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Species diversity of *Epichloë* symbiotic with two grasses from southern Argentinean Patagonia

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Abstract: In this work we performed morphological and molecular phylogenetic analyses (based on sequences of calmodulin M [*calM*], translation-elongation factor 1-α [*tefA*] and β-tubulin [*tubB*] genes) to characterize the diversity of *Epichloë* endophytes in *Bromus setifolius* and *Phleum alpinum*. The phylogenies obtained from the three genes were congruent and allowed differentiation of three lineages of endophytes that also presented morphological differences. One lineage corresponds to the previously described species *Epichloë tembladerae*, which is present in a wide range of native grasses from Argentina including *B. setifolius* and *P. alpinum*. Another genotype isolated only from *B. setifolius* is a non-hybrid endophyte, a rare condition for the South American *Epichloë* endophytes. Isolates of this genotype, described herein as a new variety, *Epichloë typhina* var. *aonikenkana*, presented waxy colonies at maturity and a low production of conidia. The third lineage, exclusively found in isolates from *P. alpinum*, is a hybrid between *E. typhina* and a common ancestor of *E. amarillans* and *E. baconii*. Isolates of this lineage produce abundant conidia that are variable in shape and size. Based on its unique phylogenetic position and morphology, we propose the new species, *Epichloë cabralii* for this lineage. The new combinations *Epichloë tembladerae* and *E. pampeana* also are proposed for the previously described *Neotyphodium tembladerae* and *Neotyphodium pampeanum* species.

Key words: *Bromus setifolius*, endophyte diversity, *Epichloë*, *Neotyphodium*, *Phleum alpinum*, phylogeny

Introduction

Species of *Epichloë* (Fr.) Tul. & C. Tul. (Hypocreales, Clavicipitaceae), whose asexual relatives formerly were classified in the anamorph genus *Neotyphodium*, are endophytic fungal symbionts of many cool-season grasses of the subfamily Pooideae (Schardl 2010). These endophytes grow asymptomatically in the apoplast of the aerial tissues of the host plants, but sexual species may produce stromata that choke the inflorescences, causing total or partial sterility of the host (choke disease) (Schardl 2010). On these stromata, spermatia are produced and eventually *Botanophila* fly species transport spermatia between stromata of opposite mating types (Bultman and Leuchtmann 2003). Perithecia with ascospores then are produced, and the ascospores are responsible for the contagious dispersal to other plants (Chung and Schardl 1997). The asexual and some of the sexual *Epichloë* species colonize the ovaries of developing flowers and are disseminated with the seed of the host (Philipson and Christey 1986). When seeds germinate the mycelium colonizes the meristems of the daughter plant (vertical transmission). Epiphyllous growth and production of conidia have been reported on several hosts (White et al. 1996, Craven et al. 2001, Moon et al. 2002, Tadych and White 2007). Tadych et al. (2012) demonstrated that under experimental conditions, hyphae derived from conidia of *Epichloë poae* Tadych, Ambrose, Belanger & White, which we consider to be a synonym of *E. typhina*, are able to infect endophyte-free seedlings of *Poa secunda*, suggesting that horizontal transmission of asexual endophytes could be a natural infection mechanism. However, other asexual species, such as *Neotyphodium*

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lolii and *N. coenophialum*, appear incapable of infecting plants in this way and therefore may be strictly transmitted vertically (Latch and Christensen 1985).

Due to importance of grasses in the agroecosystems, these symbioses have been well studied. Endophyte-infected grasses present such advantageous characteristics as increased growth (Iannone and Cabral 2006, Iannone et al. 2012b but see Faeth et al. 2004) and enhanced resistance to biotic and abiotic stresses (Malinowski and Belesky 2000, Clay and Schardl 2002). Infected plants also are protected against insect and vertebrate herbivores (Raisbeck et al. 1991, Bush et al. 1997, Schardl et al. 2006, Schardl et al. 2007) because several classes of alkaloids, namely, lolines, peramine, indole-diterpenes and ergot alkaloids, are produced by the *Epichloë* spp. (Schardl et al. 2004). Alkaloid profiles vary widely among species (Young et al. 2009, Schardl et al. 2012).

Most of the asexual *Epichloë* endophytes are hybrids derived from two *Epichloë* species and, in some cases, three parental species, although rarely two strains of one parental species are involved (Tsai et al. 1994; Moon et al. 2000, 2004; Ghimire et al. 2011). *Epichloë* species that produce the teleomorph have been detected only in the northern hemisphere, and only anamorphs of these endophytes have been found in the southern hemisphere (Cabral et al. 1999; Moon et al. 2002, 2007; Gentile et al. 2005; Iannone et al. 2009; Iannone et al. 2011). Five asexual species, two of them native to Argentina, have been described from the southern hemisphere (Cabral et al. 1999; Moon et al. 2002, 2007; Iannone et al. 2012a). The hybrids (*Epichloë festucae* × *Epichloë typhina*) predominate in this region. One such hybrid species, *Epichloë tembladerae* (*Neotyphodium tembladerae* Cabral & White), is the most common and widely distributed *Epichloë* endophyte, infecting 19 grass species (Iannone et al. 2012a), whereas *Epichloë pampeana* (*Neotyphodium pampeanum* Iannone & Cabral) infects plants in some populations of *Bromus auleticus* (Iannone et al. 2009).

Gentile et al. (2005) and Iannone et al. (2012a) reported the presence of *Epichloë* endophytes in *Bromus setifolius* and *Phleum alpinum* that would present a different evolutionary origin to that of the previously described species in this region. *Bromus setifolius* is a perennial species that inhabits regions south of the 35th parallel in Argentina and Chile. Its habitat includes dry steppes above 2500 m in the central Andes, Patagonian steppes and temperate forests of *Nothofagus* in the Andes mountains and foothills, also in Patagonia (Gutiérrez and Pensiero 1998). *Phleum alpinum* is a widespread species in Europe, Asia and America, inhabiting woods and humid grasslands (Stewart et al. 2009, 2011). In Argentina, *P. alpinum* also inhabits river and stream banks in temperate altitudes of the central Andes.

In the present work we study the diversity and the evolutionary origins of *Epichloë* species in populations of *B. setifolius* and *P. alpinum* from Argentina. Based on their evolutionary origins as well as differences in morphology between isolates, we propose the formal description of a new species and a new variety. Also, we formally propose the new combinations *Epichloë pampeana* and *E. tembladerae* for *Neotyphodium pampeanum* and *N. tembladerae* (previously described endophytes from Argentina) respectively, according to the ICN for algae, fungi and plants, article 59 (McNeill et al. 2012).

MATERIALS AND METHODS

Plant collection.—Collection trips were conducted in Santa Cruz and Tierra del Fuego provinces in southern Argentinean Patagonia where *B. setifolius* and *P. alpinum* had been collected (Gentile et al. 2005, Novas et al. 2007). We sampled previously described populations as well as new ones. The studied area extends from the Atlantic coast to the Cordillera de los Andes in the south of Santa Cruz province and the entire Argentinean territory of Tierra del Fuego Island (FIG. 1, TABLE I). This area includes humid grasslands, arid and semi-arid steppes and woods of *Nothofagus* tree species. At each collection site (TABLE I) 10–20 plants (when possible), each more than 5 m apart, were randomly collected. Tillers (1–3) of each plant were screened by microscopic observation of culm piths or leaf sheaths after staining with aniline blue in lactophenol (Clark et al. 1983) to determine endophyte status. Endophyte infections in seeds were confirmed by microscopic observation after softening in 10% NaOH and staining with aniline blue in lactophenol.

Endophyte isolation and morphological characterization.—Caryopses and pieces of leaves or culms from endophyte-infected plants were surface sterilized by washing 1 min in 70% ethanol, followed by a 15 min wash in 2.5–3% sodium hypochlorite (50% commercial bleach), and a 1 min wash in 50% ethanol. After sterilization, caryopses and leaves were cut and placed on potato dextrose agar (PDA) plates, incubated in darkness at 24 C and checked regularly for endophytic growth for up to 2 mo. In some cases the fungus emerged from several segments or seeds from the same plant; however, only one isolate per plant was considered for this study because the isolates from each individual plant did not exhibit morphological differences. Single-spore cultures of each isolate were obtained as described by Iannone et al. (2009) to eliminate the possibility of contamination or heterokaryosis (Craven et al. 2001). In those isolates that did not produce conidia, hyphal tips (2 mm long) were taken from cultures grown in water agar (WA) plates and transferred to PDA plates. To examine the macroscopic characteristics of endophyte cultures, 2 mm^2 agar blocks were cut from the margins of actively growing

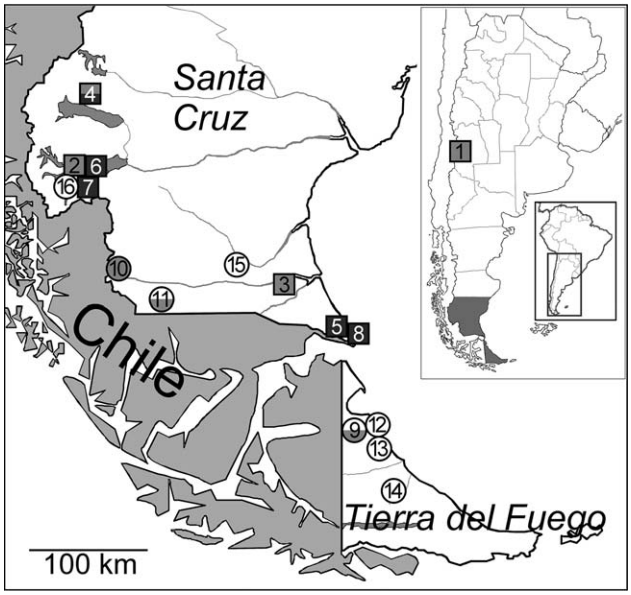

FIG. 1. The surveyed area and the placement of the *Bromus setifolius* (squares) and *Phleum alpinum* (circles) collection sites. Dark gray-filled symbols indicate populations infected with *E. typhina* var. *aonikenkana*, light gray-filled symbols indicate populations infected with *E. tembladerae* and white-filled symbols indicate populations infected with *E. cabralii*. Populations where more than one endophyte species was detected are indicated by double color symbols as described above.

colonies and transferred to PDA plates. Cultures were incubated 20–30 d at 24 C in darkness. Colony characteristics and diameter measurements were taken after 30 d. For microscopic characterization, pieces of actively growing mycelium also were transferred to 2% WA plates. Cultures were allowed to grow 30 d at 24 C in darkness. Blocks of agar (1 cm^2) were mounted on slides and stained with aniline blue or calcofluor white M2R and observed at up to 1000×. Thirty conidia (when possible) and conidiogenous cells from each isolate were measured and photographed with a Karl Zeiss Axioskope microscope. The morphology of isolates was compared with other *Epichloë* spp. isolates from Argentina (Cabral et al. 1999, Iannone et al. 2009, Iannone et al. 2011) and descriptions from literature.

DNA isolation.—Nineteen isolates from *B. setifolius* and 15 from *P. alpinum* (including those studied by Gentile et al. (2005), representative of populations inhabiting different environments (TABLE I), were chosen for DNA extraction. Total genomic DNA was isolated with the kit ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research, Irvine, California) 10–50 mg actively growing fresh mycelium of single-spore cultures grown on PDA plates overlaid with cellophane. DNA samples were quantified by fluorometry with the Quant-iT DNA HS assay kit (Invitrogen, Carlsbad, California).

Amplification of gene fragments.—PCR reactions were performed in 50 μL volumes containing 5 μL 10× PCR buffer (Applied Biosystems, Foster City, California), 1.5 mM $MgCl_2$, 125 μM each of dATP, dCTP, dGTP and dTTP, 200 nM of each primer, 0.025 U/μL AmpliTaq Gold DNA polymerase (Applied Biosystems) and 2–5 ng of fungal genomic DNA.

The PCR temperature cycling program consisted of one denaturalization at 94 C for 2 min followed by 40 cycles of denaturalization at 94 C for 45 s, annealing at temperatures established for each gene (see below) for 45 s and amplification for 1 min at 72 C, followed by one incubation at 72 C for 10 min. Because most of the endophytes from Argentina are hybrids from at least two sexual parents (Moon et al. 2002, Gentile et al. 2005), two alleles of each gene were expected to be present for every gene in each isolate. Each parental haplotype was selectively amplified with species-specific primers designed with a selective nucleotide at the 3′ end. Amplified products were visualized by electrophoresis in 1% agarose gels stained with ethidium bromide or gel red.

Amplification of calmodulin gene (calM). Calmodulin (FIG. 2) is a highly conserved gene found in all eukaryotic lineages. Calmodulin protein functions as a multifunctional intercellular Ca^{2+} receptor that binds to several other proteins to alter their activity (Chin and Means, 2000). Specific primers (TABLE II) were designed for the amplification of a segment of *calM* gene (FIG. 2) of endophytes from *B. setifolius* and *P. alpinum* and previously described *Neotyphodium* and *Epichloë* species (reference taxa). The *calM* gene sequence of *E. festucae* E2368, obtained from *E. festucae* genome project (http://csbio-l.csr.uky.edu/ef2011/) (contig00054:120266..121965) was used as reference for primer design. Primer *cal-exon1d* hybridizes upstream of the start codon (AUG) (FIG. 2). Primer *cal-exon7u* hybridizes downstream of the stop codon (FIG. 2). These primers were used for PCR amplification of *calM* portions from different strains of sexual and asexual non-hybrid *Epichloë* species. The annealing temperature for PCR reactions performed with this pair of primers was 50 C, and a fragment of approximately 1000 bp from the *calM* gene was amplified from all *Epichloë* species. The allele from *E. typhina* could be amplified only in a few strains of those endophytes expected to have a common ancestor with *E. typhina* (*E. tembladerae*, *E. pampeana*, *N. coenophialum*, *N. uncinatum*). To sequence the allele derived from *E. typhina* from those expected hybrid endophytes, a primer was designed with a selective nucleotide at the 3′ end, on a polymorphic site on intron 1 (primer *cal-intron1d-ty*) (TABLE II, FIG. 2) that was conserved for all the sequences of the *E. typhina*-derived allele previously obtained from some of the hybrid strains but that differs in the known sequences from other haploid *Epichloë* species. PCR reactions to amplify the *E. typhina* allele were performed with primers *cal-intron1d-ty* and *cal-exon7u* as describe above, but annealing temperature was 54 C. To get the whole sequence of both DNA strands, the primers used for selective amplification were used in combination with two internal (non-selective) primers. These primers were designed on exon 4 (*cal-exon4d* and *cal exon-4u*) (FIG. 2, TABLE II).

For the triple hybrid, *Neotyphodium coenophialum* (*Lolium*-associated endophyte (LAE) (Schardl et al. 2008) × *E. festucae* × *E. typhina*), the copies derived from LAE and *E.*

TABLE I. Isolates and geographic origins of *Epichloë* species of *Bromus setifolius* and *Phleum alpinum* examined in this study

Isolate	Population	Host	Endophyte	Origin (locality, department, province)	Environment
2381	1	*B. setifolius*	*E. tembladerae*	Las Leñas, Malargüe, Mendoza	shrub-steppe
BAFC4007	1	*B. setifolius*	*E. tembladerae*	Las Leñas, Malargüe, Mendoza	shrub-steppe
BAFC532	1	*B. setifolius*	*E. tembladerae*	Las Leñas, Malargüe, Mendoza	shrub-steppe
BAFC649	2	*B. setifolius*	*E. tembladerae*	Rio Mitre, Lago Argentino, Santa Cruz	grassland-forest
BAFC713	2	*B. setifolius*	*E. tembladerae*	Rio Mitre, Lago Argentino, Santa Cruz	grassland-forest
BAFC4009	2	*B. setifolius*	*E. tembladerae*	Rio Mitre, Lago Argentino, Santa Cruz	grassland-forest
BAFC4008	3	*B. setifolius*	*E. tembladerae*	Guer Aike, Guer Aike, Santa Cruz	grassy steppe
2640	4	*B. setifolius*	*E. tembladerae*	Rio Cangrejos, Corpen Aike, Santa Cruz	shrub-steppe
BAFC4004	4	*B. setifolius*	*E. tembladerae*	Rio Cangrejos, Corpen Aike, Santa Cruz	shrub-steppe
BAFC4005	4	*B. setifolius*	*E. tembladerae*	Rio Cangrejos, Corpen Aike, Santa Cruz	shrub-steppe
2670	4	*B. setifolius*	*E. tembladerae*	Rio Cangrejos, Corpen Aike, Santa Cruz	shrub-steppe
2642	4	*B. setifolius*	*E. t. aonikenkana*	Rio Cangrejos, Corpen Aike, Santa Cruz	shrub-steppe
BAFC508	5	*B. setifolius*	*E. t. aonikenkana*	Estancia El Condor, Guer Aike, Santa Cruz	humid steppe
BAFC420	6	*B. setifolius*	*E. t. aonikenkana*	Estancia Alice, Lago Argentino, Santa Cruz	humid steppe
BAFC719	7	*B. setifolius*	*E. t. aonikenkana*	Río Rico, Lago Argentino, Santa Cruz	humid steppe
BAFC4010	8	*B. setifolius*	*E. t. aonikenkana*	Monte Dinero, Guer Aike, Santa Cruz	humid steppe
BAFC4011	8	*B. setifolius*	*E. t. aonikenkana*	Monte Dinero, Guer Aike, Santa Cruz	humid steppe
BAFC4012	8	*B. setifolius*	*E. t. aonikenkana*	Monte Dinero, Guer Aike, Santa Cruz	humid steppe
BAFC4013	8	*B. setifolius*	*E. t. aonikenkana*	Monte Dinero, Guer Aike, Santa Cruz	humid steppe
BAFC755	9	*P. alpinum*	*E. tembladerae*	María Behety, Río Grande, Tierra del Fuego	humid steppe
2350	9	*P. alpinum*	*E. tembladerae*	María Behety, Río Grande, Tierra del Fuego	humid steppe
2351	9	*P. alpinum*	*E. cabralii*	María Behety, Río Grande, Tierra del Fuego	humid steppe
BAFC4002	10	*P. alpinum*	*E. tembladerae*	Rio Turbio, Guer Aike, Santa Cruz	humid steppe
2630	11	*P. alpinum*	*E. tembladerae*	El Zurdo, Guer Aike, Santa Cruz	humid steppe
BAFC4001	11	*P. alpinum*	*E. cabralii*	El Zurdo, Guer Aike, Santa Cruz	humid steppe
682	12	*P. alpinum*	*E. cabralii*	Cabo Domingo, Río Grande, Tierra del Fuego	humid steppe
BAFC3998	12	*P. alpinum*	*E. cabralii*	Cabo Domingo, Río Grande, Tierra del Fuego	humid steppe
BAFC3995	12	*P. alpinum*	*E. cabralii*	Cabo Domingo, Río Grande, Tierra del Fuego	humid steppe
BAFC3996	13	*P. alpinum*	*E. cabralii*	Río Grande, Río Grande, Tierra del Fuego	humid steppe
BAFC3997	13	*P. alpinum*	*E. cabralii*	Río Grande, Río Grande, Tierra del Fuego	humid steppe
2549	14	*P. alpinum*	*E. cabralii*	Río Grande, Tierra del Fuego	forest
BAFC3999	15	*P. alpinum*	*E. cabralii*	Las Horquetas, Guer Aike, Santa Cruz	grassland
BAFC4000	15	*P. alpinum*	*E. cabralii*	Las Horquetas, Guer Aike, Santa Cruz	grassland
BAFC4003	16	*P. alpinum*	*E. cabralii*	Los Glaciares Natl. Park, Santa Cruz	forest

festucae were amplified with primers *cal-exon1d* and *cal-exon7u*. Then, the PCR products were cloned in *E. coli* with TOPO TA cloning kit (Invitrogen), according to instructions provided by the manufacturer. Ten transformant colonies were randomly selected, grown for plasmid DNA extraction and sequenced with primers *M13 forward*, *M13 reverse* and the two internal primers designed for *calM* sequencing.

Amplification of tefA *and* tubB *gene fragments.* Intron-rich regions of the β-tubulin (*tubB*) and translation elongation factor 1-α (*tefA*) genes of endophytes isolated from *B. setifolius* and *P. alpinum* also were amplified by PCR with primers *tub2-exon1d-1* × *tub2-exon4u-2* and *tef1-exon1d-1* × *tef1-exon6u-1* respectively (Moon et al. 2002). Sequences of *tefA* and *tubB* genes of *Neotyphodium* and *Epichloë* reference

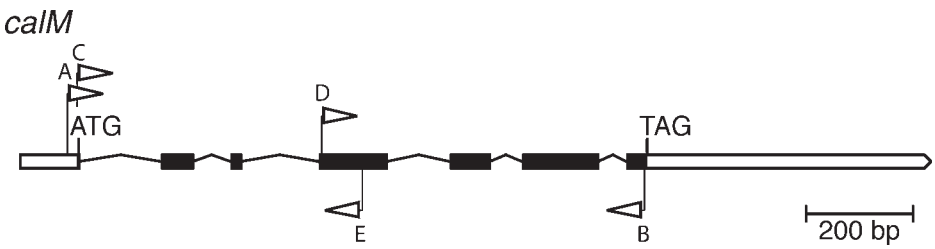

FIG. 2. *calM* gene structure showing the amplified and sequenced regions. Lines and boxes indicate intron and coding sequences respectively. Arrows indicate the amplification and sequencing primer positions and orientations (see TABLE II).

TABLE II. PCR and sequencing primers used in this study

Primer[a]	Primer sequence (5′–3′)	Use[b]	Comb.[c]	PCR product	T[d]
A) *cal-exon1d*	TAT CAA ATT TTC CAC CAT GG	Amp.-Seq.	A × B	non selective *calM* in non-hybrids	50
B) *cal-exon7u*	TAC TTC TGC ATC ATA AGC T	Amp.-Seq.	A × B	*E. festucae*, *E. amarillans-E. baconii* allele in hybrids with *E. typhina*	50
C) *cal-intron1d-ty*	GGT AAG TCC TTC ATT CAG	Amp.-Seq.	C × B	*E. typhina* allele in *E. typhina* × *E. festucae/E. baconii-E. amarillans* hybrids	54
D) *cal-exon4d*	TCA CCA CTA AAG AGC TAG G	Seq.			55
E) *cal-exon4u*	ATC ATA TCT TGC AAT TCG G	Seq.			55

[a] Primer nomenclature and orientation (u = upstream, d = downstream).
[b] Amp. = PCR amplification, seq. = DNA sequencing.
[c] Comb. = primer combination for PCR.
[d] T = annealing temperature (Celsius).

species were obtained from the NCBI GenBank. When two alleles were present, as was typical of interspecific hybrids, products from both alleles were unselectively amplified, such that the sequence traces from hybrid isolates clearly indicated the presence of multiple alleles for both *tubB* and *tefA*. In such cases the individual haplotypes were amplified with species-specific primers as described in Iannone et al. (2009). The approximate sizes of the expected products were 480 bp and 800 bp for *tubB* and *tefA* respectively.

DNA sequencing.—PCR products were purified with the PureLink™ Quick PCR Purification Kit (Invitrogen). Sequencing reactions were performed with a BigDye® Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems). Both DNA strands were sequenced with the corresponding primers described by Gentile et al. (2005). Sequences were obtained on an ABI 3730xl DNA Analyzer and then run with the software Sequence Scanner (Applied Biosystems). Contiguous sequences were assembled with Vector NTI (Invitrogen). Gene sequences of *calM*, *tubB* and *tefA* were deposited in GenBank under these accession numbers: KF533931–KF533995, KF533996–KF534055 and KF534056–KF534111 respectively.

Sequence alignment and phylogenetic analysis.—For each gene, sequences were aligned with Clustal W (Thompson et al. 1994) for multiple alignment of BioEdit 7.0.5. Alignments were checked by eye for ambiguities and corrected by hand if necessary. Alignments and phylogenetic analyses for *calM*, *tubB* and *tefA* are deposited in TreeBASE under accession numbers S14315, S14316 and S14314.

Maximum parsimony (MP) analyses were performed by heuristic search in WINCLADA 0.9.9 (Nixon 1999). Character states were unordered and unweighted, and trees were built by 1000 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. Maximum likelihood (ML) analyses were performed with MEGA 5 (Tamura et al. 2011), and 1000 bootstrap replicates were run. Bayesian analysis with estimation of posterior probabilities of nodes was conducted with MrBayes 3.2 (Ronquist et al. 2012). Four chains (three heated at temp = 0.2) were run for 1 000 000 generations, saving one out of every 100 trees (mcmc ngen = 1 000 000, samplefreq = 100, nchains = 4). The first 2500 trees were discarded as burn-in, and the consensus tree and posterior probabilities were determined from the remaining 7500 trees. The evolutionary models for ML and MrBayes analyses were Kimura 2-parameter + Gamma (K2+G) for *calM* and *tubB* genes and K2 for *tefA*, established as the best evolutionary models with MEGA 5 (Tamura et al. 2011).

RESULTS

Endophyte isolation and morphological characterization.—A total of 27 isolates were obtained from eight populations of *B. setifolius* from Santa Cruz. Sixteen isolates were obtained from *P. alpinum* plants collected in four localities in Tierra del Fuego and three localities from Santa Cruz province. Plants of both species were not detected inhabiting the same locality.

Growing on PDA agar, all the isolates presented velvety to slightly cottony, white to yellowish colonies 15–35 mm diam in 30 d (FIG. 3). However, according to differences observed in microscopic characteristics and in the appearance of the colonies in old cultures, three morphotypes were established. Morphotype 1 included isolates obtained from *B. setifolius* and *P. alpinum* plants. These isolates presented conidiogenous cells variable in size, 20–100 µm long with an abundant production of allantoid to moon shaped conidia 7–12 µm long (FIG. 3A–E). Morphotype 2 included isolates only from *P. alpinum* (FIG. 3F–H) and differed slightly from the former morphotype by the abundance of bigger and lemon-shaped to allantoid conidia (7.5-14 µm long). Morphotype 3 contained only isolates from *B. setifolius* (FIG. 3I–K), in which mycelia seems to be hydrophilic becoming wet when water was poured on the colony to get suspensions of conidia, and the colonies looked waxy in more than 35 d old cultures. Whereas some isolates were sterile, other isolates produced sparse allantoid conidia 5–7 µm long.

Phylogenetic analyses (see below), consistent with the morphological characteristics, indicated that the

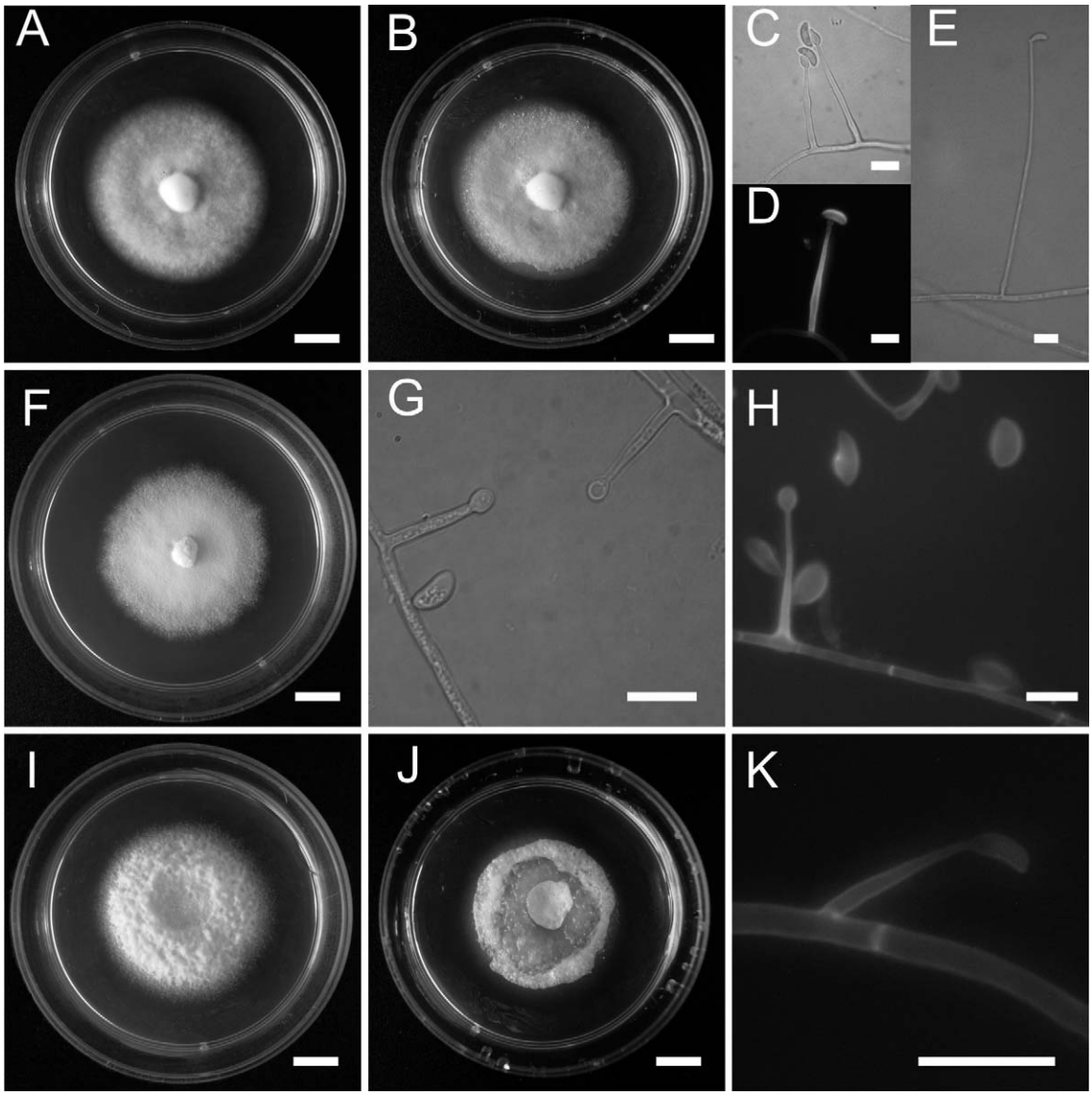

FIG. 3. Colony and conidial morphology of *E. tembladerae* (morphotype 1) (A–E) from *P. alpinum*, *E. cabralii* (morphotype 2) (F–H) from *P. alpinum* and *E. typhina* var. *aonikenkana* (morphotype 3) (I–K) from *B. setifolius*. A, B, F, I, J. Colonies after 30 d on PDA at 24 C. B. hydrophobic colony of *E. tembladerae* after pouring water on it. J. hydrophilic colony of *E. typhina* var *aonikenkana* after pouring water on it. C, D, E, G, H, K. Conidia and conidiogenous cells. Cultures for the observation of conidial ontogeny were grown on water agar, calcofluor white stained and observed with white and UV light. Bars : A, B, F, I, J = 10 mm; C, D, E, G, H, K = 10 μm.

isolates of morphotype 1 belonged to the previously described species, *E. tembladerae* (Cabral et al. 1999). Morphotype 2 was considered a new species, *Epichloë cabralii* and morphotype 3 was considered a new variety of *E. typhina*, *E. typhina* var. *aonikenkana*.

Phylogenetic analyses.—Calmodulin gene phylogeny. PCR amplification of *calM* of sexual and haploid *Epichloë* spp. isolates rendered only one product from each isolate. In contrast, two copies (allele-1 and allele-2) were identified from *N. uncinatum*, the endophytes isolated from *P. alpinum*, some isolates from *B. setifolius* and isolates from other hosts from Argentina. Three different alleles could be identified from *N. coenophialum*. Even though PCR reactions were performed with putatively non-selective primers, only one allele of each gene was obtained from those isolates from *B. setifolius* considered *E. typhina* var. *aonikenkana*.

The *calM* gene sequence alignment was 942 positions long, and 89 characters were parsimony informative. Phylogenies obtained by MP, MrBayes

and ML methods were congruent and showed the same two main clades. One clade included sequences obtained from the reference strains of *E. typhina* and their asexual derivatives (*E. typhina* clade), whereas the other clade included sequences of *E. festucae*, *E. baconii* and *E. amarillans* and their hybrids (FIG. 4). The sequences obtained from *E. bromicola* and one of the copies of *N. uncinatum* were grouped in a basal clade (FIG. 4).

All strains from Argentina presented one allele, derived from *E. typhina*, which grouped a well supported clade that we call the ''*E. typhina* poae clade'' in that it includes sequences from those *E. typhina* strains described as *E. poae* by Tadych et al. (2012). The other allele, when present, was placed in the clades that included the sequences from *E. festucae* or *E. amarillans/E. baconii*.

The sequences obtained from endophytes isolated from *B. setifolius* and *P. alpinum* were placed in different clades, establishing three different lineages that corresponded with their morphological distinctions (FIGS. 3, 4). One lineage (morphotype 1) was detected in both host species and included isolates that showed two different haplotypes of the *calM* gene. The allele-1 was placed in the *E. festucae* clade, in a well supported subclade that included the allele-1 of *E. tembladerae* reference strains from Argentina. The allele-2 of these isolates was clustered with the allele-2 of *E. tembladerae* reference strains in the *E. typhina* poae clade. Thus, these isolates were considered to belong to the hybrid species *E. tembladerae* (*E. festucae* × *E. typhina*).

A different lineage was represented by some of the endophytes isolated from *P. alpinum* (morphotype 2) that also showed two alleles. Allele-1 of these isolates was included in a well supported clade that shared a common ancestor with *E. amarillans* plus *E. baconii* and one of the alleles of the hybrid *N. coenophialum*. The allele-2 sequences fell in the *E. typhina* complex, in a basal polytomy of the *E. typhina* poae clade that included sequences from all the endophytes from Argentina, as well as sequences from *N. coenophialum* and *N. uncinatum* and *E. typhina* from *Poa nemoralis*. This result indicated that the isolates in this lineage were hybrids between *E. typhina* and the common ancestor of *E. amarillans* and *E. baconii*. We propose that these endophytes be described as a new species, *Epichloë cabralii* sp. nov.

The third lineage of endophytes was isolated from *B. setifolius* plants from Santa Cruz province and included those isolates considered morphotype 3. In contrast to other endophytes from Argentina, these presented only one allele of *calM* and were placed in the *E. typhina* poae clade, within which they were in a well supported subclade sister to allele-2 of *E. tembladerae*. Thus, these isolates are proposed to represent a new variety, *E. typhina* var. *aonikenkana*.

Allele-1 of *Epichloë pampeana* strains was clustered in a well supported subclade in a clade that also included allele-1 of *E. tembladerae*, allele-1 of *N. coenophialum* and the sole allele of *E. festucae* strains. Allele-2 of *E. pampeana* fell in a polytomy in the *E. typhina* poae clade, within which also were included the allele-2 of *E. tembladerae* and *E. typhina* var. *aonikenkana* subclades, allele-2 of *N. uncinatum* and allele-3 of *N. coenophialum* (FIG. 4).

Neotyphodium coenophialum presented three different copies (alleles) of *calM*. Allele-1 related this species with *E. festucae*, allele-2 with *E. baconii* and *E. amarillans* and allele-3 with *E. typhina*. These results agreed with the earlier conclusion that this species was a hybrid with three ancestral species (Tsai et al. 1994, Moon et al. 2004).

Phylogenies of tubB *and* tefA *genes.*—All isolates considered to be *E. tembladerae* and *E. cabralii* presented two different alleles of each gene, whereas isolates considered *E. typhina* var. *aonikenkana* presented only one allele of each gene. The phylogenies of *tubB* (not shown) and *tefA* (FIG. 5) genes, obtained by MP, MrBayes and ML algorithms, were mostly congruent with the phylogeny of *calM* (FIG. 4). The *tubB* phylogeny of each allele clustered apart the sequences of the three different lineages in three different subclades (similarly to the *calM* phylogeny). However, the topology of the tree was not totally resolved and the ancestral relationships among some of the subclades could not be clearly established.

The *tefA* phylogenetic tree gene (FIG. 5) yielded the same clades as the *calM* phylogeny but presented a better resolution of the evolutionary relationships among the lineages described above. As observed in the *calM* phylogeny, two principal clades were obtained with sequences from Argentinean *Epichloë* species. One of these clades included the sexual species *E. baconii*, *E. amarillans* and *E. festucae*, with allele-1 of *E. cabralii* isolates sister to *E. amarillans*. This clade also included allele-1 of *E. tembladerae*, *E. pampeana*, *N. coenophialum* and *N. australiense* grouped with *E. festucae* sequences. The allele-2 of *N. coenophialum* and *E. baconii* were placed in the basal position of this clade. The other principal clade included sequences of *Epichloë typhina* and its related sexual species *E. sylvatica* and *E. clarkii*. In this clade, allele-2 of *E. pampeana*, *E. tembladerae*, *E. cabralii* and the sequences from *E. typhina* var *aonikenkana* were distributed in four well supported subclades, sharing a common ancestor with *N. huerfanum*, *N. typhinum* var *canariense*, one of the ancestors of *N. australiense*

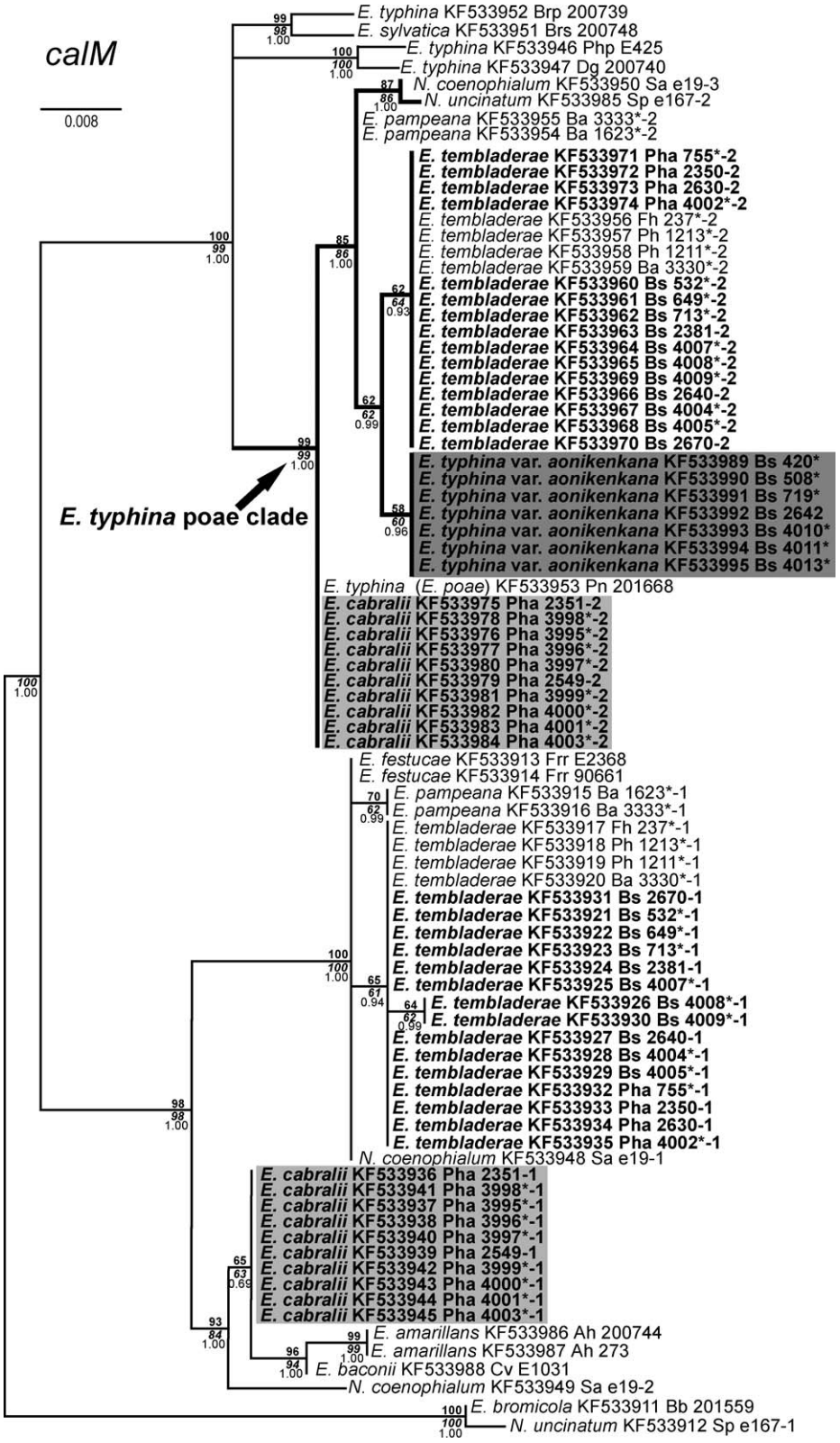

FIG. 4. MrBayes phylogenetic tree of *Epichloë* and *Neotyphodium* species based on *calM* sequences. For hybrid endophytes, different alleles are indicated by 1, 2 or 3 after the isolate designation. New endophyte taxa of *Phleum alpinum* and *Bromus setifolius* are indicated with shading (*E. typhina* var. *aonikenkana* in dark gray and *E. cabralii* in light gray). Bayesian posterior probabilities, MP (> 50%) bootstrap support (italic bold) and ML bootstrap support (bold) are shown in each node. GenBank

and *E. typhina* from *Poa nemoralis* in the *E. typhina* poae clade. The *E. tembladerae* isolates from *P. alpinum* were clustered in a subclade apart from sequences of other *E. tembladerae* isolates. Inside *E. typhina* var. *aonikenkana* clade, three isolates (Bs2642, BAFC4010, BAFC4011) were clustered in a well supported subclade, but this grouping was not related to the geographic origin of these isolates (TABLE I).

TAXONOMY

Epichloë cabralii Iannone, Rossi & Schardl, sp. nov. FIG. 3F–H

MycoBank MB805169.

Colony diameter reaching 20–30 mm after 30 d of growth at 24 C on PDA. Colony velvety with irregular borders, raised from the agar surface, moderately to highly convoluted in the central zone, white to slightly yellowish in the central zone. Colony reverse brown in the center to tan in the margins. Hyphae septate, 1.5–2.5 μm wide. Conidiogenous cells hyaline, orthotropic, determinate, sometimes branched, without septum at the base of the branch, 20–40 μm long, straight to lightly flexuous, 1.5–2.0 μm wide at the base tapering to 0.5–1.2 at the tip. Conidia shape variable, highly asymmetric, lemon-shaped to allantoid, 7.5–14 (20) μm long, two or three conidia produced by each conidiogenous cell, usually proliferating attached to the conidiogenous cell to originate a vegetative hyphae or a new conidiogenous cell.

Holotype: Argentina, BAFC 52290, BAFCcult 3998 (ex-type), infecting *Phleum alpinum* from Cabo Domingo, Departamento Río Grande, Tierra del Fuego, Argentina. 53°41′S, 67°50′W. *Collector:* Dr Daniel Cabral.

Other names for cultures derive from the ex-type culture are: DC2539 in Dr Cabral's culture collection or 7902 in Dr Schardl's culture collection.

Etymology: in memory of Dr Daniel Cabral, who pioneered studies of fungal endophytes in Argentina.

Known distribution: This species is distributed as an endophytic fungus of *Phleum alpinum* in prairies and steppes of the northern part of the Argentinean territory of Tierra del Fuego Island and grasslands and forests in the south of Santa Cruz province in Argentina.

Epichloë pampeana (Iannone & Cabral) Iannone & Schardl, comb. nov.

≡ *Neotyphodium pampeanum* Iannone & Cabral 2009. *Mycologia* 101:347.

MycoBank MB805170

Epichloë tembladerae (Cabral & J.F. White) Iannone & Schardl, comb. nov.

≡ *Neotyphodium tembladerae* Cabral & J.F. White 1999. *Mycologia* 91:321.

MycoBank MB805176

Epichloë typhina var aonikenkana Iannone, Rossi & Schardl, var. nov. FIG. 3I–K

MycoBank MB805168

Colony diameter 25–35 mm after 30 d at 24 C on PDA. Colony cottony, raised from the agar surface, white to slightly yellowish in the central zone with irregular borders, moderately convoluted in the central zone. Mycelium hydrophilic, becoming wet when pouring water on it. Colony reverse pale brown in the center to tan in the margins. Hyphae septate, 1.5–2.5 μm wide. Conidiogenous cells phialidic, hyaline, orthotropic, determinate, unbranched, septum at the base rarely present, 20–40 μm long, straight to lightly flexuous, 1.5–2.0 μm wide at the base tapering to 0.5–1.2 at the tip. Conidia scarce, variable in shape, mostly allantoid, 5–7(8) μm long, one or rarely two conidia produced by each conidiogenous cell. In cultures on PDA older than 30 d, colonies become waxy.

Holotype: Argentina, BAFC 52289, BAFCcult 420 (ex-type), infecting *Bromus setifolius* from Departamento Lago Argentino, Santa Cruz province, Argentina. 50°20′S, 72°27′W. *Collector:* Dr Daniel Cabral.

Other name for cultures derived from the ex-type culture is: DC2370 in Dr Cabral's culture collection.

Etymology: the specific epithet comes from the Latin form of Aónikenk the name that the native inhabitants of southern Patagonia gave themselves.

Known distribution: This species is distributed as an endophytic fungus of *Bromus setifolius* in steppes of the southern part of Santa Cruz province in Argentina.

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sequence accession numbers are indicated between species and host names. Abbreviations for the names of the host species are: Ah = *Agrostis hiemalis*, Ba = *Bromus auleticus*, Bb = *Bromus benekenii*, Brp = *Brachypodium pinnatum*, Brs = *Brachypodium sylvaticum*, Bs = *Bromus setifolius*, Cv = *Calamagrostis villosa*, Dg = *Dactylis glomerata*, Fh = *Festuca hieronymi*, Frr = *Festuca rubra* var. *rubra*, Pha = *Phleum alpinum*, Ph = *Poa huecu*, Pn = *Poa nemoralis*, Pp = *Poa pratensis*, Sa = *Schedonorus arundinaceus*, Sp = *Schedonorus pratensis*. Asterisks indicate accession number of the cultures in the BAFC culture collection.

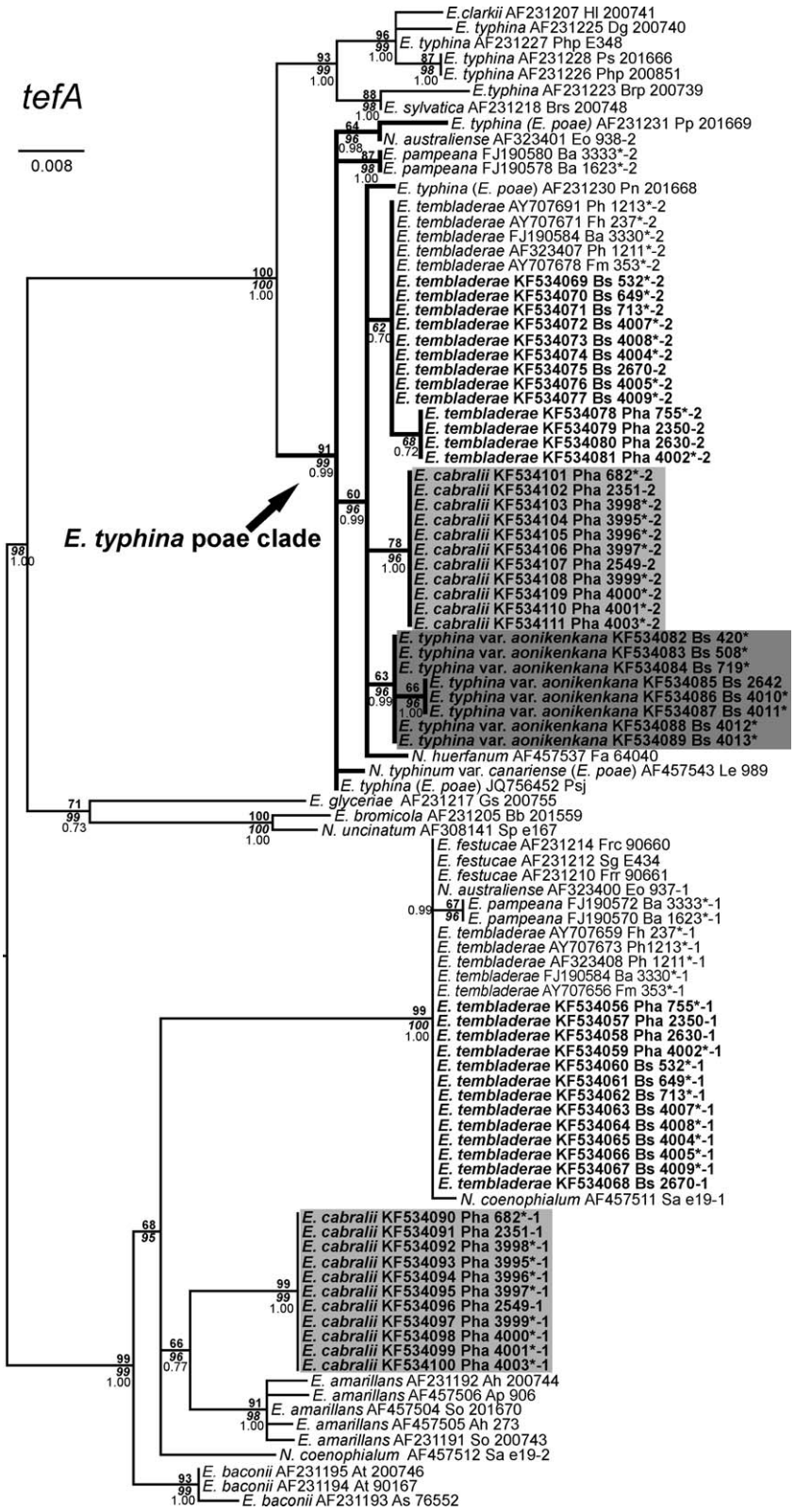

FIG. 5. MrBayes phylogenetic tree of *Epichloë* and *Neotyphodium* species based on partial *tefA* sequences. For hybrid endophytes, different alleles are indicated by 1 or 2 after the isolate designation. New endophyte taxa from hosts *Phleum alpinum* and *Bromus setifolius* are indicated with shading (*E. typhina* var. *aonikenkana* in dark gray and *E. cabralii* in light gray). Bayesian posterior probabilities, MP (> 50%) bootstrap support (italic bold) and ML bootstrap support (bold) are

DISCUSSION

Previous studies on endophytes from Argentina suggested a wide host range for *E. tembladerae* and the existence of genetic diversity among asexual *Epichloë* endophytes from this region (Cabral et al. 1999, Gentile et al. 2005, Iannone et al. 2009, Iannone et al. 2012a). In the present work we add *calM* phylogeny to the previously employed *tefA* and *tubB* phylogenies of endophytes from Argentina. Based on multigene phylogeny, we show new, strong evidence about the existence of such diversity and we formally describe the new species *Epichloë cabralii* sp. nov. from *Phleum alpinum* and a new variety of *E. typhina* from *Bromus setifolius*, two grasses from southern Patagonia.

The existence of *E. cabralii* and *E. typhina* var. *aonikenkana* was suggested in works of our group (Gentile et al. 2005, Iannone et al. 2012b) and confirmed with the isolation and characterization of more endophytes from *B. setifolius* and *P. alpinum*. These taxa are particularly interesting because, whereas other *Epichloë* endophytes from Argentina (*E. tembladerae* and *E. pampeana*) are hybrids between *E. typhina* and *E. festucae*, the two new lineages described here present different evolutionary origins.

Although the ancestry relationships among *E. cabralii*, *E. amarillans* and *E. baconii* are not totally resolved in *calM* and *tubB* phylogenies, our *tefA* phylogenies indicate that *E. cabralii* is a hybrid between *E. typhina* and the ancestor of *E. amarillans*. *E. cabralii* is the only hybrid species that shares common ancestors with *E. typhina* and *E. amarillans* and, up to now, this is the only species from the southern hemisphere that presents a common ancestor with *E. amarillans* among their parents. In this sense, it is noteworthy that the sexual species that contributed with their genomes in the origin of *E. cabralii* have sexual extants known only in the northern hemisphere. However, to our knowledge *Epichloë* endophytes have not been reported in *P. alpinum* in that region.

Phylogenies of *calM*, *tefA* and *tubB* grouped *E. typhina* var. *aonikenkana* with high support in a distinct subclade within the *E. typhina* poae clade (FIGS. 4, 5). This variety could be differentiated from other endophytes by this phylogenetic placement, because most of the isolates were sterile or produced conidia smaller than 7 µm long, and by the waxy aspect of the colony in old cultures. In addition, its non-hybrid condition differentiates this taxon from other previously described in Argentina.

The possible scenarios for the origin and the establishment of the hybrid endophytes in a region where sexual species have not been reported have been extensively discussed by Gentile et al. (2005) and Iannone et al. (2009). Our results show that, as previously reported by Iannone et al. (2009), different individuals of one grass species may host different *Epichloë* endophytes, as in the case of *Bromus auleticus*. These results suggest that the symbiosis between these host species and *Epichloë* endophytes was established in more than one event.

The simplest hypothesis for the presence of *E. tembladerae* in *B. setifolius* and *P. alpinum* is that these two species first established a symbiosis with an ancestor of those extant *E. typhina* strains considered to be *E. poae* by Tadych et al. (2012). Later, infections of both grasses by *E. festucae* gave rise to *E. tembladerae* in these hosts. In the same way *E. cabralii* could have been originated after the infection by an ancestor of *E. amarillans* in some plants of *P. alpinum* already infected with *E. typhina*.

Based on the recent discoveries of horizontal transmission presented by Tadych et al. (2012) where seedlings from endophyte-free (E-) seeds, germinated aseptically, in the presence of actively growing hyphae of *E. poae* became infected, the possibility of horizontal transmission should be considered. In this scenario, whereas in some plants of *P. alpinum* and *B. setifolius* the symbiosis with *E. cabralii* and *E. typhina* var. *aonikenkana* was established as described before other plants were infected by *E. tembladerae* through horizontal transmission, perhaps via propagules on the phylloplane from other host plants living in sympatry (i.e. *Festuca magellanica*, *Festuca argentina*). However, we do not have evidence of the capability of *E. tembladerae* to infect plants of another host species under laboratory or natural conditions. In addition,

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shown in each node. GenBank sequence accession numbers are indicated between species and host names. Abbreviations for the names of the host species are: Ah = *Agrostis hiemalis*, Ap = *Agrostis perennans*, As = *Agrostis stolonifera*, At = *Agrostis tenuis*, Ba = *Bromus auleticus*, Bb = *Bromus benekenii*, Brp = *Brachypodium pinnatum*, Brs = *Brachypodium sylvaticum*, Bs = *Bromus setifolius*, Dg = *Dactylis glomerata*, Eo = *Echinopogon ovatus*, Fa = *Festuca arizonica*, Fh = *Festuca hieronymi*, Fm = *Festuca magellanica*, Frc = *Festuca rubra commutata*, Frr = *Festuca rubra rubra*, Gs = *Glyceria striata*, Hl = *Holcus lanatus*, Le = *Lolium edwardii*, Lg = *Lolium giganteum*, Pha = *Phleum alpinum*, Php = *Phleum pratense*, Ph = *Poa huecu*, Pn = *Poa nemoralis*, Pp = *Poa pratensis*, Ps = *Poa secunda*, Sa = *Schedonorus arundinaceus*, Sp = *Schedonorus pratensis*, So = *Sphenopholis obtusata*. Asterisks indicate accession number of the cultures in the BAFC culture collection.

many attempts failed to establish new associations among endophytes and grasses that are not their original hosts and positive results have only been obtained when mycelium is introduced in artificially wounded seedlings (Latch and Christensen 1985).

Geographic distribution of the endophytes.—Although the number of individuals studied in this work is not enough to characterize the endophyte species at the population level, the results allowed preliminary identification of the distribution areas of both new taxa. *Epichloë typhina* var. *aonikenkana* seems to be present in most endophyte-infected populations of *B. setifolius* studied in the south of Santa Cruz province. These populations are distributed from grassy steppes close to the Atlantic Ocean (where it is the only endophyte species associated with *B. setifolius*) to the Cordillera de los Andes, where it seems to be less frequent than *E. tembladerae*. On the other hand, *E. tembladerae* seems to have a wider distribution area in this host, from steppes, grasslands and forests near the Cordillera de los Andes to Mendoza province in the central Andes of Argentina (FIG. 1). Thus, *E. tembladerae* and *E. typhina* var. *aonikenkana* co-exist in populations of *B. setifolius* in southwestern Santa Cruz province. *E. cabralii* was detected in all of the studied populations of *Phleum alpinum* and seems to be the dominant species in this grass species (11/15 studied isolates). *Epichloë tembladerae* seems to be less abundant in *P. alpinum* (4/15 studied isolates) but could be found in some populations co-existing with *E. cabralii*. The co-existence of different *Epichloë* species in the same mixed populations is considered rare but has been described before by several authors (Sullivan and Faeth 2004, Oberhofer and Leuchtmann 2012).

Host-endophyte genetic diversity and frequency of infection.—The incidence of *Epichloë* spp. in populations of *Bromus setifolius* and *Phleum alpinum* and their distribution in southern Argentina was studied by Novas et al. (2007). These authors showed that the incidence was 0–100%. They also found a strong correlation between endophyte incidence and the environmental characteristics. However, in that work the genotypes of the endophytes were not studied. The results of the present work indicate that in some of those populations different plants may be infected by different endophytes species. The incidence of endophytes in natural populations is affected both by the fitness of the infected plants, by the efficiency of the transmission of the endophyte from mother to daughter plants (Saikkonen et al. 2002, Afkhami and Rudgers 2008) and, for those endophytes that may be capable of it, the efficiency of horizontal transmission. Both fitness and transmissibility depend on the interactions between the genotypes of the host plants and their endophytes. Thus, it will be of interest in future to test whether endophyte-free plants in the studied populations lack compatibility with any of the endophyte species found in those host species and to determine the effects of the inoculation of the different endophytes in the fitness of the plants from different populations.

In addition, *B. setifolius* and *P. alpinum* are polyploid species and different varieties have been described. *Bromus setifolius* var. *setifolius* (2n = 28), *B. setifolius* var. *brevifolius* (2n = 70) and *B. setifolius* var. *pictus* (2n = 70) have been reported in the south of Argentina. In *P. alpinum* several diploid and tetraploid cytotypes were described; however only a tetraploid cytotype seems to be present in South America (Stewart et al. 2009, 2011). Thus, more studies should be conducted to study whether any association exists among the cytotype of the hosts and the endophyte species, the frequency of endophyte infection or the distributions of infected plants among populations of these two host species.

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