Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular Phylogenetics and Evolution 35 (2005) 196–208
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Origin, divergence, and phylogeny of epichloë endophytes of native Argentine grasses

Agustina Gentile [a,b], María Susana Rossi [b,1], Daniel Cabral [a,1], Kelly D. Craven [c], Christopher L. Schardl [c,*,1]

[a] *Laboratorio de Micología, PRHIDEB-CONICET, Depart. Biodiversidad y Biología Exp., Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina*
[b] *Depart. Fisiología, Biología Molecular y Celular, IFIBYNE-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina*
[c] *Department of Plant Pathology, University of Kentucky, Lexington, KY 40546-0312, USA*

Received 27 April 2004; revised 12 October 2004

Abstract

The epichloë endophytes are systemic, constitutive, and often vertically transmitted fungal symbionts of grass species in subfamily Poöideae. Prior studies indicate that several asexual epichloë endophytes (*Neotyphodium* species) have evolved directly from sexual (*Epichloë*) species, whereas others evolved by hybridization between two or more endophyte species. In this paper, we investigate the phylogenies of 27 *Neotyphodium* spp. isolates from 10 native grass species (in 4 tribes) in 22 populations throughout Argentina. Relationships among these fungi and a worldwide collection of epichloë endophytes were estimated by phylogenetic analysis of sequences from variable portions (mainly introns) of genes for β-tubulin (*tub2*) and translation elongation factor 1-α (*tef1*). Most of the Argentine endophyte isolates were interspecific hybrids of *Epichloë festucae* and *E. typhina*. Only one isolate was a hybrid of a different ancestry, and three isolates were apparently non-hybrid endophytes. These results indicate that interspecific hybridization, which promotes genetic variation, was common during the evolution of the endophytes of Argentine grasses.

Keywords: *Epichloë*; Endophyte phylogeny; Poaceae; *Neotyphodium*; Hybrid fungi

1. Introduction

The epichloë endophytes are an important group of filamentous fungi that inhabit the aerial tissues of cool-season grasses. This group of fungi comprises sexual species (*Epichloë* Tul. spp.) and their asexual derivatives (*Neotyphodium* Glenn, Bacon, and Hanlin spp.) (Glenn et al., 1996). The *Epichloë* sexual cycle provides the sole or primary means of horizontal transmission (Chung and Schardl, 1997). In contrast, transmission of the asexual endophytes to the next generation of the grass generally occurs via seeds, in which hyphae penetrate the developing embryo (Freeman, 1904). The symbioses between these endophytes and their hosts are considered constitutive mutualisms (Carroll, 1988) in reference to their persistence throughout the life of the plant and to host benefits including protection from biotic and abiotic stresses (Clay and Schardl, 2002; Malinowski and Belesky, 2000).

The endophyte–grass associations are highly relevant to animal health and grass biotechnology. Some grasses infected with endophytes—such as *Lolium arundinaceum* (=*Festuca arundinacea*) with *Neotyphodium coenophialum*, and *Lolium perenne* with *Neotyphodium lolii*—accumulate alkaloids toxic to grazing mammals (Bacon et al., 1986; Raisbeck et al., 1991). However, alkaloids

* Corresponding author. Fax: +1 859 323 1961.
E-mail address: schardl@uky.edu (C.L. Schardl).
[1] These authors contributed equally to this work.

1055-7903/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2005.01.008

specifically toxic to insects are also produced, and endophytes enhance resistance to some nematodes and fungal pathogens (reviewed in Clay and Schardl, 2002; Schardl et al., 2004). Physiological changes reported to occur in infected plants include increased photosynthetic rate, response to elevated CO_2, improved N-utilization, and improved mineral phosphate uptake (reviewed in Malinowski and Belesky, 2000). Abiotic stress factors, such as drought, are also found to have reduced detrimental effects on certain endophyte-infected grasses (Arechavaleta et al., 1989). The range of protective benefits and physiological enhancements, together with the typically high degree of host specificity of endophyte isolates, suggests considerable co-adaptation of grass hosts and epichloë endophytes (Clay and Schardl, 2002).

Grasses toxic to grazing mammals have been known in Argentina since the beginning of the last century. A cattle disease known as *tembladera* is associated with the range grass *Festuca hieronymi*. Rivas and Zanolli (1909), investigating the cause of *tembladera*, were among the first in the world to associate an endophytic fungus with livestock toxicosis. Nearly seven decades later, Bacon et al. (1977) concluded that an endophytic fungus was involved in tall fescue toxicosis associated with *L. arundinaceum* in pastures. As an asexual fungus (Fungi Imperfecti), this species was classified together with related endophytes and the asexual spore state of *Epichloë typhina* in form genus *Neotyphodium* (Glenn et al., 1996).

During the past 10 years, the endophytes of native cool-season grasses have been studied in Argentina. Endophyte strains from several populations of toxic grasses (*Festuca argentina*; *Poa huecu* and *Festuca hieronymi*) and from non-toxic grasses (*Poa rigidifolia*, *Festuca magellanica*, *Melica stuckertii*, among others) have been isolated (Bertoni et al., 1993; Cabral and Bertoni, 1991; Cabral and Lugo, 1993, 1994; White et al., 1996). All of the isolates show similar micro- and macromorphological characters in culture that justify their classification in the genus *Neotyphodium*. None of the isolates has presented teleomorphic forms (Gentile and Cabral, unpublished results), which among the *Epichloë* species consist of conspicuous stromata surrounding the immature inflorescences. Thus, until now the sexual stage has not been found in Argentina or elsewhere in the Southern Hemisphere.

As with the Northern Hemisphere endophytes, morphological characters of Southern Hemisphere endophytes are insufficient to differentiate species. To clarify their relationships, Cabral et al. (1999) analyzed isozymes and DNA sequences in endophyte isolates from three toxic grasses, and compared these molecular data with those of sexual and asexual endophytes from Northern Hemisphere hosts. As result of this study, a new species was named *N. tembladerae* Cabral and White (Cabral et al., 1999). An endophyte of another Argentine grass, *Bromus setifolius*, has also been identified with a morphological relationship to *N. tembladerae* (White et al., 2001).

Recent studies have revealed unexpectedly high levels of hybridization among *Neotyphodium* spp. (Moon et al., 2004). Among these hybrids are three Southern Hemisphere endophytes, including the *N. tembladerae* ex type from *Poa huecu*, as well as *N. australiense* and *N. melicicola* from grasses indigenous to Australia and South Africa, respectively (Moon et al., 2002). The discovery of hybrid endophytes in the Southern Hemisphere encouraged us to study the evolutionary origins of endophytes isolated from a much wider taxonomic range of Argentine grasses. Here we investigate the relationships of 27 isolates, from 5 host genera (10 species), with each other and with a worldwide collection of sexual and asexual isolates.

2. Materials and methods

2.1. Isolation of endophytes and morphological evaluation

The endophyte-infected grasses, sampled populations and provinces, and fungal isolates are listed in Table 1. Endophytes were isolated from grasses collected from 10 different provinces of Argentina. Caryopses and pieces of leaves from endophyte-infected grasses were surface-sterilized by briefly washing in 50% ethanol, followed by a 1–3 min wash in 2.5–3% sodium hypochlorite (50% commercial bleach), and a 2 min wash in a 70% ethanol (Clark et al., 1983). After sterilization, caryopses and leaves were cut and placed on potato dextrose agar (PDA, Merck) plates, incubated in the dark at 23 °C, and checked regularly for endophytic growth for up to 2 months. Endophytes were single-spore isolated three times on PDA to eliminate the possibility of contamination or heterokaryosis (Craven et al., 2001).

To examine the macroscopic characteristics of endophyte cultures, small agar blocks were cut from the margins of actively growing colonies and transferred to PDA plates. Mitotic spore (conidium) dimensions from ≈1-month-old PDA-grown cultures were assessed by a microscopic examination (Zeiss Axioscope) with calcofluor as a stain (Romero and Minter, 1988). Sizes of at least 10 conidia were determined from each isolate.

2.2. DNA isolation and PCR amplification

Total fungal DNA was isolated using the microwave small scale DNA extraction method of Goodwin and Lee (1993) with minor modifications. Fresh mycelium (3–15 mg) from PDA-plate culture was thoroughly ground using an autoclaved plastic mini-pestle in a microcentrifuge tube with 100 µl of lysis buffer (50 mM Tris–HCl, pH 7.2, 50 mM EDTA, 3% SDS, and 1% of

Table 1
Endophyte-infected grasses, population, province, and fungal isolates from Argentina

Host species	Host tribe	Population and geographic origin	Province	Isolate[a]	Average colony size[b]	Genes analyzed[c]
Bromus auleticus	Bromeae	Van Praett	La Pampa	Ba308	7.2	*tef1*, *tub2*
B. auleticus	Bromeae	Colón	Entre Ríos	Ba355[d]	10.4	*tef1*, *tub2*
Bromus setifolius	Bromeae	Ea. Alice (Site 24)	Santa Cruz	**Bs420**	23.9	*tef1*, *tub2*
B. setifolius	Bromeae	Ea. El Condor tub2 (Site 5)	Santa Cruz	**Bs508**	27.6	*tef1*, *tub2*
B. setifolius	Bromeae	Río Rico (Site 18)	Santa Cruz	**Bs719**	27.5	*tef1*, *tub2*
B. setifolius	Bromeae	Ruta 220 a Las Leñas	Mendoza	Bs649[d]	8.2	*tub2*
B. setifolius	Bromeae	Ruta 11. Glaciar P. Moreno (Site 16)	Santa Cruz	Bs532[d]	20.8	*tef1*, *tub2*
B. setifolius	Bromeae	Ruta 11. Glaciar P. Moreno (Site 16)	Santa Cruz	Bs713[d]	18.0	*tef1*, *tub2*
Festuca argentina	Poeae	Aluminé	Neuquén	Fa2203[d]	13.6	*tub2*
Festuca hieronymi	Poeae	Cuesta del Obispo	Salta	Fh344[d]	17.9	*tub2*
F. hieronymi	Poeae	Ruta 42-Desví o a Frías	Catamarca	Fh734[d]	17.4	*tef1*, *tub2*
F. hieronymi	Poeae	Cuesta del Portezuelo	Catamarca	Fh737[d]	n.d.	*tub2*
Festuca magellanica	Poeae	Las Horquetas, Ruta 5	Santa Cruz	Fm353[d]	10.2	*tef1*, *tub2*
F. magellanica	Poeae	Ruta 220 Las Leñas-Valle Hermoso	Mendoza	Fm351[d]	17.2	*tub2*
F. magellanica	Poeae	Ruta 220 Las Leñas-Valle Hermoso	Mendoza	Fm745[d]	10.2	*tub2*
Festuca superba	Poeae	Yala	Jujuy	Fs347	10.4	*tef1*, *tub2*
F. superba	Poeae	Yala	Jujuy	Fs349	11.1	*tef1*, *tub2*
Melica stuckertii	Meliceae	Capilla del Monte	Córdoba	Ms803[d]	19.2	*tef1*, *tub2*
Phleum commutatum	Aveneae	Cabo Domingo	Tierra del Fuego	Phc682	19.5	*tef1*, *tub2*
Ph. commutatum	Aveneae	Ea. María Behety	Tierra del Fuego	Phc755[d]	15.8	*tub2*
Poa huecu	Poeae	Cajón Almanza	Neuquén	Ph2267[d]	11.25	*tub2*
P. huecu	Poeae	Cajón Almanza	Neuquén	Ph1213[d]	11.4	*tef1*
Poa rigidifolia	Poeae	Paso Garibaldi	Tierra del Fuego	Pr641	18.5	*tub2*
P. rigidifolia	Poeae	Río Grande	Tierra del Fuego	Pr345	15.8	*tef1*, *tub2*
P. rigidifolia	Poeae	Río Grande	Tierra del Fuego	Pr198[d]	20.1	*tef1*, *tub2*
P. rigidifolia	Poeae	Lago Yehuin	Tierra del Fuego	Pr642	28.4	*tef1*, *tub2*
P. rigidifolia	Poeae	Cabo Vírgenes	Santa Cruz	Pr219	28.2	*tef1*

[a] For clarity these designations are abbreviated host species followed by BAFC. (Buenos Aires Facultad de Ciencias) isolate collection number. Isolates indicated in bold were apparent non-hybrids.
[b] Average measure (mm) taken from 4 replicates of each isolate at 21 days growth at 23 °C on potato dextrose agar; n.d., not determined.
[c] GenBank Accession Nos. AY707651–AY707746.
[d] *N. tembladerae* isolates.

2-mercaptoethanol). Tubes were covered loosely with plastic food wrap and heated in a microwave oven at full power for a total of 30 s, in bursts of 15, 10, and 5 s to prevent boiling. Immediately, 300 µl of additional lysis buffer was added to each sample tube, which was then incubated at 80 °C for 10 min. Extraction was performed with 200 µl phenol and then with 200 µl chloroform. DNA was precipitated with 0.5 vol isopropanol and 15 µl of 5 M sodium acetate, and pelleted by centrifugation. The DNA was redissolved in 50 µl of sterile water, quantified by fluorometry and agarose-gel electrophoresis, and stored at 5 °C.

Intron-rich portions of the β-tubulin (*tub2*) and translation elongation factor 1-α (*tef1*) genes were amplified by PCR using primers listed in Table 2. Fig. 1 shows the regions of primer annealing. The approximate sizes of the PCR products were 560 and 860 bp for *tub2* and *tef1*, respectively. Reactions were performed in 50 µl volumes containing 10 mM PCR buffer (Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, 125 µM each of dATP, dCTP, dGTP, and dTTP, 200 nM each primer, 0.025 U μl^{-1} AmpliTaq Gold DNA Polymerase (Applied Biosystems), and 10 ng of fungal genomic DNA. Reactions were carried out in a PE Applied Biosystems DNA thermal cycler programmed for an initial incubation at 94 °C (9 min), followed by 40 cycles at 94 °C (1 min), 52 °C (1 min), and 72 °C (2 min); then a final extension reaction at 72 °C (5 min). Amplified products were separated by electrophoresis in 1% agarose gels.

2.3. DNA sequencing

PCR products were purified using a QIAquick PCR purification Kit (Qiagen, Valencia, CA). Purified products were concentrated and quantified by using a 100 bp ladder (Bioline) as standard. Sequencing reactions were performed using a CEQ2000 Dye Terminator Cycle Sequencing Kit (Beckman–Coulter, USA). Both strands of the DNA were sequenced by using the corresponding primers (Table 2 and Fig. 1). Sequences were obtained on a CEQ8000 Genetic Analyzer (Beckman–Coulter) and analyzed using CEQ2000 DNA Analysis software set to detect heterozygotes. Contiguous sequences were assembled by using Phrap on a Red Hat Linux (v.7.1) platform, and with BioEdit.

The presence of two dye-terminator peaks at any single nucleotide position was considered to be the possible result of two different alleles of the gene, characteristic of hybrid endophytes (Moon et al., 2004). Generally, there were indications of several such polymorphisms as well

Table 2
PCR and sequencing primers used for *tub2* and *tef1* sequence analyses

Primer[a]	Primer sequence (5′–3′)	Main application[b]
tub2-exon1d (1d)	GAG AAA ATG CGT GAG ATT GT	A
tub2-exon4u (4u)	TGG TCA ACC AGC TCA GCA CC	A
tub2-sela 110–134 d (A)	CCG CCG AGC CCG GCC ACG AA	SA/A
tub2-selc 110–134 d (B)	CCG CCG AGC CCG GCC ACG AC	SA/A
tub2-sela 614–595 u (C)	AGA AGC CTG TCA CAT AGA TT	SA/A
tub2-selc 614–595 u (D)	AGA AGC CTG TCA CAT GGA TG	SA/A
tub2-exon3d (3d)	CAA ATT GGT GCT GCT TTC TGG	S
tub2-exon3u (3u)	TCG TTG AAG TAG ACA CTC AT	S
tef1-exon1d (1d)	GGG TAA GGA CGA AAA GAC TCA	A
tef1-exon6u (6u)	CGG CAG CGA TAA TCA GGA TAG	A
tef1-selt 126–146 d (a)	CCG AAA TTC ACG TAC TGA CT	SA/A
tef1-sela 126–146 d (b)	CCG AAA TTC ACG TAC TGA CA	SA/A
tef1-selt 881–862 u (e)	GAT GCG CGT TAA TGA TAC AT	SA/A
tef1-selg 881–862 u (f)	GAT GCG CGT TAA TGA TAC AG	SA/A
tef1-selc 918–898 u (c)	TCT TGA TGA AAT CAC GGT GC	SA/A
tef1-sela 918–898 u (d)	TCT TGA TGA AAT CAC GGT GA	SA/A
tef1-selt 403–452 d (g)	TTC GGT CGC GGG GCT CAG TC	S
tef1-sela 403–452 d (g)	TTC GGT CGC GGG GCT CAG TA	S
tef1-selt 522–505 u (h)	GAA TGT CAT GTC ATG TC	S
tef1-sela 527–501 u (h)	AGA GGT GGG CCA CGC GAA TGT ATG	S
tef1-exon4.2d (4.2d)	ATC GAG AAG TTC GAG AAG GT	S
tef1-exon4.2u (4.2u)	TTG CAG CGA GTG AAC ATC GG	S

[a] Primer nomenclature describes gene (*tub2* or *tef1*), gene region, primer number, and orientation (u, upstream and d, downstream). The name of each selective amplification primer used to amplify one of the hybrid gene copies is suffixed "sel," followed by the 3′-terminal nucleotide, then by nucleotide positions (relative to the start codon) where the primer hybridized to the gene sequence, and finally by d (for downstream) or u (for upstream) to indicate the direction of primer extension. Abbreviations in parentheses correspond to labels in Fig. 1.
[b] A, PCR amplification; S, DNA sequencing, and SA, selective amplification.

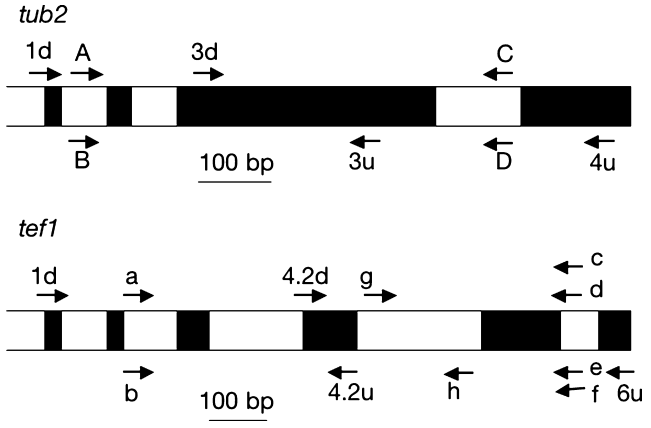

Fig. 1. The amplified and sequenced regions of the two genes *tub2* and *tef1*. White and black boxes indicate introns and coding sequences, respectively. Arrows indicate the amplification and sequencing primer positions and orientations. Primers designated with numbers are those used in amplification and internal sequencing and the others designated with letters are those for selective amplifications (see Table 2).

as indels in the intron sequences. Selective primers incorporating a selective nucleotide at the 3′ end were designed and used to amplify DNA specifically from each allele (Table 2).

2.4. Sequence alignment and phylogenetic analysis

Sequences were aligned using the PILEUP program of the Wisconsin Package (GCG, 1998) and ClustalX (Thompson et al., 1997). Sequences also included in these analyses were from representative *Epichloë* spp. and *Neotyphodium* spp. isolates listed in Table 3. Alignments were checked by eye for ambiguities and adjusted if necessary. Alignment gaps were treated as missing information.

Maximum-likelihood (ML) analysis was conducted in PAUP* 4.0b10 (Swofford, 1998), with a GTR (general time reversible) + I (proportion of invariable sites) + G (gamma distribution of rate substitutions) model, whose parameters were estimated from the routine proposed by Sullivan et al. (1997). For *tub2* and *tef1* sequences, the proportions of sites assumed to be invariable were 0.356553 and 0.381605, and the estimated shape parameters of the gamma distributions were 0.789502 and 4.242062, respectively.

Maximum parsimony (MP) analyses were performed by heuristic search in PAUP* 4.0b10 (Swofford, 1998), in which character states were unordered and unweighted, and trees were built by 100 iterations of random taxon addition with a different number seed for each iteration. To assess the robustness of the topology, 1000 bootstrap replicates were run by MP.

ML analysis of a concatenated *tef1* + *tub2* dataset with estimation of posterior probabilities of nodes was conducted with MrBayes version 3 (Ronquist and Huelsenbeck, 2003), using a GTR + I + G model (lset nst = 6, rates = gamma). Four chains (three heated at temp = 0.2) were run for 450,000 generations, saving one out of every 100 trees (mcmc ngen = 450,000, printfreq = 10,000,

Table 3
Additional isolates represented in phylogenetic analyses[a]

Species	Isolate[b]	Host	Host tribe	Geographic origin[c]
Epichloë amarillans	Agh200744	*Agrostis hyemalis*	Aveneae	NA
E. amarillans	Spo200743	*Sphenopholis obtusata*	Aveneae	NA
E. baconii	Ags76552	*Agrostis stolonifera*	Aveneae	EU
E. baconii	Agt90167	*Agrostis tenuis*	Aveneae	EU
E. baconii	Cv200745	*Calamagrostis villosa*	Aveneae	NA
E. brachyelytri	Bre200752	*Brachyelytrum erectum*	Brachyelytreae	NA
E. brachyelytri	Bre201561	*Br. erectum*	Brachyelytreae	EU
E. bromicola	Be200749	*Bromus erectus*	Bromeae	EU
E. bromicola	Br201558	*Bromus ramosus*	Bromeae	EU
E. clarkii	Hla200742	*Holcus lanatus*	Poeae	EU
E. elymi	Ec201551	*Elymus canadensis*	Triticeae	NA
E. elymi	Ev200850	*Elymus virginicus*	Triticeae	NA
E. festucae	Frc90660	*Festuca rubra* subsp. *commutata*	Poeae	EU
E. festucae	Frr90661	*F. rubra* subsp. *rubra*	Poeae	EU
E. festucae	LgMYA-434	*Lolium giganteum*	Poeae	EU
E. glyceriae	Gs200747	*Glyceria striata*	Meliceae	NA
E. glyceriae	Gs200755	*G. striata*	Meliceae	NA
E. sylvatica	Bps200748	*Brachypodium sylvaticum*	Brachypodieae	EU
E. sylvatica	Bps200751	*Bp. sylvaticum*	Brachypodieae	EU
E. typhina	Ao200738	*Anthoxanthum odoratum*	Aveneae	EU
E. typhina	Bpp200739	*Brachypodium pinnatum*	Brachypodieae	EU
E. typhina	Dg200740	*Dactylis glomerata*	Poeae	EU
E. typhina	Php200851	*Phleum pratense*	Aveneae	EU
E. typhina	Pn201667	*Poa nemoralis*	Poeae	EU
E. typhina	Pn201668	*P. nemoralis*	Poeae	EU
E. typhina	Pp201669	*Poa pratensis*	Poeae	EU
E. typhina	Ps201666	*Poa silvicola*	Poeae	EU
E. typhina	Lpp200736	*Lolium perenne* subsp. *perenne*	Poeae	EU
Neotyphodium aotearoae	EovMYA-1234	*Echinopogon ovatus*	Aveneae	NZ
N. australiense	EovMYA-1195	*E. ovatus*	Aveneae	AU
N. huerfanum	Faz64040	*Festuca arizonica*	Poeae	NA
N. lolii	LppUKY-135	*L. perenne* subsp. *perenne*	Poeae	EU
N. occultans	LprUKY-992	*L. perenne* subsp. *rigidum*	Poeae	EU
Neotyphodium sp.	FobUKY-866	*Festuca obtusa*	Poeae	NA
N. tembladerae	Ph200844	*Poa huecu*	Poeae	SA
N. typhinum	PsyUKY-1097	*Poa sylvestris*	Poeae	NA
N. uncinatum	LprMYA-431	*Lolium pratense*	Poeae	EU

[a] Sequence accession numbers are published in Moon et al. (2004).
[b] Isolates designations are prefixed with host abbreviations followed by a laboratory (UKY) or ATCC accession number.
[c] Geographic origins of symbiota: AU, Australia; EU, Europe; NA, North America; NZ, New Zealand; and SA, South America.

samplefreq = 100, nchains = 4). The first 2000 trees (200,000 generations) were discarded as burn-in, and the consensus tree and posterior support values were determined from the remaining 2500 trees.

3. Results

Almost all endophytes isolated from surface-disinfected plant tissues had *Neotyphodium* morphologies. In addition, three *Acremonium*-like endophytes were consistently isolated from *Bromus auleticus*, but these were not examined further for this study. All of the *Neotyphodium* isolates analyzed by microscopic examination exhibited slow to moderate growth on PDA (Table 1), with colonies white to yellowish in color, and cottony to cerebroid in growth morphology. Phialidic conidia were allantoid, lunate to uncinate and the conidiogenous cells were solitary and arose perpendicularly from the hyphae. These are typical morphological features associated with the genus *Neotyphodium* and the *Neotyphodium* anamorphs of *Epichloë* species (Glenn et al., 1996). Moreover, most of the isolates were similar enough to be considered the same species, and fit the description of *N. tembladerae* (Cabral et al., 1999). Within this species, we could include isolates from *Festuca magellanica* (Fm745, Fm351, and Fm353), *Festuca hieronymi* (Fh344, Fh734, and Fh737), *Festuca argentina* (Fa2203), *Melica stuckertii* (Ms803), *Poa rigidifolia* (Pr198), *Poa huecu* (Ph2267 and Ph1213), *Phleum commutatum* (Phc755), *Bromus setifolius* (Bs532, Bs649, and Bs713), and *Bromus auleticus* (Ba355). However, we found some isolates with differences when compared with *N. tembladerae*. For example, Pr345, Fs347, Fs349, and Ba308, among others, had white colonies but grew slowly and lacked abundant aerial mycelium. Their colonies presented a brain-like

and waxy morphology. Another isolate, Phc682, presented a yellowish colony with irregular edges and crystals on the surface.

None of the isolates was sterile, but in some cases we observed very few conidia (isolates Bs420, Bs508, and Bs719), whereas in others (Ms803, Bs532, Bs713, and Fm353) the conidia were abundant. For all, conidium morphology was consistent with the description of the genus *Neotyphodium* (Glenn et al., 1996), varying from lunate to reniform. One isolate (Ba308) had a particular conidium morphology similar to the description of *N. uncinatum*, but apparently a different conidium ontogeny (Iannone, 2003). The conidia of all Argentine isolates were larger than previously reported for the sexual species of the genus *Epichloë*, varying from 6 to 10 μm long × 3 to 4 μm wide. The dimensions of the conidiogenous cells were variable between isolates. Some were thin and long (Pr642 and Bs719), whereas others were short with protuberances (Fm351 and Fsu349).

PCR amplification of *tub2* and *tef1* genes yielded products of the expected size ranges for each *Neotyphodium* isolate. Direct sequencing of these products most often revealed that more than one template was present, because haplotype polymorphisms, including both nucleotide substitutions and indels, were evident from the chromatographs. Therefore, fragments from individual alleles were amplified separately by use of selective primers (Table 2) in the PCRs. Sequences of the products indicated that all isolates with morphological similarities to *N. tembladerae* had two alleles, with relationships similar to those of the *N. tembladerae* ex type isolate (Moon et al., 2002). Only the endophyte isolates Bs420, Bs508 and Bs719, all from *Bromus setifolius* plants in the Patagonia region (Santa Cruz province), appeared to have single alleles for *tub2* and *tef1*.

Aligned *tub2* sequences were ≈600 characters, 105 of which were variable, and 63 of these were parsimony-informative. The ≈850-character long *tef1* sequence alignment included 173 variable sites, of which 101 were parsimony-informative. Maximum-likelihood (ML) trees inferred from the aligned *tub2* and *tef1* data sets were largely congruent (Figs. 2 and 3). Most of the haplotypes from Argentine isolates fell into either of two main clades, one associated with *Epichloë festucae*, and the other with *E. typhina*.

All isolates had a haplotype in the *E. typhina* clade, and all except four isolates had a haplotype in the *E. festucae* clade (Figs. 2 and 3). Those lacking an *E. festucae*-related haplotype were the aforementioned isolates Bs420, Bs508, and Bs719 from *Bromus setifolius*, and Phc682 from *Ph. commutatum*. The latter had, in addition to the *E. typhina*-related haplotype, a *tub2* haplotype that grouped together with the sequence from an *E. baconii* isolate (Fig. 2). Isolate Phc682 also had a second *tef1* haplotype, which was placed in a basal position in the *Epichloë amarillans* clade (Fig. 3).

Maximum parsimony (MP) and maximum-likelihood (ML) trees inferred from both, *tub2* and *tef1*, data sets were congruent, although MP bootstrap trees poorly resolved phylogenetic relationships among the *E. festucae*-derived as well as *E. typhina*-derived haplotypes of the South American endophytes (Figs. 2 and 3). This was as expected because such branches involved very few polymorphic sites. Nevertheless several well-supported branches separated the *E. typhina* and *E. festucae* clades that included haplotypes from Argentine hybrids.

In both the *tub2* and *tef1* trees there was greater sequence variability in the *E. typhina* clade than in the *E. festucae* clade (Figs. 2 and 3). In addition, the relationships among members of the *E. typhina* clade were better resolved in the *tef1* tree than in the *tub2* tree. In each tree a subclade included sequences from the ex type isolate of *N. tembladerae* and several other isolates, all of which fit the morphological description of *N. tembladerae*. Identical genotypes were discerned for *N. tembladerae* Fa2203, Fh344, Fm353, Ph2267, Pr198, Phc755, Ba355, Bs713, and Ms803, among others, from host tribes Poeae, Aveneae, Bromeae, and Meliceae. In contrast, the *E. typhina*-related haplotypes of isolate Ba308 from *Bromus auleticus*, isolates Fs347 and Fs349 from *Festuca superba*, and isolates Pr345, Pr641, and Pr642 from *Poa rigidifolia* were not included in this subclade.

Non-hybrid endophytes appeared to be much rarer and had a much more restricted distribution among hosts and regions than the hybrids. Apparent non-hybrids were identified in only a single host species, *Bromus setifolius* (although three hybrid isolates were also obtained from this host). Two of the non-hybrids (Bs420 and Bs719) were obtained from two populations 20 km apart from each other and both approximately 300 km from the population from which the third non-hybrid (Bs508) was isolated, all in Patagonia. Taking into account the non-hybrid genotype of three isolates from *B. setifolius*, their fast growth in culture (Table 1), and their grouping in a subclade nested within the *E. typhina* clade, we consider these isolates as belonging to the species *N. typhinum*. A further evaluation is needed to assess whether they should be distinguished as a variety of this species.

The *tub2* and *tef1* datasets were concatenated and analyzed by MP bootstrap as well as by ML implemented in MrBayes (Fig. 4). Sequences from Phc682 were omitted from the concatenated data set because its *E. amarillans*-related *tef1* allele and *E. baconii*-related *tub2* allele may have been derived from different ancestors. Because *E. typhina* and *E. festucae* are European species, but are closely related (apparently ancestral) to asexual endophytes elsewhere, the dataset was supplemented with sequences from the following North American isolates: *N. typhinum* isolates PaUKY-187 from *Poa ampla* and PsyUKY-1097 from *Poa sylvestris*, *Neotyphodium huerfanum* isolate Faz64040 from *Festuca arizo-*

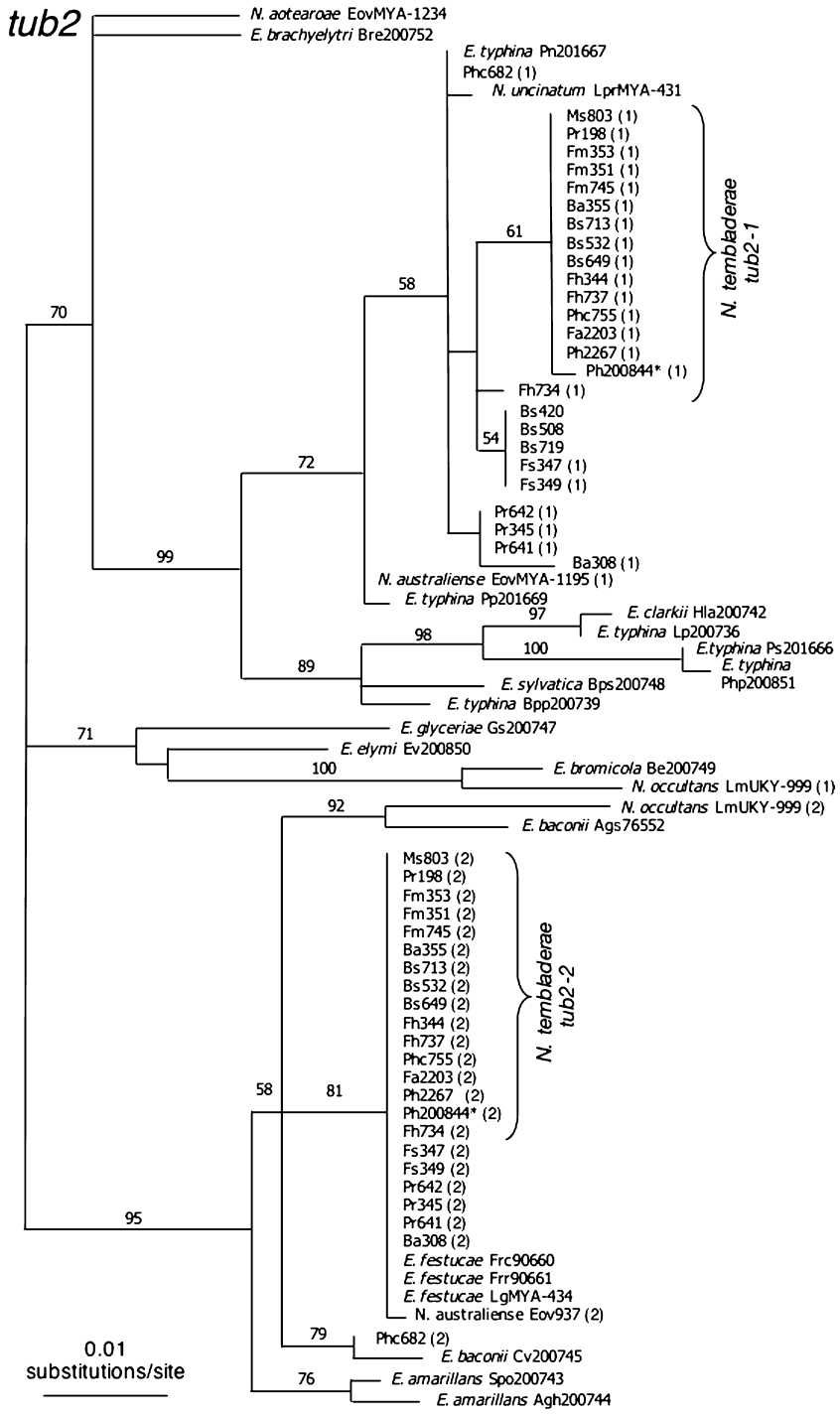

Fig. 2. Maximum-likelihood tree of *Epichloë*/*Neotyphodium* haplotypes based on *tub2* gene sequences. Model of character evolution was general time reversible (GTR), G and I. Argentinean host designations are as follows: Fm (*Festuca magellanica*), Fh (*Festuca hieronymi*), Fa (*Festuca argentina*), Fs (*Festuca superba*), Bs (*Bromus setifolius*), Ba (*Bromus auleticus*), Ph (*Poa huecu*), Pr (*Poa rigidifolia*), Phc (*Phleum commutatum*), and Ms (*Melica stuckertii*). Numbers following these host abbreviations refer to the BAFC (Buenos Aires Facultad de Ciencias Exactas y. Naturales) accession numbers. Other endophytes are designated as in Table 3. Whenever an isolate had two haplotypes, the haplotypes are numbered in parentheses after each isolate designation. Asterisks (∗) indicate haplotypes from the *N. tembladerae* ex type. The tree is midpoint-rooted and MP bootstrap percentages (>50%) are indicated on the branches.

nica, and an *E. festucae*-related isolate (FobUKY-866) from *Festuca obtusa* (Moon et al., 2004). Also included were additional sequences from several *Epichloë* species plus the non-hybrid European species *N. lolii*. Inclusion of these additional sequences caused no change in tree topology and had little effect on branch support. As was the case for individual gene trees, MP bootstrap analysis of the concatenated data indicated strong support for

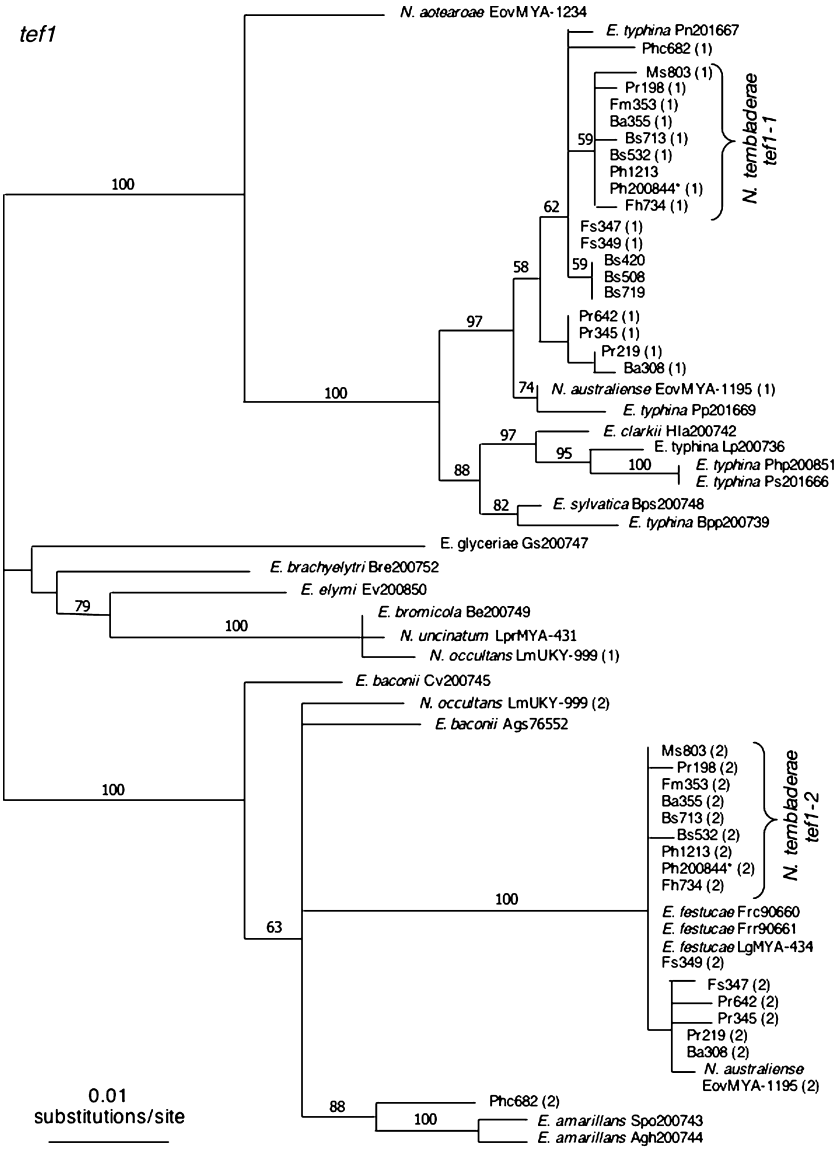

Fig. 3. Maximum-likelihood tree of *Epichloë*/*Neotyphodium* haplotypes based on *tefI* gene sequences. Model of character evolution was general time reversible (GTR), G and I. Isolate numbers, hosts, and haplotype designations are as in Fig. 2. Asterisks (∗) indicate haplotypes from the *N. tembladerae* ex type. The tree is midpoint rooted and MP bootstrap percentages (>50%) are indicated on the branches.

most branches separating major clades and different *Epichloë* species, but weak to moderate support for branches within each of the two clades of Argentine haplotypes. Bayesian posterior probabilities were usually high for most clades, including the clade of *E. typhina*-related haplotypes in *N. tembladerae* isolates ($p = 0.99$). This clade was contained within a larger clade that included *E. typhina*-related haplotypes from other Argentine isolates, *E. typhina* from the European host *Poa nemoralis*, and some non-hybrid isolates from North American grasses (*N. huerfanum* from *Festuca arizonica* and an *N. typhinum* from *Poa ampla*). Likewise, the *E. festucae* genotypes were closely related to each other, asexual relatives in Europe (*N. lolii*) and North America (FobUKY-866), and the *E. festucae*-related haplotypes of most Argentine hybrids.

4. Discussion

We have performed phylogenetic analysis of *tub2* and *tef1* sequences to infer the genomic constitution and elucidate the evolutionary origins of the endophytes from 10 different host species from Argentina. Phylogenies derived from both genes support hybrid origins from *E. festucae* and *E. typhina* of the majority of the isolates in this study. Out of the 27 Argentine isolates, 23 could be characterized as *E. typhina* × *E. festucae* hybrids. These hybrids had two haplotypes each of *tub2* and *tef1*, whereby one haplotype grouped with *E. typhina* and the other with *E. festucae*. Such groupings were strongly supported by MP bootstrap and Bayesian posterior probabilities. The distribution of different endophyte genotypes was unprecedented in that there were hybrids

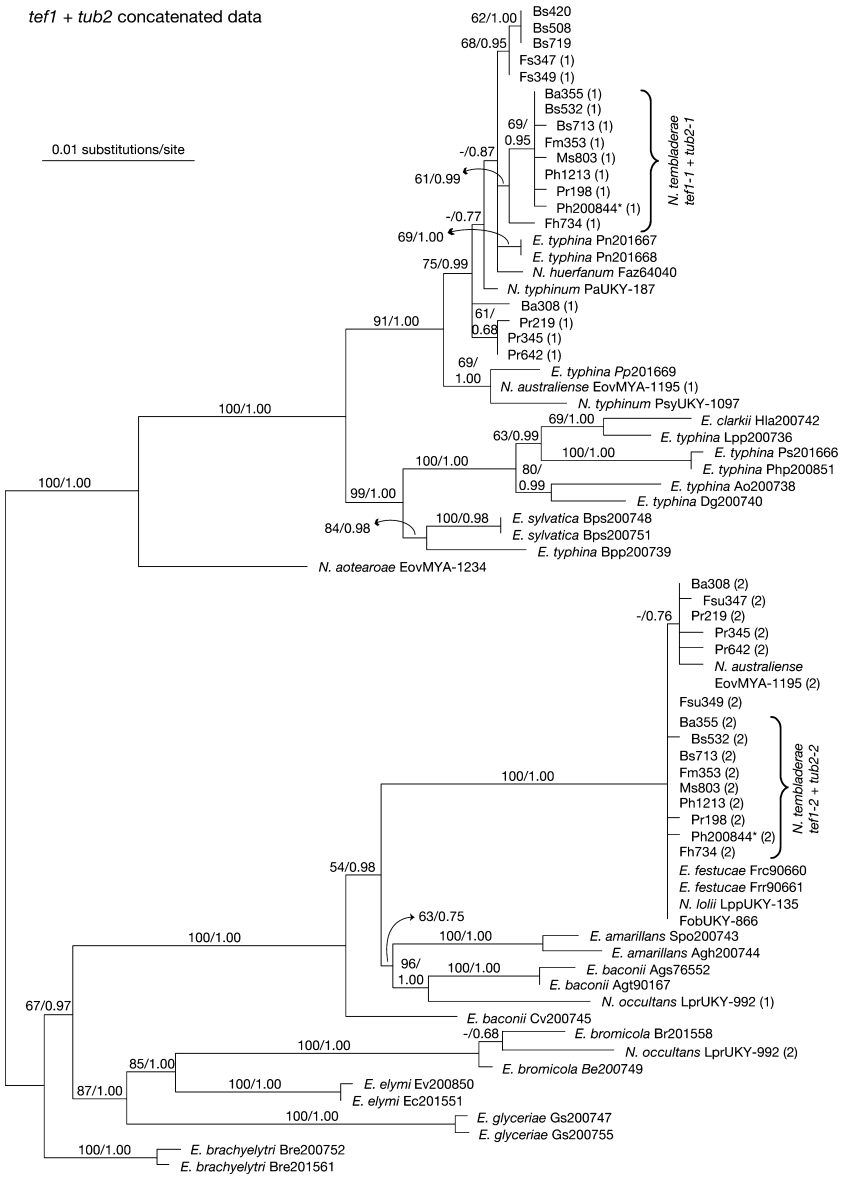

Fig. 4. Maximum-likelihood consensus tree inferred by MrBayes 3.0 on concatenated *tub2* and *tef1* sequences. Numbers on branches are bootstrap support (MP search; dashes indicate <50%) followed by Bayesian posterior probability values. Ln likelihood = −4491.52205. Estimated base frequencies: A, 0.253419; C, 0.263753; G, 0.227499; and T, 0.255329. Estimated value of gamma shape parameter = 0.373177.

from different grass tribes with genotypes that were indistinguishable from one another. This was particularly evident for isolates referable to *N. tembladerae* based on morphological and phylogenetic criteria. Although there was variation particularly among the *E. typhina*-derived haplotypes, the *N. tembladerae* haplotypes tended to group together with the sole exception of Fh734 in the *tub2* tree. Isolates of *N. tembladerae* were identified in host tribes Poeae (*Festuca* and *Poa* spp.), Aveneae (*Ph. commutatum*), Bromeae (*Bromus* spp.), and Meliceae (*Melica stuckertii*). These tribes nearly span the phylogenetic diversity of the grass subfamily Poöideae (Catalán et al., 1997). Nevertheless, Argentine endophytes exhibited considerable diversity, considering polymorphisms among *E. typhina* × *E. festucae* hybrids, the identification of an isolate from *Ph. commutatum* with a different hybrid origin (discussed below), and the identification as well of three isolates from *Bromus setifolius* (Bs420, Bs508, and Bs719) that were apparently non-hybrid.

One of the isolates from *Ph. commutatum* (Phc755) had the *N. tembladerae* genotype, but the other (Phc682) had a unique genotype that indicated a distinct hybrid origin. Isolate Phc682 had *E. typhina*-related *tub2* and *tef1* haplotypes, but also possessed a second haplotype of each gene that, unlike *N. tembladerae*, was well outside the *E. festucae* clade. The placement of the second *tub2* haplotype and the second *tef1* haplotype did not precisely correspond, with the former associated with an *E. baconii* isolate, and the latter grouping together with

the *E. amarillans* clade. Given the close relationship between *E. amarillans* and *E. baconii*, it is not possible to establish whether this isolate had contributions from distinct *E. baconii*- and *E. amarillans*-related ancestors. The alternative is that one of its ancestors may have had these particular *tub2* and *tef1* haplotypes. Nevertheless, three-ancestor hybrids have a precedent among *Neotyphodium* species. Each of the endophytes *N. coenophialum* (Tsai et al., 1994) and *N. chisosum* (Moon et al., 2004) clearly exhibit genomic contributions from three different *Epichloë* spp. The implication of one or two ancestors related to *E. amarillans* and *E. baconii* is of interest on consideration of host and geographical relationships. Both of these *Epichloë* species are associated with host genera in tribe Aveneae (only *E. typhina* is also found in this host tribe), and *Ph. commutatum* is a member of the same tribe. Whereas *E. amarillans* has been found only in North America, *E. baconii* appears to be restricted to Europe. Conceivably, this component of the Phc682 genome may have been derived from an ancestral relationship with a common ancestor to *E. amarillans* and *E. baconii*, possibly established on another continent, then followed by migration and eventual hybridization with *E. typhina*.

In this work we isolated each strain from a single tiller, then single-spore isolated three times, thereby excluding the possibility of co-cultivation and simultaneous DNA purification from different endophytes. Also, nuclear staining of a large number of *Epichloë* spp. and *Neotyphodium* spp. isolates (including the ex type *N. tembladerae* isolate Ph200844) has consistently indicated that the conidia are uninucleate (Moon et al., 2004). Therefore, these single-spore isolates almost certainly represent hybrid nuclear rather than heterokaryon genotypes.

The discovery of many hybrid *Neotyphodium* species from diverse grasses in a single geographic area is novel. It seems that hybridization has been a common mechanism during the evolution of these endophytes. The frequent occurrence of hybrid asexual endophytes in many different grass hosts suggests a positive selective pressure favoring the hybrids. One possibility is that hybridization plays a role similar to sexual recombination, enhancing genetic variability needed for adaptive evolution, or perhaps masking accumulated deleterious mutations (Moon et al., 2004). Alternatively, as Rice (2002) points out, the fittest class of individuals may be subjected to an ever-decreasing representation in the population unless replenished by a process such as sexual recombination. Without a sexual option for these endophytes, the major contribution to the fittest class may instead be via a parasexual process, and even interspecific hybridization.

It is interesting to note that, even though 24 out of 27 isolates in this study were hybrids, and there was considerable sequence variation among the *E. typhina*-derived haplotypes, there was never any evidence of *E. typhina* × *E. typhina* diploid recombinant. Such an endophyte should have been easy to detect in this and previous studies, where the majority of interspecific hybrids have *E. typhina* as one of the ancestors (Moon et al., 2004). Up to now no intraspecific diploidized strain has been found. Perhaps such hybrids would tend to undergo sexual recombination and haploidize. However, the *Epichloë* sexual cycle first requires formation of a distinct ectophytic structure—the stroma—which is completely absent from hybrids and even some non-hybrids such as *N. huerfanum* and *N. lolii*. Alternatively, intraspecific parasexual hybridization may be followed by chromosome losses leading to haploidization, whereas for unknown reasons the interspecific hybrids tend to remain diploid or partially diploid. If this is the case, such intraspecific hybridization may be common (and may help compensate for fitness erosion in asexual lineages), yet remains undetected due to haploidization.

Previous studies have suggested that spore sizes may indicate ploidy. To date, all *Neotyphodium* spp. hybrids have exhibited conidium sizes larger than those of the haploid *Epichloë* spp. and non-hybrid *Neotyphodium* spp. (C.D. Moon, K.D. Craven, and C.L. Schardl, unpublished data; Kuldau et al., 1999). Conidium sizes for the isolates from Argentine grasses fell within the ranges seen for other hybrid *Neotyphodium* spp. and were larger than those of *Epichloë* spp. (A. Gentile and D. Cabral, unpublished data). Interestingly, the apparently non-hybrid Argentine isolates had spore sizes within the range of the hybrids. Either spore sizes are a poorer indication of ploidy than expected from past observations, or the ploidy or genome size of Bs420, Bs508, and Bs719 is greater than was evident from our analysis of *tub2* and *tef1* relationships.

The wide distribution among diverse hosts of phylogenetically related asexual endophytes is surprising because contagious spread involves meiotically derived spores (ascospores) (Brem and Leuchtmann, 1999; Chung and Schardl, 1997), infection of host plants with asexual propagules (conidia and mycelia) is very difficult and unlikely to occur in nature (Latch and Christensen, 1985; Schardl, 1996), and experimental studies have indicated that most *Neotyphodium* sp. isolates are host-specific (Christensen, 1995). The observation that diverse grasses harbor endophytes with similar hybrid genotypes could reflect independent colonization events of South American grasses from multiple lineages of related sexual endophytes. The sequence diversity of the *E. typhina*-derived haplotypes suggests that this is the case. However, there has been no sexual *Epichloë* species identified in South America to permit an evaluation of this hypothesis. We can speculate on several scenarios by which these *Neotyphodium* hybrids may have evolved and distributed among their diverse hosts in the region. If we assume that *E. typhina* and *E. festucae* were never

present in South America, the hybrids might have originated in the Northern Hemisphere, then migrated southward within their hosts. Another possibility is that sexual forms were distributed in South America some time in the past but have since gone extinct. An alternative scenario assumes that the ancestral sexual species are indeed present in South America, remaining to be discovered. There may be scarce and restricted areas where the sexual species occur and hybridize, and the hybrids may then spread over a wider geographical area. In this respect, it is worth noting that one of the North American *N. typhinum* isolates, PaUKY-187, produces stromata on its host. There are no reports that these stromata can complete the sexual cycle, but perhaps in the location from which it was collected (the Yukon Valley), the opposite mating type may be present and the *Poa ampla* endophyte may indeed be sexual. Similarly, in South America it is conceivable that the sexual species have a more restricted range than the asexual species, either because opposite mating types occur together in fewer locations, or because of restricted distribution of their "pollinators." For example, it is known that *Epichloë* spp. in the Northern Hemisphere depend upon *Botanophila* spp. flies for spermatization (Bultman et al., 1998; Kohlmeyer and Kohlmeyer, 1974). If these flies are distributed in restricted areas, the sexual *Epichloë* species can only persist in those areas. Other environmental variables might also limit sexual expression. Asexual endophytes, being strictly and efficiently transmitted vertically via seeds, should not be excluded by such factors from any habitat of their hosts.

Finally, we consider the possibility that the asexual endophytes might be horizontally transmitted. The discovery of *Neotyphodium* conidia production over the epiphyllous plane of some grasses (White et al., 1996) raises the possibility that these fungi could be horizontally spread from plant to plant via conidia produced on the leaf surface. However, this appears to be a very rare characteristic, which we have seen only on the phylloplane of one plant of *Poa rigidifolia*. Furthermore, experimental introduction of endophytes into host grasses is notoriously difficult and requires invasive techniques (Latch and Christensen, 1985). Nevertheless, more extensive population analyses and experimental inoculations with the Argentine isolates would be warranted to investigate the possibility of horizontal transmission of *N. tembladerae* and related endophytes.

Hybrid epichloë endophytes have likely originated through parasexual anastomosis between *Epichloë*/*Neotyphodium* species (Moon et al., 2004; Schardl et al., 1994; Tsai et al., 1994), requiring that the two ancestral endophyte species occupy the same host at the same time. However, a common observation is that, when a grass host is infected with two different epichloë endophyte strains, the strains rapidly segregate among tillers (Christensen et al., 2000; Meijer and Leuchtmann, 1999; Wille et al., 1999). Therefore, hybridization is likely to be a rare event. Yet the ubiquitous distribution of hybrid endophytes among the Argentine grasses suggests that such hybrids may have a selective advantage in environments typical of the region. Furthermore, since the maintenance of symbionts that rely on vertical transmission depends on fitness of their hosts (Ewald, 1987), it seems likely that the hosts often benefit more from harboring hybrid compared to non-hybrid endophytes. If so, there are various possible reasons for such an advantage. Hybridization clearly was crucial during distribution of fungal endophytes among locations and hosts, and might have thereby facilitated co-evolution, optimizing compatibility with their hosts. Each mutualistic grass–endophyte symbiosis appears to require close co-adaptation of host and endophyte. This is evident when endophytes are moved between host species, resulting in highly unstable associations or causing defensive or necrotic responses by the plants, in stark contrast to the benign interactions of natural associations (Christensen, 1995). Although it has not been proven for most host grasses that endophyte infections are positively selected, the numerous documented benefits to grasses of epichloë endophytes suggest that this may be the case. Even if such benefits are not realized in all environments or at all times, the extreme stability and efficient vertical transmission of the endophytes assure their persistence in nature (Saikkonen et al., 2002). Given the evolutionary processes typifying the grasses (often including interspecific hybridization), endophytes must also evolve. The possible role of hybridization in evolution and host interactions of other plant-associated fungi (mainly pathogens) has recently been reviewed (Schardl and Craven, 2003). Thus, hybridization may be key to both the ubiquity and the remarkable co-adaptation of epichloë endophyte–grass symbioses.

Acknowledgments

This research was supported by CONICET and Universidad de Buenos Aires grants, PRHIDEB-CONICET (Publication No. 159), and by the Harry E. Wheeler Endowment to the University of Kentucky. A.G. held a fellowship from the Universidad de Buenos Aires. D.C. and M.S.R. are members of the Carrera del Investigador Científico, CONICET, Argentina. Sequence analysis was conducted at the University of Kentucky Advanced Genetic Technologies Center, supported by USDA-CSREES Grants 2000-34431-8975 and 2001-34457-10343, and USDA-NRI Grant 2002-35311-11716. We thank A. Leuchtmann for supplying cultures from Switzerland and elsewhere in Europe. We are grateful to A.D. Byrd, W. Hollin, K.G. Lindstrom, M.V. Novas, and C. Slamovits for able assistance.

References

Arechavaleta, M., Bacon, C.W., Hoveland, C.S., Radcliffe, D.E., 1989. Effect of tall fescue endophyte on plant response to environmental stress. Agron. J. 81, 83–90.

Bacon, C.W., Porter, J.K., Robbins, J.D., Lutrell, E.S., 1977. *Epichloë typhina* from toxic tall fescue grasses. Appl. Environ. Microbiol. 34, 576–581.

Bacon, C.W., Philip, C.L., Porter, J.K., Robbins, J.D., 1986. Ergot toxicity from endophyte-infected grasses: A review. Agron. J. 78, 106–116.

Bertoni, M.D., Cabral, D., Romero, N., 1993. Endofitos fúngicos en especies sudamericanas de *Festuca* (Poaceae). Boletín de la Asociación Argentina de Botánica 2, 25–34.

Brem, D., Leuchtmann, A., 1999. High prevalence of horizontal transmission of the fungal endophyte *Epichloë sylvatica*. Bull. Geobot. Inst. ETH 65, 3–12.

Bultman, T.L., White Jr., J.F., Bowdish, T.I., Welch, A.M., 1998. A new kind of mutualism between fungi and insects. Mycol. Res. 102, 235–238.

Cabral, D., Bertoni, M.D., 1991. Una nueva especie de *Acremonium*: endofito de *Festuca argentina*. Resúmenes de las Jornadas Argentinas de Botánica.

Cabral, D., Lugo, M.A., 1993. Los endofitos de *Festuca hieronymi* y *Poa huecu*: dos plantas autóctonas tóxicas para el ganado. VI Congreso de Micología y XVI Jornadas Argentinas de Micología. 20–23 Octubre. Poster num. 1091.

Cabral, D., Lugo, M.A., 1994. Los endofitos fúngicos en gramíneas de Argentina. VI Congreso Latinoamericano de Botánica. 2–8 Octubre. Libro de resumenes, p. 106.

Cabral, D., Cafaro, M.J., Saidman, B.O., Lugo, M.A., Reddy, P.V., White Jr., J.F., 1999. Evidence supporting the occurrence of a new species of endophyte in some South American grasses. Mycologia 91, 315–325.

Carroll, G., 1988. Fungal endophytes in stems and leaves: From latent pathogen to mutualistic symbiont. Ecology 69, 2–9.

Catalán, P., Kellogg, E.A., Olmstead, R.G., 1997. Phylogeny of Poaceae subfamily Pooideae based on chloroplast ndhF gene sequences. Mol. Phylogenet. Evol. 8, 150–166.

Christensen, M.J., 1995. Variation in the ability of *Acremonium* endophytes of *Lolium perenne*, *Festuca arundinacea* and *F. pratensis* to form compatible associations in the three grasses. Mycol. Res. 99, 466–470.

Christensen, M.J., Simpson, W.R., Samarrai, T., 2000. Infection of tall fescue and perennial ryegrass plants by combinations of different *Neotyphodium* endophytes. Mycol. Res. 104, 974–978.

Chung, K.R., Schardl, C.L., 1997. Sexual cycle and horizontal transmission of the grass symbiont, *Epichloë typhina*. Mycol. Res. 101, 295–301.

Clark, E.H., White Jr., J.F., Patterson, R.M., 1983. Improved histochemical techniques for the detection of *A. coenophialum* in tall fescue and methods of in vitro culture of the fungus. J. Microbiol. Meth. 1, 149–155.

Clay, K., Schardl, C.L., 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am. Nat. 160, 99–127.

Craven, K.D., Blankenship, J.D., Leuchtmann, A., Hignight, K., Schardl, C.L., 2001. Hybrid fungal endophytes symbiotic with the grass *Lolium pratense*. Sydowia 53, 44–73.

Ewald, P.W., 1987. Transmission modes and evolution of the parasitism–mutualism continuum. Ann. NY Acad. Sci. 503, 295–306.

Freeman, E.M., 1904. The seed fungus of *Lolium temulentum* L., the darnel. Philos. Trans. R. Soc. Lond. B 214, 1–28.

GCG, 1998. Wisconsin Package, version 10.0. Genetics Computer Group (GCG), Madison, Wisconsin.

Glenn, A.E., Bacon, C.W., Price, R., Hanlin, R.T., 1996. Molecular phylogeny of *Acremonium* and its taxonomic implications. Mycologia 88, 369–383.

Goodwin, D.C., Lee, S.B., 1993. Microwave miniprep of total genomic DNA from fungi, plants, protists and animals for PCR. BioTechniques 15, 438–444.

Iannone, L.J., 2003. Caracterización del endofito de *Bromus auleticus* y sus efectos sobre la biología del hospedante. Tesis de Licenciatura. FCEyN, UBA.

Kohlmeyer, J., Kohlmeyer, E., 1974. Distribution of *Epichloë typhina* (Ascomycetes) and its parasitic fly. Mycologia 66, 77–86.

Kuldau, G.A., Tsai, H.F., Schardl, C.L., 1999. Genome sizes of *Epichloë* species and anamorphic hybrids. Mycologia 91, 776–782.

Latch, G.C.M., Christensen, M.J., 1985. Artificial infections of grasses with endophytes. Ann. Appl. Biol. 107, 17–24.

Malinowski, D.P., Belesky, D.P., 2000. Adaptations of endophyte-infected cool-season grasses to environmental stresses: Mechanisms of drought and mineral stress tolerance. Crop Sci. 40, 923–940.

Meijer, G., Leuchtmann, A., 1999. Multistrain infections of the grass *Brachypodium sylvaticum* by its fungal endophyte *Epichloë sylvatica*. New Phytol. 141, 355–368.

Moon, C.D., Miles, C.O., Jarlfors, U., Schardl, C.L., 2002. The evolutionary origins of three new *Neotyphodium* endophyte species from grasses indigenous to the Southern Hemisphere. Mycologia 94, 694–711.

Moon, C.D., Craven, K.D., Leuchtmann, A., Clement, S.L., Schardl, C.L., 2004. Prevalence of interspecific hybrids amongst asexual fungal endophytes of grasses. Mol. Ecol. 13, 1455–1467.

Raisbeck, M.F., Rottinghaus, G.E., Kendall, J.D., 1991. Effects of naturally occurring mycotoxins on ruminants. In: Smith, E.R., Henderson, S. (Eds.), Mycotoxins and Animal Foods. CRC Press, Boca Raton, FL, pp. 647–677.

Rice, W.R., 2002. Experimental tests of the adaptive significance of sexual recombination. Nat. Rev. Genet. 3, 241–251.

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

Romero, A.I., Minter, D.W., 1988. Fluorescence microscopy: An aid to the elucidation of Ascomycete structures. Trans. Br. Mycol. Soc. 90, 457–470.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Saikkonen, K., Ion, D., Gyllenberg, M., 2002. The persistence of vertically transmitted fungi in grass metapopulations. Proc. R. Soc. Lond. B 269, 1397–1403.

Schardl, C.L., 1996. *Epichloë* species: Fungal symbionts of grasses. Annu. Rev. Phytopathol. 34, 109–130.

Schardl, C.L., Craven, K.D., 2003. Interspecific hybridization in plant-associated fungi and oomycetes: A review. Mol. Ecol. 12, 2861–2873.

Schardl, C.L., Leuchtmann, A., Spiering, M.J., 2004. Symbioses of grasses with seedborne fungal endophytes. Annu. Rev. Plant Biol. 55, 315–340.

Schardl, C.L., Leuchtmann, A., Tsai, H.F., Collett, M.A., Watt, D.M., Scott, D.B., 1994. Origin of a fungal symbiont of perennial ryegrass by interspecific hybridization of a mutualist with the ryegrass choke pathogen, *Epichloë typhina*. Genetics 136, 1307–1317.

Sullivan, J., Markert, J.A., Kilpatrick, C.W., 1997. Phylogeography and molecular systematics of the *Peromyscus aztecus* species group (Rodentia: Muridae) inferred using parsimony and likelihood. Syst. Biol. 46, 426–440.

Swofford, D.L., 1998. PAUP*. Phylogenetic analysis using parsimony (* and another methods). Sinauer Associates, Sunderland, MA.

Thompson, J.D., Gibson, T.J., Pleuniak, F., Janemougin, E., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 24, 4876–4882.

Tsai, H.F., Liu, J.S., Staben, C., Christensen, M.J., Latch, G.C.M., Siegel, M.R., Schardl, C.L., 1994. Evolutionary diversification of fun-

gal endophytes of tall fescue grass by hybridization with *Epichloë* species. Proc. Natl. Acad. Sci. USA 91, 2542–2546.

White Jr., J.F., Martin, T.I., Cabral, D., 1996. Endophyte–host associations in grasses. XXIV. Conidia formation by *Acremonium* endophytes on the phylloplanes of *Agrostis hiemalis* and *Poa rigidifolia*. Mycologia 88, 174–178.

White Jr., J.F., Sullivan, R.F., Balady, G.A., Gianfagna, T.J., Yue, Q., Meyer, W.A., Cabral, D., 2001. A fungal endosymbiont of the grass *Bromus setifolius*: Distribution in some populations, identification and examination of beneficial properties. Symbiosis 31, 241–257.

Wille, P.A., Aeschbacher, R.A., Boller, T., 1999. Distribution of fungal endophyte genotypes in doubly infected host grasses. Plant J. 18, 349–358.