Nucleotide polymorphisms in three genes support host and geographic speciation in tree pathogens belonging to *Gremmeniella* spp.

M. Dusabenyagasani, G. Laflamme, and R.C. Hamelin

Abstract: We detected nucleotide polymorphisms within the genus *Gremmeniella* in DNA sequences of β-tubulin, glyceraldehyde phosphate dehydrogenase, and mitochondrial small subunit rRNA (mtSSU rRNA) genes. A group-I intron was present in strains originating from fir (*Abies* spp.) in the mtSSU rRNA locus. This intron in the mtSSU rRNA locus of strains isolated from *Abies sachalinensis* (Fridr. Schmidt) M.T. Mast in Asia was also found in strains isolated from *Abies balsamea* (L.) Mill. in North America. Phylogenetic analyses yielded trees that grouped strains by host of origin with strong branch support. Asian strains of *Gremmeniella abietina* (Lagerberg) Morelet var. *abietina* isolated from fir (*A. sachalinensis*) were more closely related to *G. abietina* var. *balsamea* from North America, which is found on spruce (*Picea* spp.) and balsam fir, and European and North American races of *G. abietina* var. *abietina* from pines (*Pinus* spp.) were distantly related. Likewise, North American isolates of *Gremmeniella laricina* (Ettinger) O. Petrini, L.E. Petrini, G. Laflamme, & G.B. Ouellette, a pathogen of larch, was more closely related to *G. laricina* from Europe than to *G. abietina* var. *abietina* from North America. These data suggest that host specialization might have been the leading evolutionary force shaping *Gremmeniella* spp., with geographic separation acting as a secondary factor.

Key words: *Gremmeniella*, geographic separation, host specialization, mitochondrial rRNA, nuclear genes.

Résumé : Nous avons détecté des polymorphismes de nucléotides à l'intérieur du genre *Gremmeniella*, au sein des séquences de l'ADN des gènes nucléaires codant pour la β-tubuline et la glycéraldehyde-phosphate deshydrogénase (GPD), et du gène mitochondrien codant pour la petite sous-unité de l'ARN ribosomique (mtSSU rRNA). Un intron du groupe I localisé dans le locus mtSSU rRNA était aussi présent chez les isolats du sapin (*Abies* spp.). Cet intron dans le locus mtSSU rRNA présent chez les isolats originaires de l'*Abies sachalinensis* (Fridr. Schmidt) M.T. Mast en Asie fut aussi trouvé chez les isolats originaires de l'*Abies balsamea* (L.) Mill. en Amérique du Nord. Les analyses phylogénétiques ont produit des arbres dont les embranchements statistiquement significatifs supportent le regroupement des isolats par leur hôte d'origine. Les isolats asiatiques du *Gremmeniella abietina* (Lagerberg) Morelet var. *abietina* isolés du sapin (*A. sachalinensis*) sont plus étroitement reliés au *G. abietina* var. *balsamea* d'Amérique du Nord, lequel attaque l'épinette (*Picea* spp.) et le sapin baumier, qu'à des races européennes et nord-américaines du *G. abietina* var. *abietina* qui sont des pathogènes des pins (*Pinus* spp.). De même, les isolats nord-américains du *Gremmeniella laricina* (Ettinger) O. Petrini, L.E. Petrini, G. Laflamme, & G.B. Ouellette, un champignon pathogène du mélèze, sont plus étroitement reliés au *G. laricina* d'Europe qu'au *G. abietina* var. *abietina* d'Amérique du Nord. Ces données suggèrent que la spécialisation à l'hôte peut avoir été la force évolutive dominante qui a façonné les *Gremmeniella* spp., avec une séparation géographique comme facteur secondaire.

Mots clés : *Gremmeniella*, séparation géographique, spécialisation à l'hôte, ARNr mitochondrien, gènes nucléaires.

Introduction

Several coniferous tree species in the northern hemisphere are susceptible to scleroderris canker, caused by fungal pathogens in the genus *Gremmeniella* (Donaubauer 1972). The disease can affect pines, spruces, firs, larches, and juniper, but economic damage is most significant on pines in northern Europe and northern North America (Donaubauer 1972).

The taxonomic status and evolutionary relationship among some *Gremmeniella* spp. are not clear, in part because of the absence of distinguishing morphological characters.

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Within the genus *Gremmeniella*, three species have been recognized: *G. abietina* (Lagerberg) Morelet, *G. laricina* (Ettinger) O. Petrini, L.E. Petrini, G. Laflamme, & G.B. Ouellette, and *G. juniperina* L. Holm & K. Holm (Petrini et al. 1989). The latter two species appear to be host specific on the *Larix* and *Juniperus* genera, respectively. *Gremmeniella abietina* is comprised of two described varieties: variety *abietina* found mostly on pines and variety *balsamea* found on spruces and firs (Petrini et al. 1989).

Gremmeniella abietina var. *abietina* has been further divided into three races based on serology (Dorworth and Krywienczyk 1975). The separation of these races has been confirmed by comparisons of soluble proteins, random amplified polymorphic DNA (RAPD) profiles, and nuclear rDNA length and restriction profiles (Benhamou et al. 1984; Ouellette et al. 1988; Hamelin et al. 1993; Bernier et al. 1994). The North American race occurs in North America mainly on *Pinus resinosa* Ait., *Pinus banksiana* Lamb., and *Pinus contorta* Dougl. ex Loud., where it causes damage on portions of trees that remain under snow cover during winter. The European race generally infects the entire crown of several hosts belonging mainly to *Pinus* spp. in Europe and North America. The Asian race has been found only in Japan on *Abies sachalinensis* (Fridr. Schmidt) M.T. Mast (Yokota 1975).

There are several unanswered questions concerning the epidemiology of the disease. In North America, the European race of *G. abietina* var. *abietina* was discovered initially 30 years ago in New York State and has since spread throughout pine stands in the northeastern United States and eastern Canadian provinces (Dorworth et al. 1977; Skilling et al. 1984, 1986; Laflamme and Lachance 1987). This pathogen has since been the target of quarantines to prevent further spread (Barrett 1984).

Increased knowledge about the population genetics, phylogeny, species diversity, and taxonomy of this pathogen could help in understanding the epidemiology and potential impact of the disease and could have an impact on the design and application of quarantines. The North American population of the European race of *G. abietina* var. *abietina* most likely originated from at least two distinct founder populations. One population was established on continental North America in 1975, while the other was established in Newfoundland in 1979 (Lachance 1979; Singh et al. 1980; Hamelin et al. 1998). The European race has been reported to be comprised of at least three biotypes, only one of which is known to be present in North America (Hellgren and Högberg 1995; Hamelin et al. 1996; Dusabenyagasani et al. 1998). This may be particularly significant in light of the high susceptibility of lodgepole pine to one of the biotypes currently only present in Fennoscandia (Karlman et al. 1994).

A phylogenetic analysis of *Gremmeniella* spp. using partial sequences of the nuclear ribosomal RNA gene has revealed some inconsistencies between the current serology-based taxonomic divisions and those inferred from the DNA sequence-based phylogeny (Hamelin and Rail 1997). These inconsistencies could have an important impact on quarantine policy in Canada, as currently quarantine is aimed at the European race of *Gremmeniella abietina* var. *abietina*. It is, therefore, important to better document the relationship among the various taxa within the genus.

Previous investigations on *Gremmeniella* have used a variety of biochemical approaches, including protein (Ouellette et al. 1988) and fatty acid profiles (Müller and Uotila 1997), isozymes (Lecours et al. 1994), restriction fragment length polymorphisms (Bernier et al. 1994), random amplified polymorphic DNA (RAPD) and random amplified microsatellites (RAMS) (Hamelin et al. 1993; Hellgren and Högberg 1995; Hamelin et al. 1996; Hantula and Müller 1997; Hamelin et al. 1998), sequence tagged sites (STS) (Dusabenyagasani et al. 1998), and rDNA sequence analysis (Hamelin and Rail 1997). In this paper, we report a DNA sequence survey of exons and introns in two nuclear genes and in one mitochondrial gene. Our objective was to study the phylogenetic and evolutionary relationships within *Gremmeniella* by sampling nuclear and mitochondrial genes and comparing sequence variability in coding and noncoding regions to determine the importance of geographic isolation, and host specialization and adaptation as evolutionary forces.

Material and methods

Fungal samples and DNA extractions

Eighteen strains, selected from the research isolate collection of G. Laflamme, and previously characterized by RAPDs or rDNA restriction profiles, and internal transcribed spacer (ITS) sequencing were used (Table 1). Fungal cultures and DNA extractions with the cetyltrimethylammonium bromide (CTAB) method were performed as previously described (Hamelin and Rail 1997).

DNA amplification and sequencing

DNA regions from β-tubulin and the mitochondrial small subunit rRNA genes were amplified with the primer pairs Bt1a–Bt1b and MS1–MS2, respectively (White et al. 1990; Glass and Donaldson 1995). For glyceraldehyde phosphate dehydrogenase (GPD), primers GPD3′in (5′-TTG CCG TTA AGC TCT GGA AT-3′) and GPD5′ex2 (5′-ATT GGC CGC/T ATC GTC TTC CG-3′) were designed based on conserved regions of genomic DNA sequences from the GenBank database. Polymerase chain reactions (PCR) were performed in a MJ Research PTC-100 thermal cycler (Watertown, Mass.). PCR conditions for β-tubulin were as described in Glass and Donaldson (1995). The cycling protocol for amplifying GPD included a 10-min hot start at 99°C, followed by 35 cycles of 30 s of DNA denaturation at 94°C, 45 s of primers annealing at 50°C, 2 min of primers extension at 72°C, and finally a last 10-min extension step at 72°C. PCR were carried out in a 25-μL volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 400 μM deoxynucleoside triphosphates (dNTPs), 1 μM each of GPD3′in and GPD5′ex2 primers, 1 unit of *Taq* DNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany), and 10 ng of template DNA. PCR for the mtSSU rRNA gene were identical to that for amplifying GPD except that 3.5 mM $MgCl_2$ was used. The cycling protocol was as described for GPD locus except that the hot start step was replaced by an initial 3-min DNA denaturation at 94°C and that 30 cycles instead of 35 were used. The PCR products were purified from gel with the Agarose Gel DNA extraction kit (Boehringer Mannheim GmbH, Mannheim, Germany)

Table 1. Characteristics of strains of *Gremmeniella* spp. used in this study.

Strain	Biotype	Host	Geographic origin	Abbreviation[b]	GenBank accession No. β-tubulin	GPD	mtSSU rRNA
Gremmeniella laricina							
M1070	—[a]	*Larix decidua*	Switzerland	GLEU-1070	AF282827	AY004156	AF284841
CF82-639	—	*Larix decidua*	Quebec	GLNA-639	AF282828	AY004157	AF284842
CF84-484	—	*Larix laricina*	Quebec	GLNA-484	AF282829	AY004158	AF284843
G. abietina* var. *balsamea							
CF87-0065	—	*Picea mariana*	Quebec	GABP-65	AF282839	AY004167	AF284852
CF82-0493	—	*Abies balsamea*	Quebec	GABA-493	AF282840	AY004168	AF284853
CF84-0301	—	*Abies balsamea*	Quebec	GABA-301	AF282841	AY004169	AF284854
***G. abietina* var. *abietina* Asian race**							
JA72-0001	—	*Abies sachalinensis*	Japan	GAAS-72	AF282843	AY004170	AF284855
JA70-0002	—	*Abies sachalinensis*	Japan	GAAS-70	AF282842	AY004171	AF284856
***G. abietina* var. *abietina* European race**							
P80	European	*Pinus mugo*	Germany	GAEU-P80	AF282830	AY004159	AF282844
P85	European	*Pinus ponderosa*	Germany	GAEU-P85	AF282831	—	—
Fi0003	Fennoscandian	*Pinus sylvestris*	Finland	GAEU-FI03	AF282832	AY004160	AF284845
M1024	Alpine	*Pinus cembra*	Switzerland	GAEU-M1024	AF282833	AY004161	AF284846
US15	European	*Pinus sylvestris*	U.S.A.	GAEU-US15	AF282834	AY004162	AF284847
J7	Alpine	*Pinus cembra*	Italy	GAEU-J7	AF282835	—	AF284848
***G. abietina* var. *abietina* North American race**							
C51	—	*Pinus contorta*	Alberta	GANA-C51	AF282836	AY004163	AF284849
C62	—	*Pinus contorta*	British Columbia	GANA-C62	AF282837	AY004164	AF284850
NB71-530	—	*Pinus sylvestris*	New Brunswick	GANA-530	AF282838	AY004165	AF284851
NS72-0600	—	*Pinus banksiana*	Nova Scotia	GANA-600	—	AY004166	—

[a]Not applicable.
[b]GLEU, *Gremmeniella laricina* from Europe; GLNA, *G. laricina* from North America; GABP, *G. abietina* var. *balsamea* from *Picea* spp.; GABA, *G. abietina* var. *balsamea* from *Abies* spp.; GAAS, *G. abietina* var. *abietina* Asian race; GAEU, *G. abietina* var. *abietina* European race; GANA, *G. abietina* var. *abietina* North American race.

according to the manufacturer's specifications. Then, both strands of purified DNA fragments were directly sequenced with Applied Biosystems 373 DNA Sequencer (Applied Biosystems Inc., Foster City, Calif.).

Data analysis

Manually edited sequences were aligned with the algorithm CLUSTAL W (Thompson et al. 1994). To determine if data sets from the different genes could be pooled, tests of homogeneity between β-tubulin and GPD data sets were performed with PAUP (Swofford 1997). The combined data set was used to infer phylogenetic relationships with parsimony analysis with the heuristic search option. Statistical confidence for branches was estimated with 1000 bootstrap replications of the data set using PAUP. Pairwise genetic distance analysis was performed with the Kimura distance measure with the DNADIST algorithm of the PHYLIP software package version 3.56c (Felsenstein 1993). A phylogenetic tree, with *Neurospora crassa* as an outgroup, was constructed from this distance matrix using the neighbor-joining method (Saitou and Nei 1987). Branch confidence was estimated by 100 bootstrap replications. The mtSSU rRNA data set was not included in these analyses because of the lack of sequence data at this locus for the outgroup.

To compare evolutionary rates in the various lineages, synonymous and nonsynonymous rates of nucleotide polymorphisms were estimated using SITES (Hey and Wakeley 1997). Nei's nucleotide divergence and mean pairwise differences were calculated among pairs of taxa with SITES. Similarities of the mtSSU rRNA insert with already published sequence data were examined with BLASTn and BLASTx (Altschul et al. 1990), open reading frame (ORF) searches, and secondary structure comparison with group-I introns using this insert sequence as a query.

Results

We sequenced a 477-bp DNA fragment containing a 54-bp intron for β-tubulin in *Gremmeniella* spp. The two fragments of exonic sequences available comprised 138 and 285 base pairs (bp), respectively. A total of 30 polymorphic sites were obtained. The proportion of sites that were polymorphic among the 18 isolates was 4.9% in each of the two exons and 17.6% in the intron (Table 2).

The number of fixed polymorphic sites varied from one in *G. abietina* var. *abietina* North American race to seven in *G. laricina* (Table 1). Most polymorphic sites observed were fixed within strains grouped by host of origin. For example, four polymorphic sites were fixed within *G. abietina* var. *abietina* (from *Pinus* spp.), including strains from Europe and North America (Table 2).

Nucleotide divergence was estimated to compare divergence within and among accepted taxonomic groups. The nucleotide net divergence per base pair between the Asian race of *G. abietina* var. *abietina* and *G. abietina* var. *balsamea*, and within *G. abietina* var. *balsamea*, was 0.0084,

Table 2. (A) Sequence polymorphism and (B) proportions of polymorphic sites in two exons and one intron at the β-tubulin locus of *Gremmeniella* spp.

(A) Sequence polymorphism

	Position in exon 1 (138 bp)[a]							Position in intron (54 bp)									Position in exon 2 (285 bp)													
Strain[b]	4	10	52	73	109	124	130	145	158	164	165	167	168	176	187	190	196	202	208	226	234	238	256	277	292	299	322	323	342	391
GLEU-1070	T	A	G	T	C	C	C	T	C	C	T	A	A	T	T	T	C	G	T	T	G	C	C	T	A	C	G	C	T	C
GLNA-639	T	A	G	T	C	C	C	T	C	C	T	A	A	T	T	T	T	G	T	T	G	C	C	T	A	C	G	C	A	C
GLNA-484	T	A	G	T	C	C	C	T	C	C	T	A	A	T	T	T	T	G	T	T	G	C	C	T	A	C	G	C	A	C
GABP-65	T	C	A	C	C	C	C	C	A	C	T	A	G	T	C	C	T	G	C	T	G	C	G	T	A	T	T	T	T	C
GABA-493	C	C	A	C	C	C	C	C	C	C	T	A	G	C	C	C	T	A	C	T	G	C	C	T	A	C	T	T	T	C
GABA-301	C	C	A	C	C	C	C	C	C	C	T	A	G	C	C	C	T	A	C	T	G	C	C	T	A	C	T	T	T	C
GAAS-70	T	C	A	C	C	C	C	C	C	C	C	A	G	T	C	C	T	G	C	C	A	C	C	T	A	C	T	T	T	C
GAAS-72	T	C	A	C	C	C	C	C	C	C	C	A	G	T	C	C	T	G	C	C	A	C	C	T	A	C	T	T	T	C
GAEU-P80	T	C	A	C	T	T	T	C	C	A	T	G	G	T	C	C	T	G	T	T	G	T	C	C	G	C	T	C	T	C
GAEU-P85	T	C	A	C	T	T	T	C	C	A	T	G	G	T	C	C	T	G	T	T	G	T	C	C	G	C	T	C	T	C
GAEU-FI03	T	C	A	C	T	T	T	C	C	A	T	G	G	T	C	C	T	G	T	T	G	T	C	C	G	C	T	C	T	C
GAEU-MI024	T	C	A	C	T	T	T	C	C	A	T	G	G	T	C	C	T	G	T	T	G	T	C	C	G	C	T	C	T	C
GAEU-US15	T	C	A	C	T	T	T	C	C	A	T	G	G	T	C	C	T	G	T	T	G	T	C	C	G	C	T	C	T	C
GANA-C51	T	C	A	C	T	T	T	C	C	A	T	A	A	T	C	C	T	G	T	T	G	C	C	C	G	C	T	C	T	T
GANA-C62	T	C	A	C	T	T	T	C	C	A	T	A	A	T	C	C	T	G	T	T	G	C	C	C	G	C	T	C	T	T
GANA-530	T	C	A	C	T	T	T	C	C	A	T	A	A	T	C	C	T	G	T	T	G	C	C	C	G	C	T	C	T	T

(B) Proportions of polymorphic sites

	Exon 1	Intron	Exon 2
Proportion of polymorphic sites	0.049	0.176	0.049

Note: Proportion of polymorphic sites (synonymous), 0.208; proportion of polymorphic sites (nonsynonymous), 0.006.

[a]Values in parentheses are numbers of base pairs in the sequence.

[b]See Table 1 for strain characteristics.

Table 3. Nucleotide divergence at the β-tubulin locus.

	G. laricina	*G. abietina* var. *abietina*	*G. abietina* var. *balsamea*	*G. abietina* var. *abietina* (Asian)
G. laricina	0.0028[a]	0.0335[c]	0.0294	0.0294
G. abietina var. *abietina*	0.0296[b]	0.0050	0.0273	0.0273
G. abietina var. *balsamea*	0.0238	0.0206	0.0084	0.0126
G. abietina var. *abietina* (Asian)	0.0280	0.0247	0.0084	0.0000

[a]Values on the diagonal are means within taxon pairwise differences (Saitou and Nei 1987).
[b]Values below the diagonal: net mean pairwise differences.
[c]Values above the diagonal are means among taxa pairwise differences.

confirming that these taxa are closely related. Furthermore, the nucleotide net divergence between *G. abietina* var. *abietina* Asian race and *G. abietina* var. *balsamea* was four times lower than the nucleotide net divergence of at least 0.0247, between the *G. abietina* var. *abietina* Asian race and other members of the variety *abietina* (Table 3).

We obtained 666 bp of sequence data for the GPD locus including 78 bp of the first exon, an intron of 72 bp, and 516 bp of the second exon. We observed a total of 34 polymorphic sites, including two indels located in the intron. The percentages of polymorphic sites of 3.8 and 3.7% were quite similar for the two exons. In the intron, the percentages of polymorphic sites of 12.3% (or 15.1%, when indels are included) were three (four) times higher and close to the proportion of synonymous sites in the exons (Table 4). Both variable and fixed polymorphic sites within taxa were present (Table 4). As observed for β-tubulin, nucleotide net divergence per base pair values revealed that the *G. abietina* var. *abietina* Asian race was closer to the variety *balsamea* than to other members of the variety *abietina* (Table 5).

Most of the sequence polymorphisms in β-tubulin and GPD were observed among strains grouped by host of origin. These groupings roughly correspond to accepted current taxonomic groups (with the exception of the Asian race of *G. abietina* var. *abietina*). However, within the variety level we also observed sequence polymorphisms among strains from different geographic origins. One substitution in the GPD sequence and two substitutions in the β-tubulin sequence were observed between the North American and European strains of *G. laricina*. Two substitutions in the GPD sequence and four substitutions in the β-tubulin sequence were present between the North American and European races of *G. abietina* var. *abietina*, and additional single nucleotide substitutions were observed within the North American strains (Tables 2 and 4).

We identified fixed polymorphic sites in *G. laricina*, *G. abietina* var. *balsamea*, and the Asian race of *G. abietina* var. *abietina* in the mtSSU rRNA sequences. The mitochondrial rRNA gene region sampled appeared to be less variable than β-tubulin and GPD. The proportion of polymorphic sites, 0.4% (0.6% in the intron), was comparable with the proportions of polymorphic nucleotides observed for nonsynonymous sites in the exons of β-tubulin and GPD genes.

We also observed a length polymorphism attributable to a 1395-bp insertion in all isolates from *Abies* (Table 6). A BLASTn search of the GenBank database using the 1395-bp sequence as the query revealed that this intron shared 74% sequence similarity (*E* value = $2e^{-62}$) with a group-I intron previously identified in the *Sclerotinia sclerotiorum* mtSSU rRNA genes (Carbone et al. 1995). In addition, significant similarities in secondary structure and ORFs between this intron and the subgroup-IC introns from coding genes of fungal species such as *Podospora anserina* (*E* value = $3e^{-54}$) and *Neurospora crassa* (*E* value = $6e^{-54}$) were also found (Cummings and Domenico 1988; Nelson and Macino 1987).

Maximum parsimony and distance analyses using *Neurospora crassa* as an outgroup produced phylogenetic trees with similar topologies (Fig. 1 and data not shown). The test of homogeneity was not significant ($P \leq 0.01$) and allowed us to pool the sequence data from β-tubulin and GPD genes. The analysis of the combined data set identified three well-supported clusters corresponding to three host genus groups, i.e., *Larix*, *Pinus*, and *Abies–Picea*. These three groups were strongly supported: 96 and 100% bootstraps for the distance method and 70 and 99% bootstraps for the parsimony method (Fig. 1 and data not shown).

Discussion

This study was designed to compare sequence variability among members of the *Gremmeniella* genus and to analyze their evolutionary relationships. One of our hypotheses was whether or not geographic isolation has been more important in the evolution of this genus than host specialization. This question is relevant, not only to better understand evolution in this important group of conifer pathogens, but because it also has some implications for assessing the utility of the current taxonomy and its application to quarantine standards. Although most species and varieties have been named according to their host of origin, some taxa (e.g., the three races of *G. abietina* var. *abietina*) were named according to their geographic origin (Dorworth and Krywienczyk 1975). Pathogenicity experiments suggest that host preferences exist between *G. abietina* var. *abietina* and *G. abietina* var. *balsamea* (Laflamme et al. 1996). Our results strongly support the hypothesis that host specialization has been a major driving force in the evolution and speciation of this group. However, late secondary differentiation within regions could explain the lower levels of sequence variation found between geographic origins.

Our results that indicate that the Asian race of *G. abietina* var. *abietina* is more closely related to *G. abietina* var. *balsamea* than to the European and the North American races of *G. abietina* var. *abietina* are in conflict with the current taxonomy (Dorworth and Krywienczyk 1975). This conflict has been alluded to in a study using protein profiles and morphological characters showing that the Asian race

Table 4. (A) Sequence polymorphism and (B) proportions of polymorphic sites in two exons and one intron at the glyceraldehyde

(A) Sequence polymorphism

	Position in exon 1 (78 bp)[a]			Position in intron (72 bp)										
Strain[b]	15	30	36	105	114	115	116	118	120	126	129	143	147	149
GLEU-1070	G	T	C	G	A	—	T	T	A	C	A	C	C	C
GLNA-639	G	T	C	G	A	—	T	T	A	C	A	C	C	T
GLNA-484	G	T	C	G	A	—	T	T	A	C	A	C	C	T
GABP-65	C	T	T	A	A	T	T	T	A	C	—	T	G	T
GABA-493	C	T	T	A	A	T	T	T	A	T	—	T	G	T
GABA-301	C	T	T	A	A	T	T	T	A	T	—	T	G	T
GAAS-70	G	C	T	A	G	T	T	C	A	C	A	T	G	T
GAAS-72	G	C	T	A	G	T	T	C	A	C	A	T	G	T
GAEU-P80	C	T	T	A	A	T	C	T	A	C	A	T	C	T
GAEU-FI03	C	T	T	A	A	T	C	T	A	C	A	T	C	T
GAEU-M1024	C	T	T	A	A	T	C	T	A	C	A	T	C	T
GAEU-US15	C	T	T	A	A	T	C	T	A	C	A	T	C	T
GANA-C51	C	T	T	A	A	T	C	T	G	C	A	T	C	T
GANA-C62	C	T	T	A	A	T	C	T	G	C	A	T	C	T
GANA-530	C	T	T	A	A	T	C	T	A	C	A	T	C	T
GANA-NS72	C	T	T	A	A	T	C	T	G	C	A	T	C	T

(B) Proportions of polymorphic sites

	Exon 1	Intron	Exon 2
Proportion of polymorphic sites	0.038	0.123 (0.151)[c]	0.037

Note: Proportion of polymorphic sites (synonymous), 0.137; proportion of polymorphic sites (nonsynonymous), 0.007.

[a]Values in parentheses are the numbers of base pairs in the sequence.

[b]See Table 1 for strain characteristics.

[c]Proportion of polymorphic sites in parentheses includes indels.

Table 5. Nucleotide divergence at the GPD locus.

	G. laricina	*G. abietina* var. *abietina*	*G. abietina* var. *balsamea*	*G. abietina* var. *abietina* (Asian)
G. laricina	0.0020[a]	0.0287[c]	0.0201	0.0220
G. abietina var. *abietina*	0.0262[b]	0.0029	0.0220	0.0270
G. abietina var. *balsamea*	0.0175	0.0191	0.0030	0.0100
G. abietina var. *abietina* (Asian)	0.0210	0.0256	0.0085	0.0000

[a]Values on the diagonal are means within taxon pairwise differences (Saitou and Nei 1987).

[b]Values below the diagonal are net mean pairwise differences.

[c]Values above the diagonal are means among taxa pairwise differences.

was in the same megacluster containing subgroups corresponding to taxa from *Picea*, *Abies*, and *Larix* (Petrini et al. 1989) and in a study of sequences from the nuclear rRNA gene (Hamelin and Rail 1997). Altogether, these results provide further support to the hypothesis of speciation by host specialization in *Gremmeniella* spp., since the Asian race occurs in Japan on *A. sachalinensis* (Yokota 1975), and *G. abietina* var. *balsamea* occurs on *Abies* and *Picea* in North America; thus, the affiliation of the Asian race to the variety *abietina* is weaker (Petrini et al. 1989; Hamelin and Rail 1997).

One surprising result of the current study is the absence of polymorphism among the three known biotypes of the European race of *G. abietina* var. *abietina*. Previous work using RAPDs, RAMS, and microsatellite repeats indicated the presence of one biotype in the Alps, another in northern Europe, and a third biotype broadly distributed throughout Europe and present in eastern North America (Hellgren and Högberg 1995; Hamelin et al. 1996; Hantula and Müller 1997; Dusabenyagasani et al. 1998). DNA markers (i.e., RAPDs, RAMS, and STS) used to differentiate these biotypes sampled anonymous genomic regions most likely in noncoding regions. These genomic regions are likely to evolve faster than the genes analyzed in the current study. Crossing experiments between two of these biotypes produced fruiting structures but yielded mostly unviable progeny (Uotila 1997; Uotila et al. 2000).

The most likely explanation for these observations was that geographical isolation and ecotypic adaptation after the end of the last glaciation might have allowed the accumulation of sufficient post-zygotic incompatibilities that inhibit successful recombination (Dusabenyagasani et al. 1998). The absence of polymorphisms within the European race of *G. abietina* var. *abietina* in the genomic regions sampled by our study indicates that this speciation event may be too recent for mutations to have accumulated in the more

phosphate dehydrogenase locus of *Gremmeniella* spp.

Position in exon 2 (516 bp)																		
158	184	214	220	221	280	337	340	373	397	400	445	469	481	496	502	538	599	658
A	A	C	A	T	A	C	G	G	C	C	G	T	A	C	T	G	G	T
C	A	C	A	T	A	C	G	G	C	C	G	T	A	C	T	G	G	T
A	A	C	A	T	A	C	G	G	C	C	G	T	A	C	T	G	G	T
A	A	C	G	T	A	C	A	G	G	C	T	C	A	T	T	G	T	T
A	A	C	A	T	G	C	A	G	G	C	T	C	A	T	T	G	T	T
A	A	C	A	T	G	C	A	G	G	C	T	C	A	T	T	G	T	T
A	A	C	A	T	A	C	A	G	G	C	T	C	G	T	T	G	T	T
A	A	C	A	T	A	C	A	G	G	C	T	C	G	T	T	G	T	T
A	C	T	A	C	A	C	A	A	G	T	G	C	A	C	C	A	T	C
A	C	T	A	C	A	C	A	A	G	T	G	C	A	C	C	A	T	C
A	C	T	A	C	A	C	A	A	G	T	G	C	A	C	C	A	T	C
A	C	T	A	C	A	C	A	A	G	T	G	C	A	C	C	A	T	C
A	C	T	A	C	A	A	A	A	G	T	G	C	A	T	C	A	T	C
A	C	T	A	C	A	A	A	A	A	T	G	C	A	T	C	A	T	C
A	C	T	A	C	A	A	A	A	G	T	G	C	A	C	C	A	T	C
A	C	T	A	C	A	A	A	A	G	T	G	C	A	C	C	A	T	C

Table 6. Sequence polymorphism at the mitochondrial small subunit rRNA locus of *Gremmeniella* spp.

	Position of polymorphic site					
Strain[a]	78	136	226	384	2220	2225
GLEU-1070	T	—[b]	A	—	T	T
GLNA-639	T	—	A	—	T	T
GLNA-484	T	—	A	—	T	T
GABP-65	T	C	T	—	T	—
GABA-493	T	C	T	I[c]	T	—
GABA-301	T	C	T	I	T	—
GAAS-70	A	C	T	I	C	—
GAAS-72	A	C	T	I	C	—
GAEU-P80	T	—	T	—	T	—
GAEU-FI03	T	—	T	—	T	—
GAEU-M1024	T	—	T	—	T	—
GAEU-US15	T	—	T	—	T	—
GAEU-J7	T	—	T	—	T	—
GANA-C51	T	—	T	—	T	—
GANA-C62	T	—	T	—	T	—
GANA-530	T	—	T	—	T	—

Note: The sequence is 714 bp long. Proportion of polymorphic sites, 0.004 (0.007). Value in parentheses includes indels.

[a]See Table 1 for strain characteristics.

[b]Indel.

[c]1395-bp intron.

conserved genomic regions studied, even though polymorphisms characterizing biotypes are present in more variable regions sampled by the markers used elsewhere (Hellgren and Högberg 1995; Hamelin et al. 1996; Hantula and Müller 1997; Dusabenyagasani et al. 1998; Hamelin et al. 1998). Nevertheless, geographic differentiation and incipient speciation may be taking place within *Gremmeniella* spp. as indicated by the nucleotide divergence between European and North American races of *G. abietina* var. *abietina* and between European and North American isolates of *G. laricina*.

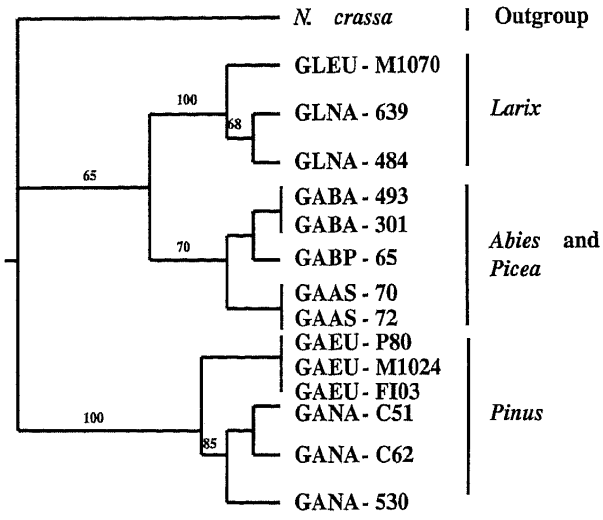
Several nucleotide substitutions in β-tubulin and GPD as well as the 1395-bp insertion in mtSSU rRNA also suggest that host specialization is probably taking place within *G. abietina* var. *balsamea* on *Abies* and *Picea*. These results parallel those of rDNA restriction analysis, which reported the presence of small differences among these groups (Bernier et al. 1994). In addition to these molecular differences, morphological traits in culture have recently been described that suggest that *G. abietina* var. *balsamea* from *Abies* spp. and *G. abietina* var. *balsamea* from *Picea*

Table 7. Sequence polymorphism in the intron of the mitochondrial small subunit rRNA locus of *Gremmeniella* spp. on *Abies*.

	Position of polymorphic site							
Strain[a]	558	685	830	1004	1162	1217	1370	1747
GABA-493	C	T	A	T	A	C	A	A
GABA-301	C	T	A	T	A	C	A	A
GAAS-70	T	C	G	A	T	G	T	T
GAAS-72	T	C	G	A	T	G	T	T

Note: The sequence is 1395 bp long. Proportion of polymorphic sites, 0.01.
[a]See Table 1 for strain characteristics.

Fig. 1. Phylogenetic relationships of *Gremmeniella* species inferred from a combined data set of β-tubulin and GPD sequences with the neighbor-joining method using *Neurospora crassa* as an outgroup. Numbers above the branches indicate the percentage of bootstrap replications that support the branch.
GLEU, *Gremmeniella laricina* from Europe; GLNA, *G. laricina* from North America; GABA, *G. abietina* var. *balsamea* from *Abies* spp.; GABP, *G. abietina* var. *balsamea* from *Picea* spp.; GAAS, *G. abietina* var. *abietina* Asian race; GAEU, *G. abietina* var. *abietina* European race; GANA, *G. abietina* var. *abietina* North American race.



spp. may represent distinct taxonomic entities (Laflamme 2002).

The 1395-bp insertion that we found in strains from *Abies* had significant sequence, ORF, and secondary structure similarities to group-I introns described in protein and rRNA encoding genes in nuclear and mitochondrial genomes from various fungal species (Nelson and Macino 1987; Cummings and Domenico 1988; Carbone et al. 1995; Shinohara et al. 1996; Gonzalez et al. 1997; Chambergo et al. 2002). As reported in other fungi, this insert might have been acquired by strains from *Abies* through horizontal transmission (Hibbert 1996). It is interesting to note the highly conserved sequences of this intron in the isolates of *Abies* from North America and Japan (Table 7), suggesting that the acquisition of this intron occurred before geographic isolation but after host specialization.

This study has shown that the current *abietina* and *balsamea* varieties and the different races are distinct. The data on sequence divergence of multiple data sets (β-tubulin, GPD, ITS, and mtSSU rRNA; this study, Hamelin and Rail 1997) produce congruent phylogenetic results and indicate that these taxa are genetically and biologically isolated. This result supports the presence of several phylogenetic groups in *Gremmeniella* as suggested by Laflamme (2002) and morphological observations are underway to further substantiate these differences and support the hypothesis that these taxa represent distinct groups.

It is also interesting to compare the nucleotide divergence reported here with those of well-characterized model systems, such as *Drosophila* species, for which extensive crossing studies have been done to define the species. For example, nucleotide divergence at the period locus between *Drosophila melanogaster* and *Drosophila simulans* of 2.6% (Kliman and Hey 1993) is comparable with nucleotide divergence values of 2.9 and 2.6% (β-tubulin and GPD, respectively) between *G. abietina* var. *abietina* and *G. abietina* var. *balsamea*, on the one hand, and of 2.8% and 1.9% (β-tubulin and GPD, respectively) between *G. abietina* and *G. laricina*.

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