Molecular characterization of foreign and domestic isolates of Rhizoctonia solani for the biological control of leafy spurge Euphorbia esula
by Deanna Lynne Nash

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University


Abstract:
Fungal isolates belonging to the genus Rhizoctonia are often very diverse and difficult to characterize. The most recognized method for identification and characterization is anastomosis testing, and more recently molecular characterization using regions of ribosomal RNA genes. The present study sought to determine the phylogenetic relationship among foreign and domestic Rhizoctonia isolates being screened as possible biocontrol agents of Euphorbia esula. Restriction enzyme analysis (REA) of a 28s rDNA region, and sequence data of internal transcribed spacer (ITS) regions were used to infer phylogeny. The results obtained from REA and from the sequence data of ITS regions are similar, and the results of both methods indicate that several foreign isolates are very similar to domestic isolates.

# MOLECULAR CHARACTERIZATION OF FOREIGN AND DOMESTIC ISOLATES OF *RHIZOCTONIA SOLANI* FOR THE BIOLOGICAL CONTROL OF LEAFY SPURGE *EUPHORBIA ESULA*

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A thesis submitted in partial fulfillment
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APPROVAL

Of a thesis submitted by

Deanna Lynne Nash

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the college of graduate studies.

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Signature Deanna Lynn Cash

Date Jan 12, 1998

*I dedicate this work to my family,*
*for their patience, because some things...*
*arrive in their own mysterious hour,*
*on their own mysterious terms.*

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## ABSTRACT

Fungal isolates belonging to the genus *Rhizoctonia* are often very diverse and difficult to characterize. The most recognized method for identification and characterization is anastomosis testing, and more recently molecular characterization using regions of ribosomal RNA genes. The present study sought to determine the phylogenetic relationship among foreign and domestic *Rhizoctonia* isolates being screened as possible biocontrol agents of *Euphorbia esula*. Restriction enzyme analysis (REA) of a 28s rDNA region, and sequence data of internal transcribed spacer (ITS) regions were used to infer phylogeny. The results obtained from REA and from the sequence data of ITS regions are similar, and the results of both methods indicate that several foreign isolates are very similar to domestic isolates.

# CHAPTER 1

## MOLECULAR CHARACTERIZATION OF *RHIZOCTONIA SOLANI* FOR THE BIOLOGICAL CONTROL OF LEAFY SPURGE *EUPHORBIA ESULA*: INTRODUCTION

Economic losses based on the infestation of the common weed *Euphorbia esula (E. esula)* alone are estimated at more than $100 million in the United States (5). Due to the fact that conventional methods for controlling *E. esula* (leafy spurge) are extremely costly and that it is difficult to achieve long term control, we are looking at alternative solutions such as biological control. The biological control agent that we are particularly interested in is the soil-borne fungus *Rhizoctonia solani (R. solani)* due to its ability to decrease stands of *E. esula* (8). In order to consider the release of this agent as a biological control agent, it must be better characterized using molecular tools.

### The Plant *Euphorbia esula*

The perennial plant leafy spurge, *E. esula*, belonging to the family *Euphorbiacea*, is a plant species native to Eurasia. The introduction of *E. esula* to the United States from Eurasia, occurred in approximately 1827 through the importation of impure seed (37). Leafy spurge is present on all continents with the exception of Australia (23), but only in North America, in a region encompassing southern Canada and the north central United States, is the presence of *E. esula* considered a serious problem. In these areas alone, nearly 2.5

million acres are infested by *E. esula* (23,37). The infestation chokes out native plant and grass species, thereby depleting valuable grazing areas that wildlife and livestock depend on for survival. *E. esula* is considered to be an irritant foodstuff for animals due to a white milky latex that is present in all parts of the plant. The latex, which seals any wounds that the plant may endure, can cause blisters and dermatitis in both man and cattle, as well as serve as a mouth and digestive tract irritant leading to scours and death in cattle (23; 28). Economic losses due to the infestation of *E. esula* are estimated at more than $100 million in the affected states (5).

The flowers of this plant are small, yellowish-green and arranged in bountiful small clusters. The plant itself may reach three feet in height and reproduce by either root stalks or seed. The seeds of *E. esula* are oblong shaped, grayish to purple in color and are contained in a tri-compartmental capsule that explodes when dry, projecting seeds as far away as fifteen feet from the plant. Once established, *E. esula* is very difficult to control due to it's extensive root system, with root depths exceeding fourteen feet, and extremely hardy seeds, able to germinate after lying dormant in soils for up to eight years (23,28,37).

## Conventional Control of *Euphorbia esula*

It is extremely difficult to achieve long-term control of *E. esula*. The most appropriate control of *E. esula* is assessed on a case by case basis. The options include chemicals, cultivation, sheep grazing, insects and pathogens, or a combination of these techniques used over a long period of time. The two most commonly used chemicals to control *E. esula* are the herbicides 2,4-D, dicamba (brand name Banvel®) and picloram (brand name Tordon™) (23). Both broadleaf herbicides work well, but application is prohibited if *E.*

*esula* occurs around water. Cultivation practices, such as cropping, can also diminish *E. esula* populations, but may not be a practical choice for pasture and rangeland situations. The use of sheep grazing does not eradicate *E. esula,* but provides a reduction in stands in addition to being a well suited control measure along waterways. The use of insects and pathogens for the biological control of *E. esula* is another measure that is effective, but generally takes longer to obtain visible results (5,16,23,28). The term biological control agent encompasses any native biotic factors that may suppress the population of another organism, in this case, *E. esula*. Currently the trend is to implement ecologically sound biological control measures to achieve long-term control of *E. esula.*

## *Rhizoctonia solani* as a Biological Control Agent

In the search for biological control agents, an increasing number of fungi are being collected and screened for their potential use against plant pathogens and weeds. However, before the release of such agents can be considered, a responsible assessment of the risks involved must be made. For imported pathogens, quarantine guidelines, such as extensive host range, pathogenicity testing and the development of genetic markers, which allow characterization and monitoring of the released biological control agent, must be met (32).

Strains of *Rhizoctonia solani (R. solani)* are among the few pathogens in the U.S. that are capable of reducing *E. esula* populations naturally and through field applications, as a biological control agent. Field observations, where *R. solani* is involved in abating populations of *E. esula*, confirm that surrounding grasses are largely unaffected by strains of *R. solani* that are pathogenic to leafy spurge (8). Observations by Caesar et al. (10) are that diseased plants of *Euphorbia* in Eurasia are pervasive and may provide a larger pool of highly virulent, well adapted, and narrow host range strains for the control of *E. esula*. Greenhouse tests

conducted by Caesar et al. (11) using *Fusarium* species show that the onset of symptoms was in general more rapid using foreign strains than domestic strains. As found earlier, the most virulent domestic strains pathogenic to *E. esula* were in association with root damage caused by insect biological control agents. Because *Fusarium* spp and *Rhizoctonia* spp both reduce stands of *E. esula*, and insect associated *Fusarium* spp collected in Eurasia are more effective at causing disease due to a more rapid onset of symptoms, insect associated foreign strains of *R. solani* may also be excellent candidates as biological control agents of this noxious weed (10,11). The ideal *R. solani* candidate selected for biological control is one that exhibits aggressiveness towards *E. esula* while maintaining a narrow host range.

## Classification of the *Rhizoctonia solani* Complex

Fungi named *R. solani* occur world-wide, are economically important plant pathogens, and may actually include several different species (21). These plant pathogens have an extended host range that infect over 150 plant species, including agriculturally important crops as well as weed species. *R. solani* causes a variety of diseases including root rot, cankers, damping-off, fruit decay and foliage disease. These fungi do not produce spores, and therefore must be identified on the basis of their mycelial characteristics (2,26).

Historically, fungi classified as *Rhizoctonia* species have been lumped together and classified as such due to their lack of distinctive taxonomic features (24,34). With this classification scheme, it is not surprising that *Rhizoctonia* species are extremely diverse and may belong to several different orders of basidiomycetes as well as ascomycetes. Because of the importance of numerous *Rhizoctonia* species as plant pathogens, an assortment of practical approaches have been developed for identifying these species. These approaches are needed because conventional taxonomic measures are difficult to apply to *Rhizoctonia*, due to its

high levels of phenotypic variation and the inability of most strains to fruit in culture (34). Isolates of *R. solani* vary greatly in their cultural appearance, growth characteristics and pathogenicity towards plants as well as virulence (20). To date, the most unambiguous method for the characterization of *Rhizoctonia* is based on the occurrence of hyphal cell wall fusion between related isolates, and is called anastomosis grouping.

## Hyphal Anastomosis Grouping

In a typical anastomosis reaction test, two different fungal isolates are placed on agar near each other but not touching. Isolates belonging to the same anastomosis group (AG) are generally attracted to each other, evident by hyphal growth toward each other, that leads to hyphal fusion and the killing reaction (35). Isolates belonging to different AGs typically do not undergo hyphal fusion with each other, and isolates belonging to the same AG do not always anastomose with each other at equal frequencies. Some isolates cannot be assigned to an AG because they have not been observed to anastomose with any representatives of known AGs (24). At least 12 AGs have been delineated within the *Rhizoctonia solani* complex, and in practice, when an isolate is unable to anastomose with existing representatives, it is assigned to a new AG. Table 1 lists the classification criteria of hyphal anastomosis reactions of MacNish et al. Even with such straightforward criteria to follow, determining the anastomosis group of an *R. solani* isolate can be very difficult. Environmental conditions such as humidity, temperature, light, and nutritional requirements need to be perfect for hyphal fusion to occur (13).

An intraspecific group is a further subdivision of isolates within an AG that anastomose with each other more frequently, or else possess other unique, distinguishing characters. Such distinguishing characteristics may include host-range specificity, nutrient utilization

or genetic differences based on molecular markers (20,34).

Though the AG classification scheme described allows a method for dividing *R. solani* isolates into more distinct groups, it does not offer other significant pieces of information such as distinguishing pathogenic isolates from nonpathogenic isolates. AG grouping as an indicator of genetic relatedness has been substantiated by DNA/DNA hybridization (34), though the mechanisms of anastomosis are not fully understood (24), which perpetuates an ill-defined *Rhizoctonia solani* complex.

Table 1. Classification of hyphal anastomosis reactions using the criteria of MacNish et al. to delimit anastamosis groups (AG) in *Rhizoctonia solani* (13).

| Reaction | Phenotype | Nature of genetic relationship between isolates |
|---|---|---|
| C0: No interaction | Hyphae grow past each other, no recognition | Isolates have no genetic relationship and belong to different AG |
| C1: Hyphal contact only | No evidence of wall or membrane contact, reaction may or may not be accompanied by cell death | Isolates have a distant genetic relationship and belong to either the same or different AG |
| C2: Killing reaction | Wall fusion (anastomosis) evident, with cell death of anastomosing and adjacent cells, somatic incompatibility response often macroscopically visible | Isolates represent genetically distinct individuals that belong to the same AG |
| C3: Perfect fusion | Wall and membrane fusion evident, point of anastomosis not clearly visible, cell death absent | Isolates are genetically identical or closely related, individuals belong to the same AG and may represent clones |

## Systematics of *Rhizoctonia*

The term *systematics* is the comparative study of biotic diversity at any level (18). Systematics of *Rhizoctonia* is hindered by the ambiguity and shortage of credible, morphological characteristics to soundly characterize this genus. Therefore molecular approaches offer a new avenue for the characterization of *Rhizoctonia*. Looking at molecular data can yield much information for establishing classification systems and phylogenetic relationships at the species level (34). In particular, conserved evolutionary markers, such as the genes coding for ribosomal RNA, appear to be appropriate for comparisons of evolution at the species level because the rRNA genes evolve at a slower rate than other parts of the genome (1,6,35).

Several other properties of the rRNA gene make this gene a useful launching point for comparative analysis. Ribosomal DNA genes in basidiomycetes are tandemly repeated multigene families containing both genic and nongenic, or spacer regions. Each repeat contains a copy of the 18s, 5.8s, and 28s ribosomal subunit RNAs in addition to two spacer regions (internal transcribed spacer or ITS). As shown in Figure 1, the 5.8s rDNA gene is typically flanked by ITS1 and ITS2, which separates the 5.8s rDNA from the 18s and 28s genes (31). This gene in particular possesses highly conserved gene regions that are valuable in identifying organisms, as well as exhibiting regions such as the ITS regions that are considerably variable. Foremost, there is a homolog of this gene in every living cell, making it a well suited gene region as a basis for molecular comparison. In addition, genetic exchange between organisms has not been shown to occur in the rRNA operon, strengthening the rationale for using this region (38). Examinations of the variable ITS regions can often reveal differences present among separate isolates of the same species that may not present themselves when only examining the ribosomal subunit regions (15,20,29).

Having chosen the ribosomal subunit genes and the ITS regions to base our molecular comparisons, we elected to amplify them using the polymerase chain reaction (PCR). The PCR allowed us to exponentially amplify the rDNA region of interest. The specific portion to be amplified was governed by the choice of the oligonucleotide primers. One advantage that exists by using PCR is that it enabled us to amplify homologous gene segments in each of the isolates for comparison. A second advantage of using the PCR is the relatively short length of time needed to complete the reaction. Typically, each PCR run using the thermal cycler was under four hours and we were able to use crude DNA samples. Theoretically, the PCR is sensitive enough to detect a single target molecule for amplification without having to use other potentially harmful methods that, for example, may involve radioactivity (17).

The PCR was the initial step for each of the two methods we chose to analyze our isolates. The first method was the creation of a restriction fragment length polymorphism (RFLP) library using restriction enzyme analysis (REA), and the second method was sequence analysis. Both PCRs were identical except for the oligonucleotide primers chosen to amplify different portions of the rRNA gene (see Table 2).

Table 2. Oligonucleotides primer sequences used to amplify rRNA gene of *Rhizoctonia solani* isolates.

| Primer | Nucleotide sequence 5'--3' |
|---|---|
| LR7 | TACTACCACCAAGATCT |
| LROR | ACCCGCTGAACTTAAGC |
| ITS4 | TCCTCCGCTTATTGATATGC |
| ITS5 | GGAAGTAAAAGTCGTAACAAGG |

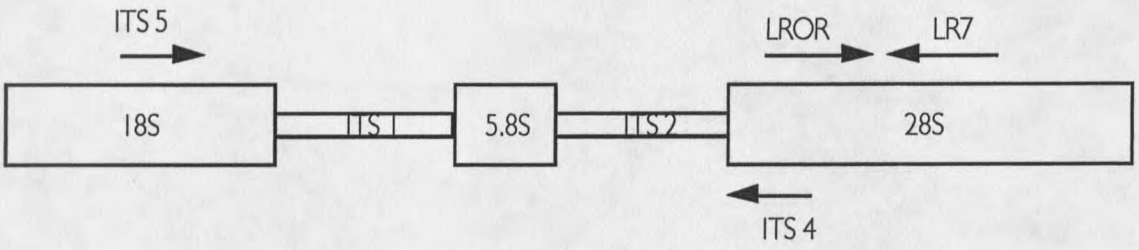


Figure 1. Schematic representation of the internal transcribed spacer regions. The boxes represent the ribosomal subunits. The arrows indicate the direction of the oligonucleotide primers (4).

# CHAPTER 2

# COLLECTION OF FOREIGN AND DOMESTIC *RHIZOCTONIA* ISOLATES FOR MOLECULAR CHARACTERIZATION

## Introduction

Our objective in collecting both foreign and domestic isolates for molecular characterization stems from the fact that *E. esula* originated from Eurasia. There, *E. esula* populations are kept in check by a natural complex of insect and plant pathogens, including *R. solani* (8). We were looking for foreign and domestic isolates that appeared to be closely related. Ideally, we wanted to find foreign isolates that are related to isolates that already exist in the U.S., that show aggressiveness towards *E. esula,* and exhibit a narrow host range. Release of such isolates as biocontrol agents, in essence, would not be as risky as releasing an isolate or a new pathogen that does not already exist in the U.S. Equally as interesting is the basic information gained and possible future correlations that can be made by not only comparing the isolates to each other, but by using all the information we can gather to better characterize *R. solani*.

## Field Materials and Methods

Surveys for disease within stands of *Euphorbia* spp. were undertaken in Eurasia 1993, 1994, 1995 and 1996. Entire plants were uprooted, placed in plastic bags and stored in an insulated container, then were cultured as soon as possible. Samples of *Euphorbia* which

exhibited wilting or stem necrosis were examined for decay in crown tissue or dead root buds, then washed thoroughly. The sample was then vigorously wiped with sterile cotton saturated with 0.5% NaOCl. Tissue from the sample was excised with a sterile scalpel and placed on acidified potato dextrose agar ( PDA) for isolation. The cultures obtained were initially determined to be *Rhizoctonia* spp. based on microscopic characteristics, such as hyphal branching at 90° angles, and constriction at the septa, as well as cultural characteristics, including rapid growth, colony morphology, and sclerotia formation. Apparent *Rhizoctonia* spp. were hyphal-tipped and transferred to agar again (8).

The *Rhizoctonia* isolates from the diseased plants were evaluated for their ability to anastomose with thirteen isolates representing *R. solani*. Field isolates and tester strains were coupled on 2% water agar plates at a distance of 2.5 centimeters apart and assessed for anastomosis. To better visualize anastomosis reactions, hyphae were stained with a Methyl blue/lactophenol solution. The nuclear condition of the isolates was determined by vegetative cell staining with a solution of 4, 6-diamidino-2-phenyl indole (DAPI) (8).

## Results

Tables 3 and 4 summarize information obtained for the 41 isolates collected. The tables list the *R. solani* sample, the original host of isolation, the geographic location, the identity determined by anastomosis testing and staining, and whether or not insects were present. Pathogenicity and virulence testing on all the isolates is incomplete (data not shown). Preliminary results indicate that isolates 01 and 06 may be good biological control candidates because they exhibited high aggressiveness to roots of *E. esula* (7).

Table 3. Identification and Features of Domestic Isolates of *Rhizoctonia solani*

| Sample # | Designation | Identity of *Rhizoctonia* spp | Host | Insect Presence | Location |
|---|---|---|---|---|---|
| 01 | 94-Lyman | AG-4 | *Euphorbia esula* | Yes | Lyman Creek Bozeman, MT |
| 02 | 94-WSS-M | AG-4 | *Euphorbia esula* | No | White Sulfur Springs, MT |
| 03 | 94-Miss-M | AG-4 | *Euphorbia esula* | No | Missoula, MT |
| 04 | 94-CC#2JL | AG-4 | *Euphorbia esula* | No | Cabin Creek, MT |
| 05 | 94-6J Rhiz. | AG-4 | *Euphorbia esula* | No | Fort Benton, MT |
| 06 | 93-2 | AG-4 | *Euphorbia esula* | No | Sidney, MT |
| 07 | 93-1 | AG-4 | *Euphorbia esula* | No | Sidney, MT |
| 08 | 94-20 | binucleate | *Euphorbia esula* | No | Two Dot, MT |
| 09 | 94-11 | multinucleate | *Euphorbia esula* | No | Devil's Tower, WY |
| 10 | 95-3 | binucleate | *Euphorbia esula* | Yes | Stillwater, MT |
| 11 | 95-6 | multinucleate | *Euphorbia esula* | No | Knutson Creek, Theodore Roosevelt National Park (TRNP), ND |
| 12 | tester isolate | AG-4-HGI | n/a | No | n/a |
| 13 | 95-5 | binucleate | *Euphorbia esula* | No | TRNP, ND |
| 14 | 95-10 | binucleate | *Euphorbia esula* | No | Two Dot, MT |
| 15 | 95-9 | binucleate | *Centaurea maculosa* | No | Hill Co, MT |
| 16 | tester isolate | AG-4 | n/a | No | n/a |
| 17 | KVAM 4 | multinucleate | *Euphorbia esula* | Yes | Lambert, MT |
| 18 | KVAM 2 | multinucleate | *Euphorbia esula* | Yes | Lambert, MT |
| 19 | KVAM 5X01 | binucleate | *Euphorbia esula* | Yes | Lambert, MT |
| 20 | 96-2 | binucleate | *Euphorbia esula* | Yes | Fremont Co, WY |
| 21 | 96 Johno 3 | multinucleate | *Euphorbia esula* | Yes | Savage, MT |

Table 4. Identification and Features of Foreign Isolates of *Rhizoctonia solani*

| Sample # | Designation | Identity of *Rhizoctonia* spp | Host | Insect Presence | Location |
|---|---|---|---|---|---|
| 22 | 93f-23C | multinucleate/ AG-4 | *E. esula/virgata* | No | Volgograd, Russia |
| 23 | 93f-16c | binucleate | *E. stepposa* | No | Novomarieskaya, Russia |
| 24 | 93f-23A | multinucleate | *E. esula/virgata* | No | Volgograd, Russia |
| 25 | 94f-15 | binucleate | *E. esula/virgata* | No | Spakovskoe, Russia |
| 26 | 94f-38 | binucleate | *E. esula/virgata* | Yes | Cavaillon, France |
| 27 | 95f-14 | binucleate | *E. esula/virgata* | Yes | Fuzesabony, Hungary |
| 28 | 95f-13 | multinucleate | *E. esula/virgata* | Yes | Puspokladany, Hungary |
| 29 | 95f-20 | multinucleate | *E. esula/virgata* | Yes | Izsa, Slovak Republic |
| 30 | 94f-18 | multinucleate | *C. diffusa* | Yes | Novomarieskaya, Russia |
| 31 | 94f-38a | binucleate | *E. esula/virgata* | No | Cavaillon, France |
| 32 | 94f-38b | binucleate | *E. esula/virgata* | No | Cavaillon, France |
| 33 | 94f-3N | binucleate | *Centaurea maculosa* | Yes | Spakovskoe Sniish, Russia |
| 34 | 95f-18 | binucleate | *E. stepposa* | No | Vesprem, Hungary |
| 35 | 95f-16 | binucleate | *Centaurea maculosa* | Yes | Vesprem, Hungary |
| 36 | 95f-19 | binucleate | *Centaurea maculosa* | Yes | Izsa, Slovak Republic |
| 37 | 95f-10 | multinucleate | *E. esula/virgata* | Yes | Balmasurvos, Hungary |
| 38 | 96f-10 | binucleate | *E. esula/virgata* | No | Xilinhot, China |
| 39 | 96f-14 | binucleate | *E. esula/virgata* | Yes | Inner Mongolia, China |
| 40 | 96f-17 | multinucleate | *E. esula/virgata* | Yes | Bautou, China |
| 41 | 96f-12 | binucleate | *E. esula/virgata* | No | Inner Mongolia, China |

# CHAPTER 3

## RESTRICTION ENZYME ANALYSIS TO CREATE A RESTRICTION FRAGMENT LENGTH POLYMORPHISM LIBRARY

### Introduction

The first approach we employed to characterize *R. solani* involved the PCR to amplify regions of rDNA to create our restriction fragment length polymorphism (RFLP) fingerprint library using restriction enzyme analysis (REA). REA is based on the capability of restriction enzymes to identify DNA sequences that are 4 to 8 bases in length and cleave the double helix at these sites. The number of restriction sites that exist for each isolate determines the number of fragments generated for each isolate and the distance between each restriction site governs the size of the fragments produced by these cuts. The products of these restriction digests are separated by standard agarose gel electrophoresis. The banding patterns produced by these restriction enzyme cuts are reproducible but usually vary somewhat between gels. Banding patterns produced by this method are known as the RFLP fingerprints. The banding pattern or RFLP fingerprint will change if changes in the DNA occur such as a point mutation, that would result in the loss or gain of a restriction site, or chromosomal rearrangement (38). By comparing the RFLP fingerprints, we can estimate the genetic similarity of the isolates (30,36).

## Materials and Methods

### Fungal cultures

A total of 41 foreign and domestic *Rhizoctonia solani* isolates, representing anastomosis group 4, intraspecific groups 1 and 2, were analyzed. All isolates were maintained on potato dextrose agar at 4°C. Each isolate was grown in 250 ml potato dextrose broth at room temperature with shaking for seven days.

### DNA preparation

Mycelia from each isolate was sterile filtered and dehydrated on a lyopholizer for 24 hours. The dehydrated isolates were then ground with 12.5 grams of non-sterile sand to a fine powder in a sterilized mortar with a pestle. The DNA was prepared using the "Sand Prep " method (14). The powder-sand mixtures were transferred to polypropylene centrifugation tubes containing nine ml of extraction buffer and gently mixed by rolling the tubes back and forth on the bench top. The tubes were placed on their sides and gently rocked on an electric rocker for at least one hour at 60° C. After a one hour incubation, 6 ml of chloroform-octanol (24:1) were added to each tube. Each tube was then gently rolled on its side for 5 minutes and centrifuged for ten minutes at 4000 rpm. The aqueous layer of each tube was poured into new tubes each containing 1 ml 10% CTAB (56 ml $H_2O$, 10 g CTAB, 33ml 2.5M NaCl), then balanced with the addition of at least 4 ml of chloroform-octanol and centrifuged for ten minutes at 4000 rpm. Following centrifugation, the aqueous layer of each sample was pipetted into centrifugation tubes containing 6 ml isopropanol. The DNA in these tubes were allowed to precipitate for 30 minutes and then centrifuged for 20 minutes at 7000 rpm. The aqueous layer of each sample was discarded and the pellets that

remained were left to dry on the bench top overnight. The dried DNA pellets were then suspended in 1 ml TE buffer and stored at -20°C until later use.

Polymerase Chain Reaction

PCR reactions were performed using the oligonucleotide primers LROR and LR7 (see Figure 1 and Table 2) with template DNA obtained from the Sand Prep DNA extraction method. The DNA samples were diluted 1:100 before performing PCR in order to obtain the greatest yield of PCR products. Thirty PCR cycles were performed on an automated thermal cycler, using the following parameters: 1 minute denaturation step at 94°C, 1 minute annealing step at 50°C, and 1 minute primer extension step at 72°C followed by a final 7 minute incubation step at 72°C to ensure complete polymerization of any remaining PCR products (36). PCR products were examined on a 2% ethidium bromide-stained agarose gel over an ultraviolet light transilluminator, and photographed.

Restriction Enzyme Analysis of PCR Products

Restriction enzyme analysis was performed directly on the PCR products according to the manufacturer's directions (New England Biolabs). The restriction enzymes used to digest the PCR products are as follows: *Ava*II, *Hha*1, *Hpa*II, *Msp*1 and *Taq*1. The RFLPs were analyzed by separation of the fragments using electrophoresis on a 4% agarose gel. Figure 2 shows an example of RFLP banding patterns obtained from restriction enzyme *Hha*1. Four gels per restriction enzyme were run to accommodate all 41 isolates, and photos of these gels are found in Appendix A. The 1kb DNA ladder standard was run with each group of isolates for each restriction enzyme. The fragments of the standard are of known size, so the fragment size was plotted against the distance travelled from the origin. An

example of a calibration curve of the 1kb DNA ladder is shown in Figure 3 for the *Hha*1 gel shown in Figure 2. A calibration curve for each gel and for each restriction enzyme was plotted. This allowed us to determine the size of the fragments produced for each isolate by each restriction enzyme.

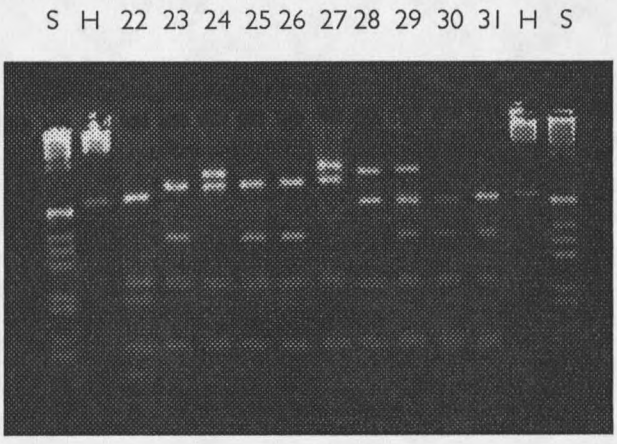


Figure 2. RFLP fingerprints produced by the digestion of LROR, LR7 amplified rDNA with restriction enzyme *Hha*1 analyzed on a 4% agarose gel. Isolate numbers and ladder identification are shown. The 1kb DNA ladder is labeled "S" and the *Hin*dIII lambda ladder is labeled "H".

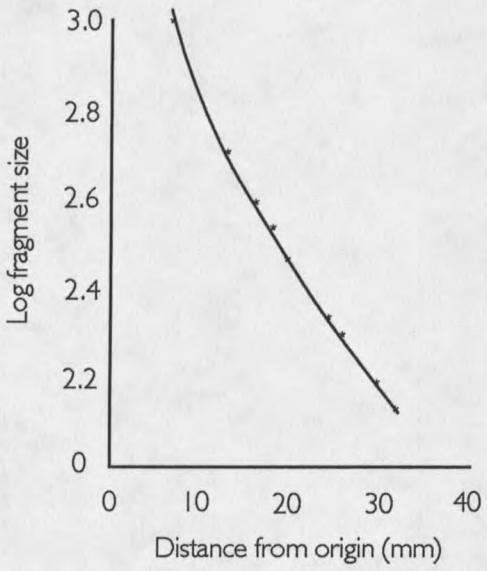


Figure 3. Calibration curve based on the 1kb DNA ladder for the RFLP fingerprint shown in Figure 2 (18).

Letter designations for RFLP banding patterns were assigned to all isolates for each restriction enzyme used. Isolates with identical RFLP banding patterns were placed in the same letter group. If the patterns of the lettered groups were the same for all six restriction enzymes, the isolates were placed in the same class. This is summarized in Tables 5 & 6.

Table 5. Genotype classes and restriction phenotypes of the domestic isolates.

| Sample # | Class | Restriction phenotype of fragment digested with: | | | | | |
|---|---|---|---|---|---|---|---|
| | | *AvaII* | *HhaI* | *HpaII* | *MspI* | *Sau3AI* | *TaqI* |
| 01 | I | A | A | A | A | A | A |
| 02 | I | A | A | A | A | A | A |
| 03 | I | A | A | A | A | A | A |
| 04 | I | A | A | A | A | A | A |
| 05 | I | A | A | A | A | A | A |
| 06 | I | A | A | A | A | A | A |
| 07 | II | B | B | B | B | B | A |
| 08 | III | A | A | C | C | C | A |
| 09 | IV | A | A | A | A | C | A |
| 10 | III | A | A | C | C | C | A |
| 11 | I | A | A | A | A | A | A |
| 12 | V | A | C | D | D | C | B |
| 13 | VI | C | C | E | E | D | C |
| 14 | VII | A | D | F | F | E | A |
| 15 | VIII | D | E | G | G | B | D |
| 16 | IX | A | F | H | H | F | D |
| 17 | I | A | A | A | A | A | A |
| 18 | X | A | A | A | A | A | E |
| 19 | XI | D | F | I | I | K | D |
| 20 | XII | A | A | C | C | G | A |
| 21 | XIII | E | G | J | J | H | F |

Table 6. Genotype classes and restriction phenotypes of the foreign isolates.

| Sample # | Class | Restriction phenotype of fragment digested with: | | | | | |
|---|---|---|---|---|---|---|---|
| | | *AvaII* | *HhaI* | *HpaII* | *MspI* | *Sau3AI* | *TaqI* |
| 22 | XIV | A | C | A | A | A | A |
| 23 | XV | A | C | N | N | C | A |
| 24 | XVI | A | D | A | A | A | A |
| 25 | XVII | A | E | C | C | C | A |
| 26 | XVIII | G | F | O | O | L | J |
| 27 | III | A | A | C | C | C | A |
| 28 | I | A | A | A | A | A | A |
| 29 | XIX | A | F | N | N | C | A |
| 30 | XX | A | A | P | P | K | D |
| 31 | XXI | D | G | G | G | I | K |
| 32 | XXII | D | F | G | G | I | G |
| 33 | VIII | D | E | G | G | B | D |
| 34 | XXIII | C | H | K | K | J | H |
| 35 | XXIV | D | F | G | G | K | D |
| 36 | VIII | D | E | G | G | B | D |
| 37 | X | A | A | A | A | A | E |
| 38 | XXV | A | A | C | C | C | E |
| 39 | XXV | A | A | C | C | C | E |
| 40 | XXVI | F | I | L | L | C | I |
| 41 | XXVII | A | J | M | M | C | A |

A distance matrix was calculated by using the equation

$$D = 1 - 2(\sum n_{xy})/(\sum n_x + \sum n_y),$$

where $D$ represents the distance to be entered into the matrix; $n_x$ represents the number of fragments found in the first of the two isolates being compared; $n_y$ represents the number of fragments found in the second of the two isolates being compared; and $n_{xy}$ represents the number of restriction fragments the two isolates have in common. The $\sum$ sign indicates the summation of the values for each restriction digest (19,36). The RFLP banding patterns and classes aided in the construction of a distance matrix. Figure 4 is an example of one "cell" used in calculating the matrix. Appendix B contains the entire collection of cell calculations in