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Studies in Diatrypaceae: the new species *Eutypa microasca* and investigation of ligninolytic enzyme production

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The extracellular production of ligninolytic enzymes by xylariaceous and diatrypaceous species from Argentina was investigated. Among them, a new species, *Eutypa microasca*, was evaluated. Its morphological characteristics (e.g.: very small asci) along with the molecular analyses, support the hypothesis of the new taxon. Ligninolytic enzyme production by several saprotrophic and endophytic strains belonging to the genera *Xylaria, Eutypella, Eutypa* and *Peroneutypa* was assessed on solid medium supplemented with different dyes (Poly R-478, Azure B and Malachite Green) and under submerged fermentation in copper amended malt extract/glucose medium. All the strains produced laccase in liquid cultures, and seven out of nine also produced Mn-peroxidase (highest titres detected 113 U/l). Only five of the strains assayed could not decolorize Malachite Green. This is the first report on laccase and Mn-peroxidase production by Diatrypaceae and contributes to the knowledge on ligninolytic enzyme production in Xylariaceae.

Keywords: enzyme assay, Eutypella, laccase, Peroneutypa, Xylaria.

The family Diatrypaceae was established by Nitschke (1867). Since then, several authors have reviewed both the family and the generic concepts (Tiffany & Gilman 1965, Glawe & Rogers 1984, Rappaz 1987, Vasilyeva & Stephenson 2004, 2005, Carmaran *et al.* 2006). Kirk & al. (2008) reported 13 genera for this family, but recent studies (Acero *et al* 2004, Trouillas *et al.* 2011), using molecular approaches, have suggested that the current taxonomic scheme for the *Diatrypaceae* does not reflect the evolutionary relationships of the genera proposed up today. *Peroneutypa* Berl. (Carmaran *et al.* 2006), represents in our knowledge the unique genus that appears as a monophyletic group in the different phylogenetic analyses (Acero *et al.* 2004, Carmaran *et al.* 2009, Trouillas *et al.* 2011).

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Several taxonomic studies try to classify diatrypaceous fungi in North America (Tiffany & Gilman 1965, Glawe & Rogers 1984, Vasilyeva & Stephenson 2004, 2005). Trouillas *et al.* (2011) investigated the diversity and host range of diatrypaceous fungi from prominent wine grape in Australia. In Argentina, Spegazzini (1898, 1910, 1925) began to study the biodiversity of the *Diatrypaceae* and described 31 species and several varieties. In recent years, the knowledge on diatrypaceous fungi in this country has increased substantially (Carmarán & Romero 1992; Carmarán 2002; Romero & Carmarán 2003; Pildain *et al.* 2005; Carmaran *et al.* 2006, 2009).

The present study is part of an extensive project aiming at describing and characterizing the diatrypaceous fungi on native and cultivated woody plants in Argentina.

Members of this family are predominantly saprotrophic on angiosperm bark, few of them were reported as pathogens (Trouillas & Gubler 2004, Trouillas *et al.* 2011) or endophytes in petioles (Carroll & Carroll 1978) and woody tissue (De Errasti *et al.* 2010).

In spite of the association of members of this family with wood, only a few studies have evaluated lignocellulose degradation in this group of microorganisms. A previous work by Shary *et al.* (2007) has shown that they are soft-rot decay fungi, and Worrall *et al.* (1997) have suggested that *Eutypella parasitica* and *Cryptosphaeria lignyota* have the ability to cause white-rot decay. *Cryptovalsa halosarceicola* proved its capacity of solubilizing significant amounts of lignin, comparable to those of white-rot Basidiomycota (Bucher *et al.* 2004). As far as we know, ligninolytic enzyme production by diatrypaceous fungi has been evaluated only on solid media (Pildain *et al.* 2005). Information on the enzymes involved in the degradation process and quantitative analyses of enzyme production in order to corroborate their white-rot behaviour is still missing. To our knowledge there are no reports on enzyme production under submerged fermentation.

In the present paper, a new species of this family is described. Additionally, we carried out a study on several diatrypaceous fungi including saprotrophic and endophytic strains in order to assess ligninolytic enzyme production on solid media and under submerged fermentation, and to determine if diverse ecological strategies could be related with differences in enzyme profile production.

Materials and methods

Identification and morphological characterization

Two replicates per strain were grown on MEA and Leonian-agar in the dark at 25 °C. Colony characteristics were observed every 7 days during 4 weeks according to Glawe & Rogers (1984) and Pildain *et al.* (2005). Measurements for at least 30 conidia, 30 ascospores and 10 asci were made at 400× and 1000× magnification. For the determination of the number of nuclei of

the conidia mycelia and conidiomata, 4 weeks-old cultures on Leonian agar, were stained with Acridine Orange (400 µM) and 4',6-diamidino-2 phenylindole (DAPI). To visualize the nuclei with Acridine Orange, the method of Sándor *et al.* (2000) slightly modified was followed. Mycelia were covered 5 min with Acridine Orange-ethanol 0.01 %, followed by two rinses in Phosphate Buffered Saline (PBS) 100 mM pH 7.4 during 30 sec. For DAPI, samples were incubated with acetic acid 60 % in PBS during 30 min and stained with DAPI during 15 min in the dark. The samples were observed under confocal microscope Olympus FV300.

Samples for light microscopy were prepared from moistened specimens mounted in distillated water, 5 % KOH, Phloxine and Melzer's reagent. Samples for epifluorescence light microscopy (EFM) were prepared in 0.05 % Calcofluor (Romero & Minter 1988). Photographs were taken with an Olympus c-5060 wide zoom digital camera.

Molecular analyses

For genotypic analyses the strains were grown on malt peptone (MP) broth using 10 % (v/v) of malt extract (Brix 10) and 0.1 % (w/v) Bacto peptone (Difco), 2 ml of medium in 15 ml tubes. The cultures were incubated at 25 °C for 21 days in light/darkness. DNA was extracted from the cells by using the UltraCleanTM Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Solana Beach, USA), according to the manufacturer's instructions. The ITS region of the nuc-DNA was amplified using the universal primers ITS1 and ITS4 (White *et al.* 1990), while a fragment of the β-tubulin gene was amplified using the primers T1 and Bt2b (Glass & Donaldson 1995, O'Donnell & Cigelnik 1997). The best amplification results were achieved by adding 6 % bovine serum albumin (BSA, Promega Corp.) to the PCR reaction mix. PCR products were purified using a QIAGEN Gel Extraction kit (QIAGEN Inc.). Both strands of each fragment were sequenced at the Molecular Biology Facility of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (Buenos Aires, Argentina). Additionally 59 sequences obtained from GenBank were included in the analysis, representing 58 species of the family Diatrypaceae. *Xylaria curta* (DQ322144) and *Xylaria berteri* (AY951763) were chosen as outgroups according to Trouillas & Gubler (2004). DNA sequences were edited with the software program BioEdit sequence alignment editor, version 7.0.5.3 (Hall 1999). The phylogenetic analysis of the sequence data was performed using maximum parsimony on NONA version 2.0 (Goloboff 2005) with all the characters equally weighted and gaps scored as missing data. Overall, 3.5 % of the data matrix cells were scored as gaps. A bootstrap analysis was performed with 1000 replications. Bayesian inference was calculated with MrBayes v. 3.1.2 with default settings (Huelsenbeck & Ronquist 2001). Two Markov chains were run from random starting trees for 5 million generations and sampled every 250 generations. The first 25000 generations were discarded as burn-in.

Isolates and enzyme assays

The screening test was carried out with 15 native Argentinean strains of *Diatrypaceae* (Xylariales, Ascomycota) of the genera *Eutypella*, *Eutypa* and *Peroneutypa*. In order to establish a benchmark, two strains belonging to the genus *Xylaria* and four strains of Basidiomycota with recognized ligninolytic ability were included. A strain of *Bjerkandera*, which proved to be incapable of decolorizing the dyes assayed in a previous work (Levin *et al.* 2004) was also included as negative control. All native strains were deposited in BAFC (Holmgren *et al.* 1990). Stock cultures were maintained on malt extract agar slants at 4 °C.

Culture conditions

All the strains were inoculated in 90 mm Petri plates (20 ml medium per plate) containing malt extract (12.7 g/l), glucose (10 g/l) and agar (20 g/l) (MEA), or MEA supplemented with either 0.02 % of Poly R-478 (Sigma), 50 µM of Azure B (Sigma) or 50 µM of Malachite Green (Mallinckrodt). Inoculum consisted of a 25 mm agar plug from a 5 day-old culture grown on MEA. Uninoculated plates served as controls for abiotic decolorization. Each fungus was tested in three independent experiments on all media. The inoculated plates were incubated at 24 °C for 21–28 days. Mycelial radial growth was measured weekly in each plate. Average growth rates (cm/day) were calculated. A decolorized zone appeared when the fungus degraded the dye. Weekly measurements of the colonies and the decolorized zones (if any) were performed for each strain.

Ten isolates were selected for further assays in submerged fermentation. Cultures were incubated at 24 °C with constant agitation at 120 rpm, in 250 ml Erlenmeyer flasks with 25 ml of medium [malt extract (12.7 g/l), glucose (10 g/l), copper sulphate 300 µM)] which were inoculated with a 25 mm^2 surface agar plug from a 7 day-old colony grown on agar 2 %. Cultures were harvested periodically; aliquots of the supernatant were collected aseptically, after 35 days the whole culture was filtered through a filter paper using a Büchner funnel, and dried overnight at 70 °C. The dried mycelium was then weighed. Culture filtrates were used as enzyme sources.

Enzymatic quantitative analysis

A quantitative enzymatic assay was carried out for nine Ascomycota strains selected due to their superior ligninolytic enzyme production, assessed on solid medium. The basidiomycete *Coriolus antarcticus* also was evaluated as a positive control strain.

To detect activity in submerged cultures, supernatants were used in reaction mixtures. Laccase activity was determined measuring the increase in Abs. 420 (ε420=36/mM cm) after 10 min of incubation at 30 °C, due to the oxidation of 0.5 mM 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonate)

(ABTS) in 0.1 M sodium acetate buffer (pH 3.5) (Bucher *et al.* 2004). One unit of laccase activity (*U*) was defined as the amount of enzyme required to oxidize 1 µmol of ABTS in 1 min. Mn-peroxidase (MnP) activity was assayed with 0.1 mM $MnSO_4$ and H_2O_2 and 0.01 % phenol red as the substrate (Glenn & Gold 1985). Reactions were halted after 45 min of incubation at 30 °C, by adding NaOH 5 N, and increase in Abs. 610 was measured (ε610=22/mM cm). One unit of enzyme activity (*U*) was defined as the amount of enzyme required to oxidize 1 µmol of Phenol Red in 1 min. Lignin peroxidase (LiP) activity was assayed, by measuring the decrease in absorbance at 651 nm due to the oxidation of Azure B (Archibald 1992).

Statistical analysis

The data presented are the average of the results of three replicates with a standard error of less than 5 %. Analysis of variance was tested by the software Infostat (Di Rienzo *et al.* 2011). Differences between treatments were compared by Tukey's multiple comparison tests at 5 % level of probability.

Results

Taxonomy

Eutypa microasca Grassi & Carmarán, **sp. nov.** – Fig. 1.
MycoBank no.: MB 807020

Latin diagnosis. – Stromata sparsa. Perithecia monostycha. Asci 15–27 × 3–5 µm. Ascosporae allantoideae, hyalinae, 5–6 × 1–1,5 µm.

Holotypus. – ARGENTINA, Buenos Aires, Lomas de Zamora, Reserva Sta. Catalina, on fallen unidentified wood, Carmarán Cecilia, June 2005, BAFC 51550 (holotype, BAFC). GenBank accession number: KF964566 (ITS sequence).

Description. – Stromata effuse, frequently with a stromatic layer in inner tissue of wood. Perithecia numerous, immersed in the wood (Fig. 1 a, b), necks long, emerging separately, prominent, ostioles sulcate. Asci fusiform 15–27 × 3–5 µm. Ascospores allantoid, hyaline, 5–6 × 1–1.5 µm (Fig. 1 d–g).

Conidiomata effuse, weakly developed, melanised, associated with arising conidiophores. Conidiogenous cells cylindrical with few ramifications, sympodial growth or percurrent proliferation on MEA, with two conidia per cell, observed since the end of the first week of growth. Conidia produced holoblastically, moderately curved in the upper part (comma-like appearance, Fig. 1 h), hyaline, basal end flattened, 14–24 × 1.5 µm, observed since the end of the first week of growth. Under fluorescence with Acridine Orange and DAPI the nuclei of conidia elongated, filling a large portion of the cell, with a spirally appearance in immature conidia, however, in a significant number of conidia nuclei not observed. Culture characteristics: On MEA, the mitosporic state growing fast, covering the dish in 2 weeks (Fig. 1 c). Colonies whitish-cream, with cottony tufts around the inoculum, reverse coloration yellow at center, forming numerous conidiomata at the end of the first week of growth.

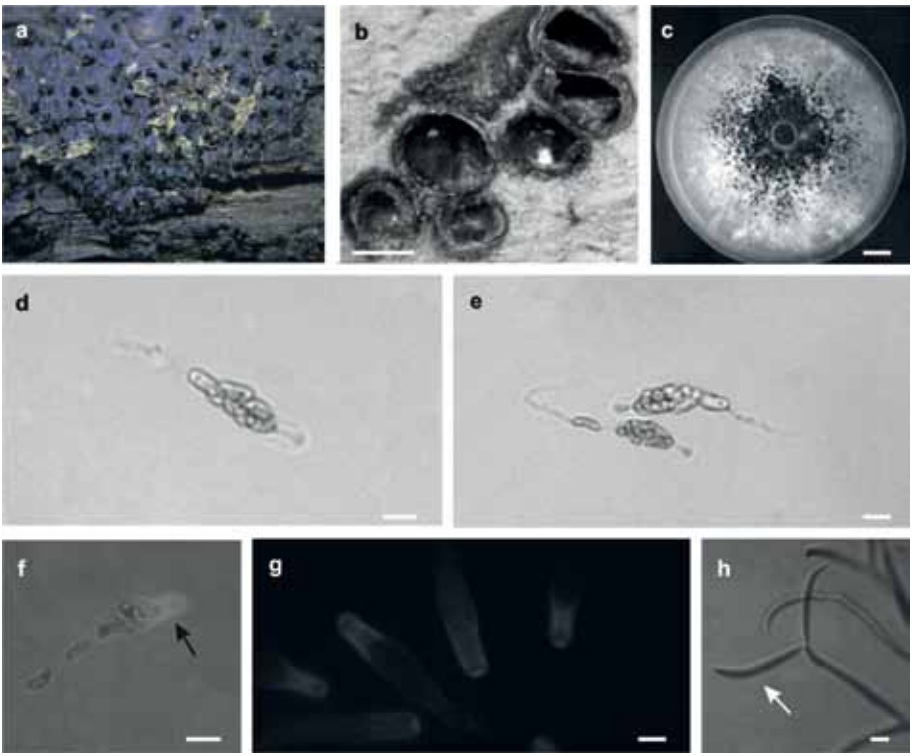

Fig. 1. *Eutypa microasca*. **a** Stroma, **b** perithecia, longitudinal section, **c** culture plate, **d**, **e** asci, **f** ascus apical apparatus under UV light, **g** ascus with fluorescence, h conidiogenous cells. Scale bars: b = 50 µm; c = 1000 mm; d–h = 5 µm.

Etymology. – Refers to the small size of asci.

Distribution and habitat. – Known from Buenos Aires, Argentina, growing on dead unidentified wood.

Material examined. – ARGENTINA, Buenos Aires, Lomas de Zamora, Reserva Sta. Catalina, 34°45´42"S, 58°27´39"W, from bark of fallen limbs, June 2005, Carmarán Cecilia, BAFC 51556 living culture BAFC cult. 3013; BAFC 51550 (holotypus) living culture BAFC cult. 3015; BAFC 51551, living culture BAFC cult. 3050.

Comments. – The morphological features observed in the material studied are very similar to those described for *Eutypa leptoplaca* (Mon) Rappaz (Rappaz 1987), a known pathogen (Carmaran *et al.* 2009), however, the stromata are effuse, very extended in the substrate and the necks are more prominent than those described for *E. leptoplaca*. The morphology of nuclei agrees with the morphology reported in other species (Jacobs *et al.* 1988). Taking into account the lack of nuclei in an important amount of cells, probably they are degenerated. These combinations of characters suggest that these materials represent a new species. Molecular analyses support the hypothesis of the new taxon.

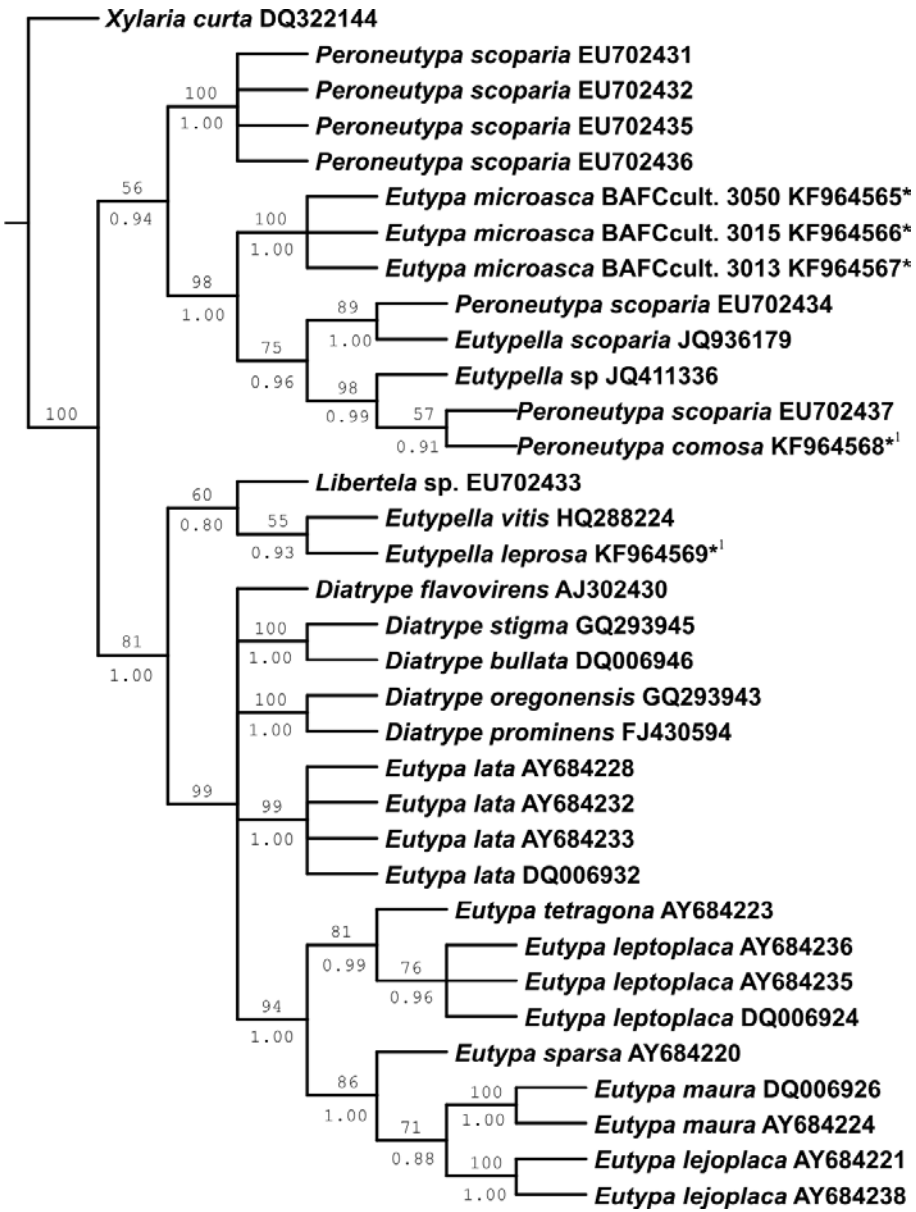

Fig. 2. One of the most parsimonious trees obtained from the phylogenetic analysis of the nrDNAs ITS sequence data set, using maximum parsimony analysis and Bayesian inference. The bootstrap values are shown above the line (> 50 %, 1.000 replications) and the Bayesian posterior probabilities (BPP) below the line. (*) Strains included into the new species. (*[1]) Sequences obtained during this work.

The maximum parsimony analysis of the ITS sequence dataset included 1230 informative characters and yielded two parsimonious trees of length (L) = 771, consistency index (Ci) = 66 and retention index (Ri) = 82. The consensus tree is shown in Fig. 2. Identical topology was recuperated from Bayesian inference (Fig. 2). The phylogenetic analysis with the β-tubulin dataset retained 107 most parsimonious trees sharing similar clade topology. One of the most parsimonious trees is presented in Fig. 3. The alignment contained 693 informative characters; the length of the β-tubulin trees was 1941 steps, with (Ci) = 69 and Ri = 87.

The taxa belonging to *Diatrypaceae* included in the analyses are strongly supported as monophyletic group (100 % bootstrap value) using *Xylaria curta* and *X. berteri* as outgroup taxa. However, taking into account the taxa belonging to the family, the topology of the tree does not agree with the accepted taxonomic rearrangement at genus level.

Both analyses showed an evident clustering of strains BAFC cult 3013, 3015 and 3050, supporting the hypothesis of a new species.

The three phylogenies were concordant with no conflict between the topologies of the trees except in the key clade that includes members of the genus *Peroneutypa*. In the analysis performed using ITS sequences, the new species appeared associated with strains belonging to the genus *Peroneutypa*. In Diatrypaceae three types of asci can be recognized according to Carmarán *et al.* (2006, 2009). In the Argentinean material presently studied some morphological characters were similar to those assigned to *Peroneutypa*, however the observed morphology of asci differed from the characteristic uniform asci described for this genus and shows the typical morphology of asci type 1, that is a thick-walled apical region, penetrated by a narrow channel with cytoplasmic strands connecting the apex with ascus cytoplasm. It is the most common type in the family. In the phylogenetic analysis performed from β-tubulin gene, the new species appeared in a separated clade close to the species with fusiform asci, supporting the morphogical observations. Taking into account the analyses performed and the morphological features, effuse stromata and necks separately emergent, we decided to place the new species in the genus *Eutypa*.

Growth and dye decolorization on solid media

Cultures of native Argentinean strains of *Diatrypaceae* (including the strains belonging to the new species), two strains belonging to the genus *Xylaria* and four strains of Basidiomycota, growing on agar complex medium containing the dyes tested, showed that 11 out of 17 Ascomycota strains evaluated and all the Basidiomycota examined, were able to grow and to decolorize all the dyes assayed (Tab. 1). Colony sizes were similar in Azure B and Poly R-478 media. Only Malachite Green (widely used as the most efficacious antifungal agent in the fish farming industry (Cha *et al.* 2001) inhibited partially the mycelial growth at the concentration used; six of the assayed Ascomycota were unable to decolorize this dye.

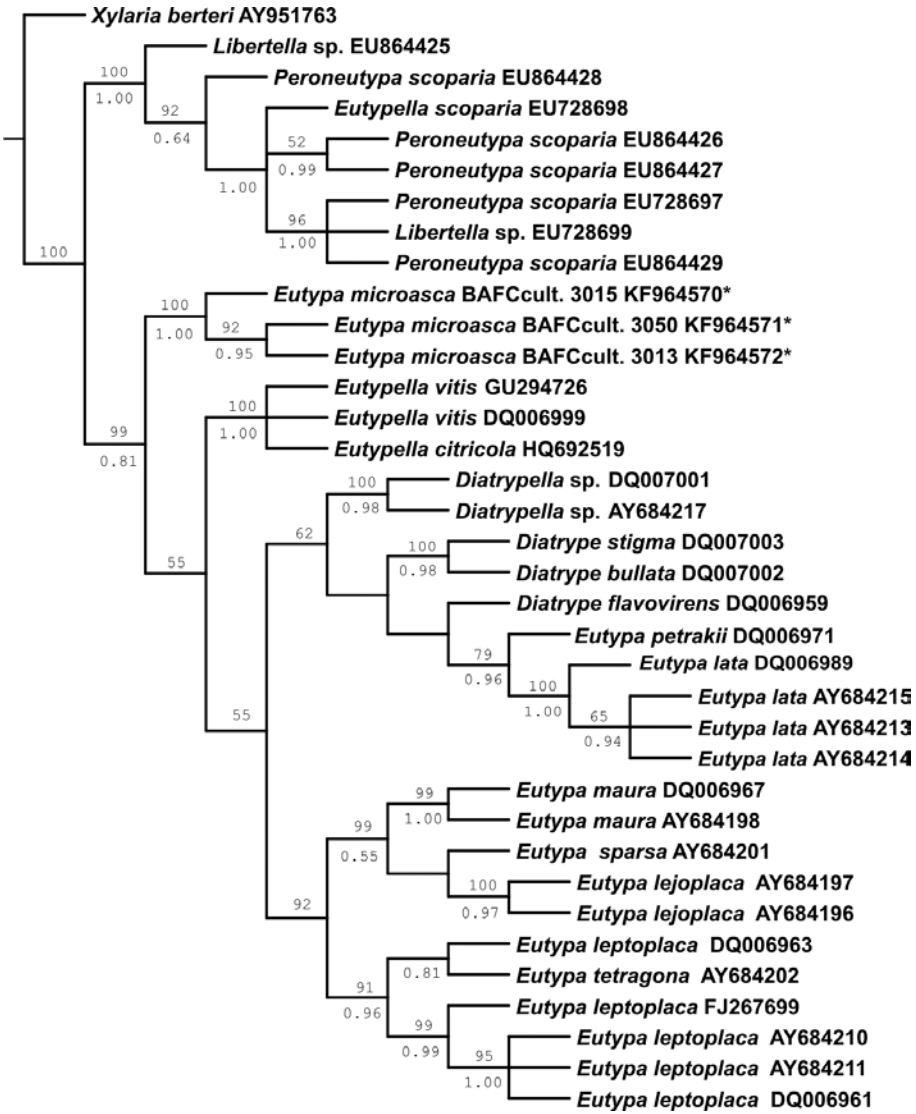

Fig. 3. One of the most parsimonious trees from the maximum parsimony and Bayesian analysis of the β-tubulin sequence data set. The bootstrap values are shown above the line (> 50 %, 1.000 replications) and the Bayesian posterior probabilities (BPP) below the line. (*) Strains included into the new species.

Plates with the lignin-model substrate Poly R-478 were used to estimate ligninolytic activity and plates containing Azure B were used to assess LiP activity. Only *Peroneutypa scoparia* 3322 did not decolorize Poly R-478 medium after 28 days of incubation. Among the basidiomycetes evaluated *Co-*

Tab. 1. Growth and solid-plate decolorization on media containing glucose (10 g/l), malt extract (12.7 g/l), agar (20 g/l) (MEA), supplemented with three different dyes: Poly-R 0.02 %; Azure B 50 µM or Malachite Green 25 µM.

Fungal strain (strain number)	Habit[3]	Growth in MEA[2]	Poly R-478[1] decolorization	Poly R-478[2] growth	Azure B[1] decolorization	Azure B[2] growth	Malachite Green[1] decolorization	Malachite Green[2] growth
Ascomycota								
Peroneutypa scoparia (146)	S	Slow	+	Slow	+++	Slow	+++	Slow
Peroneutypa comosa (393)	S	Fast	++++	Fast	++++	Fast	+++	Medium
Peroneutypa scoparia (3321)	E	Fast	+++	Fast	+++	Fast	–	Fast
Peroneutypa scoparia (3322)	E	Slow	–	Slow	+++	Slow	–	Slow
Peroneutypa scoparia (3324)	S	Medium	+++	Medium	++	Medium	+++	Slow
Peroneutypa scoparia (3325)	S	Medium	++	Medium	++	Slow	+++	Slow
Peroneutypa scoparia (3326)	S	Fast	+++	Medium	+++	Slow	–	Slow
Peroneutypa scoparia (3327)	S	Fast	+++	Fast	++++	Fast	+++	Medium
Peroneutypa scoparia (3328)	S	Mdium	++	Slow	+	Medium	–	Slow
Peroneutypa scoparia (3390)	E	Slow	++	Slow	++	Slow	–	Slow
Peroneutypa scoparia (3391)	E	Fast	+++	Fast	++++	Fast	+++	Slow
Eutypella leprosa (1744)	S	Fast	+	Fast	+++	Fast	+++	Slow
Eutypa microasca (3013)	S	Fast	+	Fast	++	Fast	+	Medium
Eutypa microasca (3015)	S	Fast	+	Fast	++	Medium	–	Slow
Eutypa microasca (3050)	S	Fast	+	Fast	++	Fast	+	Slow
Xylaria sp. (3192)	S	Medium	+	Medium	+++	Medium	+++	Medium
Xylaria sp. (3193)	S	Medium	+++	Slow	++	Medium	+++	Medium
Basidiomycota								
Coriolus antarcticus (266)	S	Fast	++++	Fast	++++	Fast	++++	Fast
Trametes trogii (463)	S	Fast	+++	Fast	++++	Fast	++++	Medium
Fomes sclerodermeus (2752)	S	Fast	++++	Fast	++++	Fast	++++	Fast
Trametes villosa (2755)	S	Fast	++++	Fast	+++	Fast	++++	Fast
Bjerkandera adusta (130)	S	Fast	–	Fast	–	Fast	–	Medium

The plates were incubated for 28 days at 28 °C.

[1] Poly R-478, Azure B and Malachite green solid-plate decolorization categories: (–) no decolorization; (+) decolorization around the inoculum; (++) decolorization less than 50 % of the plate; (+++) decolorization over 50 % of the plate; (++++) complete decolorization of the plate.

[2] Growth rate categories: Fast: > 0.6 cm/day; Medium: 0.2–0.6 cm/day; Slow: < 0.2 cm/day.

[3] Habit: S (saprotrophic) and E (endophytic).

riolus antarcticus most rapidly decolorized this medium. Although the rates of decolorization obtained with *Peroneutypa comosa* 393, *Xylaria* sp. 3193 and *P. scoparia* 3324, 3321 and 3391 were comparable to those obtained with *Trametes trogii* and *T. villosa*, all the ascomycetes assayed only partially decolorized Poly R-478 (from red to pinkish). All the strains evaluated decolorized the Azure B plates (associated with LiP activity); rates of decolorization attained by *P. comosa* 393, *P. scoparia* 3327 and 3391, were comparable to those obtained with the strains belonging to Basidiomycota. Eleven of the ascomycetes evaluated were capable of decolorizing Malachite Green. The decolorization rate obtained with *Xylaria* sp. 3192 was comparable with that attained by *Trametes villosa*.

Production of ligninolytic enzymes under submerged fermentation

Those strains with a good performance in ligninolytic enzyme production on solid medium were selected to perform a quantitative enzymatic assay.

The kinetics of *in vitro* production of ligninolytic enzymes by nine strains of ascomycetes and *Coriolus antarcticus* were studied in malt extract/glucose medium amended with copper sulphate (Tab. 2). All strains produced laccase and MnP activities with the exception of *P. scoparia* 3327

Tab. 2. Laccase and Mn-peroxidase (MnP) activities under submerged fermentation in malt extract/glucose medium amended with copper sulphate.

Fungal strain (strain number)	Laccase activity (mU/ml)	MnP activity (mU/ml)	Dry weight (mg/25 ml)
Ascomycota			
Peroneutypa scoparia (146)	6.67 ± 1.50 (21)[1]	9.14 ± 0.11 (21)	87.5 ± 3.82 [2]
Peroneutypa comosa (393)	65.80 ± 2.82 (28)	4.76 ± 0.02 (21)	120.6 ± 5.84
Peroneutypa scoparia (3322)	0.14 ± 0.03 (21)	2.20 ± 0.22 (21)	140.9 ± 7.43
Peroneutypa scoparia (3324)	2.71 ± 0.52 (21)	5.45 ± 0.88 (28)	79.1 ± 2.06
Peroneutypa scoparia (3327)	5.17 ± 0.29 (28)	ND[3]	169.2 ± 6.08
Peroneutypa scoparia (3390)	21.50 ± 1.07 (21)	ND	131.2 ± 5.44
Peroneutypa scoparia (3391)	2.03 ± 0.31 (21)	7.22 ± 1.38 (28)	124.8 ± 7.25
Xylaria sp. (3193)	14.14 ± 0.64 (28)	8.90 ± 0.19 (28)	166.1 ± 8.11
Eutypella leprosa (1744)	4.53 ± 0.66 (21)	1.88 ± 0.10 (28)	154.5 ± 8.37
Basidiomycota			
Coriolus antarcticus (266)	3935.76 ± 5.50 (21)	112.95 ± 7.20 (21)	94.5 ± 4.27

[1] The values shown correspond to the peak of enzyme production. The numbers above the columns indicate the day these maxima were achieved. The values are the mean of three replications ± SD.

[2] Growth was determined after 28 days of incubation. The values are the mean of three replications ± SD.

[3] ND: not detected.

and 3390. Low laccase and MnP activities were produced by the ascomycetes in comparison with *C. antarcticus* (3821 U/l laccase, 113 U/l MnP). Among the Ascomycota highest laccase and MnP productions were detected in *P. comosa* 393 and *P. scoparia* 146, respectively 65 and 9.1 U/l. *Peroneutypa comosa* 393 did not only produce the maximum titres of laccase *in vitro*, but also exhibited the best decolorization performance on Poly R-media. Only *P. scoparia* 3322 was incapable of decolorizing the Poly R-478; this strain also had the lowest activities of laccase, under submerged fermentation, among the strains assayed.

Discussion

The phylogenetic analyses performed showed a polyphyletic origin of the Diatrypaceae genera as was indicated by previous publications (Trouillas & Gubler 2004, Trouillas *et al.* 2011). In the present analyses we found that the phylogenetic analysis of β-tubulin shows better concordance with the morphology, but it is necessary to explore new genes to obtain more robust results.

For the first time ligninolytic enzyme production under submerged fermentation was characterized in members of the Diatrypaceae family. We found inter-generic, inter- and intra-specific differences in the ratio between the growth halo and the decolorization halo suggesting physiological differences. Similar results were obtained by Freitag & Morrell (1992) and Levin *et al.* (2004). All the strain assayed only partially decolorized Poly R-478 (from red to pinkish), indicating a slightly different enzymology in these fungi. Pointing *et al.* (2003), Urairuj *et al.* (2003), and Luo (2005) evaluated the capacity of xylariaceous fungi for Poly R-478 decolorization, some of the strains evaluated were able to decolorize Poly R-478 and even Urairuj *et al.* (2003) proved the capacity of one of them to decolorize Poly R-478 in liquid culture, attaining 91 % decolorization after 12 days. The eight diatrypaceous fungi assayed by Pildain *et al.* (2005) were also capable of decolorizing Poly R-478. According to de Koker *et al.* (2000), fungi decolorizing Poly R-478 could be placed in different groups: fungi with strong LiP and MnP activities, fungi with strong MnP activity and fungi with strong laccase activity.

The high incidence of ligninolytic enzyme production (as determined by the Poly R-478 assay) indicated that at least these members of the Diatrypaceae are physiologically capable of producing lignin decay in wood. Nonetheless, the relatively weak decolorization may reflect comparatively low levels of enzyme production by diatrypaceous fungi and evidence from lignin solubilization rates in wood substrates supports this (Abe 1989, Worrall *et al.* 1997, Pointing *et al.* 2003, 2005) where lignin solubilisation typically occurs at much lower levels than in Basidiomycota.

Little is known about the mechanisms of lignocellulose degradation by ascomycetes, in contrast to those of the better-studied lignocellulolytic basidiomycetes that degrade wood (Shary *et al.* 2007). Nevertheless, it is clear that some soft-rot fungi can degrade lignin, because they erode the second-

ary cell wall and decrease the content of acid-insoluble material (Klason lignin) in angiosperm wood (Nilsson *et al.* 1989, Worrall *et al.* 1997). Some ascomycetes have also been shown to mineralize radiolabeled synthetic lignins, but the low extents of $^{14}CO_2$ production (less than 10 % of the lignin) indicate that the ligninolytic capabilities of ascomycetes are more limited than those of white-rot basidiomycetes (Liers *et al.* 2006). Laccase appears to be the dominant enzyme for ligninolysis in Ascomycota (Luo 2005, Liers *et al.* 2006). Soft rot fungi belonging to the Xylariaceae are known to substantially degrade hardwood by means of various hydrolases, including feruloyl esterases and laccase. A novel glycoside hydrolase that combines glycosyl hydrolase with esterase activities was recently purified from cultures of *Xylaria polymorpha* and may help this soft rot fungus to degrade lignocelluloses (Nghi *et al.* 2012). The xylariaceous fungi had demonstrated their ability to produce laccase, that could act directly on phenolic lignin, while MnP and LiP are not common in these organisms (Luo 2005, Liers *et al.* 2011). LiPs are able to oxidize and cleave the recalcitrant nonphenolic structures that compose the bulk of lignin (Tien & Kirk 1983). Nevertheless, *Daldinia concentrica* was capable of attacking the recalcitrant nonphenolic structures in lignin (Shary *et al.* 2007). Liers *et al.* (2006) described peroxidase production by *X. polymorpha* growing in complex liquid media based on soybean meal. Laccase and MnP production under submerged fermentation is reported for the first time in diatrypaceous fungi in the present work. However, these enzyme activities were not characterized after subsequent purification steps.

In spite of the ability demonstrated by all the strains to decolorize Azure B in solid media, none of them produced LiP under submerged fermentation. Several diatrypaceaeous and xylariaceous fungi had already demonstrated their ability to decolorize Azure B (Pointing *et al.* 2003, Pildain *et al.* 2005). But up to date LiP has only been reported in *Chrysonillia sitophila* (anamorph of *Neurospora* spp) (Durán *et al.* 1987) and more recently in *Xylaria* sp. CY829 (Luo 2005).

The presence of ligninolytic peroxidases suggests that certain Ascomycota may possess high redox potential enzymes that are usually seen in the ligninolytic systems of white-rot Basidiomycota.

Several studies have shown that endophytes are capable of producing wood decay enzymes (Oses *et al.* 2006). Recently Yuan *et al.* (2011) demonstrated that endophytic strains from foliar tissue exhibited a remarkable diversity of laccase genes, identified in culturable endophytes at inter- and intra-specific levels, and postulated that these diversity of laccase genes confers them an ecological advantage in competition for nutrients. The differences registered in laccase production by three different endophytic strains of *P. scoparia* (BAFC 3322, 3390, 3391) in our work support this hypothesis. Promputtha *et al.* (2010) suggested that endophytes can switch lifestyle to saprobes. These authors proved that each counterpart of endophyte and saprobe (same species) produced the same carbohydrases. The capacity of endo-

phytic fungi to produce enzymes that degrade cellulose and lignin is also a possible strategy that allows endophytes to decay host tissue and persist as saprobes after host senescence. In our study the wood endophytic strains were found to produce the same ligninolytic enzymes as their saprotrophic counterparts, a pattern seen also in foliar endophytes.

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