosomal RNA encoding DNA has several advantages that make it an ideal region to sequence for phylogenetic analysis. Its rate of evolution is appropriate for studies at the specific and generic levels. It is phyogenetically interpretable, i.e., the sequences are relatively simple to align and large enough to offer potentially enough characters for phylogenetic reconstruction. Given the short length of the ITS and the highly conserved nature of the flanking ribosomal subunit genes, the ITS region is easily amplified from small amounts of genomic DNA by the polymerase chain reaction (2).

The internal transcribed spacer (ITS) regions of rDNA have proven particularly useful for separation of fungal taxa at the species to genus level, because the rate of accumulation of mutations in these regions often approximates to the rate of speciation (3, 7).

Efforts to characterize pathogens using molecular approaches have shown that considerable variation exists. Random amplified polymorphic DNA (RAPD) markers were used by Sivaramakrishnan (14), to characterize six host genotype-specific

pathotypes of *Sclerospora graminicola*. Two oligonucleotide primers A8 and C5 showed polymorphism among the different pathotypes. The polymorphisms were due to the presence or absence of fragments of specific size rather than the total number of fragments. The six pathotypes were classified into five groups based on the banding patterns on agarose gels using the A8 primer and cluster analysis for the genetic similarities. The results were claimed to confirm the existence of distinct host genotype-specific virulence in *Sclerospora graminicola* pathotypes (12).

Genetic variability in six host genotypes-specific-pathotypes of pearl millet downy mildew pathogen *Sclerospora graminicola* was also studied at the molecular level using mini and micro-satellites. The results indicated that the use of simple sequence repeats like $(GATA)_4$ which is highly polymorphic may be useful for the rapid and efficient identification and genetic analysis of the pathogen population (10).

A certain level of caution is necessary in interpreting these results. Given that the pathogen cannot be grown in pure culture, the alternative explanation for both of these studies is that the variability seen resulted from the presence of different contaminating fungi or bacteria in the samples, rather than from within-species variation as claimed by the authors.

Sivaramakrishnan, (Personal Communication) successfully used spore samples of *S.graminicola* isolates from pearl millet seedlings grown in the greenhouse for DNA extraction and characterization using Amplified Fragment Length Polymorphism (AFLP). Among the 12 primer combinations [4EcoRI(2+) and 6MseI(3+)] used in AFLP, 5 gave highly polymorphic patterns. A dendrogram constructed from the similarity index data on the AFLP markers classified the 15 isolates into four major clusters. The same isolates maintained on new hosts for a single or multiple generations showed a number of differences in the AFLP patterns, suggesting relatively rare genotypes within the original spore population become predominant when associated with a different host cultivar. In these studies, relatively pure samples of the fungus could be obtained, so that differences in amplified bands are likely to reflect differences in *Sclerospora graminicola*.

When field isolates are used as in the case for African isolates, the differences in amplified bands are not as likely to reflect differences in *Sclerospora graminicola*. The ITS sequences obtained from field isolates from some of the African samples showed that the DNA samples that were used for PCR amplification were not obtained from pure *Sclerospora graminicola* spores, that is, contamination by other organisms was observed (Shankar, Personal Communication).

CHAPTER II
MATERIALS AND METHODS

Sample collection. Small pieces (2x5 cm, wxl) of MIRACLOTH (Calbiochem, USA) were wetted in distilled water just before the collection of spores. Each cloth piece was placed over the sporulating pearl millet leaf and swiped gently to collect the spores. The Miracloth pieces were transferred to 1.5 ml tubes containing 1-2ml ethanol and left at 4^0C overnight before being shipped to the US. The spore samples, collected from various locations in West Africa (Mali, Burkina Faso, Niger and Nigeria) were provided by Dr. Dale Hess. Spore samples of *Sclerospora graminicola* isolates from greenhouse-grown pearl millet seedlings were used for DNA extraction in India. DNA extracted from these samples was provided by Dr.Sivaramakrishnan.

Spore materials. A total population of fifteen DNA samples was used in this study. The spore samples were collected from various locations in India and Africa. The samples India 44-5, India 44-6 and India 44-15 were collected from greenhouse inoculated plants. The samples Mali 4, Mali 12 were collected from Cinzana Village in Mali while Mali 1 was collected from Sanogola Village in Mail. The samples from Burkina Faso, BFaso1 and BFaso 2 were collected from Farako Ba and BFaso 17 was collected from Bobo Dioulasso. Those from Niger, Niger 4, Niger 8 and Niger12 were collected from Tara. The samples from Nigeria, Nigeria 40, Nigeria 47, Nigeria 93 were collected from Samaru/Zaria.

DNA extraction. The suspended spores were collected in a 1.5 ml tube by centrifugation at 12,000 rpm for 10 min. The spores collected by centrifugation were lyophilized before extraction. Genomic DNA was extracted following the protocol designed by (Nucleon PhytoPure DNA Extraction Kit). The procedure was modified according to the amount of spore suspension obtained. Lyophilized samples were ground in liquid nitrogen 1.5 ml in centrifuge tubes to a free flowing powder. Each sample was lysed with 150ul of Reagent 1, followed by addition of 50ul of Reagent 2. A homogeneous mixture was obtained by inverting the sample tubes several times. The

samples were incubated for 10 minutes at 65^0C in a shaking water-bath and placed on ice for 20 minutes.

The protein was extracted with 500ul of chloroform, followed by addition of 100ul of PhytoPure resin and the samples were shaken for 10 minutes on a tilt shaker. The aqueous phase was obtained by centrifugation at 1300g for 10 minutes and transferred to a micro centrifuge tube. DNA was precipitated with 150ul of cold isopropanol. DNA was pelleted in the microcentrifuge at 4000g for 5 minutes, washed with 70% ethanol and centrifuge again at 4000g for 5 minutes. The DNA pellets were air dried for 10 minutes and resuspended in 50ul of sterile water.

PCR amplification with conserved ITS primers. *S.graminicola* DNA samples from India, Mali, Burkina Faso, Niger and Nigeria were amplified using a nested PCR in which DNA was first amplified using ITS primers 1 and 6 followed by amplification with primers 3 and 4 (Table 1). These primers are based on the highly conserved regions of the 18S, 5.8S and 28S rRNA genes that flank the ITS region of the rDNA in several fungi and were designed to amplify the non-coding regions (ITS) between them (Fig.1). A 10ul PCR reaction mixture contained 1X Ready Mix RED Taq (Sigma-Aldrich, Saint Louis, Missouri), 2.5mM $MgCl_2$ (Sigma), 1.0uM primer ITS 1, 1.0 uM primer ITS 6 and 2.5 ng of template. All the PCR primers used in this project were synthesized at the Gene Technologies Lab, Texas A&M University. The reactions were subjected to PCR amplification using a Techne Progene Thermal Cycler for 28 cycles. The PCR conditions used were as follows: initial denaturing for 3 minutes at 94^0C followed by cycles of 1 minute for denaturing at 94^0C, 1 minute for primer annealing at 50^0C and 2 minutes for primer extension at 72^0C.

The nested PCR was carried out using the same PCR protocol, after substituting ITS primers 3 and 4 for ITS primers 1 and 6. The product from the first PCR reaction was diluted 1:100 and 1 ul of it was substituted for template DNA, in the second PCR reaction. The PCR products were evaluated on a 1% agarose gel. The ITS region was amplified by using the protocol as previously described in a similar study in rose downy mildew pathogen *Peronospora sparsa* (10).

Table 1. PCR primers designed for this study and their sequences

Primer	Primer sequence (5' ----> 3')
ITS 1	TCCGTAGGTGAACCTGCGG
ITS3	GCATCGATGAAGAACGCAGC
ITS 4	TCCTCCGCTTATTGATATGC
ITS 6	CACTTTTCAAAGTGCTTTTCATCTTTC

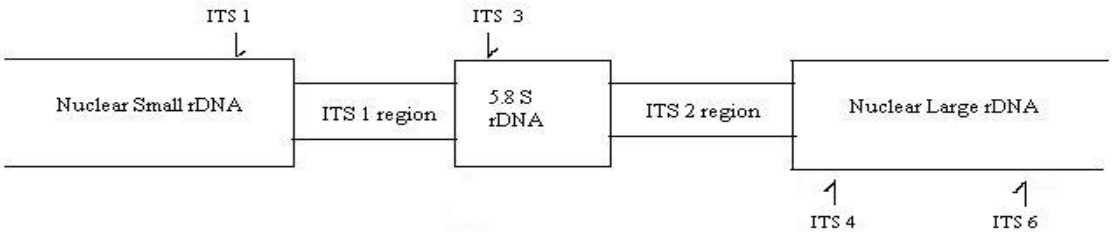

Figure 1. Locations of PCR primers for internal transcribed spacers on the nuclear rDNA map.

Cloning. Individual bands from the ITS 2 products amplified using ITS primers 3 and 4 were excised from a 1% agarose gel and purified using the Qiagen Gel Extraction Kit (Qiagen, Chatsworth, CA) as directed by the manufacturer. Only one DNA band at about 400 bp that was common to all the samples tested, was cut from the gel, purified and cloned into pUC-4 vector and transformed into chemically competent *E.coli* cells using the Topo TA Cloning System (Invitrogen) as directed by the manufacturer. The colonies were randomly selected on Luria-Bertani (LB) agar plates

and grown in tubes of LB medium with 50 mg/ml ampicillin. Plasmid DNA was extracted from 1.5 ml of the bacterial culture using the Qiagen Spin Plasmid Miniprep Kit (Qiagen, Chatsworth, CA) as directed by the manufacturer. The sample BFaso1 from the Burkina Faso, did not yield any colonies hence, were not considered for further analysis.

Sequencing. All the plasmid DNA was sequenced by automated sequencing in the Gene Technologies Laboratory at Texas A&M University. The sequences were compared to other known fungal ITS 2 regions using the BLAST search service provided by Genbank. Comparisons were made: a) including both the 5.8s and beginning of the 28s rDNA regions that are the sites for conserved primers 3 and 4 respectively, or b) the ITS 2 region alone after trimming these more highly conserved regions. The sequences were aligned using CLUSTAL W, PAUP and CLUSTAL X.

Outgroup selection. Since, no ancestral sequence was available an "outgroup" was used as a reference to measure distances. *Cladosporium herbarum* was chosen for the outgroup because it didn't belong to the group of interest.

Phylogenetic analysis. Phylogenies were generated using PAUP* 4.0. Bootstrap analysis was implemented using 1000 replicates of heuristic searches. Phylogenetic analysis of fragment sequences was used to determine the relationship among the isolates of *S.graminicola* . The ITS 2 regions of other known Oomycetes (*Peronospora, Phytophthora* and *Pythium*) were included to construct a bootstrap consensus of a parsimonious tree.

CHAPTER III
RESULTS

DNA amplification using a nested PCR with universal primers (ITS1 and 6 and ITS 3 and 4) resulted in PCR products of various lengths for the isolates tested (Fig.2). India 44-6, India 44-15, BFaso1, BFaso 17, Mali1, Nigeria 40, Nigeria 47 and Nigeria 93 showed a single fragment length of approximately 400bp. India 44-5, BFaso2, Mali 4, Mali 12,Niger 4, Niger 8 and Niger 12 showed two band lengths, one approximately 400 bp and the other between 400-600bp. Since, the 400bp fragment was uniform among all the samples this length of the ITS region was excised for cloning (Fig2.)

Sequences of the fourteen putative samples of *Sclerospora graminicola* were obtained for independent clones of the ITS region amplified with the conserved primers ITS 3 and 4 **(**see Appendix 1; Gene Technologies Lab, Texas A&M University).

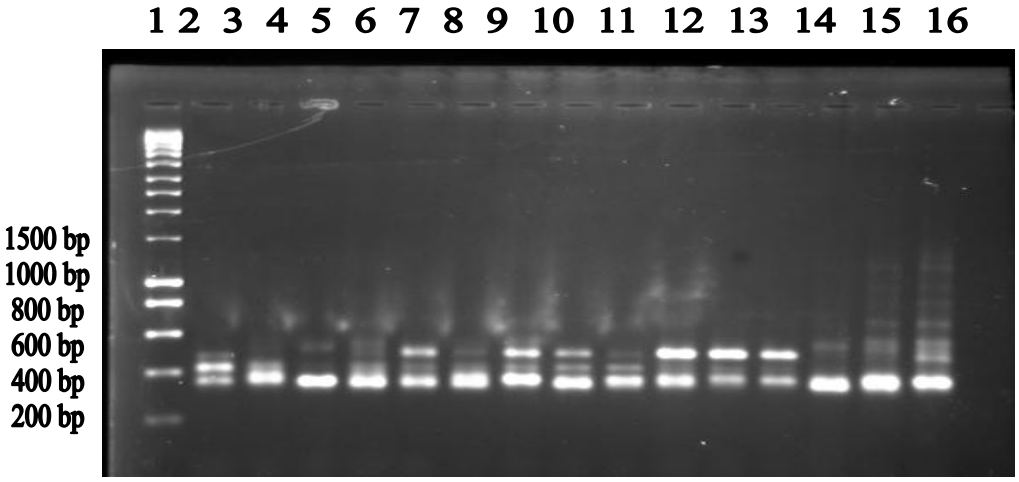

Figure 2. Nested PCR amplification with conserved ITS 1 and ITS 6 primers and ITS 3 and ITS 4 primers respectively. Lane 1(ladder), Lanes 2-4(India 44-5, India 44-6, India 44-15), Lanes 5-7 (BFaso 1, BFaso 2, BFaso 17), Lanes 8-10 (Mali 4, Mali 12, Mali 1), Lanes 11-13(Niger4, Niger 8, Niger 12) and Lanes 14-16 (Nigeria 40, Nigeria 47, Nigeria 93). The 400bp band was cloned and sequenced from all the samples.

The results of BLAST searches with selected sequences are shown in (Table 2), using the entire sequence between the conserved primers ITS 3 and 4 and (Table 3), using only the ITS 2 regions. Only the highest scoring matches are reported in the tables. None of the fourteen sequences derived from nested PCR showed matches to previously reported oomycetes, even if the advanced BLAST version was used to permit listing up to 1000 matches. However, BLAST comparison of another clone derived directly from amplification with ITS 3 and 4 primers does show homology to *Peronospora* and *Phythophthora,* (high score 63-63) based primarily on homology in the 5.8s region of the clone.

Table 2. Results of BLAST searches using selected sequences between primers ITS 3 and ITS 4.

Sample	Blast Hit	Score	E-value
India 44-5	*Sporidiobolus pararoseus* strain	769	0.0
	Sporidiobolus pararoseus strain	753	0.0
Mali 4	*Cryptococcus flavus* strainCBS 3	688	0.0
	Uncultured fungus clone D13 18S	262	3e-67
Nigeria 47	*Cryptococcus laurentii* internal	622	e-176
	Cryptococcus laurentii st	622	e-176
Niger 8	*Pseudozyma parantartica* genes	811	0.0
	Pseudozyma antartica st	753	0.0

Table 3. Results of BLAST searches using selected sequences showing only the ITS 2 region.

Sample	Blast Hit	Score	E-value
India 44-5	*Sporidiobolus pararoseus* strain	690	0.0
	Sporidiobolus pararoseus strain	674	0.0
Mali 4	*Cryptococcus flavus* strainCBS 3	609	e-171
	Uncultured fungus clone D13 18S	222	3e-55
Nigeria 47	*Cryptococcus laurentii* internal	543	e-152
	Cryptococcus laurentii st	543	e-152
Niger 8	*Pseudozyma parantartica* genes	773	0.0
	Pseudozyma antartica st	700	0.0

Phylogenetic analysis of the ITS data set resulted in the bootstrap consensus of a parsimonious tree (Fig 3).

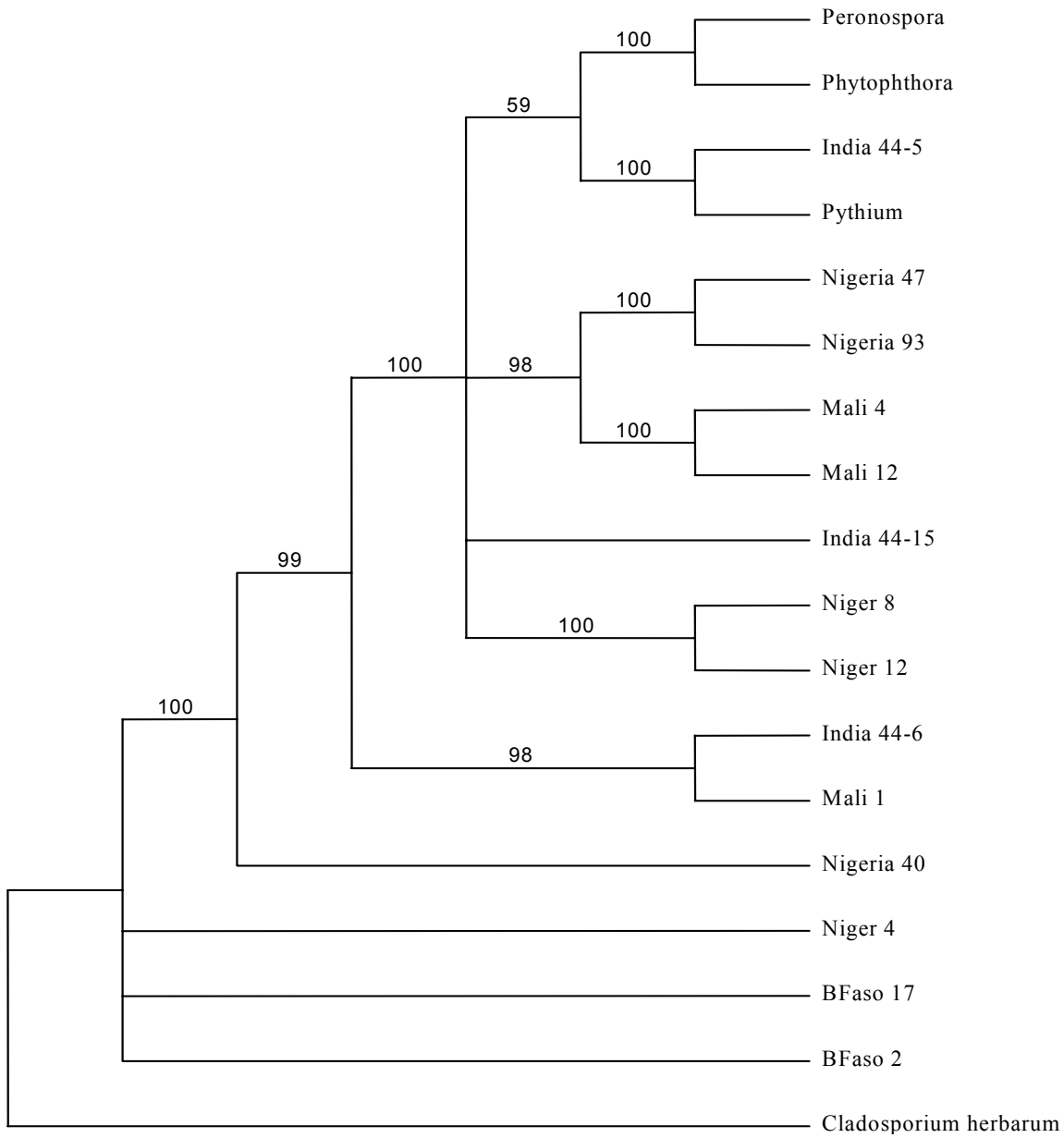

Figure 3. A bootstrap consensus of a parsimonious tree based on sequences of the ITS region of the rDNA. Numbers above represent the bootstrap values. The outgroup used for constructing this tree is *Cladosporium herbarum.*

Our study aimed at amplifying the ITS region from the different samples of DNA expected to be *Sclerospora graminicola* from India and Africa. All the samples from India, Mali, Niger and Nigeria with the exception of Niger 4 fell into a clade with known Oomycetes (*Peronospora, Phytophthora* and *Pythium*; Fig3).

Furthermore, it can also be seen that the isolates of *S.graminicola* from India, Mali, Nigeria and Niger, (except Niger 4) exhibit equal or greater diversity amongst themselves than as is exhibited among the established Oomycete species. Thus, either *Sclerospora graminicola* is quite diverse in these populations or else the DNA isolated from these samples comes from different species.

The outgroup selected for this analysis *Cladosporium herbarum* (Ascomycete) is also known to be a very common saprophyte. Based on previous results we expected the spore samples of at least a few field isolates might show the presence of this saprophyte(Shankar, Personal Communication). This was confirmed by observing (Fig3) that the ITS-2 sequences of samples Niger 4 and those from Burkina Faso, BFaso 2 and BFaso 17 are identical and share a close relation with the ITS 2 of *Cladosporium herbarum.* These samples were therefore considered contaminants of *Cladosporium herbarum* of a related type.

The alignment of the ITS 2 sequences of all the samples from India, Mali, Burkina Faso, Niger, Nigeria, the Oomycetes (*Peronospora, Phytophthora* and *Pythium)* and the outgroup *Cladosporium herbarum* using CLUSTAL W can be observed in Appendix 2.

CHAPTER IV
DISCUSSION

Sclerospora graminicola causes downy mildew, one of the most important diseases affecting Pearl Millet in Africa and India. Despite the economic and ecological importance of the downy mildews, understanding of their evolution and taxonomy is poor and the current approach to species concepts is "inadequate and potentially misleading"(6).A recurrent problem in downy mildew characterization is their biotropic habit that has hampered detailed morphological, physiological, biochemical and molecular studies. In *Peronospora,* which is by far the largest genus of downy mildews, more than 83 species have been listed in the British Isles alone. Classifications such as this have resulted in inconsistent and often inadequate species definitions, and it is widely accepted that a more rigorous analysis of the downy mildew and related genera is required (4).

Genetic diversity in fungal plant pathogens that attack pearl millet has been identified by ICRISAT as a primary constraint preventing stable food production in Africa. In the case of downy mildew of pearl millet, ICRISAT scientists have previously identified numerous sources of genetic resistance in the host. They also found that races of the pathogen (pathotypes) exist that can overcome specific resistance genes. An elite cultivar bred for resistance to downy mildew in one location was highly susceptible if released in other locations. Thakur (13) reported that the pearlmillet genotype 852B, resistant to the ICRISAT Center population of *S.graminicola*, became susceptible to the Mysore population. If such a cultivar, bred for resistance becomes susceptible to pathogen population within a country, then introducing it and similar varieties to another continent would probably provide diverse pathogen population. This led to the question, "Is the population of *S. graminicola* in various countries in Africa where downy mildew occurs similar to the one in Patancheru where improved cultivars have been deployed?" Previous studies have shown that Pearl Millet originated in Africa (10), hence it would

be logical to assume that the greatest diversity of a co-evolving pathogen would also be found in Africa.

The original objective of this study when it was started was to develop simple, rapid and accurate DNA- based tests that would define the population structure of *Sclerospora graminicola.* A number of approaches were taken to achieve this target. The lack of knowledge about the population and diversity in Africa suggested that DNA sequence homology, as detected by RFLP analysis, would provide an optimal starting point.

However, since the fungus cannot be grown in culture and live spores cannot be imported to the US where samples could be expanded by growth under greenhouse conditions, samples were limited to amount of DNA available from a single lesion. This often produced less than 400 ng of DNA, dictating that PCR techniques be employed. (Clint Magill, Personal Communication). As a consequence, the question of diversity had to be approached differently.

In India, spore samples of *S. graminicola* isolates from greenhouse grown pearl millet seedlings were characterized using Amplified Fragment Length Polymorphism (AFLP). A dendrogram constructed from the similarity index data on the AFLP markers classified 15 isolates into four major clusters. The same isolates maintained on new hosts for a single or multiple generations showed a number of differences in the AFLP patterns, suggesting relatively rare genotypes within the original spore population become predominant when associated with a different host cultivar (Sivaramakrishnan, Personal Communication).

The rapid shifts in AFLP patterns from downy mildew isolated in India, further strengthens the belief that when a hybrid developed there is introduced into Africa, races of *S. graminicola* that already exist in local populations are able to infect and reproduce on the introduced cultivar. Such an adaptation might reveal variation in the pathogen population that would have otherwise gone undetected.

Another important question that can often be addressed by an examination of diversity concerns the life cycle of the fungus itself. Does it reproduce sexually or

asexually in nature? If it reproduces sexually then genetic recombination would result in a higher degree of variation than that expected for asexual (clonal) reproduction. *S. graminicola* as, an oomycete fungus is predominantly heterothallic. Therefore, outbreeding and extensive variation within and between isolates from geographical areas can be expected. The Oomycetes are almost certainly diploid and the pathogen population may contain unexpressed variation that could allow it to respond rapidly to steep selection pressure (8).

Hence, this study became one aimed at detecting the presence of any variation in the isolates collected from India and Africa by characterizing them at the DNA level. This was carried out by sequencing the Internal Transcribed Spacer (ITS-2) region of the ribosomal RNA gene tandem repeat (rDNA) so that phylogenetic analysis could be used to compare these sequences and to examine their relationship to other known Oomycetes.

PCR amplification of the ITS region has become a popular choice for phylogenetic analysis of closely related species and populations. This popularity stems from the derivation of conserved primers located in the coding regions flanking the ITS. Both direct sequencing (provided there is no heterogeneity within the multiple copies within a species) and cloning of PCR products can be used for ITS analysis (11).

While outcrossing and sexual recombination are expected to cause an increase in diversity for most regions of the genome, since the multiple copies of rDNA in a species tend to remain identical via concerted evolution, recombination between *S.graminicola* races or pathotypes would likely lead to homogenization of the ITS-2 sequence. In many cases, the ITS sequences can be used to develop species-specific PCR primers that can be used for diagnostic purposes.

In the present study, conserved flanking sequences within the ribosomal genes were used as primers for PCR amplification of the ITS region. In India 44-5, BFaso2, Mali 4, Mali 12, Niger 4, Niger 8 and Niger 12 length polymorphism was revealed by the presence of multiple bands. The presence of such a polymorphism is probably due to length heterogeneity in the ITS region. The above reasoning is based on previous study

involving a group of *Peronosclerospora* species that cause downy mildew of sorghum, maize and sugarcane. In *P. maydis*, length heterogeneity was revealed by the presence of multiple bands following PCR amplification and verified RFLP analysis using rDNA probes. Since, the possibility of length differences in the 5.8s rDNA in fungi is remote, this observation can most certainly be attributed to length variation in the ITS 2 of the rDNA repeat unit of this genus (15).

The observations reported here can be interpreted in two very different scenarios. In the first scenario it is assumed that the sequences amplified do in fact originate from the downy mildew pathogen. Collections made from a lesion can be expected to provide a great preponderance of downy mildew spores and thus of *S. graminicola* DNA compared to any other fungal contaminants that may be present. The primers used in this study have previously been used to amplify ITS-2 sequences from other downy mildews, including *Peronospora sparsa* and several *Peronosclerosporas*. Hence, the bulk of the products formed and cloned should originate from the primary source of DNA.

Clustal alignments followed by bootstrap analysis of the 5.8s and ITS-2 region sequences revealed both expected and unexpected relationships. For the most part, isolates from the same country are quite similar in sequence and in some cases identical. However, while the samples from India, Mali, Niger and Nigeria formed a monophyletic group or clade with other known Oomycetes, ITS sequences from different countries were generally as different from each other as the sequences for different defined species (*Peronospora, Phytophthora* and *Pythium*). Within this clade considerable diversity is observed among both the Indian and African populations. The level of differences seen suggest that the populations are evolving independently, with no exchange of genetic material to maintain a common sequence for ITS-2. India 44-15 falls into a clade with the populations from India and Africa but it is unique. Inspection of the aligned sequences suggests that it has an unusual number of insertions and deletions, a further indication of independent evolution.

The three samples Niger 4, BFaso 2 and BFaso 17 fell into an entirely different clade with *Cladosporium herbarum*, the outgroup selected for this study. Previous studies have

demonstrated that the samples collected from field locations in Africa contained traces of other fungi. This leads one to hypothesize that DNA from fungi other than the target fungus is present in the test sample. In our case DNA from the saprophyte *Cladosporium herbarum* might provide a better match for ITS primer binding, leading to preferential amplification of the contaminant.

The original expectation was that all the sequences from India would group together with greater differences separating the African isolates. This would then have fit the hypothesis that the F-1 hybrids developed in India tend to be susceptible in Africa because the pathogen population in Africa is very diverse compared to the one existing in India. But, the consensus tree shows otherwise (Fig 3). One can see that the populations from India, which were relatively the most pure samples, were scattered at different positions throughout the tree. This leads to the conclusion that there was as much variation within the samples from India as was detected in the samples from Africa. Most sequences from specific countries of Africa cluster more together, that is at least two out of three samples that were amplified and sequenced were highly homologous. While many more samples would have to be sequenced to estimate the full range of diversity, it is clear that considerable diversity exists in both the Indian and African populations. From the limited data available, it is possible that frequent exchange in pearl millet germplasm between the continents has been accompanied by exchange of *S.graminicola.* In addition, the breeding for single gene resistance in pearl millet that has occurred in India may have hastened the evolution of races and lowered the frequency of genetic exchange in the pathogen, giving rise to the different ITS sequences detected. Perhaps the best option for breeders under this scenario is to select for quantitatively expressed forms of resistance (tolerance) in multiple locations with the expectation that different quantitative loci (QTLs) will be identified that can be combined using marker assisted selection to provide a more durable form of resistance.

What if the population does not belong to *S.graminicola* and is a mixed population? Where does that lead us? This gives rise to the alternative scenario. This scenario stems from the very high expectation values obtained when specific sequences are submitted

for BLAST analysis (Tables 2 and 3). BLAST scores reflect the "best" matches between sequences based on the longest stretches of near identity, so even though they are not a direct method for phylogenetic analysis, they do tend to identify close relatives.

Based on the BLAST results one can also hypothesize that though most of the samples, fell into a clade with known Oomycete fungi, the phylogenetic tree may actually be comparing species that are the most common saprophytes or contaminants in the area tested. The ITS primers 3 and 4 were originally made when sequences from only a few fungi were available, including *Neurospora* and yeast (16). When these primers or reverse primer 6 which was made using information from numerous fungal 28s rDNA sequences are subjected to BLAST searches using the "search for nearly exact matches" feature, over 1,000 exact matches are found, primarily from ascomycetes and basidiomycetes, but no oomycetes appear in the list. Primer 1 does show several *Phytophthora* sequences among the exact matches. Thus, even though these primers have been used to amplify the ITS regions of several oomycetes, including *Peronospora* and *Peronosclerospora,* they could preferentially amplify the comparable regions of any contaminating fungi.

Since both the outer and inner primers are homologous to many other fungal ITS sequences, it is possible that each of the fourteen products that were cloned and sequenced are not derived from *S. graminicola*, especially when considered with the observation that several non-nested amplifications resulted in clones with significant homology to known Oomycetes. It was also observed that the sequences of two clones picked from a single sample were very diverse in nature and some of them showed homology to the defined Oomycete species. Such a clonal diversity is indicative of more than one population of fungal DNA being present in a given sample. Based, on these observations, the alternative scenario can be equally justified. However, it should also be pointed out that when sequence homology with other oomycetes was seen, it was practically limited to the 5.8s region, which is highly conserved among all fungi. Thus it is not possible to conclude with certainty that contamination with other fungi is a better

explanation for the results obtained than significant variation in the ITS-2 region of the pearl millet downy mildew pathogen.

Some of the future directions that can be taken to further study the relationship between the samples from India and Africa are as follows. More primer combinations and larger sample size are necessary to draw a final conclusion on the genetic relatedness of India and African populations of *S. graminicola*. The data presented here also suggests that monophyly does not follow geographic divisions and further reiterates the existing diversity within a region. The ITS-2 sequences published with this study can be used to develop further primers that are more specific to the donor organism. Based on earlier studies, specific primers have been developed for other oomycete fungi. Once a larger sample is studied, more clones can be identified that may show homology to other Oomycetes. Those sequences can be used to derive further specific primers or those clones can be used to make probes to detect species using RFLPs and other samples could be hybridized to them. This would facilitate in identifying and screening the most pure samples. In our present study we have targeted only one region that is the ITS-2 region. It would probably facilitate looking for other genes which are more conserved than the ITS. One of the attractions of nuclear rDNA genes and spacers is that they occur in high copy number, in tandem, and the uniformity of these copies is generally maintained through concerted evolution. Glass (5) has identified other genes (e.g., beta-tubulin, histones and plasma membrane ATPase) in filamentous ascomycetes that may provide information at the species level due to variation in their introns or the third base of the codons, but to our knowledge there is no similar information available in literature regarding Oomycete fungi.

The most significant outcomes of this research will be the necessity of developing a collection or amplification method that is specific for the target pathogen and to raise cautions concerning results from any studies where contamination can affect results. This is especially true where PCR amplification is used. Comparisons made with RAPD primers or AFLPs where all fragments are tailed with common primer sites could be extremely misleading unless DNA from a single source is present.

To our knowledge, this is the first time a phylogenetic approach has been taken to compare the Indian and African populations of the study of *S. graminicola.*

CHAPTER V
SUMMARY

DNA was obtained from fifteen isolates, India 44-5, India 44-6, India 44-15(India), Mali 4, Mali 12, Mali 1 (Mali), BFaso1, BFaso 2, BFaso 17 (Burkina Faso), Niger 4, Niger 8, Niger 12(Niger) and Nigeria 40, Nigeria 47, Nigeria 93 (Nigeria). A nested PCR protocol was designed for the amplification of the ITS region. The universal paired conserved primers were used to amplify each of these isolates. A 400 bp fragment common to all the amplifications was excised and purified. This product was cloned into a plasmid vector and tansformed into *E.coli* to obtain sufficient quantities for sequencing. The sequences obtained were compared against ITS regions of other known Oomycetes using CLUSTAL X.

Phylogenetic analysis of the ITS data set resulted in the bootstrap consensus of a parsimonious tree. The ITS region of all the samples with the exception of three from Niger 4, Burkina Faso (BFaso 2, BFaso 17) clustered together in one clade with the ITS 2 of known Oomycetes (*Peronospora*, *Phytophthora* and *Pythium*). This monophyletic group showed almost equal or greater diversity among themselves than there exists between the known Oomycetes. The outgroup selected for this study was *Cladosporium herbarum* and the ITS 2 region showed a close relation to Niger 4, BFaso 2 and BFaso 17 which then judged contaminants of this or a related form.

Two scenarios were considered as possible explanations for the high degree of differences in the ITS-2 sequences that were obtained. The first is that there are multiple, independently evolving populations of *S. graminicola* which readily explains the problem in developing stable resistance in pearl millet. The second explanation is that the primers used preferentially amplified the ITS-2 region of contaminating fungi, and the contaminants differed among samples. Additional research will be required to determine if either or both scenarios may be correct.

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APPENDIX 1

INDIA 44-5

←ITS 3 primer—>

GCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
TGAACGCATCTTGCGCTCTCTGGTATTCCGGAGAGCATGTCTGTTTGAGTGTCATGAATTCTTCAACCCAA
TCTTTTCTTGTAATCGATTGGTGTTTGGATTCTGAGCGTTGCTGGCGTTTGCCTAGCTCGTTCGTAATACA
TTAGCATCCCTAATACAAGTTTGGATTGACTTGGCGTAATAGACTATTCGCTAAGGATTCGGTGGAGACAT
CGAGCCAACTTCATTAAGGAAGCTCCTAATTTAAAAGTCTACCTTTTGATTAGATCTCAAATCAGGCAGGA
TTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

←——ITS 4 primer———>

INDIA 44-6

←ITS 3 primer—>

GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAGTCATCGAATCTT
TGAACGCACATTGCGCCCGCCAGTATCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAGGCCCC
CAGTGCCTGGCGTTGGAGATCGGCCGTGCGGCACGCAAGCCCTCCGGGGCCTGCGGCGCCGGGCCGGCTCC
GAAATCTAGTGGCGGTCTCGCTGTAGTCCTCCTCTGCGTAGTAGCACAACCTCGCAGTTGGAACGCGGCGG
TGGCCTTGCCGTTAAACATCCCACTTCTGAAAGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAA
GCATATCAATAAGCGGAGGA

←——ITS 4 primer———>

INDIA 44-15

←ITS 3 primer—>

GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
TGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTT
TGCTTGGTGTTGGGCGTCTTGTCTCCAGTTCGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACT
GGTTTCGGAGCGCAGCACAAGTCGCGCTCTCTTCCAGCCAAGGTCAGCACCCACGAAGCCTTTTTTCAACT
TTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

←——ITS 4 primer———>

BURKINA FASO 2

←ITS 3 primer—>

GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
TGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCT
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TTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGGCTACGCCGTAAAACAAACCCATTTCTAAGGTTGACC
TCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

←——ITS 4 primer———>

BURKINA FASO 17

←ITS 3 primer—>

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TTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGGCTACGCCGTAAAACAAACCCATTTCTAAGGTTGACC
TCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

←——ITS 4 primer———>

MALI 4

←—ITS 3 primer —>

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CAGAAGCCCCGACCTAGCGTAATAAGTTTCGCTGGAGAGGGTGTGGATGACTGCTTACAATCGCCCTTGGG
CAATCTTTTGACTCTGGCCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

←—ITS 4 primer———>

MALI 12

←—ITS 3 primer —>

GCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
TGAACGCACCTTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTTGAGTGTCATGTAGACTCAACCTCCC
CGGTTTATGATCGGGACGGGTTGGATGTGGGCGCTGCCGATTGCCGGCTCGCCTTAAATGTCTTAGCGGGC
TCAGAAGCCCCGACCTAGCGTAATAAGTTTCGCTGGCGAGGGTGCGGATGACCGCTCACAACTGCCCTCGG
GCACCTTTTGACTCTGGCCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

←—ITS 4 primer———>

MALI 1

←—ITS 3 primer —>

GCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
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GTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAAAACCCCAACTTCTGAATATTGA
CCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

←—ITS 4 primer———>

NIGER 4

←—ITS 3 primer —>

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TTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGGCTACGCCGTAAAACAAACCCATTTCTAAGGTTGACC
TCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

←—ITS 4 primer———>

NIGER 8

←—ITS 3 primer —>

GCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATTGCAGAAGTGAATCATCGAATCTTTGAA
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CGGCCTCAGATTGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

←—ITS 4 primer———>

NIGER 12

←—ITS 3 primer —>

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←—ITS 4 primer———>

NIGERIA 40

←—ITS 3 primer —>

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GGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

←—ITS 4 primer———>

NIGERIA 47

←—ITS 3 primer —>

GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
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CCTGCCATCGCGCACTTTTAGACTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAA
GCATATCAATAAGCGGAGGA

←—ITS 4 primer———>

NIGERIA 93

←—ITS 3 primer —>

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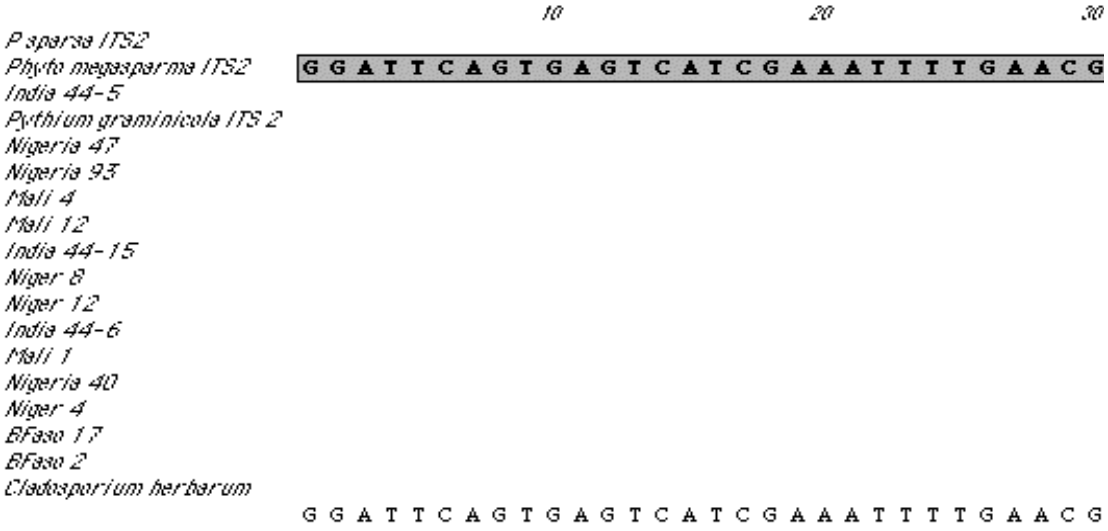
←—ITS 4 primer———>

APPENDIX 2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20
30
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Phyto megasperma ITS2
India 44-5
Pythium graminicola ITS 2
Nigeria 47
Nigeria 93
Mali 4
Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
G G A T T C A G T G A G T C A T C G A A A T T T T G A A C G
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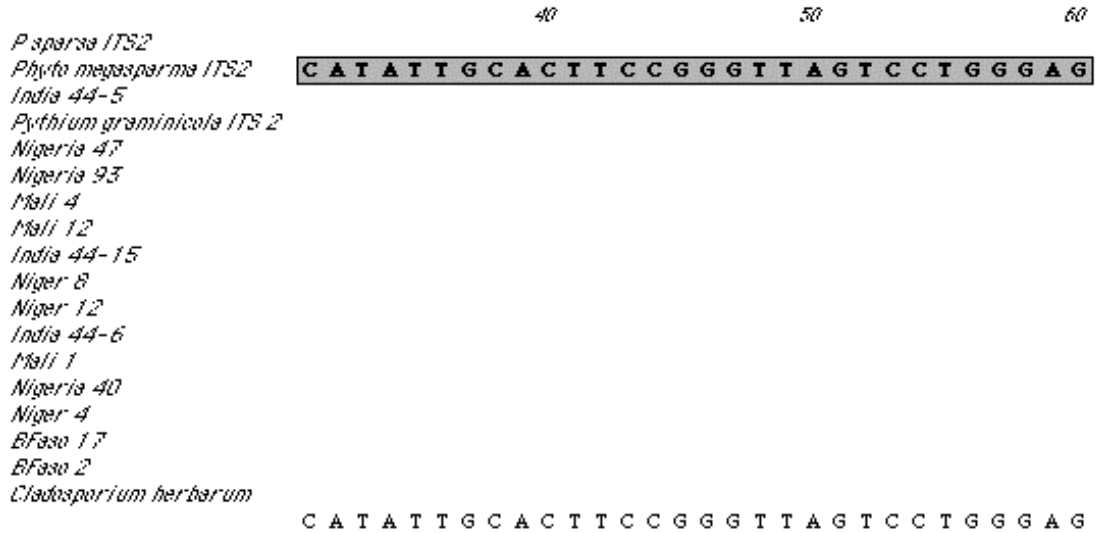
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50
60
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Phyto megasperma ITS2
India 44-5
Pythium graminicola ITS 2
Nigeria 47
Nigeria 93
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Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
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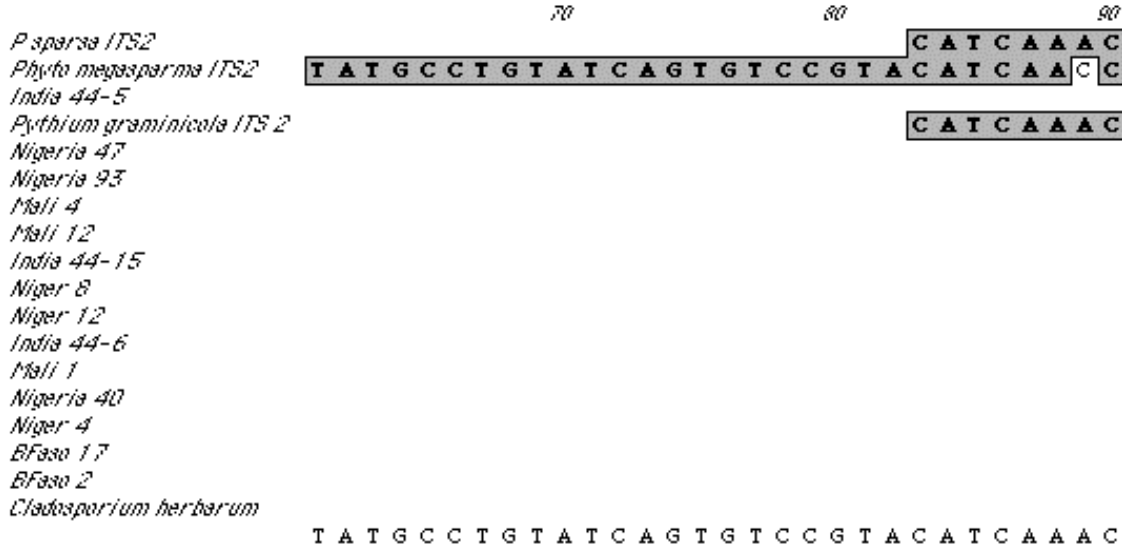
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80
90
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India 44-5
Pythium graminicola ITS 2
Nigeria 47
Nigeria 93
Mali 4
Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
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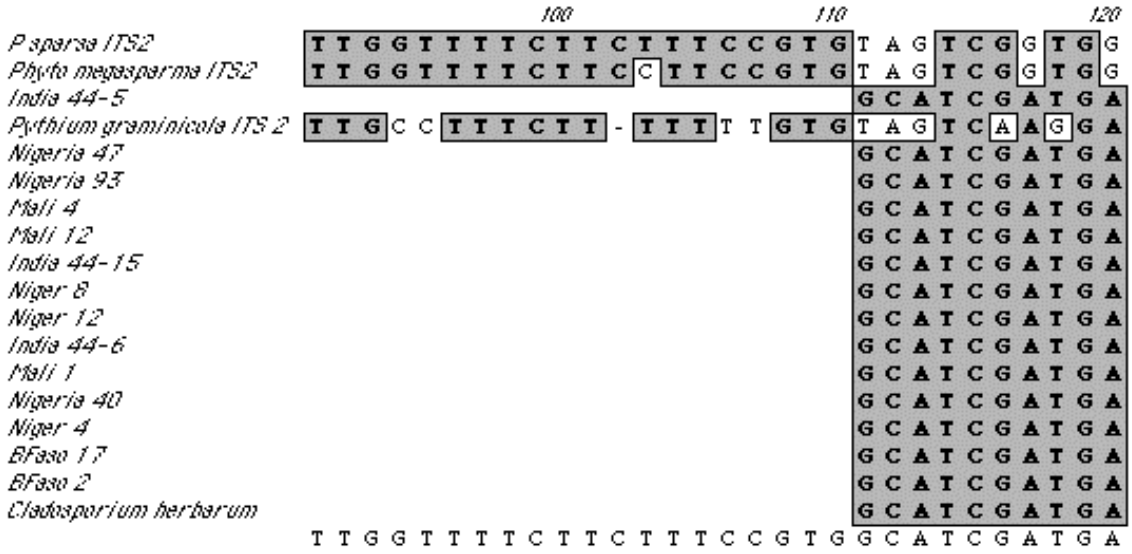
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110
120
P sparsa ITS2
Phyto megasperma ITS2
India 44-5
Pythium graminicola ITS 2
Nigeria 47
Nigeria 93
Mali 4
Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
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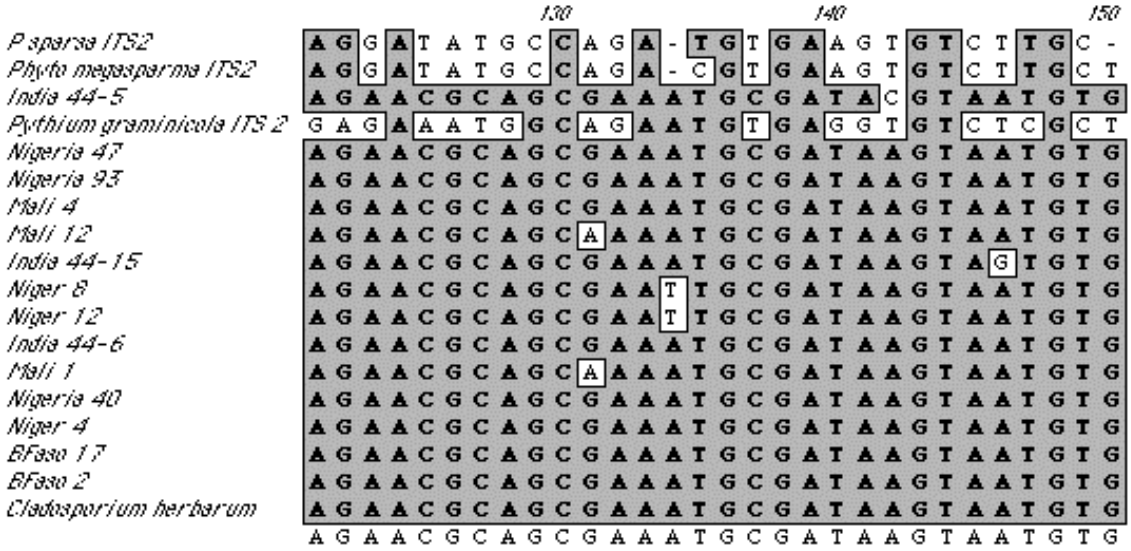
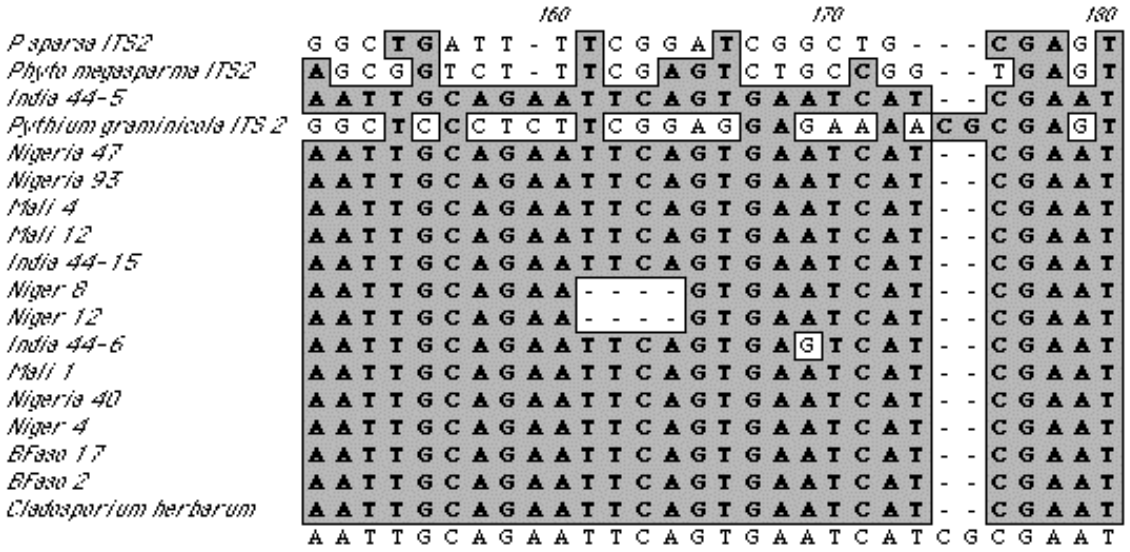

130
140
150
P sparsa ITS2
Phyto megasperma ITS2
India 44-5
Pythium graminicola ITS 2
Nigeria 47
Nigeria 93
Mali 4
Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
A G A A C G C A G C G A A A T G C G A T A A G T A A T G T G


160
170
180
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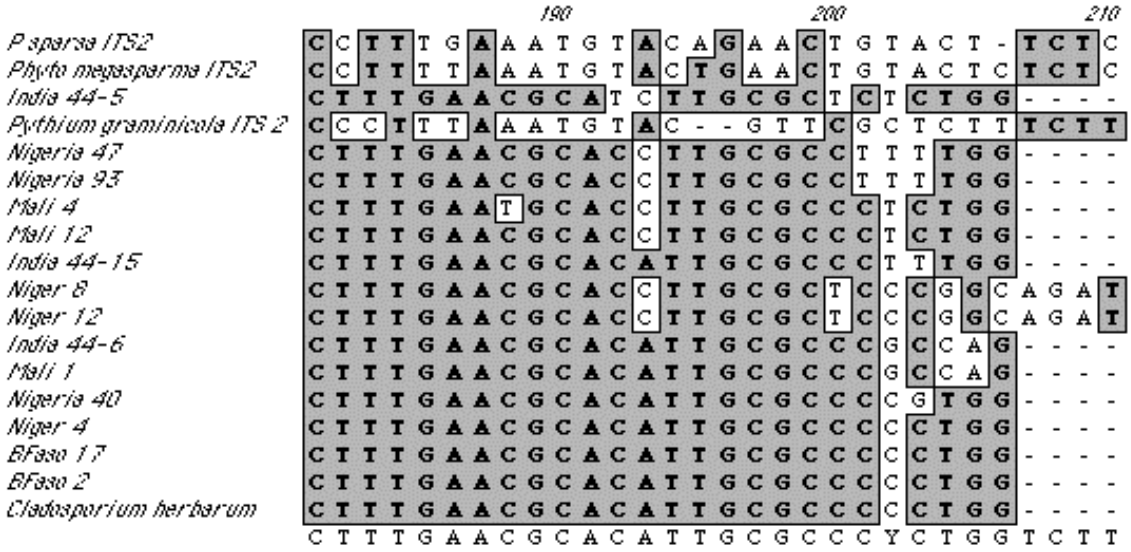
Phyto megasperma ITS2
India 44-5
Pythium graminicola ITS 2
Nigeria 47
Nigeria 93
Mali 4
Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
A A T T G C A G A A T T C A G T G A A T C A T C G C G A A T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India 44-5
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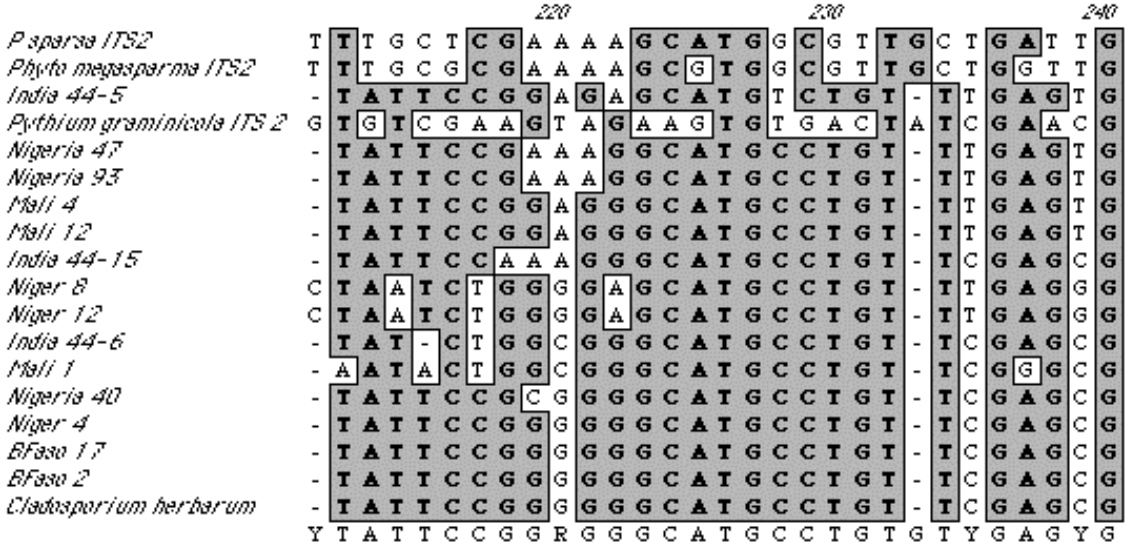
Nigeria 93
Mali 4
Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
C T T T G A A C G C A C A T T G C G C C C Y C T G G T C T T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India 44-5
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Mali 4
Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
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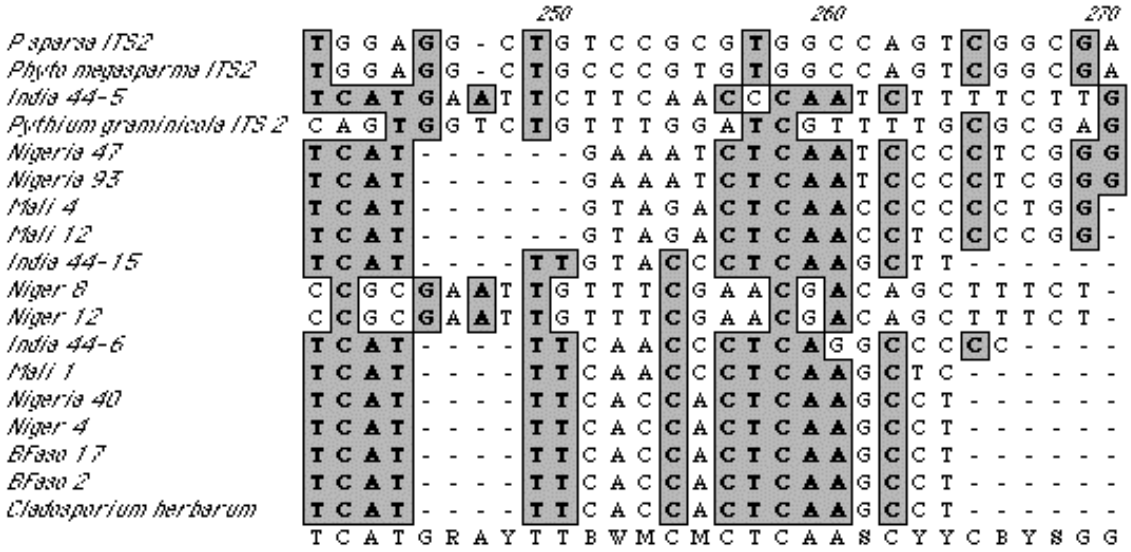
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India 44-5
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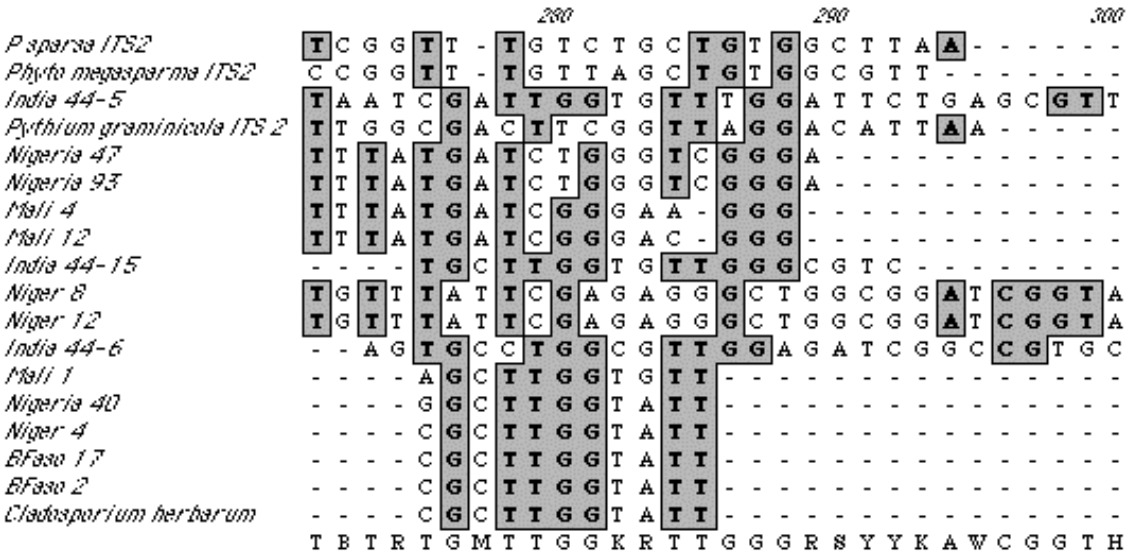
Mali 4
Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
T C A T G R A Y T T B W M C M C T C A A S C Y Y C B Y S G G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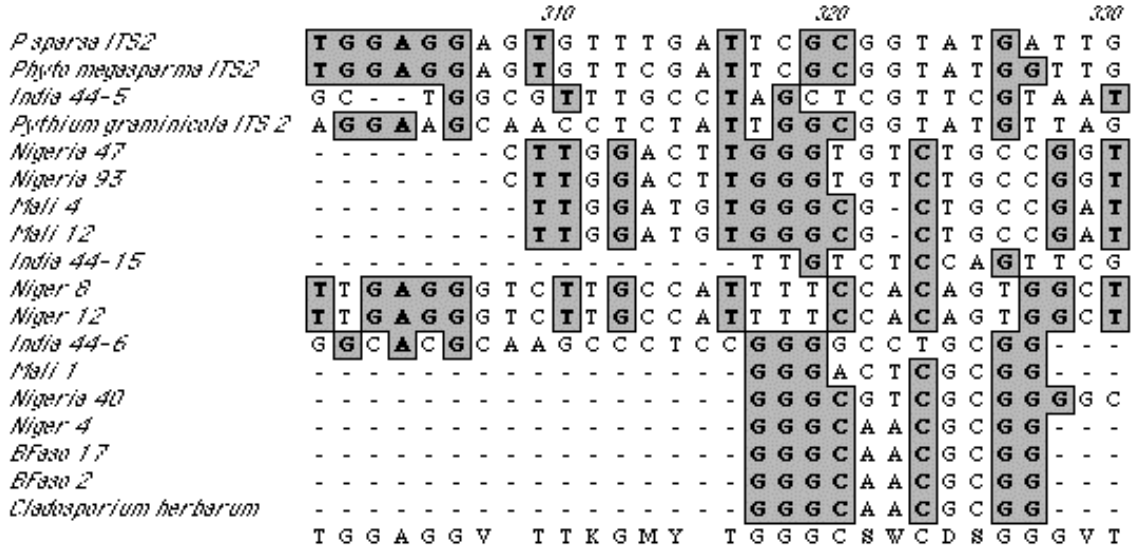
Mali 4
Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
T B T R T G M T T G G K R T T G G G R S Y Y K A W C G G T H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Mali 12
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India 44-6
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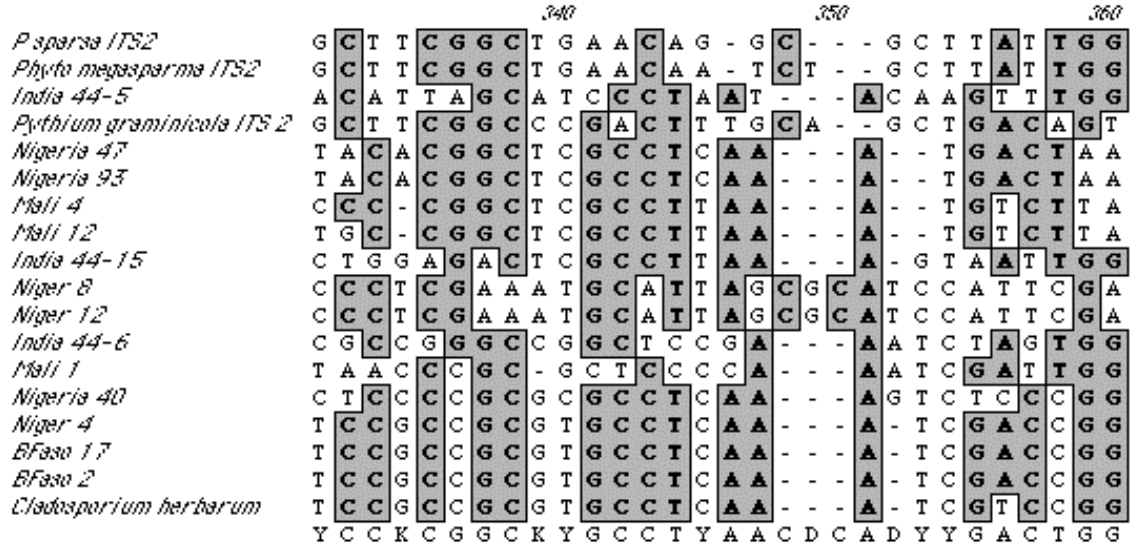
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum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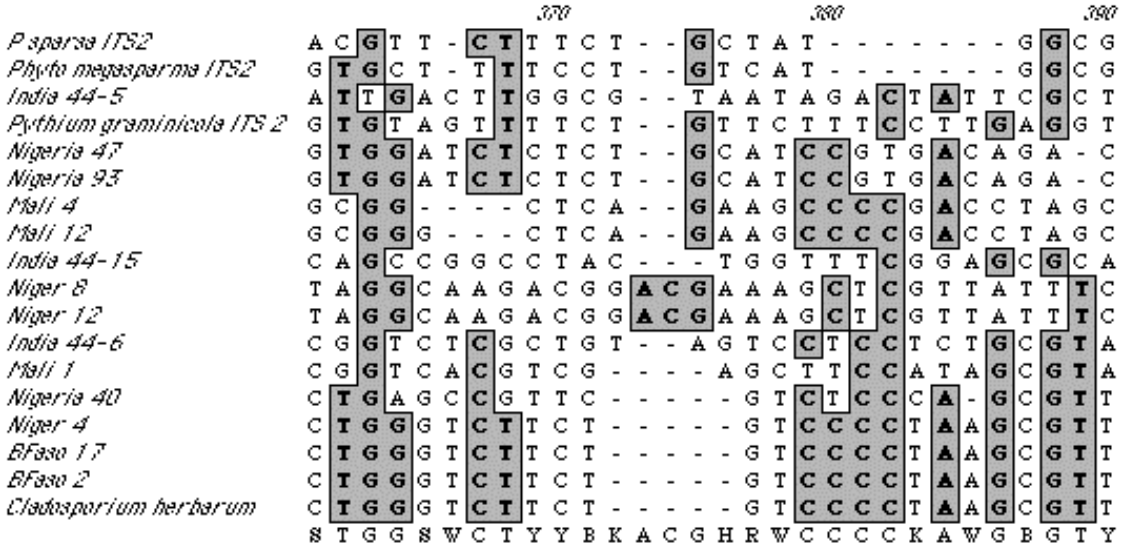
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum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Mali 4
Mali 12
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Mali 1
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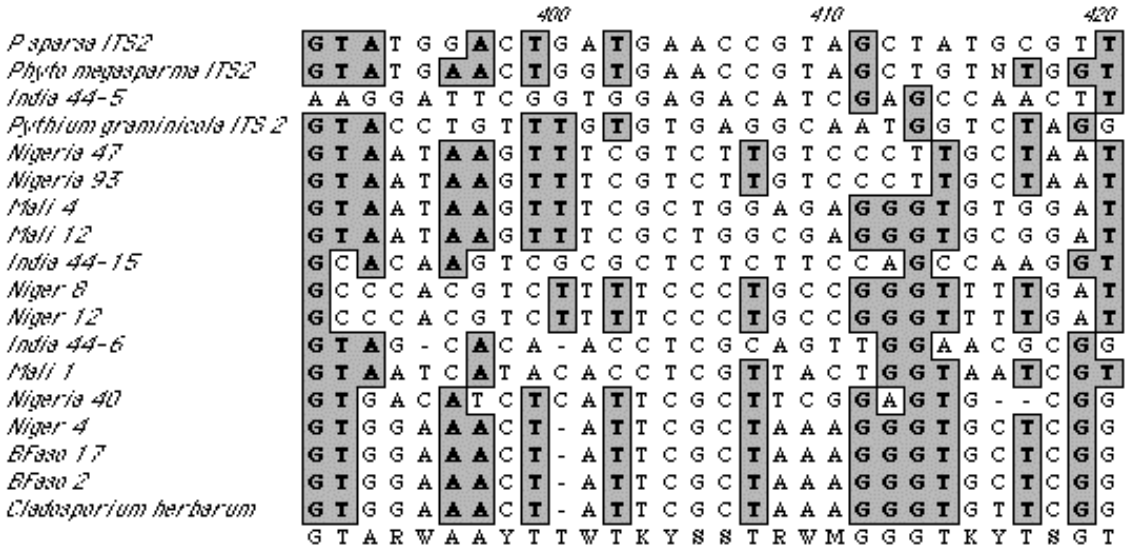
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum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India 44-5
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Nigeria 93
Mali 4
Mali 12
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Niger 8
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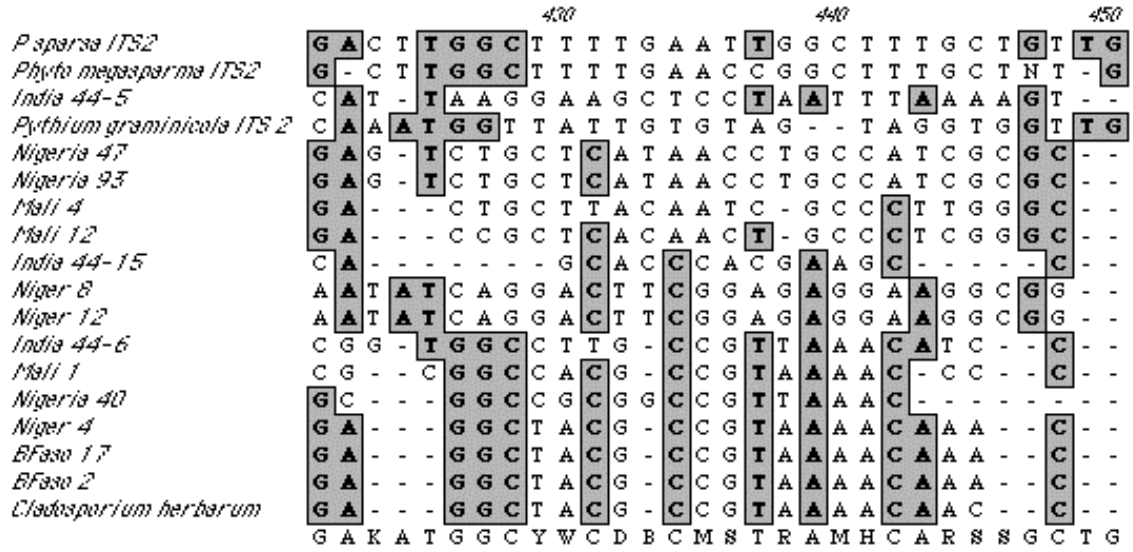
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum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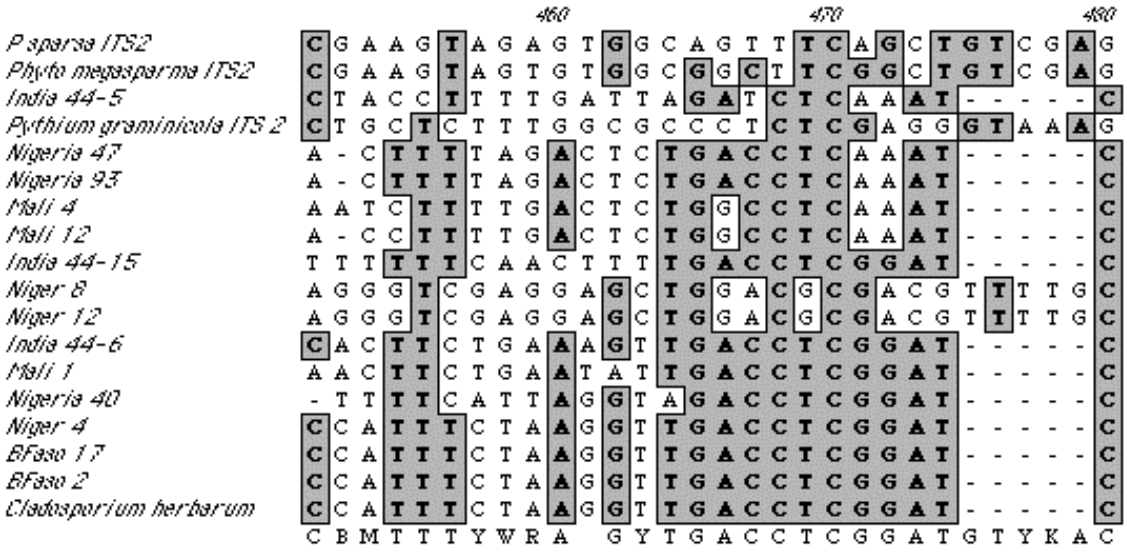
Mali 4
Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
G A K A T G G C Y W C D B C M S T R A M H C A R S S G C T G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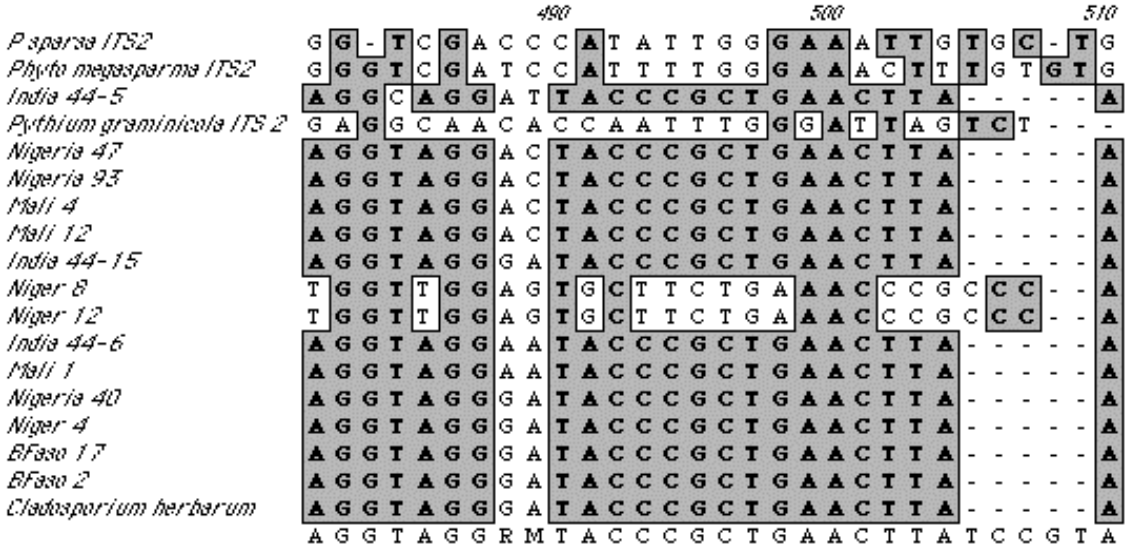
Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
C B M T T T Y W R A G Y T G A C C T C G G A T G T Y K A C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India 44-5
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Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
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BFaso 17
BFaso 2
Cladosporium herbarum
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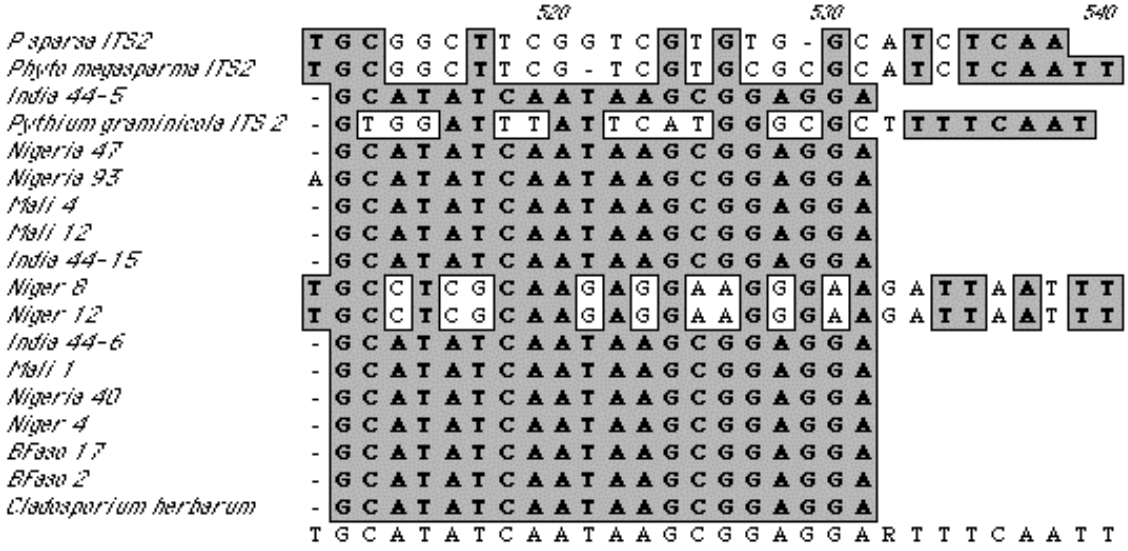
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India 44-15
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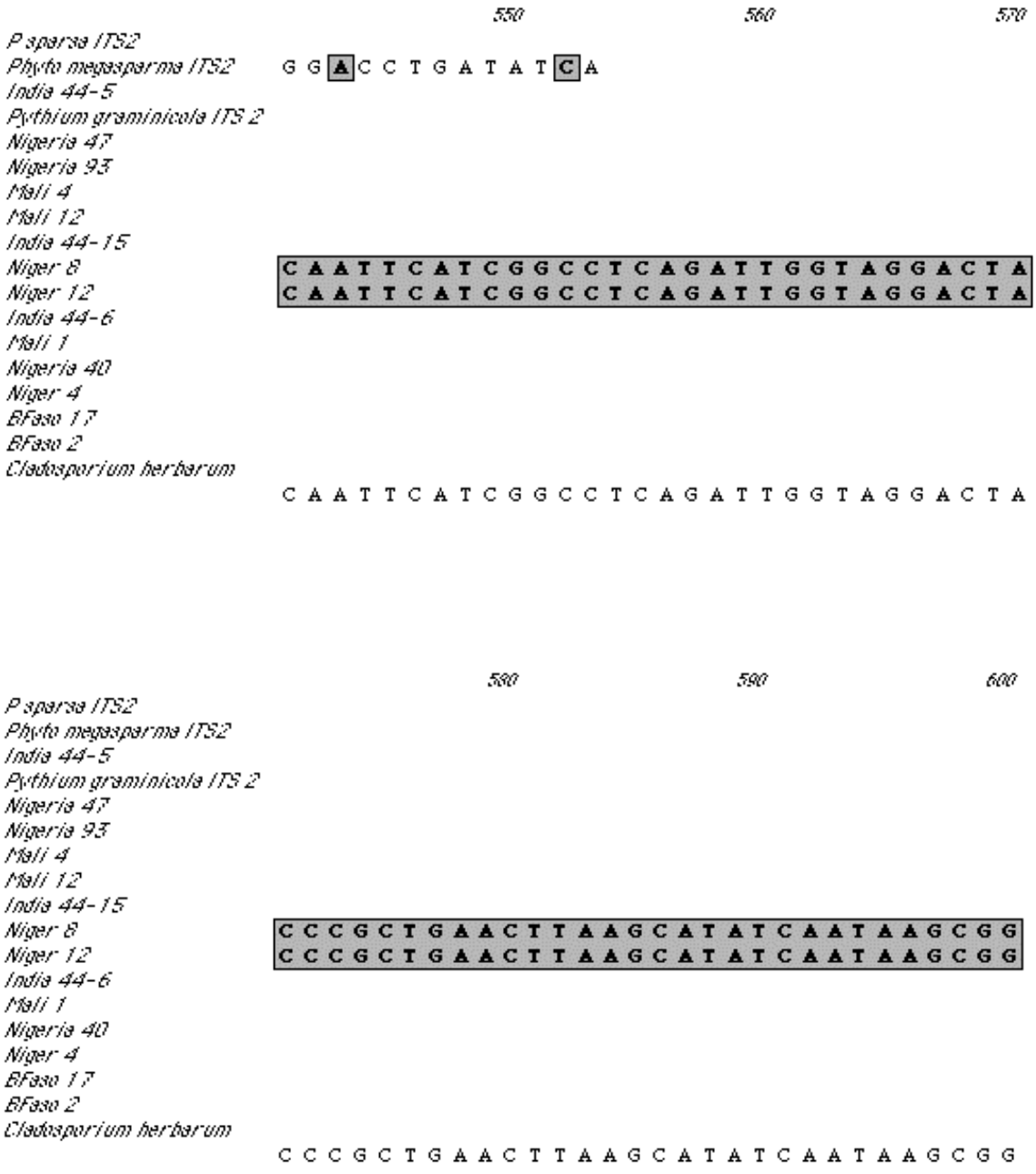
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
T G C A T A T C A A T A A G C G G A G G A R T T T C A A T T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Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
G G A C C T G A T A T C A
C A A T T C A T C G G C C T C A G A T T G G T A G G A C T A
C A A T T C A T C G G C C T C A G A T T G G T A G G A C T A
C A A T T C A T C G G C C T C A G A T T G G T A G G A C T A
580
590
600
C C C G C T G A A C T T A A G C A T A T C A A T A A G C G G
C C C G C T G A A C T T A A G C A T A T C A A T A A G C G G
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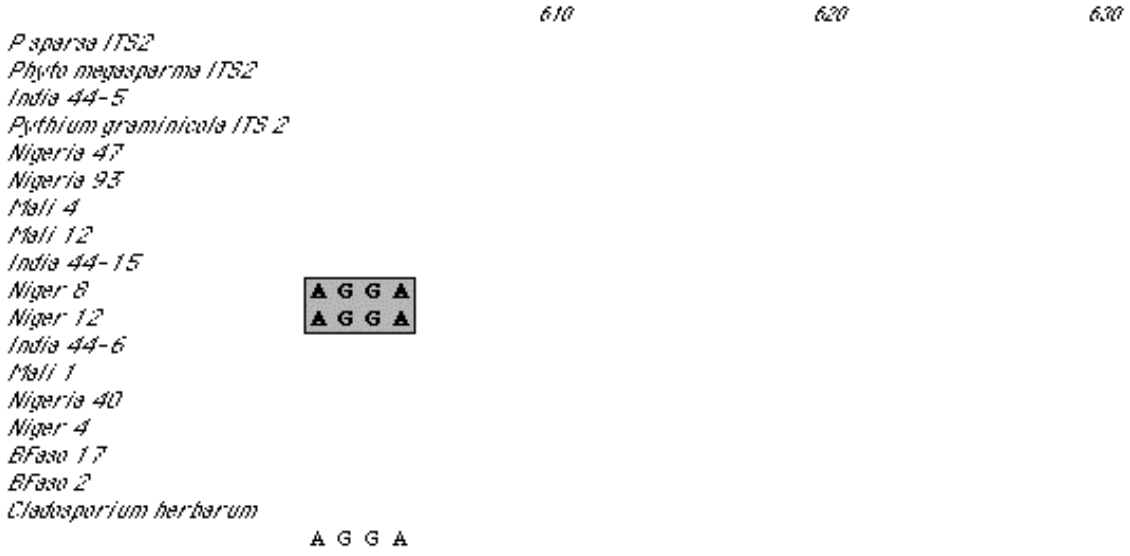
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620
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Mali 4
Mali 12
India 44-15
Niger 8
Niger 12
India 44-6
Mali 1
Nigeria 40
Niger 4
BFaso 17
BFaso 2
Cladosporium herbarum
A G G A
A G G A
A G G A

VITA

Aparna Viswanathan was born November 22,1973 in Varanasi, India. After graduating from Father Agnel School in 1991, she attended Gujarat Agricultural University in Anand, India and graduated with a BSc. in agriculture in 1991 and MSc. in plant breeding and genetics in 1998. After graduation, she began work on her master's degree in the Department of Plant Pathology and Microbiology at Texas A&M University under Dr. Clint Magill, supported first by Research Assistantship from a USAID funded project and later by teaching assistantships from the Department of Biology. Aparna enjoyed her time in research and teaching undgraduate labs in botany. She holds a position as a Research Technologist in Massachusetts General Hospital in Boston. She maybe reached through Texas A&M University, Department of Plant Pathology and Microbiology, College Station, TX 77843-2132.