# USE OF NUCLEIC ACID AND ISOENZYME POLYMORPHISMS FOR TAXONOMIC AND POPULATION STUDIES OF *GREMMENIELLA* SPP.

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## Summary

*Scleroderris* canker is a serious disease of conifers in Europe, North America and Japan, and is caused by species and varieties of *Gremmeniella* (Ascomycotina). We used nucleic acid and isoenzyme polymorphisms to investigate both intra- and interspecific variability in a worldwide collection of *Gremmeniella* isolates previously characterized by morphological, serological and electrophoretic methods. Ribosomal DNA (rDNA) gene polymorphisms and random amplified polymorphic DNAs (RAPDs) were detected following amplification by the polymerase chain reaction (PCR). Polymorphisms were also detected in eight isoenzyme systems after electrophoresis on starch, polyacrylamide and cellulose acetate gels. Phylogenetic analysis of length polymorphisms and restriction sites in the small subunit and internal transcribed spacer of PCR-amplified rDNA genes suggested the occurrence of seven distinct groups within *Gremmeniella*. They are: 1) *G. abietina* var. *abietina* (*Gaa*), North American race; 2) *Gaa*, European race; 3) *Gaa*, Asian race; 4) *G. laricina* (*Gl*) from North America; 5) *Gl* from Europe; 6) *G. abietina* var. *balsamea* (*Gab*) from *Abies balsamea*; and 7) *Gab* from *Picea* spp. RAPD analysis of the North American and European races of *G. abietina* allowed unequivocal classification of all specimens tested, confirmed the presence of both races in North America and the European race in Europe and the absence of inter-racial hybrids among the specimens, and substantiated the hypothesis of a recent introduction of the European race into North America. Results from isoenzyme and rDNA analysis were generally consistent, with the exception that the European and North American isolates of *G. laricina* had similar isoenzyme patterns. Analysis of rDNA and isoenzyme polymorphisms also showed that *Gremmeniella* isolates were more related to each other than to *Ascocalyx abietina*, a species which some authors consider to be closely related.

**Key words**: Scleroderris canker; PCR; RAPD, isozymes.

## Introduction

The disease known as Scleroderris canker causes serious damages of conifers, particularly in plantations. Outbreaks of this disease in several near mature plantations (red pine in particular) in Northeastern United States in the 1970's, and in parts of Europe and Japan, have prompted numerous investigations on the biology of the disease and of the pathogen(s) involved. The taxonomy of the causal agents was revised by Petrini et al. (1989, 1990) using biochemical (electrophoresis of mycelial total soluble proteins), morphological, and physiological data. They concluded that fungi causing the disease belonged to the genus *Gremmeniella* rather than *Ascocalyx* .

Ouellette et al. (1988) obtained a near 100% concordance between PAGE electrophoresis and serological methods (Dorworth et al. 1977) for the identification of races of *G. abietina* var. *abietina* on pines and *Abies sachalinensis*, namely the so-called European, North American, and Asian races. Other taxa of

the genus, however, were not easily differentiated with these methods. Therefore other means were required to more routinely and reliably identify members of the genus and to show their interrelationships.

To this end, our group has conducted analyses of isoenzyme (Lecours et al. 1994; G. B. Ouellette, unpublished) ribosomal DNA (Bernier et al 1994) and random genomic (Hamelin et al 1993) polymorphisms.

The purpose of this communication is to present an overview of results of these studies, and their application to the taxonomy of the genus.

## Material and Methods

### Isoenzymes

The isolates (from various hosts and of worldwide origin) used in this investigation are listed in Petrini et al. (1989, 1990).

Mycelium for enzyme extraction was grown in liquid V-8 medium (Benhamou et al. 1983) at 18°C and harvested 28 days after inoculation. Lyophilized samples were extracted with Feret buffer (Feret 1971), and extracts were subjected to electrophoresis on starch (Leuchtmann and Clay 1989), cellulose acetate (Hebert and Beaton 1989), and on polyacrylamide gels (Cruickshank 1983). Gels were stained for enzyme activity as described by Cruickshank (1983), Hebert and Beaton (1989), and Leuchtmann and Clay (1989).

Eight isoenzyme systems were selected for the study: fumarate hydratase (FUM, E.C. 4.2.1.2), glutamate oxaloacetate transferase (GOT, E.C. 2.6.1.1), leucine aminopeptinase (LAP, E.C. 3.4.11.1), malate dehydrogenase (MDH, E.C. 1.1.1.37), mannose phosphate isomerase (MPI, E.C. 5.3.1.8), phosphoglucose isomerase (PGI, E.C. 5.3.1.9), polygalacturonase (PG, E.C. )and phosphoglucomutase (PGM, E.C. 2.7.5.1).

Two different approaches were used for the statistical evaluation of the data. In the first, the presence or absence of a given band were scored and similarities among isolates were derived from the resulting matrix using the Jaccard similarity coefficient (Sneath and Sokal 1973). On the basis of the similarity matrix a dendrogram was constructed using the average linkage algorithm (Sneath and Sokal 1973). All computations were performed with the package Systat 5.2 (Wilkinson 1989). In the second approach, multiple correspondence analysis of the banding patterns was performed using the package SimCA 2.1 (Greenacre 1986).

### Analysis of amplified DNA

DNA was extracted from mycelium or directly from pycnidia and cryptopycnidia by a modified CTAB protocol (Zolan and Pukkila 1986). Portions of the nuclear ribosomal DNA (rDNA) genes were amplified by polymerase chain reaction (PCR), using universal primers flanking regions within the small subunit (SSU) and the internal transcribed spacer (ITS). Amplified fragments were digested with restriction enzymes. Oligonucleotides (10-mer) from Operon Technologies were used for Random Amplified Polymorphic DNA (RAPD) analyses (Williams et al. 1990). Amplification products were separated by agarose gel electrophoresis.

Phylogenetic relationships among the isolates were investigated by parsimony analysis of rDNA restriction sites, using PAUP (Swofford 1990).

## Results

### Isozymes

Isoenzyme polymorphisms were first analysed with starch gel electrophoresis. This method allowed the clear differentiation of *Gremmeniella* isolates from spruce and balsam fir from Quebec and of *Ascocalyx abietis*, and gave indication of differences between the other taxa.

Because of easier manipulation and greater reliability, tests were conducted with the newly developed cellulose acetate gels (Hebert and Beaton 1989); pectinase tests with polyacrylamide gels were also conducted (Lecours et al. 1994). A multiple correspondence analysis of these tests is illustrated in Fig. 1.

These results are in line with most of the previously proposed classification (Table 1) and some of the differences observed between this classification and results of the DNA analyses will be discussed below.

### DNA

Amplification of the rDNA-SSU revealed length polymorphisms in this region of the gene and allowed the differentiation of four groups: (i) *Ascocalyx abietis*; (ii) *Gremmeniella* isolates from *Picea* spp.; (iii) *Gremmeniella* isolates from *Abies balsamea*; and (iv) *Gremmeniella* isolates from *Abies sachalinensis*, *Larix* spp., and *Pinus* spp. These polymorphisms are believed to be the result of insertions in the SSU. Our digestion analysis revealed that extensive polymorphisms occurred within these insertions but that the remaining portion of the SSU was not variable for the restriction enzymes used.

The amplified rDNA ITS was the same length for all *Gremmeniella* specimens but was longer in *A. abietis.* Digestion of the ITS revealed several RFLPs and further differentiated the three races of *G. abietina* var. *abietina*, and the European and North American isolates of *G. laricina*. Phylogenetic analysis of length polymorphisms and of 24 restriction sites confirmed the existence of seven groups within *Gremmeniella* and showed that *Gremmeniella* isolates were more related to each other than to *Ascocalyx*.

### RAPDs

Four primers produced seven markers which differentiated between the North American and European races of *G. abietina* var. *abietina*. Two isolates which could not be assigned to a race with previous assays were unambiguously identified as belonging to either of the two races using RAPD markers.

Three types of markers were obtained: markers that were monomorphic within the species; markers that were polymorphic between the races but monomorphic within; and markers that showed intraracial polymorphisms. Levels of genetic diversity differed between the two races in North America. For example, for primer OPA-07, only two fingerprint patterns were observed in the European race in North America, while eight were found in the North American race.

Both mycelium and fruiting bodies provided suitable sources of DNA for PCR amplification. This will allow race identification without the need for culturing the fungus.

## Conclusions

The present investigations constitute a good example of studies of a taxonomic problem that included morphological, physiological, immunological, biochemical and molecular aspects. Results obtained with the different methods tested in our studies were in general agreement with each other and are summarized in Table 1, including the proposal of a new variety within *G. abietina*, on *Picea* spp. One difference between isoenzyme analyses and DNA analyses is the differentiation of European and North American isolates of *G. laricina*. Such differences are to be expected since only a fraction of the mutations occurring in the DNA will be expressed at the protein level.

It is interesting that, in spite of early reports of hybridization between the NA and EU races, we found that these races appear to represent distinct genetic entities. Intermediate types reported earlier with serological tests were found with RAPD markers to belong to either race, with no case of intermediate or hybrids. The RAPD technique is highly suitable for studying this question and verify the possible occurrence of hybridization among the other entities within *Gremmeniella*.

RAPD patterns for the European and North American races of *G.abietina* var. *abietina* from pine species in Québec were obtained directly from fruiting bodies; this should facilitate rapid identification of these pathogens directly from their hosts and aid in the study of epidemiology of the two races and their spread on the North American continent. Molecular probes being developed from the pathogens will be also quite useful for studying other aspects of the disease.

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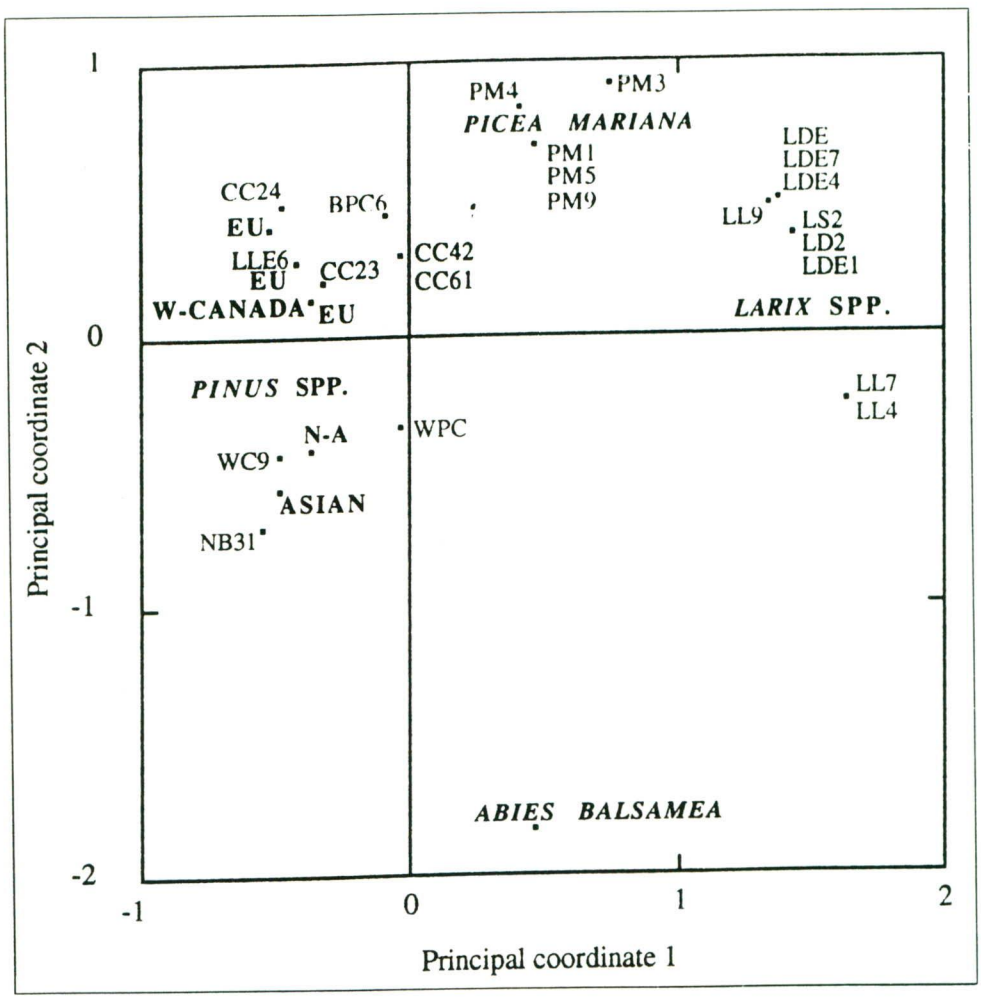


**Fig. 1**

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Table 1. Proposed classification of *Gremmeniella* spp.

*Gremmeniella*

| SPECIES | VARIETY | RACE | HOST spp. | ORIGIN |
|---|---|---|---|---|
| *laricina* | — | — | *Larix* | Quebec, Europe |
| *abietina* | *picea* | — | *Picea* | Quebec, NFld (?) |
| | *balsamea* | — | *Abies balsamea* | Quebec |
| | *abietina* | European | *Pinus* | North America, Europe |
| | | | *Larix* | Europe |
| | | North American | *Pinus* | Canada (except NFld), Northern U.S. |
| | | Asian | *A. sachalinensis* | Japan |

**Table 1.** Proposed classification of *Gremmeniella* spp.