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Fungal endophytes of needles and twigs from *Pinus taeda* and *Pinus elliotti* in Uruguay

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Pinus taeda and *Pinus elliotti* are the main coniferous species recently planted in Uruguay. These new exotic plantations constitute an important change in the typical prairie landscape of the country. Several studies about the endophytes from needles and twigs of several *Pinus* species in the northern hemisphere and also of their pathogenic fungi are published. Conversely, little information is available on endophytes and on the incidence of fungi that can cause diseases on *Pinus* species in South America mainly in Uruguay. Several years ago fungal pathogens causing needle spots were the major problem in *Pinus* spp. Plantations in Uruguay. Recently, a severe disease, pitch canker caused by *Fusarium circinatum* Nirenberg & O'Donnell was detected in *Pinus* nurseries. In this work the fungal endophytic composition of asymptomatic needles and twigs of *P. taeda* and *P. elliotti* in Uruguay and pathogens associated with spotted needles were assayed. The most common needle endophytes found in both *Pinus* species were *Lophodermium australe* Dearn and Xylariaceae, whereas in twigs *Phaeomoniella* sp. and *Botryosphaeria* sp. were the dominant taxa. *P. elliotti* evidenced a higher resistence to fungal infection than *P. taeda*.

Key words: *Xylaria* spp., *Cenangium ferruginosum, Phaeomoniella* sp., *Lophodermium australe,* needles pathogens.

During the last 30 years *ca*. one million ha have been forested with exotic species in Uruguay, *Eucalyptus* spp. are most important followed by species of *Pinus* that represents 16 % of total forested area *Pinus taeda* Linn. and *P. elliotti* Engelm. the main *Pinus* species planted (Dirección General Forestal 2007). Nearly all production is used for the national wood industry. These new exotic plantations constitute an important change in the typical prairie landscape of the country. The introduction of exotic species as propagation materials (seeds and cuttings) may constitute a phytopathological risk.

Carroll *et al*. (1977) and Carroll & Carroll (1978) were pioneers in the study of fungal endophytes from needles of several *Pinus* species in the northern hemisphere. Several other studies are available mainly related with other coniferous species (Petrini 1991, Leuchtmann *et al*.

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1992, Kowalski & Kehr 1992, Barklund & Kowalski 1996, Hata & Futai 1995, 1996, Stanosz *et al.* 1997, Sieber & Holdenrieder 1999, Ganley, Brunsfeld & Newcombe 2006). Needle colonization by endophytic fungi is characterized by a rather slow, restricted development in the middle lamella between epidermis and hypodermis after needle infection. Nutrients are gradually acquired during needle senescence for the formation of reproductive structures (Deckert *et al.* 2001). Conversely, few studies about endophytic fungi from *Pinus* twigs have been performed (Petrini & Fisher 1988) but several studies on other coniferous species are available. Endophytes of conifers may augment host defense against natural enemies e.g. *Rhabdocline parkeri* in *Pseudotsuga menziesii* (Carroll & Carroll 1978).

Arnold *et al.* (2007) examined the diversity of endophytic fungi in asymptomatic foliage of loblolly pine (*Pinus taeda*) using molecular techniques to compare culturing and culture free methods. Cultures of endophytes often lack the taxonomic characters needed for identification. Therefore, morphotaxa, based on macroscopic colony features, are frequently used as functional taxonomic units (Guo *et al.* 2003). In some cases molecular sequence data from the nuclear ribosomal internal transcribed spacer region (ITS) have been used to identify sterile cultures and to evaluate morphotaxon boundaries (Lacap *et al.* 2003).

Knowledge on fungal endophyte may constitute an important tool to evaluate the real impact of fungi associated to certain symptoms. Some infections remain latent and become pathogenic when the host is stressed. Little information is available on the incidence of fungi that can cause diseases of *Pinus taeda* Linn. and *P. elliotti* Engelm in Uruguay. *Dothistroma pini* Hulbary (= *Mycosphaerella pini* Face.) (Bettucci & Guerrero, 1970a, FAO, 2006) that causes red band needle blight; *Hypoderma desmazieri* Duby (= *Meloderma desmazieri* (Duby) Darker) (Bettucci & Guerrero 1970b, FAO 2005) causing black spots, and *Diplodia pinea* (Desm.) J. Kickx (syn. *Sphaeropsis sapinea* (Fr.: Fr.) Dyko & Sutton) causing wilting and death of tree tops in nurseries were the most common pathogens associated to *Pinus* spp. in Uruguay (Koch de Brotos *et al.* 1981). *Diplodia pinea* also causes cankers, and sapwood discoloration in standing trees and logs (Stanosz *et al.* 1999). But during the last years with the increasing area of *Pinus* plantations other problems arrived, such as pitch canker in seedlings of *P. taeda* caused by *Fusarium circinatum* Nirenberg & O'Donnell (Alonso & Bettucci 2009).

Ceratocystis and *Ophiostoma*, causal agents of blue stain on *Pinus* spp., have a high incidence on conifer plantations in other latitudes (Hansen & Lewis 1997). These fungi are often carried by insects such as bark beetles mainly Scolytidae that have a great potential in disseminating the fungi and a wide host range including conifers other than pines (Zhou *et al* 2001). These bark beetles spread in recent years in Argentine, Brazil, Uruguay and Chile (FAO, 2006).

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Another significant problem, recorded in Uruguay for the first time in 1980, the association between the wood wasp *Sirex noctilio* F. and *Amylosterum areolatum* (Fr.) Boidin, which together they may kill or damage pine trees (Carvalho 1992).

The aim of this work was to evaluate the endophytic mycobiota of symtomless needles and twigs of *P. taeda* and *P. elliotti* in Uruguay and to detect the presence of fungal pathogens associated with spotted needles.

Materials and Methods

Study area

Sampling was performed in plantations located in the North (Department of Rivera), in the North West (Department of Tacuarembó), and in the centre (Department of Durazno) of Uruguay. In the North, *P. taeda* and *P. elliotti* were planted in 1999 with a density of 580–600 trees per hectare and had been pruned three years later. In the centre *P. taeda* and to a lesser extent *P. elliottii* were planted in 1997–1998. In these plantations thinning had been done. Needles of *P. taeda* with the frequent symptom of yellow band blight were sampled as well as needles of *P. elliotti* that had a higher incidence of this symptom. Twigs from both *Pinus* species from 4 years old trees were sampled.

Asymptomatic needles from 10 trees of. *P. taeda* and 10 of *P. ellioti* were sampled at each sampling site as well as needles with spots or bands of discoloration. Specimens were transported to the laboratory in polyethylene bags, cooled and processed within 24 hours.

Fungal Isolation and identification

Needles were washed with 0.05% tween 80, surface sterilized by dipping in 80% ethanol for 1 minute, 4% sodium hypochlorite for 5 minutes and then rinsed with sterile distilled water (2 times). Needles were cut into 3 sections of 10 mm length corresponding to the basal, middle and distal portions to evaluate the distribution of endophytes in needles (Hata & Futai 1995). These segments were put onto Petri dishes containing 2% malt extract agar and chloramphenicol 0.01 mg/L, and incubated at 25 °C. A total of 100 asymptomatic *P. taeda* needles and 100 needles of *P. elliotti* were incubated. Segments of needles with yellow bands were surface sterilized as described above and 100 segments corresponding to the bands (10 segments per plate) were put onto 2 % malt extract supplemented with chloramphenicol and incubated at 25 °C.

From branches, segments of 3–4 mm were cut and bark was stripped from xylem. They were surface sterilized and incubated as described above. When fungi emerged they were counted and transferred to fresh medium for identification. Isolates were identified by macro- and micromorphological characters following the standard mycological methods. When the mycelia remained sterile or isolates

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could not be identified by morphological characteristics, identification by molecular methods was performed. DNA extraction and amplification of the ITS1-5.8S-ITS2 rDNA region was done as described by Lee & Taylor (1990) and then sequenced by MACROGEN, Korea. The sequences were blasted against GeneBank. Phylogenetic analyses were done to identify species.

Results

Needles

In total 499 isolates belonging to 33 taxa from *P. elliotti* and 517 isolates belonging to 22 taxa from *P. taeda* were obtained. In both pine species *Lophodermium australe* and species of Xylariaceae were dominant, representing more than 50% of isolates from needles of *P. elliotti* and more 85 % from those of *P. taeda*. The relative abundance of *L. australe* was 43.3 % in *P. elliottii* and 66.5% in *P taeda*. Xylariaceae had an abundance of 11.6 % and 13.7% respectively (Table 1). In needles of *P. elliotti* the highest colonization ocurred in the distal part and in *P. taeda* in the midle part.

Although slight differences were observed among anamorphic isolates of *Lophodermium* - colonies with more or less aerial mycelium and absence or presence of yellow-brown pigmentation in the culture medium- all produced similar conidia after an incubation period of 20 days under the alternation of light / dark. The similarity of the macro and micromorphological characters did not allow discriminating between species. Sexual structures were not produced in culture nor in needles or twigs placed in a moist chamber even in senescent needles collected from litter. Some isolates from several morphotypes were sequenced and molecular analysis showed high similarity either to *L. australe* or to *Lophodermium kumanicum* (Figure 1) both species are considered as endophytes.

Few fungal taxa (8 in *P. elliotti* and 7 in *P. taeda*) were obtained from needles with symptom characterized by reddish or yellow bands. The dominant species associated with this symptom was *L. australe* representing the 82% of colonies in *P. taeda* and 37 % in *P. elloitti*. The remaining taxa are ubiquitous fungi and widely distributed endophytes.

Isolates corresponding to Xylariaceae were the most frequent but they remained sterile in culture. Phylogenetic analyses of ITS sequences revealed that most of isolates corresponded to the genus *Xylaria,* mainly *Xylaria arbuscula*, *Xylaria enteroleuca, Xylaria longipes* and *Xylaria polymorpha* (Figure 2).

Cenangium ferruginosum could be isolated from *P. ellioti* in very low frequencies. The remaining isolates belong to endophyte species of several hosts and to sterile mycelia.

Fungi were unevenly distributed along the needles (distal, middle, and proximal part (Tab.1).

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Tab. 1. – Relative density of isolates of endophytic fungi present in segments of symptomless needles of *Pinus elliottii* and *Pinus taeda* (B = basal; M = middle; D = distal)

	Pinus elliotti			***Pinus taeda***		
	B	**M**	**D**	**B**	**M**	**D**
Acremonium kiliense Grütz					0.4	
Alternaria alternata (Fr.) Keissl.	25.9	1.7	4.7	8.8	0.4	
Arthrinium sphaerospermum Fuckel			0.8	1.3		
Botryosphaeria sp 416			0.4			
Cenangium ferruginosum Fr.		0.5	0.8			
Cladosporium cladosporioides (Fresen.) de Vries	17.6	2.8	1.3	8.8		0.5
Coelomycete with spermatia				1.3	0.4	0.5
Curvularia lunata var. aeria (Bat., J.A. Lima & C.T. Vasconc.) M.B. Ellis				5.0		
Didymosphaeria igniaria C. Booth		1.7	0.4			
Dreschlera raveneli (Curt.) Subram.& Jain					0.4	
Epicoccum purpurascens Ehrenb. ex Schlecht.	5.9	0.5		11.4	0.4	0.5
Fusarium anthophilum (A. Braun) Wollenw.			0.4			
Fusarium subglutinans (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas	1.2					
Geotrichum candidum Link	1.2	0.5	0.4			
Hormonema dermatioides Lagerber & Melin	5		0.4			
Lophodermium australe Dearn	25.9	50.5	53.4	43.0	84.4	72.2
Nigrospora sp.	1.2	1.7	0.8	1.3	0.4	
Nigrospora oryzae (Berk. & Broome) Petch				1.3	1.2	2.6
Nigrospora sphaerica (Sacc.) Mason		0.5				
Nodulisporium sp.			0.4			
Pestalosphaeria guepinii (Desm.) Steyaert				1.3		0.5
Pestalotiopsis guepinii (Desm.) Steyaert		0.5	0.4	6.3		
Phoma sorghina (Sacc.) Boerema, Dorenb. & Kesteren	1.2	5.5	5.5			
Phomopsis archeri B. Sutton					0.4	
Scytalidium lignicola Pesante						0.5
Torula herbarum (Pers.) Link			0.8			
Trichoderma harzianum Rifai	1.2		0.4			
Ulocladium botrytis Preuss		0.5	0.4			
Xylaria arbuscula Sacc.			0.8			
Xylaria enteroleuca (Speg.) P.M.D. Martin		2.2	1.3	1.3		0.5
Xylaria longipes Nitschke	2.4	1.1				
Xylaria polymorpha (Pers.) Grev.		0.5	0.4			
Xylaria spp.	2.4	12.2	8.1	5.0	11.0	20.1
Sterile mycelium 402		1.1				0.5
Sterile mycelium 409	2,4	5.0	6.4	1.3		0.5
Sterile mycelium 410			0.8			
Sterile mycelium 421		1.1				
Sterile mycelium 423			0.4	1.3		

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Tab. 1. – Continued

	Pinus elliotti			*Pinus taeda*		
	B	**M**	**D**	**B**	**M**	**D**
Sterile mycelium 430				1.3		0.5
Sterile mycelium E1	3.5	1.7	0.4			
Sterile mycelium E2	2.4	6.7	8.9			
Sterile mycelium E3		1.1				
Sterile mycelium T26					0.4	0.5
Total isolates	85	180	234	79	244	194
Total segments	200	200	200	200	200	200

Tab. 2. – Relative density of isolates of endophytic fungi present in twigs of *Pinus elliottii* and *Pinus taeda* (B = bark; X = xylem)

	Pinus elliotti		***Pinus taeda***	
	B	**X**	**B**	**X**
Acremonium kiliense Grütz			1.3	
Alternaria alternata (Fr.) Keissl.	5.9		1.3	
Alternaria longissima Deighton & MacGarvie			1.9	
Aureobasidium sp. T250			8.8	
Botryosphaeria sp.T251			11.3	
Cenangium ferruginossum Fr.			0.6	
Cladosporium herbarum (Pers.) Link	7.4			
Coelomycete	1.5			
Colletotrichum gloeosporioides (Penz.) Penz. & Sacc.	1.7			
Curvularia lunata (Wakker) Boedijn			2.5	
Dichomera saubineti (Mont.) Cooke			4.4	
Drechslera rostrata(Drechsler) M.J. Richardson & E.M. Fraser			3.8	
Epicoccum purpurascens Ehrenb.			1.9	
Hormonema dematioides Lagerber & Melin			7.5	
Lophodermium australe Dearn.	4.4	3.3		
Microsphaeropsis conielloides B. Sutton			2.5	
Nigrospora sacchari (Speg.) E.W. Mason			1.3	
Pestalotiopsis guepinii (Desm.) Steyaert	2.9		1.9	
Phaeomoniella sp.		96.6		86.7
Phomopsis sp.	14.7		2.5	
Sporobolomyces roseus Kluyver & C.B. Niel			12.5	
Trimmetostroma sp.	4.4			
Xylariareaceae.	19.1		0.6	
Sterile mycelium E203	7.4			
Sterile mycelium E204	17.6			
Sterile mycelium E 210	5.9			
Sterile mycelium E214	4.4			
Sterile mycelium E220	2.9			
Sterile mycelium T263			4.4	
Sterile mycelium T266			28.1	
Sterile mycelium T267			1.3	
Sterile mycelium T312				13.3
Total isolates	68	30	160	15
Total segments	100	100	100	100

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Twigs

In twigs of *Pinus elliotti* 68 isolates belonging to14 taxa were obtained from bark and 30 isolates belonging to 2 taxa from xylem. In bark tissues *Phomopsis* and species of *Xylaria* were the most abundant taxa (Tab. 2). In *Pinus taeda* 160 isolates belonging to 20 taxa from bark and 15 isolates belonging to 2 taxa from xylem were found. *Bot-*

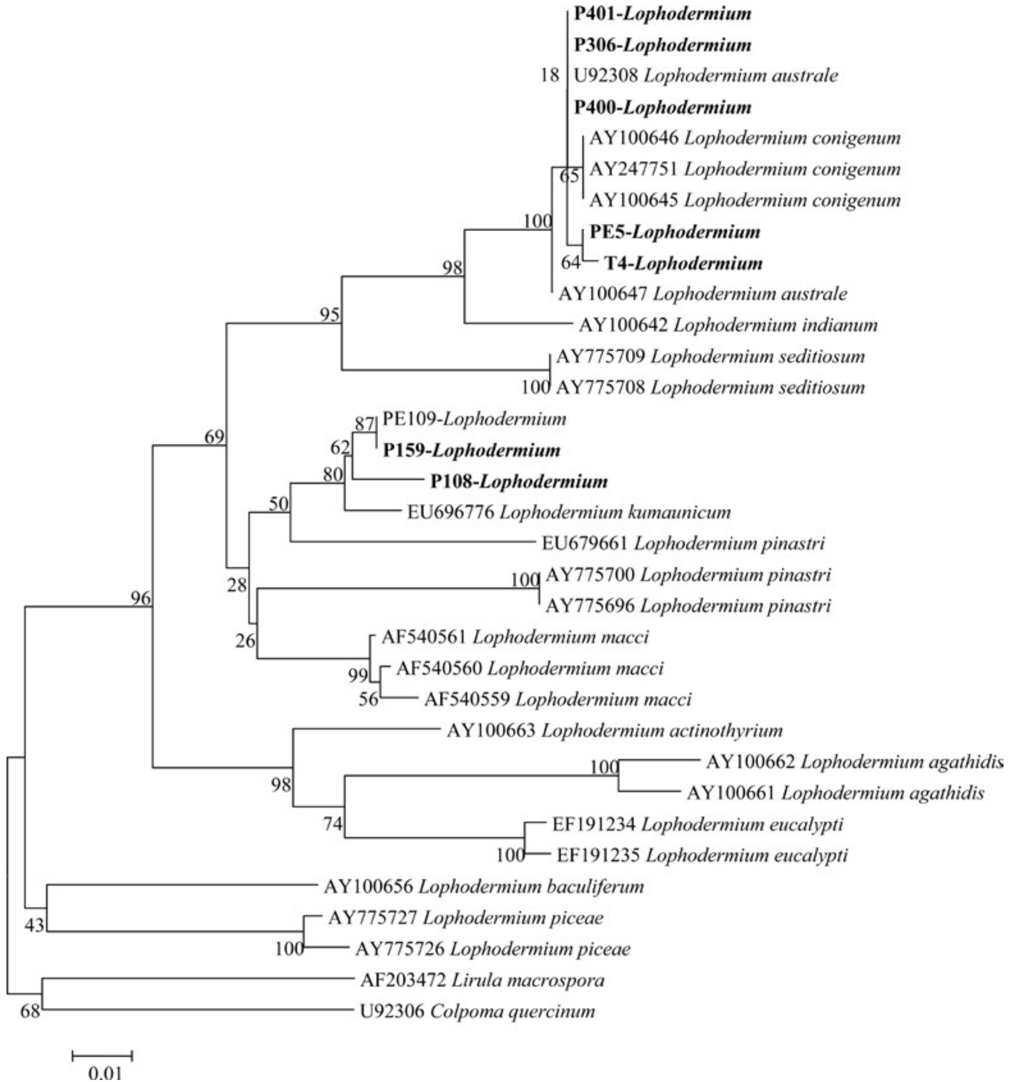

Fig. 1. – Phylogenetic tree obtained from sequence analysis of the ITS1-5.8S-ITS2 rDNA of *Lophodermium* isolates. Strains obtained in this study are indicated in bold. Numbers at nodes represent bootstrap values of 1000 replicates. Codes next to the name of the species represent accessions numbers from GenBank.

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ryosphaeria spp., *Sporobolomyces roseus* and sterile mycelia represented the 57.5 % of isolates in bark xylem of both tree species *Phaeomoniella* sp. was most common (Tab. 2; Fig. 3). The other species present in xylem were *Lophodermium australe* in *P. elliotti* and *Cenangium ferruginosum* in *P. taeda*.

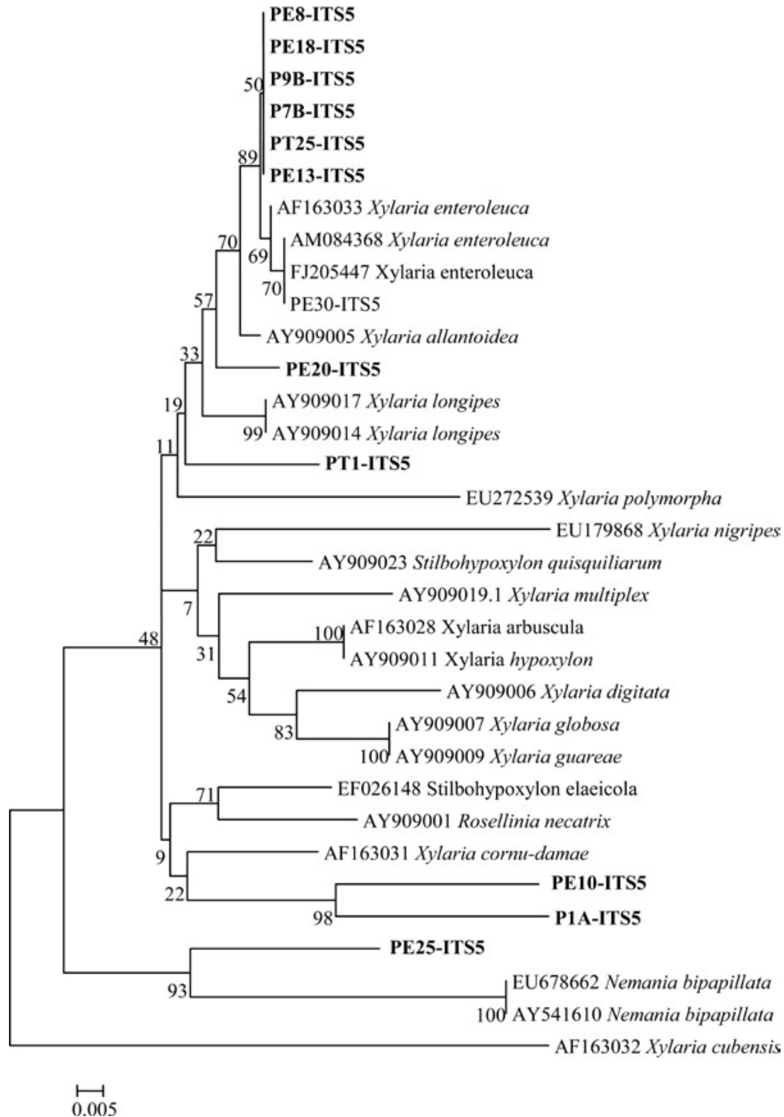

Fig. 2. – Phylogenetic tree obtained from sequence analysis of the ITS1-5.8S-ITS2 rDNA of Xylariaceae isolates. Strains obtained in this study are indicated in bold. Numbers at nodes represent bootstrap values of 1000 replicates. Codes next to the name of the species represent accessions numbers from GenBank.

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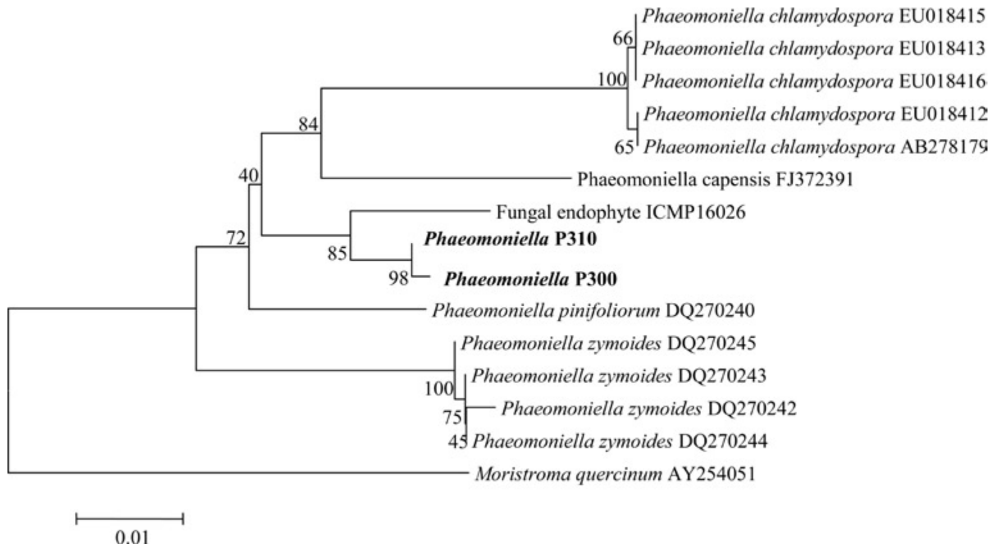

Fig 3. – Tree obtained by phylogenetic analysis of the ITS1-5.8S-ITS2 rDNA of sequences from *Phaeomoniella* isolates from xylem of twigs. Isolates analyzed in this study are indicated in bold. Numbers at nodes represent bootstrap values from 1000 replicates.

Discussion

The number of taxa obtained from each tissue confirms that the fungal endophytes of trees planted outside their geographical origin are depauperate in species composition (Espinosa-García & Langenheim 1990, Fisher 1994). In this study, several endophytic taxa of the non-native *Pinus* species are the same to those found in native plants (Bettucci *et al*. 2004). Moreover, some of the endophytes of twigs and needles are cosmopolitan. However, tissue preference was consistent and could be demonstrated by the different dominant species and the few species shared by xylem and bark.

Lophodermium australe was the most abundant species that colonized needles with and without symptoms in both pine species. In needles of *P. elliotti* and *P. taeda* the distal half had higher density of endophytes that confirms results published by Deckert & Paterson (2000). They also showed that the proximal part of the needle has a significantly lower infection rate than the distal part. The distribution of endophytic fungi could be related to the architecture and phenology of needle development (Wilson & Carroll 1994).

Xylaria spp. are the main decomposers in needles. Members of this genus are usually associated with Angiospermae and rare in conifers. The fruit bodies of *Xylaria* frequently occur on lignocellulosic litter. They play an important role in the degradation of lignocellulosic ma-

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terials particularly by their cellulolytic activity (Guo *et al.* 2003). Probably, the source of inoculum of *Xylaria enteroleuca* came from fruit bodies on debris of of Myrtaceae present in Uruguay (Bettucci et al. 2004) and on debris of *Illex paraguariensis* present in south of Brazil, Argentine, Paraguay and Uruguay (Takeda *et al.* 2003).

On the other hand, some *Lophodermium* species are known endophytes of conifers and others like *Lophodermium seditiosum* and *Lophodermium pinaster* are pathogens causing needle-cast in *Pinus* plantations in Uruguay (FAO, 2006). Some of our isolates showed a high homology with *L. australe* and others group together with *L. kumanicum* in phylogenetic analyses. Both species are considered endophytes.

Many fungal endophytes play an important role in occupying the same niche as fungal pathogens and therefore excluding them. *Lophodermium conigenum* can displace cogeneric pathogens like *L. seditosum* and *L. pinaster*. Some members of *Lophodermium* act as saprobes which decompose needles after senescence but some species have the ability to infect healthy needles and are therefore in a favorable position for colonization. The rate of colonization by saprotrophic species also increases with aging progresses reflecting a physiological change in the needles. Deckert *et al.* (2001) found that other species of *Lophodermium* are active biotrophes growing slowly during the cryptic endophytic stage. A gradual acquisition of nutrients enables the development of reproductive structures. It can be speculated that pathogenicity evolved from an endophytic life style in *Lophodermium* (Ortiz-García *et al.* 2003). The higher number of isolates of *Lophodermium* in *P. taeda* than in *P. elliotti* could be related with the resistance of needle tissues against fungal infection. The histopathology of the host/parasite relationship of *P. taeda* and *Ploioderma lethale* a Rhytismataceae infecting needles also is well documented (Jewell 2001). Inter- and intracellular hyphae can be observed in collapsed living mesophyll cell regions; intracellular hyphae are present in endodermal, transfusion, and in vascular tissues, as well as in resin ducts. In green needles of *P. elliottii* var. *elliottii*, the hyphae of *Ploioderma hedgcocki* are restricted to tissues outside the endodermis. The mesophyll cells adjacent to fungal stromata show abnormalities; conversely the mesophyll distal to the stromata no symptoms despite heavy colonization by intercellular hyphae are visible (Jewell 1994).

The endophytic fungal community in twigs was composed of species different to those found in needles. The species composition of twig bark, as in other plants, is constituted by a greater number of isolates and a higher number of taxa than in xylem.

Phaeomoniella sp. is the main species in xylem in both tree species. From phylogenetic analysis it seems that the isolates obtained here may represent a new species. The genus *Phaeomoniella* contains stem pathogenic species in *Vitis vinifera and* other plants (Abreo *et al.*

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2011, Crous & Gams 2000). Two species, *Phaeomoniella zymoides* and *Phaeomoniella pinifoliorum* were described as epiphytes of needles of pine by Lee et al. (2006).

Cenangium ferruginosum, scarcely present in healthy needles of *P. elliotti* and in bark of *P. taeda* is known from species of *Pinus*. It is a subcortical endophyte but may develop as active pathogen under unfavorable conditions as frost or drought. Some studies indicate that it can kill cortical tissue and the cambium of weakened yellowish needles during late winter and early spring (Jurc *et al*. 2000).

Several species isolated in this study were found in plantations of Angiosperms as *Vitis* spp. and in native Myrtaceae. This fact suggests that endophytic fungi in non-native *Pinus* originate from anamorphic and telemorphic fructifications developed on debris from native plants.

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