Mycologia, 91(2), 1999, pp. 315–325.

Evidence supporting the occurrence of a new species of endophyte in some South American grasses

Daniel Cabral
Matías J. Cafaro
B. Saidman
M. Lugo

Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, 1428 Buenos Aires, Argentina

Ponaka V. Reddy
James F. White, Jr.[1]

Department of Plant Pathology, Cook College-Rutgers University, New Brunswick, New Jersey, 80901

Abstract: Endophytes of several South American grasses were examined. The taxonomic status of endophytes in *Festuca argentina, F. hieronymi, Poa huecu*, and an unidentified species of *Poa* is evaluated. On the basis of morphological features, and rDNA and protease gene sequence data, it is proposed that this endophyte is different from several other endophytes examined from North American, South American and European species of *Festuca* and *Poa*. These endophytes, believed responsible for causing the 'tembladera' or 'huecú' toxicosis in animals, have also been reported to produce unique toxins. Isozyme analysis supports the distinctness of these endophytes but also demonstrates variability in populations with respect to gene polymorphisms. Taking all the data into consideration, we propose to establish a new endophyte species *Neotyphodium tembladerae* which includes endophytes found in multiple grass species. We suggest the use of a phylogenetic species concept to define species of *Neotyphodium*.

Key Words: Ascomycetes, Clavicipitaceae, molecular systematics, protease

INTRODUCTION

At the turn of the last century when endophytes were first discovered in seeds of some *Lolium* spp. their identification was a matter for speculation (Vogl 1898, Freeman 1904). Proposals about their identity included that they were smut fungi that had lost the capacity to sporulate on plants (Freeman 1906) and that they were the asymptomatic state of *Endoconidium temulentum* (teleomorph *Gloeotinia temulenta*), the blind seed disease pathogen (Steyn 1934). Neill (1941) demonstrated that the endophytes of several grasses were similar to the also endophytic ascomycete *Epichloë typhina* and suggested that they were asexual stages of that species. Morgan-Jones and Gams (1982) began to define species of endophytes using morphology of conidia and conidiogenous cells, classifying them in the genus *Acremonium* Link because of the similarity of their conidia and conidiogenous cells to those of that genus. Several species of endophytes were defined exclusively through the use of morphology of the cultural state (White 1987, Gams et al 1990). Later, Glenn et al (1996) transferred these species into the genus *Neotyphodium* Glenn, Bacon & Hanlin based on cladistic analysis of ribosomal DNA sequences.

Several studies on endophytes were conducted at the turn of the century after Vogl's discovery of the poison darnel endophyte. Among this early work was the discovery of the presence of an endophyte in the grass *Festuca hieronymi* in Argentina. Rivas and Zanolli (1909) associated toxic syndromes, called "tembladera", with an endophyte in *F. hieronymi* which they denominated *Endoconidium tembladerae*, since they believed that it was related to the *L. temulentum* endophyte which had been misidentified as *Endoconidium temulentum*. Rivas and Zanolli (1909) demonstrated the presence and in vivo morphology of the endophyte, but did not isolate the endophyte or provide Latin descriptions for their proposed new endophyte species. In this study we have examined the tembladerae endophyte (i.e., *E. tembladerae*) and endophytes of several other species of *Poa* and *Festuca*. Our aim was to evaluate and more adequately define the tembladerae endophyte through examination of protease and rDNA gene analysis, isozyme analysis, and morphology.

MATERIALS AND METHODS

Populations of endophyte-infected hosts were located in western Patagonia and northwestern Argentina (TABLE I) and were examined macroscopically for the presence of stromata as well as microscopically for presence of typical endophytic mycelium. Caryopsis and plants representing

Accepted for publication November 11, 1998.
[1] Corresponding author, email: jwhite@aesop.rutgers.edu

TABLE I. Isolates examined in this study

Host	Endophyte	BAFC[a]/rDNA[b]/protease[c]	Origin
Agrostis hiemalis	*Epichloë amarillans*	L07142[b]/AF041238[c]	Alabama, USA
Dactylis glomerata	*Epichloë typhina*	2142[a]	
Elymus canadensis	*Epichloë* sp.	L07131[b]	Missouri, USA
Festuca argentina	*N. tembladerae*	1031[a]/U80606[b]/AF041225[c], 1351[a]/U80607[b]/AF041226[c], 2004[a]	Aluminé, Neuquén, Argentina
Festuca argentina	*N. tembladerae*	0053[a], 0052[a]	Bariloche, Rio Negro, Argentina
Festuca arundinacea	*N. coenophialum*	L07140[b]/AF0412373[c]	USA
Festuca arundinacea	*N. coenophialum*	2474[a]	England
Festuca arundinacea	*N. coenophialum*	2167[a]	Switzerland
Festuca hieronymi	*N. tembladerae*	0060[a], 0065[a]/U80608[b], 0073[a]/AF041227[c], 0075[a]/AF041228[c]	Tafi, Tucuman, Argentina
Festuca langifolia	*Epichloë festucae*	L07139[b]	Switzerland
Festuca pratensis	*N. uncinatum*	L07128[b]	Switzerland
Lolium perenne	*N. lolii*	L07130[b]	Europe
Poa sp.	*N. tembladerae*	AF041230[c]	Cuesta del Obispo, Salta, Argentina
Poa ampla	*Neotyphodium* sp.	L07134[b]/AF041229[c]	Alaska, USA
Poa curta	*Neotyphodium* sp.	AF041231[c]	Oregon, USA
Poa huecu	*N. tembladerae*	2175[a]/U80612[b], 2176[a], 2178[a], 2179[a], 2184[a], 2180[a], 2181[a], 2182[a]/AF041232[c]	El Huecú, Neuquén, Argentina
Poa huecu	*N. tembladerae*	1210[a], 1212[a], 1213[a]	Cajón de Almanza, Neuquén, Argentina
Poa huecu	*N. tembladerae*	U80613[c]/AF041233[c]	Ea Yamuco, Neuquén, Argentina
Poa poecila	*Neotyphodium* sp.	AF041234[c]	Lago Yawen, Tierra del Fuego, Argentina
Poa rigidifolia	*Neotyphodium* sp.	0057[a]/U806102[b]/AF041235[c]	Paso Garibaldi, Tierra del Fuego, Argentina
Poa sylvestris	*Neotyphodium* sp.	AF041236[c]	Alabama, USA

[a] Collection number for Buenos Aires culture collection (BAFC cultures).
[b] GenBank number for rDNA.
[c] GenBank number for protease gene.

multiple separate host individuals were obtained from populations of each host species. Endophytes were isolated from 2–8 individuals from each population using previously published methods (Clark et al 1983, Bacon 1988).

DNA analysis.—DNA was extracted from rapidly growing colonies on PDA overlaid with cellophane. Mycelium was ground in liquid nitrogen and extracted using procedures of Lee and Taylor (1990). PCR (polymerase chain reaction) reactions were performed with the ITS primers ITS 5 and ITS 4 (White et al 1990). Amplifications were done in a volume of 100 μL. Final concentrations were: 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each of four deoxyribonucleotide triphosphates, 0.5 μM of each of two different primers, 2.5 U of Taq polymerase and 200 ng of template DNA. Amplifications were accomplished using a Biometra (Biometra Corp., D-37005 Gottingen, Germany) thermal cycler. Cycling conditions were: Initial denaturation 95 C for 3 min followed by 95 C for 30 s, 58 C for 1 min and 72 C for 45 s (35 cycles). Final extension was done at 72 C for 10 min. Amplifications yielded enough DNA for automated DNA sequencing.

Degenerate primers for protease gene amplification were designed based on sequences evaluated in Reddy et al (1996). The sequence of the degenerate primers were: primer 3, 5′ GAR TTY GAR GGN MGN GC 3′; and primer 4, 5′ GGN GTN GCC ATN SWN GT 3′. The symbols used for the mixed bases are: Y = C, T; M = A, C; R = A, G and N = A, C, G, T. The degeneracy of primer 3 is 786 and that of primer 4 is 1024. The amino acid sequences encoded by primers 3 and 4 are EFEGRA and TSMATP, respectively. The degenerate primers were used to amplify the protease genes with an initial annealing temperature of 64 C. The annealing temperature was decreased 1 C every 2 cycles until it reached 50 C, the lowest theoretical Tm of oligonucleotide primer 4. An additional 30 cycles at an annealing temperature of 50 C were performed. The denaturation temperature was 94 C and the primer extension temperature was 72 C for 2 min for all cycles. The resulting

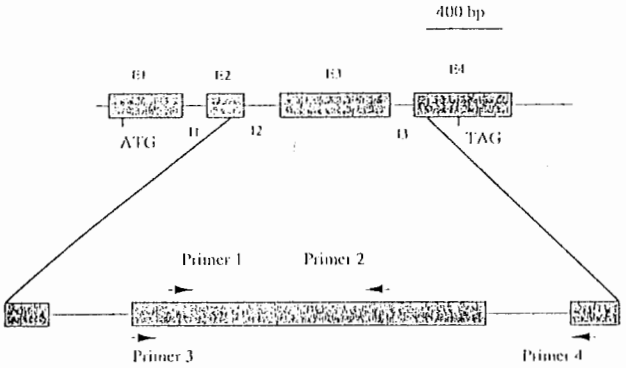


FIG. 1. Organization of the *Neotyphodium* protease gene. The gene contains three introns (I1–I3) and 4 exons (E1–E4). The PCR products (approximately 600 bp) of primers 3 and 4 were purified and reamplified using primers 1 and 2. The PCR product (approximately 300 bp) of primers 1 and 2 was sequenced.

PCR products were separated on 2% agarose gels and multiple bands were resolved with a prominent band at about 600 bp in each lane. The 600 bp band was excised from the gel and the DNA was extracted using a Qiaquick gel extraction kit (Qiagen Inc., Chatsworth, California). The eluted DNA was again reamplified using degenerate primers: primer 1 5′ AAY GTN GAY GAY AAY GGN GAY GG 3′; and primer 2 5′ TAY TTY GTN GCN GTN GCY TGM GG 3′ (Reddy et al 1996). PCR products were again separated on a 2% agarose gel and a single band of approximately 300 bp was detected.

Amplification products were run on a 1% agarose gel; bands were cut out and purified using a Qiaquick gel extraction kit (Qiagen Inc., Chatsworth, California) and sequenced using an ABI model 373A DNA sequencer (Perkin-Elmer Corporation, Norwalk, Connecticut) with a cycle sequencing kit (Amplitaq FS Dye Deoxy Terminator Cycle Sequencing kit, Perkin-Elmer Corporation). Primers ITS 5 and ITS 4 (White et al 1990) were used to sequence both strands of ITS 1, 5.8 S rRNA and ITS 2 regions. Other ITS sequences were obtained from GenBank (TABLE I). Degenerate primers 1 and 2 were used for sequencing both strands of the partial protease gene (FIG. 1).

Data analysis.—Sequences were aligned using Gene Works version 2.45 (Intelligenetics, Mountain View, California) and several variant alignments made using visual estimation. Sequence alignments were deposited in TreeBASE as SN110-366 and SN110-367. Maximum parsimony analysis of the aligned ITS1, 5.8S, and ITS2 sequences was conducted using PAUP v.3.1.1 (Swofford 1993) on a Power Macintosh 9500/132. Alignment gaps were treated as missing data. Heuristic searches were performed with the following options in effect: tree-bisection-reconnection (TBR) branch-swapping algorithm, collapsing zero length branches to yield polytomies, and saving all minimal length trees (MULPARS). To measure the relative support of the clades, bootstrap values (Efron 1982, Felsenstein 1985) were calculated using PAUP v.3.1.1. Bootstrapping was performed with 250 replications. All sequences were submitted to GenBank (TABLE I).

TABLE II. Buffer systems and electrical requirements for isozyme studies

Enzyme	EC number	Abbrev.	N electromorph	Buffers[a]
Esterase	3.1.1.1	EST	5	III
Glucose 6 phosphate dehydrogenase	1.1.1.49	G6P	4	II
Isocitrate dehydrogenase	1.1.1.42	IDH	4	III
Leucine aminopeptidase	3.4.11.1	LAP	3	III
Malate dehydrogenase	1.1.1.37	MDH	4	III
Superoxide dismutase	1.15.1.1	SOD	4	R

[a] For buffers see Wendel and Weeden 1989 and Shaw and Prasad 1970.

Isozyme analysis.—In total, 23 isolates were used (see TABLE III). Mycelium for enzyme extraction was grown in 40 mL of liquid V-8 medium (Christensen et al 1993) in 125-mL Erlenmeyer flasks and inoculated with fresh mycelium grown on PDA slants. Flasks were incubated on a rotary shaker (120 rpm) at 23 C in the dark for 14–21 d. Mycelium was vacuum filtered from the medium, lyophilized, crushed in a ceramic mortar and stored at −20 C. Enzyme extracts were prepared by suspending 40 mg of dried mycelium in 0.3 mL 0.1M Tris-HCl-PVP (pH 7.5) extraction buffer (Wendel and Weeden 1989). Extracts were absorbed onto Whatman N 3 paper wicks 2 × 1 mm.

Horizontal polyacrylamide gel electrophoresis was used in this study. Gels were prepared with 7% acrylamide and 0.2% bis-acrylamide (Sigma Chemical, St. Louis, Missouri). Paper wicks were loaded in the gel (20 per gel) and one of three buffer systems was used (TABLE II; Wendel and Weeden 1989, Shaw and Prasad 1970). Gels were run at 4 C until dye markers migrated 10–12 cm. Staining procedures followed published protocols (Wendel and Weeden 1989). The enzyme names with the enzyme commission (EC) numbers, abbreviations, along with the number of electromorphs presented and buffer systems used are given in TABLE II. Twelve enzymes were tested for activity and six were found to be suitable for isozyme analysis. All electrophoresis runs were duplicated to confirm results.

For the analysis of isozyme data, a phenetic matrix was built using electromorph data and clustering analysis by UPGMA was conducted using Nei & Li's coefficient of similarity (Nei and Li 1979, Kovach 1993). A bootstrap analysis (100 replicates) was conducted on the isozyme data using the program PHYLIP 3.5c (Felsenstein 1985).

Morphological and cultural characterization.—All isolates were grown on potato dextrose agar (PDA) under alternating light and dark cycle (12 h each) at 24 C. After 30 d of growth, measurements of diametric growth and microscopic examinations and measurements (20 for each structure) were made. A fluorescent microscope (Zeiss Axioscope) was

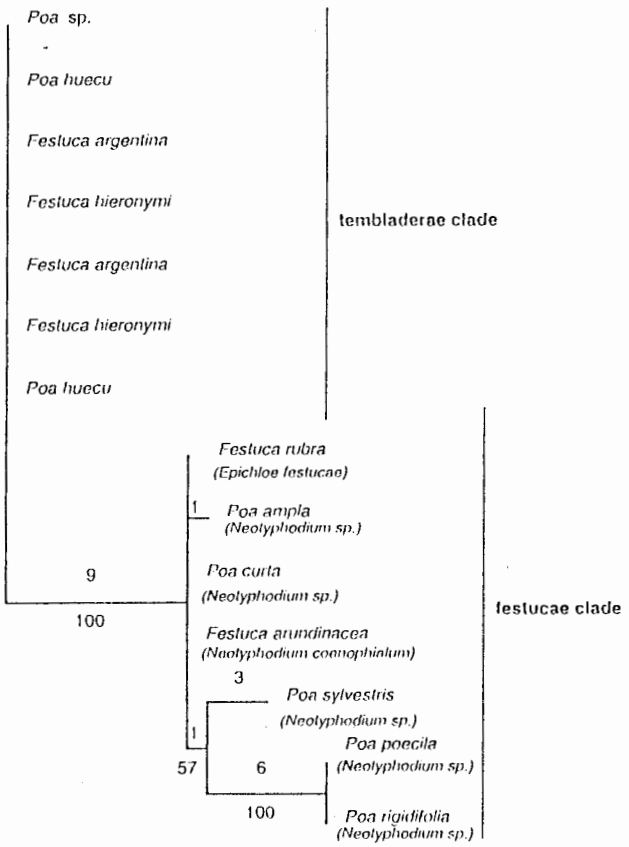


FIG. 2. Unrooted phylogram generated using PAUP showing relationship of endophytes of the tembladerae clade to other *Festuca* and *Poa* endophytes. One of two trees resulting from maximum parsimony analysis (heuristic search option) of partial protease gene sequences with HI = 0.000, CI = 1.000, and RI = 1.000. The tembladerae clade was present in both trees. Bootstrap values (250 replications) greater than 50% and the number of nucleotide changes are mapped onto tree.

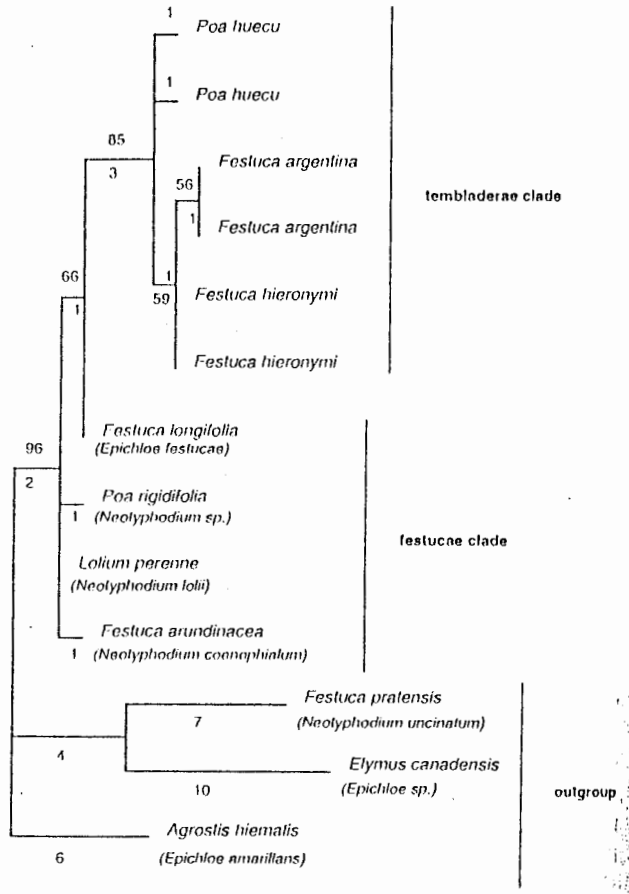


FIG. 3. Unrooted phylogram generated using PAUP showing relationship of endophytes of the tembladerae clade to other endophytes of *Festuca* and *Poa*. Tree resulting from maximum parsimony analysis (heuristic search option) of ITS sequences with HI = 0.033, CI = 0.967, and RI = 0.962. Bootstrap values (250 replications) greater than 50% and the number of nucleotide changes are mapped onto tree.

used to examine mycelium stained with Fluorescent Brightener 28 stain (Sigma, St. Louis, Missouri).

RESULTS

Maximum parsimony analysis of the protease gene sequence data of several *Festuca* and *Poa* endophytes produced two unrooted trees [both with consistency index (CI) of 1.000; retention index (RI) of 1.000; and homoplasy index (RC) of 0.000] that show two major groupings, termed tembladerae and festucae clades, with 100% bootstrap support for separating the two clades (FIG. 2). No variation was found in the sequences among isolates from different hosts in the tembladerae clade. In the festucae clade, endophytes from *Poa sylvestris*, *P. poecila*, and *Festuca rigidifolia* clustered into a subclade with 57% bootstrap support.

Maximum parsimony analysis of the ITS1, 5.8S, and ITS2 regions of the tembladerae endophytes, other endophytes included in the previous protease analysis, *Epichloë amarillans*, and an undescribed species of *Epichloë* from *Elymus canadensis* (included as outgroup) resulted in a single tree (FIG. 3) with CI of 0.967, RI of 0.962 and HI of 0.033. This tree produced a similar separation of fungi into the tembladerae and festucae clades with 85% bootstrap support for the group separation. Sequence variation was encountered among isolates of the tembladerae clade, with isolates from the same host tending to group together.

The tembladerae endophytes (i.e., those of the tembladerae clade) generally demonstrated greater numbers of polymorphs for EST and SOD than *N. coenophialum*, *E. amarillans*, and *E. typhina* (TABLE III). All of the tembladerae isolates showed identical banding patterns for enzymes MDH and SOD. Vari-

TABLE III. Number of bands exhibited for each enzyme, electromorph pattern, and phenotype number

Species or host	BAFC No.	EST	G6P	IDH	LAP	MDH	SOD	Phenotype
E. amarillans	2470	4/D	2/D	3/C	1/B	3/C	3/B	1
E. typhina	2142	4/A	1/C	2/A	1/C	1/D	2/D	2
N. coenophialum	2167	2/E	1/A	2/B	1/A	1/B	1/A	3
N. coenophialum	2474	2/E	1/A	2/B	1/A	1/B	1/A	3
Festuca hieronymi	0060	5/C	1/B	3/C	1/B	3/A	5/C	4
Festuca hieronymi	0065	5/C	1/B	3/C	1/B	3/A	5/C	4
Festuca hieronymi	0073	5/C	1/B	3/C	1/B	3/A	5/C	4
Poa huecu	2181	5/C	1/B	3/C	1/B	3/A	5/C	4
Poa huecu	2182	5/C	1/B	3/C	1/B	3/A	5/C	4
Poa huecu	2184	5/C	1/B	3/C	1/B	3/A	5/C	4
Festuca argentina	1031	5/C	1/B	1/D	1/C	3/A	5/C	5
Festuca argentina	1351	5/C	1/C	1/D	1/C	3/A	5/C	6
Festuca argentina	2004	5/C	1/C	1/D	1/C	3/A	5/C	6
Festuca argentina	0053	5/C	1/C	1/D	1/C	3/A	5/C	6
Festuca argentina	0052	5/C	1/C	1/D	1/C	3/A	5/C	6
Poa huecu	2175	5/B	1/B	3/C	1/C	3/A	5/C	7
Poa huecu	2176	5/B	1/B	3/C	1/C	3/A	5/C	7
Poa huecu	2178	5/B	1/B	3/C	1/B	3/A	5/C	8
Poa huecu	2179	5/B	1/B	3/C	1/B	3/A	5/C	8
Poa huecu	2180	5/B	1/B	3/C	1/B	3/A	5/C	8
Poa huecu	1210	5/C	1/B	3/C	1/C	3/A	5/C	9
Poa huecu	1212	5/C	1/B	3/C	1/C	3/A	5/C	9
Poa huecu	1213	5/C	1/B	3/C	1/C	3/A	5/C	9

ation was exhibited among tembladerae endophytes for LAP, IDH, G6P, and EST. Within the tembladerae clade 6 phenotypes based on isozyme banding pattern were identified (TABLE III, FIG. 4). Phenotype 4 was isolated both from *F. hieronymi* and *P. huecu*; however, *P. huecu* also demonstrated phenotypes 7, 8, and 9. The two *Epichloë* isolates showed more polymorphs for several isozymes than *N. coenophialum*. Clustering analysis (FIG. 4) shows that there is not a strict association of enzyme electromorph pattern with host species and further demonstrates the close association of the tembladerae endophytes into a clade with 100% bootstrap support.

DISCUSSION

The appropriate criteria to use for the definition of species among the endophytic Clavicipitaceae is an unsettled question. The evaluation of species in *Neotyphodium* has been accomplished almost exclusively through studies of morphology in culture (Morgan-Jones and Gams 1982, Latch et al 1984, Gams et al 1990). However, the use of morphology of the cultural states by itself to identify species groupings of *Neotyphodium* has been questioned (Schardl et al 1991, Schardl 1992). One study (White 1993) demonstrated that distinct species of *Epichloë* have very similar appearing conidial states. Schardl et al (1991) demonstrated through use of rDNA sequence analysis that the identification and definition of *Neotyphodium* endophytes through use of cultural morphology alone was untenable and subject to errors. To reduce reliance on morphological characteristics, endophyte systematists have relied on isozyme analysis (Leuchtmann and Clay 1990), randomly amplified polymorphic DNA sequences (White and Huff 1996), ribosomal DNA sequence analysis (Reddy et al 1998), and β-tubulin sequence analysis (Schardl et al 1997). For endophytes that express the stromal *Epichloë* state, reproductive compatibility has been used as an aid to distinguish species (White 1993, 1994, Leuchtmann et al 1994). However, this method for defining species of the asymptomatic endophytes is not applicable to most endophytes because stromata are required to complete crossing experiments. It cannot be applied to the tembladerae endophytes, since they do not produce stromata. A recent study of vegetative compatibility among endophytes also suggests that vegetative compatibility grouping will not provide usable information for grouping endophytes into species because distinct species of endophytes were found to be vegetatively compatible with one another (Chung and Schardl 1997). One remaining option to define meaningful species categories among the *Neotyphodium* endophytes is to adopt a phylogenetic species

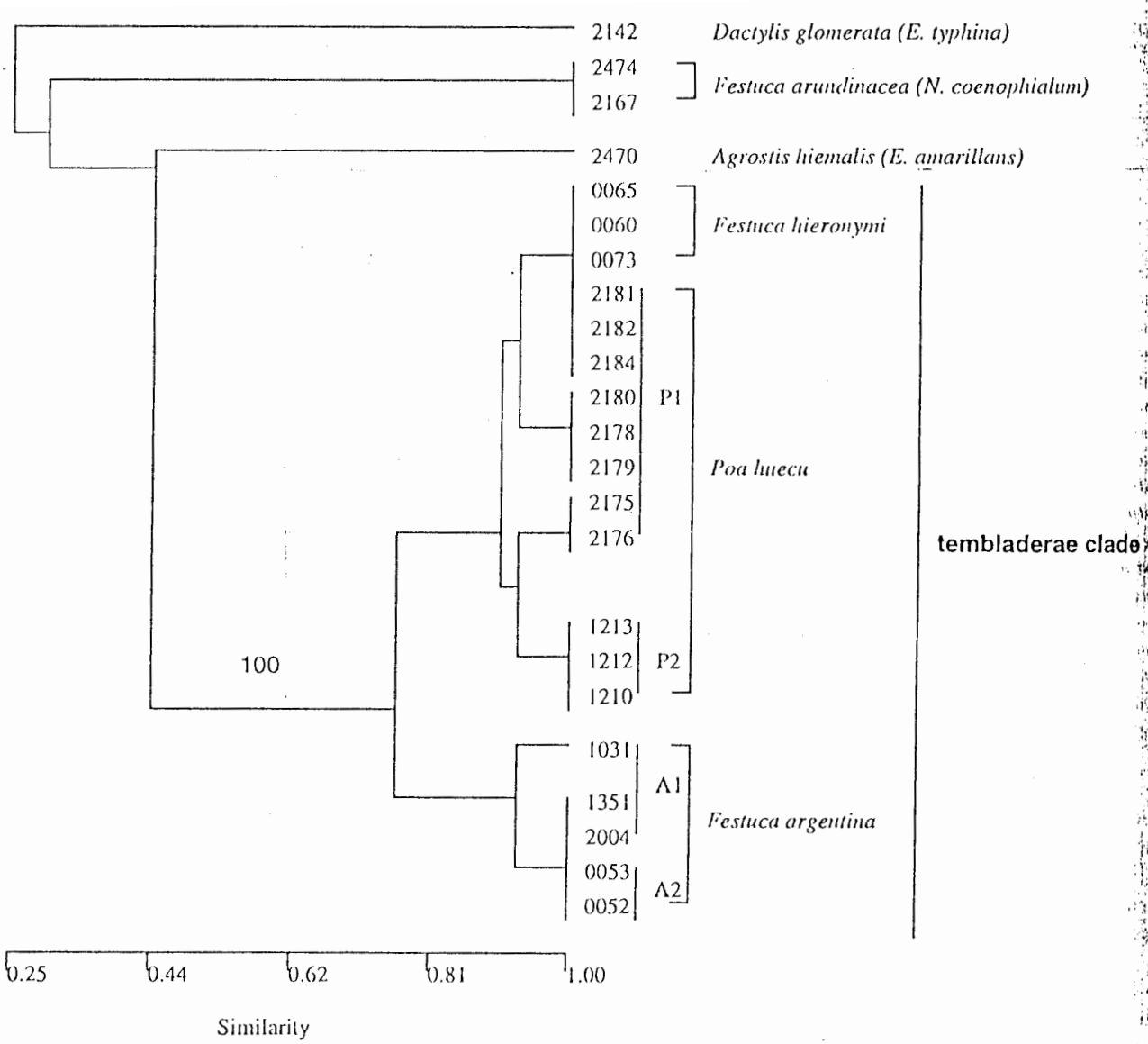


FIG. 4. Phenogram generated from isozyme electromorphs (6 enzyme systems) of 23 isolates. Location P1 is El Huecú in the province of Neuquén, Argentina; P2 is Cajón de Almanza in the province of Neuquén, Argentina; A1 is Aluminé in the province of Neuquén, Argentina; A2 is Bariloche in the province of Rio Negro, Argentina. Tree was produced using unweighted pair-group method using arithmetic averages (UPGMA). Out group included *E. typhina*, *E. amarillans*, and *N. coenophialum*. Scale represents a numerical measure of similarity.

concept and elucidate species categories using gene sequences or other data.

Selection of gene sequences for analysis of species.—The use of sequence data from multiple genes may allow better delimitation of species categories than use of single genes. The protease At1 gene that we have employed is a structural gene coding for a proteolytic enzyme of hyphal walls of species of Clavicipitaceae and is believed to play a role in the symbiosis process, often affecting virulence to hosts (St. Leger 1994, Lindstrom et al 1993). Southern blot analysis of the protease gene in several species of endophytes indicated that it is monomorphic (Lindstrom and Belanger 1994, Reddy et al 1996). Ribosomal DNA is not involved in the interaction of endophyte and host, and the ITS regions are not translated into protein; however, rDNA has not been shown to be polymorphic in species of *Neotyphodium*.

Support for species separation.—Our results suggest that endophytes of some South American grasses form a unique clade (tembladerae clade) which we propose represents a new species of endophyte. This is supported by analyses of multiple data sets that demonstrated that the tembladerae endophytes are

different from several similar previously-described endophytes (FIGS. 2–4). Morphological and developmental features of the tembladerae endophytes also suggest that they form a distinct species. The colonies of the tembladerae endophytes on PDA (FIG. 5) are similar to those of *N. coenophialum* in that they are felty and white to off-white. However, conidia are longer in *N. coenophialum* (measuring 7.5 to 13 μm) (Morgan-Jones and Gams 1982) than in the tembladerae endophytes (ranging 4 to 10 μm long; FIGS. 6–9). The tembladerae endophytes may be distinguished from *N. lolii* in the size of conidiogenous cells: conidiogenous cells of *N. lolii* are very long (ranging 8 to 110 μm long) (Latch et al 1984); while conidiogenous cells of the tembladerae endophytes are much shorter (ranging 10 to 31 μm long; see FIGS. 6, 7, 9). Chlamydospores (FIGS. 10, 11) produced in many of the cultures of the tembladerae endophytes are not encountered in most *Neotyphodium* and *Epichloë* endophytes. One exception is the European *E. baconii,* an endophyte of the grass *Agrostis stolonifera,* which has been shown to produce comparable chlamydospores in culture (White 1993). The endophytes from the tembladerae clade may be distinguished from *Epichloë baconii,* since the latter form stromata and are not known to be seed transmitted; the South American endophytes have not been found to produce stromata and appear to be exclusively seed transmitted. Thus, molecular genetic, structural, and behavioral differences separate the tembladerae isolates from other endophytes to which it seems logical to compare them. In addition, the fact that the tembladerae endophytes are all symbionts of native South American cool-season grasses is also relevant. The populations of hosts are distributed in the northern and southern hemispheres; North American and South American populations are separated by great distances and the tropics. The tropics serve as an environmental barrier, since the hosts cannot survive in warm climates and thus cannot readily move across the tropical zone. It thus is not unexpected that new species of endophytes may evolve in isolation in the southern hemisphere.

TAXONOMY

Based on the multiple differences that we have encountered between the endophytes of the tembladerae clade and previously established species of *Neotyphodium* endophytes in tribe Poideae, we propose that these collections represent a new species of endophyte and provide the following description to validate it as a new species of *Neotyphodium.*

Neotyphodium tembladerae D. Cabral et J. F. White, sp. nov. FIGS. 5–11

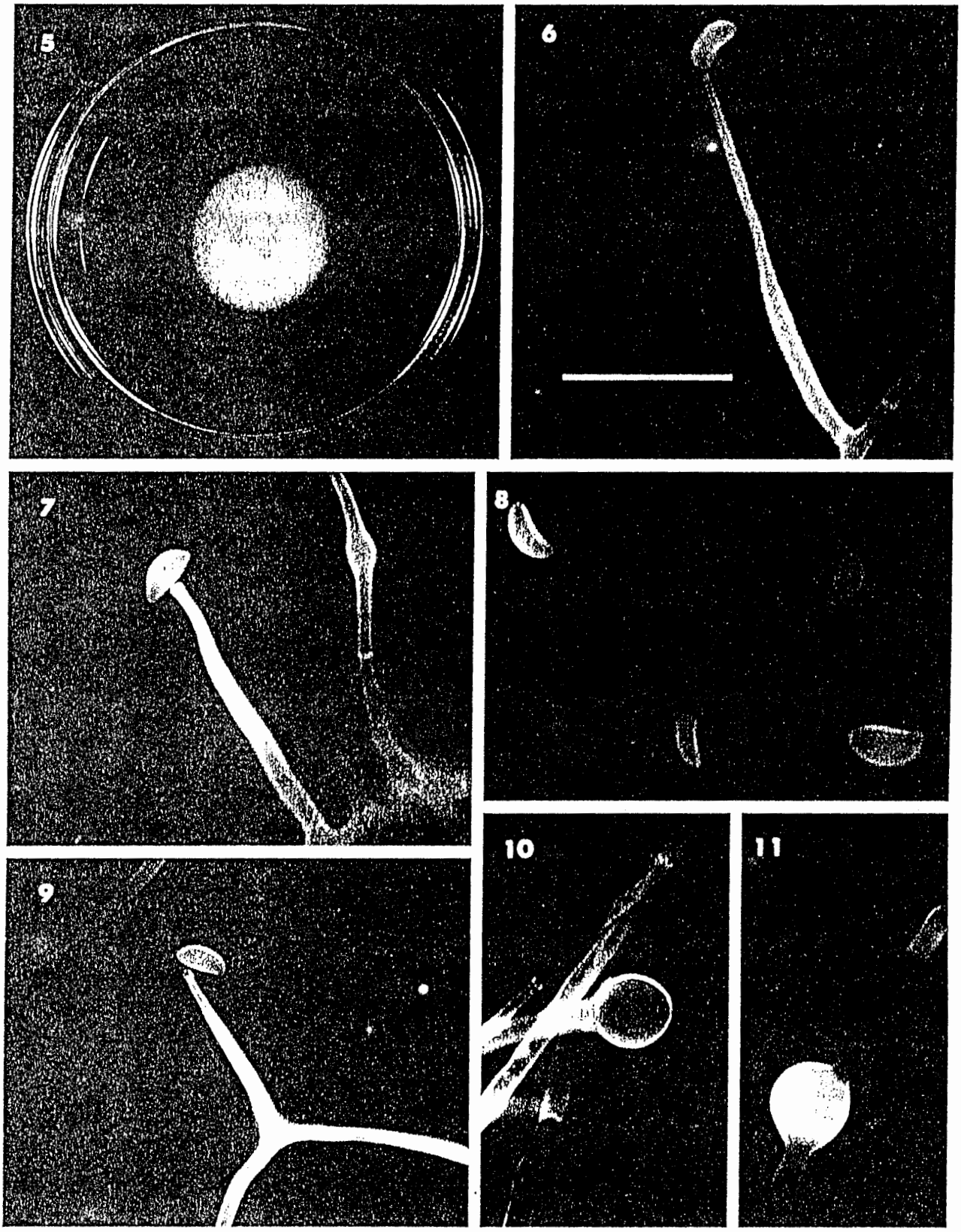
Coloniae in agaro decocto tuberorum lentissime crescunt, post 30 dies ad 24 C, 21–30 mm diametro, albae, lanosae vel cerebriformae; reverso pallide brunneo. Cellae conidiogenae ex hyphis aeriis oriundae, discretae, singulae, laterales, orthotropicae, aculeatae, hyalinae, laeviae, determinatae, 10–31 μm longae, 1.5–3.5 μm crassae ad basim, 0.5–1.8 μm crassae ad apicem. Conidia reniformia vel sigmoidia, hyalina, laevia, saepe transversaliter affixa, 4–10 × 2–4 μm. Chlamydosporae subglobosae, hyalinae, laterales vel intercalares, 5–13 × 5–9 μm. Telomorphosis ignota. In culturis ex culmis Poa huecu, Neuquén Province, Argentina, March, 1993, D. Cabral, RUTPP, HOLOTYPUS; ATCC # 200844 ex-type.

Colonies on PDA (FIG. 5) slow growing, reaching 21–30 mm after 30 d (at 24 C in alternating 12 h cycles of light and dark), white, cottony to lanose, elevated to appressed (in recently isolated colonies from *F. argentina,* colonies were brainlike but later developed aerial mycelium to resemble the colonies of isolates from the other two hosts); colony reverse brown to tan centrally to lighter at margins. Conidiogenous cells (FIGS. 6, 7, 9) forming abundantly from aerial mycelium, hyaline, unbranched, straight, smooth, sometimes with a basal septum, tapering gently from 1.5–3.5 μm wide at the base to 0.5–1.8 μm at the tip, 10–31 μm from base to tip, with a single conidium produced at the tip, often situated transversely. Conidia (FIGS. 6–9) hyaline, variable in shape, lunate, reniform, sigmoid, often swelling to obclavate or ovate in shape, measuring 4–10 × 2–4 μm. Chlamydospores (FIGS. 10, 11) seen in isolates from *F. argentina, Poa huecu,* and *Poa* sp., hyaline, smooth-walled, terminal and solitary to intercalary in chains, spherical to subspherical, measuring 5–13 × 5–9 μm. Telomorph unknown.

Etymology. In reference to the Spanish word for tremble 'tembladera.'

Geographic distribution. Neotyphodium tembladerae is distributed in South America (FIG. 12) in at least four different grass hosts from the Tropic of Capricorn (about 23°S) to as far south as 48°S. In the northern extreme of the range (23°S to 33°S), the endophyte inhabits *F. hieronymi* and *Poa* sp. The endophyte inhabits *P. huecu* in a zone 35° to 40°S; and *F. argentina* south of 40°S.

Notes. It is difficult to escape the conclusion that the tembladerae endophytes form a new species or multiple semispecies (Grant 1977). Precisely where to draw the boundaries of the new species *N. tembladerae* is uncertain. We feel that broad species categories may provide a better taxonomic base to construct a system of classification than creation of numerous smaller species categories whose biological significance is unknown. Indeed Christensen et al (1991)



FIGS. 5–11. *Neotyphodium tembladerae*. 5. 60-d-old colony from *F. hieronymi* on PDA. 6–9. Conidiogenous cells and conidia (scale bar = 10 μm). 10, 11. Chlamydospores of isolate from *F. argentina* grown on PDA (scale bar in FIG. 6).

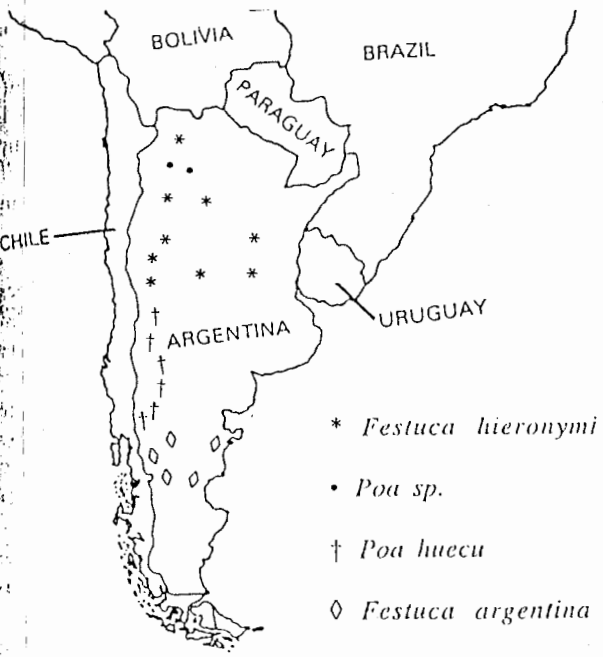


FIG. 12. Map showing confirmed locations of populations of *Neotyphodium tembladerae*. The presence of the endophyte in each host population has been verified through microscopic examination of several plants from each collection site; and for many of the sites endophytes were isolated and found to possess features consistent with *N. tembladerae* (Cabral unpubl).

suggested that the species category *N. lolii* had been defined too narrowly and that many variants in ryegrass should be recognized as *N. lolii* even though possessing differences in growth rate, colony appearance, resistance to fungicides, antibiotic activity, and secondary compound production. We also believe that minor morphological differences may not be sufficient reason to create new species. In the case of the tembladerae group of endophytes, it comes down to how the several types of data are interpreted and how variation is handled. The phylogenetic evidence clearly groups them into well-supported clades (FIGS. 2–4) and suggests that the tembladerae endophytes are distinct from other endophytes to which they were compared. However, strong evidence for dividing the tembladerae endophytes into multiple species is not apparent at present. It thus seems prudent to accept the variation evident among the isolates of the tembladerae endophytes within a single species category.

The presence of *N. tembladerae* in multiple host species suggests that horizontal spread has occurred sometime in the past. However, infective *Epichloë* stages have not been found on any grass species in South America and are assumed not to occur there (White and Cabral unpubl). It is generally believed that species of *Neotyphodium* that do not express the *Epichloë* state are incapable of horizontal transmission and instead rely on clonal transmission through seeds (White 1987, Schardl 1992, White and Huff 1996). However, recent studies (White et al 1996) suggest that at least some *Neotyphodium* endophytes possess a conidial stage that develops on surfaces of leaves of grasses that do not bear stromata. It is conceivable that dissemination through epiphyllously-produced conidia is responsible for the distribution of *N. tembladerae* in multiple grass species in South America. Additional work is required to evaluate this possibility.

Limited work has been done on identifying the secondary compounds of the South American endophyte-infected grasses (Nicora 1978). The toxicity of the tembladerae endophytes is comparable to the staggers that cattle develop when feeding on ryegrass infected by *N. lolii* where the toxic compound has been identified as lolitrem B (Porter 1994). Preliminary work conducted in New Zealand on the toxins produced by the tembladerae endophytes suggests that among the toxic compounds are indole-diterpenoid tremorgens other than lolitrem B, and unidentified ergot alkaloids (Garthwaite 1995). Studies in Argentina suggest that glycoproteins may also be involved in the toxicity of the tembladerae endophytes to mammals (Pomilio et al 1989, Casabuono 1994, Acher 1996). It seems probable that future studies of the chemistry of the tembladerae endophytes may reveal some unique secondary compounds.

The phylogenetic studies using the protease gene (FIG. 2) demonstrate that the endophytes from *Poa poecila* and *Poa rigidifolia*, also native South American species, are phylogenetically distant from the tembladerae endophytes. Indeed these grasses have not been reported to be toxic to animals and we have frequently observed these grasses being consumed by cattle.

ACKNOWLEDGMENTS

This research was supported by PRHIDEB-CONICET (Publication No. 103), NSF DEB 96-96041, and the National Geographic Society. We are grateful to Drs. Chris Schardl, Marshall Bergen, Jeffrey Stone, and an unidentified reviewer for reviewing versions of this manuscript. We thank Dr. Adrian Leuchtmann for supplying cultures for the isozyme analysis.

LITERATURE CITED

Acher A. 1996. Aislamiento e identificación del hongo endofito de *Poa huecu* Par. y busqueda de sus metabolitos

toxicos [Thesis]. Buenos Aires, Argentina: University of Buenos Aires. 65 p.

Bacon CW. 1988. Procedure for isolating the endophyte from tall fescue and screening isolates for ergot alkaloids. Appl Environ Microbiol 54:2615–2618.

Casabuono AD. 1994. Estudio químico y toxicológico de *Festuca argentina* y su comparación con *Festuca hieronymi* (familia Gramineae) [Thesis]. Buenos Aires, Argentina: University of Buenos Aires. 150 p.

Clark EM, White JF, Patterson RM. 1983. Improved histochemical techniques for the detection of *Acremonium coenophialum* in tall fescue and methods of *in vitro* culture of the fungus. J Microbiol Meth 1:149–155.

Christensen MJ, Latch GCM, Tapper BA. 1991. Variation within isolates of *Acremonium* endophytes from perennial ryegrass. Mycol Res 95:918–923.

———, Leuchtmann A, Rowan DD, Tapper BA. 1993. Taxonomy of *Acremonium* endophytes of tall fescue (*Festuca arundinacea*), meadow fescue (*Festuca pratensis*), and perennial ryegrass (*Lolium perenne*). Mycol Res 97: 1083–1092.

Chung KR, Schardl CL. 1997. Vegetative compatibility between and within *Epichloë* species. Mycologia 89:558–565.

Efron B. 1982. The jackknife, the bootstrap and other resampling plans. CBMS-NSF Regional Conference Series in Applied Mathematics, Monograph 38. Philadelphia: SIAM. 92 p.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Freeman EM. 1904. The seed fungus of *Lolium temulentum* L., the darnel. Philos Trans 196:1–29.

———. 1906. The affinties of the fungus of *Lolium temulentum* L. Ann Mycol 4:32–34.

Gams W, Petrini O, Schmidt D. 1990. *Acremonium uncinatum*, a new endophyte in *Festuca pratensis*. Mycotaxon 37:67–71.

Garthwaite L. 1995. *Toxinology and food safety research report*. Hamilton, New Zealand: AgResearch. 69 p.

Glenn AE, Bacon CW, Price R, Hanlin RT. 1996. Molecular phylogeny of *Acremonium* and its taxonomic implications. Mycologia 88:369–383.

Grant V. 1977. Organismic evolution. San Francisco: W. H. Freeman. 418 p.

Kovack WL. 1993. MSVP-A multivariate statistical package for IBM-PCs, version 2.1. Pentraeth, Wales, UK: Kovach Computing Services.

Latch GCM, Christensen MJ, Samuels GJ. 1984. Five endophytes of *Lolium* and *Festuca* in New Zealand. Mycotaxon 20:535–550.

Lee S, Taylor J. 1990. Isolation of DNA from fungal mycelia and single spores. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. New York: Academic Press. p 282–287.

Leuchtmann A, Clay K. 1990. Isozyme variation in the *Acremonium/Epichloë* fungal endophyte complex. Phytopathology 8:1133–1139.

———, Schardl CL, Siegel MR. 1994. Sexual compatibility and taxonomy of a new species of *Epichloë* symbiotic with fine fescue. Mycologia 86:802–812.

Lindstrom JT, Belanger FC. 1994. Purification and characterization of an endophytic fungal proteinase that is abundantly expressed in the infected host grass. Pl Physiol 106:7–16.

———, Sun S, Belanger FC. 1993. A novel fungal proteinase expressed in endophytic infection of *Poa* species. Pl Physiol 102:645–650.

Morgan-Jones G, Gams W. 1982. Notes on Hyphomycetes. XLI. An endophyte of *Festuca arundinacea* and the anamorph of *Epichloë typhina*, new taxa in one of two new sections of *Acremonium*. Mycotaxon 15:311–318.

Nei M, Li WH. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273.

Neill JC. 1941. The endophytes of *Lolium* and *Festuca*. New Zealand J SciTechnol 23:185–193.

Nicora EG. 1978. Flora patagonica. Parte III. Gramineae. Instituto Nacional de Technología Agropecuaria, Buenos Aires, Argentina. 563 p.

Pomilio AB, Rofi RD, Gambino MP, Mazzini CA, Debenedetti De Langenheim RT. 1989. The lethal principle of *Poa lucen* (coirón blanco): a plant indigenous to Argentina. Toxicology 27:1251.

Porter JK. 1994. Chemical constituents of grass endophytes. In: Bacon CW, White JF, eds. Biotechnology of endophytic fungi of grasses. Boca Raton, Florida: CRC Press. p 103–123.

Reddy PV, Bergen MS, Patel R, White JF. 1998. An examination of molecular phylogeny and morphology of the grass endophyte *Balansia claviceps* and similar species. Mycologia 90:108–117.

———, Lam CK, Belanger FC. 1996. Mutualistic fungal endophytes express a proteinase that is homologous to proteases suspected to be important in fungal pathogenicity. Pl Physiol 111:1209–1218.

Rivas H, Zanolli M. 1909. La tembladera. Enfermedad propia de los animales herbívoros de las regiones andinas. Government Report (locally circulated). La Plata, Argentina. 12 p.

Schardl CL. 1992. Molecular biology and evolution of grass endophytes. Nat Toxins 1:171–184.

———, Leuchtmann A, Chung K-R, Penny D, Siegel MR. 1997. Coevolution by common descent of fungal symbionts (*Epichloë* spp.) and grass hosts. Mol Biol Evol 14: 133–143.

———, Lui J-S, White JF, Finkel RA, An Q-Z, Siegel MR. 1991. Molecular phylogenetic relationships of nonpathogenic grass mycosymbionts and clavicipitaceous plant pathogens. Pl Syst Evol 178:27–41.

Shaw CR, Prasad R. 1970. Starch gel electrophoresis. A compilation of recipes. Biochem Genet 4:297–320.

St. Leger RJ. 1994. The role of cuticle-degrading proteases in fungal pathogenesis of insects. Can J Bot 73:S1119–S1125.

Steyn DG. 1934. The poisoning of human beings by weeds contained in wheat (Bread Poisoning). Farming South Africa 9:45.

Swofford DL. 1993. PAUP: phylogenetic analysis using par-

simony. Version 3.1.1. Champaign, Illinois: Illinois Natural History Survey.

Vogl A. 1898. Mehl und die anderen Mehlprodukte der Cerealien und Leguminosen. Z Nahrungsmittle Untersuch, Hyg Warenkunde 12:25–29.

Wendel JF, Weeden NF. 1989. Visualization and interpretation of plant isozymes. In: Soltis DE, Soltis PS, eds. Isozymes in plant biology. Advances in plant sciences series. Vol. 4. Portland, Oregon: Dioscorides Press. p 5–45.

White JF. 1987. Widespread distribution of endophytes in the Poaceae. Pl Dis 71:340–342.

———. 1993. Endophyte-host associations in grasses. XIX. A systematic study of some sympatric species of *Epichloë* in England. Mycologia 85:444–455.

———. 1994. Endophyte-host associations in grasses. XX. Structural and reproductive studies of *Epichloë amarillans* sp. nov. and comparisons to *E. typhina*. Mycologia 86:571–580.

———, Huff DR. 1996. Endophyte-host associations in grasses. XXIV. Some evidence to support the occurrence of endophyte microspecies complexes centered around sexually-reproducing species of *Epichloë*. Symbiosis 20:219–227.

———, Martin TI, Cabral D. 1996. Endophyte-host associations in grasses. XXII. Conidia formation by *Acremonium* endophytes on the phylloplanes of *Agrostis hiemalis* and *Poa rigidifolia*. Mycologia 88:174–178.

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Inns MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. New York: Academic Press. p 315–322.