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Culture-based study of endophytes associated with rubber trees in Peru reveals a new class of Pezizomycotina: Xylonomycetes

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ABSTRACT

Through a culture-based survey of living sapwood and leaves of rubber trees (*Hevea* spp.) in remote forests of Peru, we discovered a new major lineage of Ascomycota, equivalent to a class rank. Multilocus phylogenetic analyses reveal that this new lineage originated during the radiation of the 'Leotiomyceta', which resulted not only in the evolution of the Arthoniomycetes, Dothideomycetes, Eurotiomycetes, Geoglossomycetes, Lecanoromycetes, Leotiomycetes, Lichinomycetes, and Sordariomycetes, but also of the majority of hyperdiverse foliar endophytes. Because its origin is nested within this major burst of fungal diversification, we could not recover strong support for its phylogenetic relationship within the 'Leotiomyceta'. Congruent with their long phylogenetic history and distinctive preference for growing in sapwood, this new lineage displays unique morphological, physiological, and ecological traits relative to known endophytes and currently described members of the 'Leotiomyceta'. In marked contrast to many foliar endophytes, the strains we isolated fail to degrade cellulose and lignin *in vitro*. Discovery of the new class, herein named Xylonomycetes and originally mis-identified by ITSrDNA sequencing alone, highlights the importance of inventorying tropical endophytes from unexplored regions, using multilocus data sets to infer the phylogenetic placement of unknown strains, and the need to sample diverse plant tissues using traditional methods to enhance efforts to discover the evolutionary, taxonomic, and functional diversity of symbiotrophic fungi.

1. Introduction

Fungal endophytes are a phylogenetically and ecologically diverse group united by the functional trait of residing within plant tissues without causing any apparent symptoms of disease (Petrini, 1991). Although the roles these symbionts play in their hosts are mostly unknown (see Hyde and Soytong, 2008; Arnold, 2007; Rodriguez et al., 2009), focal species of foliar endophytic fungi – the most thoroughly studied group of endophytes – often provide ecologically important benefits to their hosts including herbivore deterrence, protection against pathogens, and the capacity to tolerate abiotic stress (e.g., Arnold et al., 2003; Rodriguez et al., 2009; Alvarez-Loayza et al., 2011; Bittleston et al., 2011). The ubiquity and hyperdiversity of endophytes are now widely accepted (Arnold et al., 2000; Hawksworth, 2001; Herre et al., 2005; Arnold, 2008; Arnold et al., 2009; U'Ren et al., 2010, 2012), as is their tendency to show tissue specificity in many cases: communities from woody tissues frequently differ from those in roots and leaves (Arnold, 2007; Rodriguez et al., 2009; Gazis and Chaverri, 2010). Recent studies have revealed that endophyte communities demonstrate strong geographic structure (e.g., Arnold and Lutzoni, 2007; Davis and Shaw, 2008; Hoffman and Arnold, 2008; U'Ren et al., 2012), such that hosts form symbioses with distinctive communities in different sites across their ranges. The vast majority of known endophytes are Ascomycota in the Sordariomycetes, Dothideomycetes, Leotiomycetes, Pezizomycetes, and Eurotiomycetes (Arnold et al., 2009).

Fungi are recognized as one of the most diverse clades of life (Rossman et al., 1998; Mueller et al., 2004). Current estimates for the global number of fungal species have risen from the often cited 1.5 million (Hawksworth, 1991) to as many as 5.1 million species (O'Brien et al., 2005; Blackwell, 2011; Rosling et al., 2011). Tropical endophytes, especially of foliage, are an important component of undescribed fungal diversity (Arnold et al., 2000; Arnold and Lutzoni, 2007; Smith et al., 2008). Recent surveys have shown that the living sapwood of trees also harbors abundant and highly diverse assemblages of endophytic fungi (Verma et al., 2007; Thomas et al., 2008; Giordano et al., 2009; Gazis and Chaverri, 2010; Hanada et al., 2010; Parfitt et al., 2010; Tayung and Jha, 2010). Biochemical properties of leaves and sapwood (e.g., water content, lignin concentration) differ markedly; therefore, it is likely that

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sapwood hosts distinctive endophytic fungi relative to the fungal communities from leaves. However, the biodiversity of sapwood endophyte communities remains extremely understudied.

Through a survey of sapwood in wild rubber trees (*Hevea* spp.) in remote, forested regions of Peru, we isolated a collection of endophytes that we found to be highly divergent from currently recognized fungal lineages. We used six loci from taxa representing all known classes of Pezizomycotina to infer the phylogenetic placement of these unusual strains. We also characterized the morphology of this new group of endophytes and synthesize these results to describe a new class of Pezizomycotina along with all lower ranks within this new lineage.

2. Materials and methods

As part of a project to characterize fungal diversity associated with rubber trees (*Hevea* spp., Euphorbiaceae), sapwood- and leaf endophytes were collected from *H. brasiliensis* and *H. guianensis* in Peru (Gazis and Chaverri, 2010; Gazis et al., 2011; Gazis et al., in preparation). Focal trees were in three localities within the Peruvian Amazon basin: (1) Los Amigos Biological Station, Madre de Dios: 12°30'43.20"S, 70°3'34.09"W, (2) Amazon Conservatory of Tropical Studies (ACTS) Biological Station, Loreto: 3°14'57.60"S, 72°54'33.30"W, and (3) Tocache rubber plantations, San Martin: 8°11'51.50"S, 76°30'50.61"W. This study focuses on a group of fourteen strains isolated from sapwood and one from an asymptomatic leaflet. Table S1 shows the collection codes and origin of each isolate.

2.1. Endophyte isolation

To isolate sapwood endophytes we used a sterilized knife to cut three slivers of ca. 3 × 6 cm of dead bark from each tree at shoulder height and from three different parts of its circumference. After exposing the living sapwood (one section at the time), three pieces of *ca.* 5 × 5 mm of tissue were excised from each exposed area and quickly transferred to Petri plates containing CMD (BBL™ cornmeal-agar +2% dextrose), which contained 1% Neomycin–Penicillin–Streptomycin (Sigma–Aldrich, St. Louis, MO, USA). Leaf endophytes were collected following Gazis and Chaverri (2010). Petri plates were kept at low temperature (*ca.* 4–8 °C) until they were processed in the laboratory (Department of Plant Sciences and Landscape Architecture, University of Maryland, College Park, USA). Once in the laboratory, plates were incubated up to 2 months and emerging colonies were subcultured in Difco™ Potato Dextrose Agar (PDA) to obtain pure isolates.

2.2. DNA extraction, PCR, and sequencing

2.2.1. ITS data acquisition and analyses

Pure cultures of endophytes were grown in Difco™ Potato Dextrose Broth (PDB) at 25 °C for 1 week. Genomic DNA was extracted from the mycelial mat using Power Plant™ DNA isolation kit (MO BIO Laboratories Inc., Solana Beach, CA, USA) according to the manufacturer's instructions with the following modifications: mycelial tissue was stored at −80 °C for several days prior to the extraction and the use of vortex was replaced by FastPrep®-24 (MP Biomedicals, Solon, OH, USA) to enhance tissue lysis.

The nuclear internal transcribed spacers and 5.8S gene (ITS) were amplified and sequenced following Gazis et al. (2011). Sequencher™ version 4.9 (Gene Codes©, MI, USA) was used to assemble bi-directional sequences into contigs and to confirm basecalls. ITS sequences were subjected to BLAST queries using the "blastn" algorithm implemented at NCBI to determine the putative identity of the strains (www.ncbi.nlm.nih.gov/BLAST) (Table S2). BLAST matched suggested affinity for lineage of lichen-forming fungi, which was surprising. Therefore, we used multilocus phylogenetic analyses to verify their taxonomic identity.

To select strains for multilocus sequencing, we grouped them into operational taxonomic units (OTUs) based on 99% ITS sequence similarity, reflecting low predicted values of interspecific variability (Lieckfeldt and Seifert, 2000; Chaverri et al., 2003; Cai et al., 2009; Pavlic et al., 2009; U'Ren et al., 2009; Rojas et al., 2010; Gazis et al., 2011). OTUs were assembled using the furthest neighbor algorithm implemented in mothur version 1.16.0 (Schloss et al., 2009: www.mothur.org). Gaps were not considered as characters and sequences were reduced to a uniform length. Genetic variation was assessed among OTUs and isolates using MEGA version 5 (Tamura et al., 2011: www.megasoftware.net) to calculate nucleotide differences and p-distances between and within OTUs using bootstrap sampling with 1000 replicates.

2.2.2. Multilocus data acquisition

Three distinct ITS haplotype groups were detected based on 99% ITS similarity. One representative of each was selected for multilocus analyses, for which we sequenced five additional loci: three ribosomal RNA-coding genes, including the nuclear small subunit (nucSSU), nuclear large subunit (nucLSU), and the mitochondrial small subunit (mitSSU); and two protein-coding genes: the largest and second largest subunits of RNA polymerase II (*RPB*1 and *RPB*2). Table S3 shows the targeted loci, the primers used in their amplification and sequencing, the resulting amplicon length, and the references for PCR conditions. All PCR reactions were assembled as follows: 2.5 μl PCR buffer (buffer IV with 15 mM MgCl2; Abgene, Rochester, NY, USA), 2.5 μl dNTP (2 mM), 2.5 μl bovine serum albumin (10 mg ml^{-1}; New England BioLabs Inc., Ipswich, MA, USA), 1.25 μl primers (10 mM), 0.15 μl Taq polymerase (5 U ml^{-1}, Denville, South Plainfield, NJ, USA), 1 μl of a 1/10 dilution of genomic DNA, and double distilled sterile water to a total volume of 25 μl. To amplify *RPB*1 and *RPB*2, we increased the volume of DNA to 3 μl and the primer to 2 μl. PCR was performed on a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA, USA). Products were purified using ExoSAP-IT® (USB Corporation, Cleveland, OH, USA.) and both strands were sequenced at Duke Genome Sequencing and Analysis Core Facility of the Institute for Genome Sciences and Policies on an ABI 3730xl DNA analyzer (PE Applied Biosystems, Foster City, CA, USA). Sequencing reactions were prepared for 10 μl total volume using 1 μl primer, 1 μl purified PCR product, 0.75 μl Big Dye (Big Dye Terminator Cycle sequencing kit, ABI PRISM version 3.1; Perkin–Elmer, Applied Biosystems, Foster City, CA, USA), 3.25 μl Big Dye buffer, and 4 μl double-distilled water. Data were edited as above and all sequences were subjected to BLAST queries of GenBank using "blastn" (ribosomal loci) or "blastx" (*RPB*1 and *RPB*2 sequences).

2.2.3. Multilocus data sets

The following data sets were assembled: a 2-locus (nucLSU + nucSSU) data set consisting of 362 taxa; 5-locus (nucLSU + nucSSU + 5.8S + *RPB*1 + *RPB*2) and 6-locus (5-locus + mitSSU) data sets consisting of 108 taxa; and a 6-locus data set consisting of 97-taxa. The 2-locus, 362-taxon data set was prepared by adding three nucLSU and nucSSU endophyte sequences into the 359-taxon alignments (241 representative Ascomycota and 118 endophytic and endolichenic isolates) for each locus generated by Arnold et al. (2009). Bootstrap analysis on each data set with 1000 bootstrap replicates and GTR + GAMMA were implemented in RAxML-VI-HPC (Stamatakis, 2006). All remaining data sets were derived from the 5-locus (nucLSU, nucSSU, 5.8S, *RPB*1 and *RPB*2), 214-taxon matrix used in James et al. (2006). In that study, ten of the eleven recognized Pezizomycotina classes (sensu Lumbsch and Huhndorf,

2010) were represented with Laboulbeniomycetes (Weir and Blackwell, 2001) excluded. For the 5-locus 108-taxon combined data set, taxa representing classes outside of Ascomycota, as well as members of Taphrinomycotina (109 taxa) were removed, and for the 5-locus 97-taxon combined data set, all members of Saccharomycotina (11 taxa) also were removed. To generate the 6-locus combined data sets (108-taxon and 97-taxon), the mitSSU sequences for all but 18 taxa present in our 108-taxon data set were retrieved from the AFTOL (www.aftol.org) and GenBank (www.ncbi.nlm.nih.gov) databases (Table S4).

All alignments were refined manually using MacClade 4.08 (Maddison and Maddison, 2005). For the nucSSU and nucLSU, the secondary structure model (Kjer, 1995) of *Saccharomyces cerevisiae* Meyen *ex* E.C. Hansen (Cannone et al., 2002) was followed. Ambiguously aligned regions (sensu Lutzoni et al., 2000) and introns were delimited and excluded from the analyses. A summary of alignment lengths and the number of included sites for each data set is shown in Table 1. The *RPB*1 and *RPB*2 genes provided the largest number of characters included in the phylogenetic analyses. Although the 5.8S region contained the second lowest proportion of ambiguously aligned characters (8%), most of the included characters were constant. Compared to the remaining ribosomal genes (except the 5.8S), *RPB*1 and *RPB*2 contained the lowest proportion of sites excluded from the analyses (11% and 17%, respectively versus 72–82%). By removing 11 taxa from the 108-taxon data set, the proportion of missing data decreased only by 2% and the number of included sites increased by 111.

2.2.4. Multilocus phylogenetic analyses

Because the mitSSU data set was not included in the phylogenetic study by James et al. (2006), and therefore was not tested for congruence against the other 5 loci (nucLSU, nucSSU, 5.8S, *RPB*1 and *RPB*2), bootstrap analysis using RAxML (1000 replicates, GTR + GAMMA model) was conducted separately on two partitions of the 108-taxon data set (mitSSU only, and the 5-locus, 108-taxon combined data set) to detect topological incongruence. Conflict was assumed to be significant if a group of taxa was supported at $\geqslant$70% as monophyletic by one data set, but supported as non-monophyletic by another data set (reciprocal 70% ML bootstrap support criterion; Mason-Gamer and Kellogg, 1996; Reeb et al., 2004). Conflict was detected for the placement of *Anisomeridium polypori*; therefore, this taxon was excluded from all analyses. The final 107- and 96-taxon combined data sets contained three representatives of rubber tree endophytes and 104 and 93 reference taxa from James et al. (2006), respectively (Table S4). The Nexus file for the 6-locus 107-taxon data set is available in TreeBASE, study ID#12729.

JMODELTEST (Posada and Buckley, 2004; Posada, 2008) was run on each locus separately (ambiguously aligned regions and introns excluded) to select the models of nucleotide substitution for Bayesian analyses. The number of substitution schemes was set to 11, base frequencies +F, rate variation among sites +I and +G, and the base tree for likelihood calculations was set to ML OPTIMIZED. Models were selected based on comparison of likelihood scores from 88 models according to the Akaike Information Criterion (AIC).

Phylogenetic relationships and internode robustness were estimated for the 6- and 5-locus, 107-taxon data sets, and the 6-locus 96-taxon data set, using maximum likelihood as implemented in RAxML-VI-HPC (Stamatakis, 2006) and using Bayesian Inference (BI) as implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). In the ML analyses, the 6- and 5-locus combined data sets were divided into 10 partitions (nucSSU, nucLSU, 5.8S, mitSSU, *RPB*1/1st, 2nd, 3rd and *RPB*2/1st, 2nd, 3rd) and nine partitions (all partitions as above except the mitSSU), respectively. The most likely tree and bootstrap support were estimated with 1000 replicates using the general time-reversible (GTR; Rodriguez et al., 1990) evolutionary model with a Gamma distribution to account for rate heterogeneity among sites (GTRGAMMA). In addition, the 6-locus 107-taxon data set was subjected to maximum likelihood bootstrap analyses using mixed models (nucleotides and amino acids) as implemented in RAxML. The Improved General Amino Acid Replacement Matrix (LG; Le and Gascuel, 2008) with empirical base frequency was selected for *RPB*1 and *RPB*2 loci using ProtTest 2.4 (“fast” strategy; Abascal et al., 2009). 1000 bootstrap replicates were completed on the 107-taxon data set divided into six partitions with GTR substitution model for each of the four ribosomal loci, LGF model for *RPB*1 and *RPB*2, and gamma distribution parameter approximated with four categories across each locus.

One set of BI was conducted on the same 10 and nine partitions as the ML and another one on a reduced number of partitions with the first, second and third positions of the *RPB*1 and *RPB*2 combined (*RPB*1 with *RPB*2/1st, *RPB*1 with *RPB*2/2nd, and *RPB*1with *RPB*2/3rd) for a total of seven partitions for the 6-locus data set, and six partitions for the 5-locus data set. All Bayesian inferences were completed with eight chains and two independent runs for a total of 50,000,000 generations, with trees sampled every 500 generations. A GTR model with an estimated proportion of invariable sites (I) and a gamma distribution approximated with four categories to account for among site rate heterogeneity was used

Table 1
Summary of datasets, including number of OTUs, length of alignments and number of characters that were included in the analyses, for each gene separately and when combined after removal of ambiguous regions. Regions that could not be aligned with confidence (ambiguous) and introns were excluded from phylogenetic searches.

Dataset	Number of OTUs	Number of characters			
		Total length	Ambiguous and/or introns	Constant	Total included
5.8S (ITS)	96	162	13	78	149
	107	162	13	76	149
nucSSU	96	5601	4520	569	1081
	107	5612	4590	546	1022
nucLSU	96	3670	2580	569	1090
	107	3695	2663	557	1032
mitSSU	96	2410	1980	159	430
	107	2531	2101	126	430
*RPB*1 (A–F)*	96	3108	318	812	2790
	107	3111	345	738	2766
*RPB*2 (7–11)*	96	2247	276	628	1971
	107	2235	375	605	1860
Combined data	96	17198	9687	2815	7511
	107	17346	10087	2648	7259

* Each codon position (1st, 2nd, and 3rd) from *RPB*1 and *RPB*2 were further partitioned.

for all partitions, except for 5.8S, where a GTR + (Zharkikh, 1994) model was selected. Convergence of log likelihood scores (–Ln) was assessed with TRACER version 1.4 (Rambaud and Drummond, 2007) and stationarity was assumed when a stable equilibrium value was reached (Huelsenbeck and Ronquist, 2001). The average standard deviation of split frequencies between runs on the same data set with different numbers of partitions were compared, and the trees resulting from the runs with lower values were selected for the next step (0.002 for the 5- and 6-gene 107-taxon data sets with seven and six partitions versus 0.003 with 10 and nine partitions, and 0.003 for the 6-gene 96-taxon data set with 10 partitions versus 0.008 with seven partitions). A burn-in sample of 15,000 trees was discarded for each run. The remaining 170,000 trees (85,000 from each run) were used to estimate posterior probabilities (PP) with the majority rule consensus tree command in PAUP* 4.0b10 (Swofford, 2002). Individual nodes were considered well supported when ML bootstrap values (BS) $\geqslant$ 70% and when PP values $\geqslant$0.95.

2.3. Morphological data

Isolates were grown on Difco™ Malt Extract Agar (MEA) and PDA for up to 1 month at 25 °C with alternating 12 h/12 h fluorescent light/darkness. Microscopic observations were made using an Olympus BX51 microscope. Measurements of continuous characters such as length and width were made using the Scion Image software beta 4.0.2 (Scion Corporation, Frederick, MD, USA). Continuous measurements (1000× magnification) were based on at least 100 measured units and are reported as the extremes (minimum and maximum) in brackets separated by the mean plus and minus one standard deviation. Images were captured with an Olympus DP71 digital camera. Some composite images were made with Helicon Focus version 4.21.5 Pro (Helicon Soft, www.heliconfocus.com). Colors were described using the mycological color chart from Rayner (1970). A dried culture of the type specimen was deposited at the US National Fungus Collections (BPI) and additional representative cultures were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Table S1).

Attempts to obtain the teleomorph state in culture were made following Rooney-Latham et al. (2005). Different strains were grown in dual cultures (PDA and MEA) for 8 weeks at 25 °C with alternating 12 h/12 h fluorescent light/darkness. In addition, isolates were grown in additional media such as Synthetic Nutrient Agar (SNA, Nirenberg, 1976) with a piece of sterile filter paper (Palm et al., 1995); oatmeal agar (OA, Difco™); and corn meal agar (CMA, Difco™).

2.4. Cellulase and ligninase assays

In-vitro assays were used to test whether strains were capable of degrading sapwood materials such as cellulose and lignin. To measure cellulase activity, 5 mm plugs of actively growing mycelium were transferred individually under sterile conditions from source cultures on PDA to carboxymethylcellulose medium (CMC-medium, prepared following Jeffries, 1987; see also Blume and Ennis, 1991) in 100 mm Petri plates (three replicate plates per isolate). One plug from each isolate was transferred at the same time to PDA to confirm viability of the mycelium. Two cellulolytic strains (DC3368 and DC0448, representing foliar endophytes obtained from the Robert L. Gilbertson Mycological Herbarium, ARIZ) were cultivated concurrently on CMC-medium in triplicate as positive controls. Plates were incubated at 22 °C under 12 h light/dark conditions and assessed at 3-day intervals over 21 d for growth and cellulase activity. On day 22, activity was assessed by flooding plates with a 0.2% w/v solution of Congo red, incubating at 22 °C for 30 min, destaining with successive washes of 1 M NaCl, and measuring the diameter of cleared areas in the CMC-medium surrounding the plug.

A similar approach was used to assess ligninase activity, except that indulin medium, which contains a commercially available substitute for lignin, was used in place of CMC-medium (Nerud et al., 1991), and a known ligninolytic strain (foliar endophyte DC3051 from ARIZ) was used as a positive control. Ligninase activity was measured by flooding plates with a 1% w/v solution of $FeCl_3$ and $K_3[Fe(CN)]_6$, incubating at 22 °C for 30 min, and rinsing with distilled water until cleared areas of the medium could be detected and scored for presence (activity) or absence (no activity).

3. Results

3.1. Preliminary identification of endophytes from sapwood using ITS

Top BLAST matches for the 15 ITS sequences from the unknown strains of sapwood endophytes from *Hevea* were all representatives of the Lecanoromycetes (based on "maximum identity"; Table S2), a class of Ascomycota containing the majority of lichen-forming fungi. However, their maximum identity scores (83–92%) were relatively low, suggestive of the distinct nature of the sequences.

ITS sequences showed little variation among these 15 strains. The distance analyses conducted with mothur revealed 1 OTU based on 98% similarity, three OTU at 99%, and nine unique OTU. Table S5 shows p-distances and number of base-pair (bp) differences between each of the three OTU groups at the 99% similarity level. No geographic clustering associated with the distance analysis was observed (Table S1).

3.2. Phylogenetic relationships of unknown endophytes from Hevea

Together, all three OTUs of *Hevea* endophytes (OTU 1–3, represented by TC269, 161, and 137, respectively; Table S1) formed a monophyletic group with significant support in all analyses. According to the 5- and 6-locus 107-taxon phylogenies, this monophyletic group is sister to the Lecanoromycetes + Lichinomycetes + Geoglossomycetes clade. This phylogenetic placement received significant support from all analyses except from BS on the 5-locus 107-taxon data set (Fig. 1). Based on the 96-taxon data set and on the 6-locus 107-taxon data set with mixed models (tree not shown), the phylogenetic placement of these sapwood isolates fall within the 'Leotiomyceta' with high support, but their specific placement within that superclass is not significantly supported (Fig. 2).

3.3. Phylogenetic relationships within Ascomycota

Eight of ten classes currently recognized within the Pezizomycotina (Lecanoromycetes, Lichinomycetes, Geoglossomycetes, Eurotiomycetes, Sordariomycetes, Leotiomycetes, Arthoniomycetes, Pezizomycetes) were delimited as monophyletic and highly supported by all phylogenetic analyses with one exception. Leotiomycetes were not monophyletic (*Leotia lubrica* was sister to Sordariomycetes; BS = 82%) when the 6 locus 107-taxon data set were analyzed with mixed models. The Dothideomycetes were recovered as monophyletic but with low support values, with the exception of the 6-locus, 96-taxon data set (PP = 0.99; Fig. 2). However, the Arthoniomycetes-Dothideomycetes clade is associated with high support values from the four separate analyses of the 107-taxon data set. The Pezizomycetes were recovered mostly as non-monophyletic (Figs. 1 and 2), and received high support (ML and BI on the 6-locus 107-taxon data set) for Orbiliomycetes being more closely related to Pezizomycetes than *Peziza* (Fig. 1).

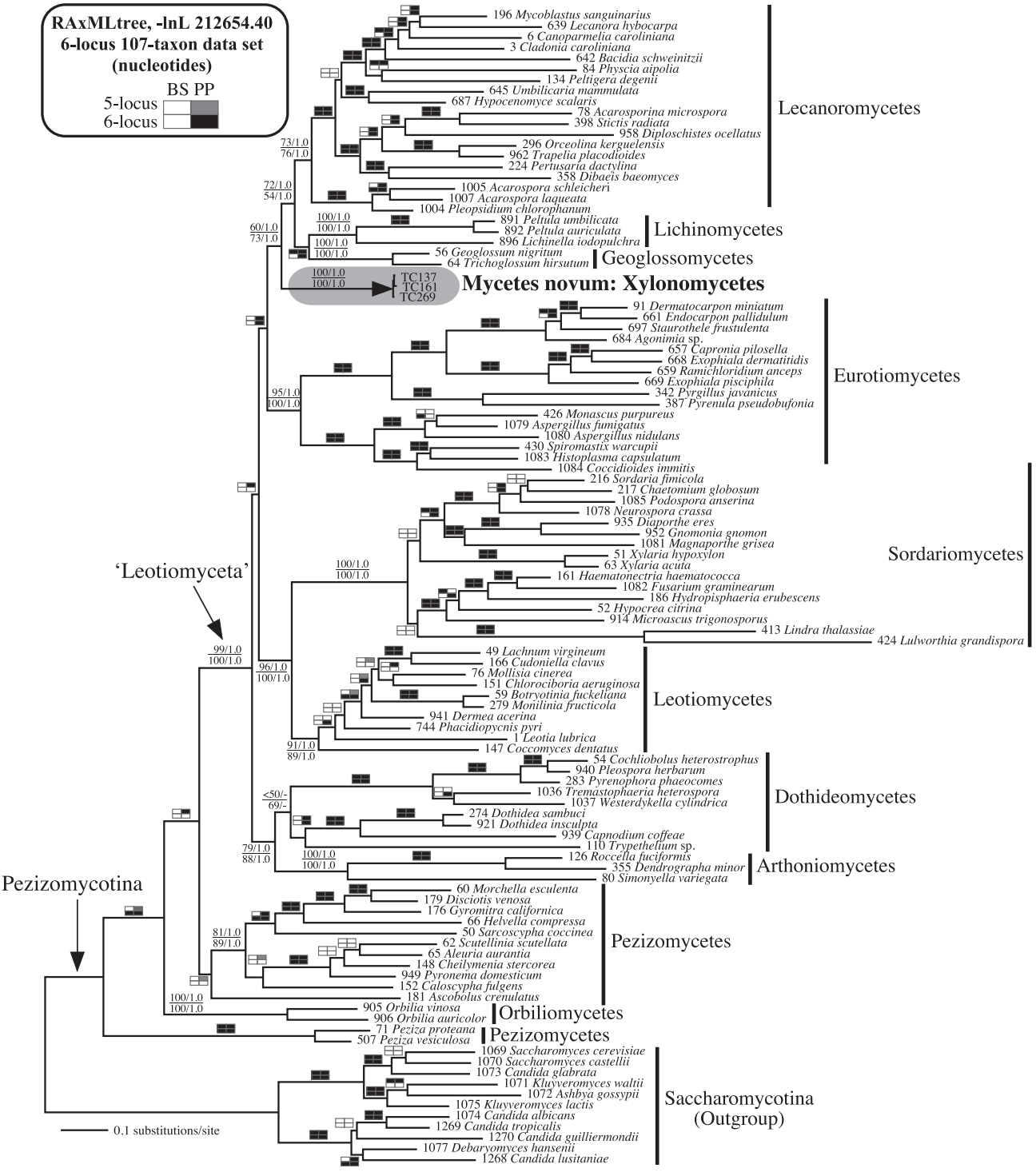

Fig. 1. Phylogenetic relationships of rubber-tree endophytic fungi isolated from their sapwood (as Mycetes novum Xylonomycetes shown in gray oval) within Pezizomycotina and 'Leotiomyceta' based on a combined 6-locus (nucLSU, nucSSU, 5.8S, mitSSU, *RPB*1 and *RPB*2) data set for 107 taxa. Support values and boxes associated with internodes represent BS (before slash or first column) and PP (after slash or second column) derived from the 5-locus 107 taxon data set (upper row) and the 6-locus 107-taxon data set (lower row). Black boxes indicate significant support (BS $\geqslant$ 70% and PP $\geqslant$ 0.95), white boxes indicate non-significant support, and gray boxes (and dashes) indicate significantly supported conflicting relationships.

Pezizomycetes were reconstructed as monophyletic with significant support (PP = 0.98) based only on BI of the 6-locus 96-taxon data set (tree not shown). Only a few significantly supported phylogenetic relationships among classes were shared by both the 107-taxon (including the BS analyses with mixed models) and 96-taxon phylogenies (Figs. 1 and 2): sister relationships between Sordariomycetes and Leotiomycetes, and between Dothideomycetes and Arthoniomycetes. Both analyses yielded high support for the monophyletic delimitation of 'Leotiomyceta'.

Overall, the 107-taxon phylogeny has a more robust phylogenetic backbone and in addition to the highly supported clades present in the 96-taxon phylogeny, it includes several other highly supported monophyletic groups: Lecanoromycetes + Lichinomycetes + Geoglossomycetes (significant BS and PP values) + Eurotiomycetes

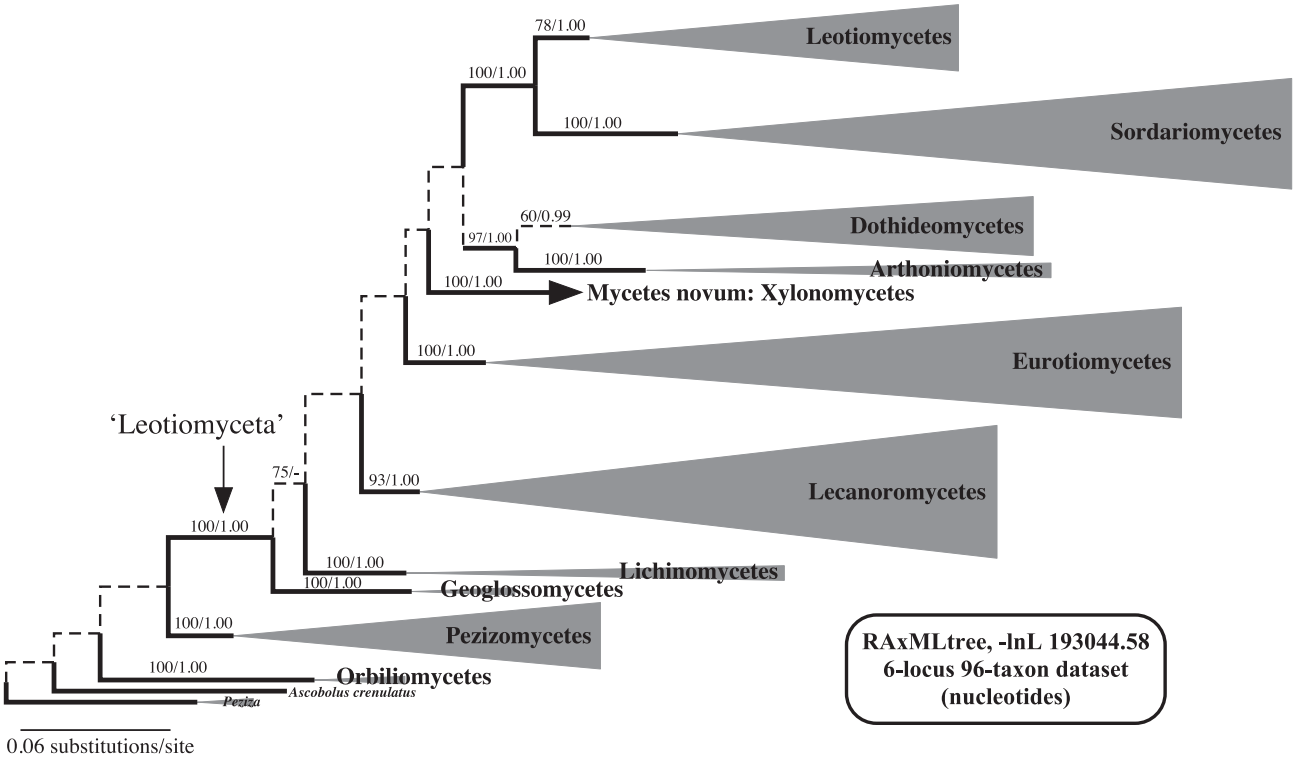

Fig. 2. Phylogenetic placement of Xylonomycetes within 'Leotiomyceta' based on a combined 6-locus (nucLSU, nucSSU, 5.8S, mitSSU, *RPB*1 and *RPB*2) data set for 96 taxa, partially derived from James et al. (2006). Clades above class level were collapsed. Support values associated with internodes represent BS (before slash) and PP (after slash). Dash indicates a significantly supported conflicting relationship between Bayesian and maximum likelihood inferences.

(significant PP values) + Sordariomycetes + Leotiomycetes (significant PP value) + Arthoniomycetes-Dothideomycetes (significant BS and PP values) + Pezizomycetes (excluding Peziza; significant PP values) + Orbiliomycetes (significant BS and PP values from the 6-locus data set). The only conflicting relationship was the placement of Geoglossomycetes as the first split within 'Leotiomyceta' in the 96-taxon data set phylogeny (BS = 74%; Fig. 2) and the 107-taxon BS analyses with mixed models (tree not shown), whereas it was shown as a sister group to the Lichinomycetes, forming a clade sister to Lecanoromycetes, in the 107-taxon phylogeny (BS and PP significant; Fig. 1).

3.4. Morphology

Macro- and microscopic descriptions are based on colonies grown on PDA and MEA after incubation at 25 °C for ca. 3–4 weeks. No teleomorph was observed in culture; therefore, descriptions are based only on the anamorph. Morphological features are shown in Fig. 3 (A–J). Conidiomata were pycnidial, astromatic, and composed of thin-walled cells forming tissue *textura angularis*. Conidial masses were liberated through the apical rupture of the pycnidia (ostiole absent). Conidiophores were absent. Conidiogenous cells were enteroblastic, phialidic, discrete, and hyaline, and presented one collarette. Conidia were apically rounded with two lateral obtuse projections appearing heart-shaped (narrower and truncated at base), hyaline when young, turning dark brick when mature, aseptate, thick-walled, smooth, and guttulated. After *ca.* 3 weeks, both types of agar acquired a vinaceous diffusing pigment, especially at the margins of the growing colony. None of the structures reacted with 3% KOH.

3.5. Cellulase and ligninase activity

All 15 isolates of this new lineage grew on PDA, and positive controls grown on the CMC- and indulin media demonstrated marked degradation of cellulose and lignin, respectively. However, none of the sapwood strains grew on the CMC-medium and no cellulolytic or ligninolytic activity was observed in proximity to the transferred plugs.

4. Discussion

In this study we used published molecular data sets with broad and, where possible, deep taxonomic representation (James et al., 2006 and Arnold et al., 2009) to assemble comprehensive data sets for the Pezizomycotina (*sensu* Schoch et al., 2009) to determine the phylogenetic affinities of endophytic strains discovered mainly from sapwood of Peruvian *Hevea* spp. Ten of the eleven accepted Pezizomycotina classes were represented in the main data set (Laboulbeniomycetes was not included). Maximum likelihood and Bayesian inference analyses of the 6 targeted loci (nucLSU, nucSSU, 5.8S, mitSSU, *RPB*1 and *RPB*2) provided strong evidence consistent with the hypothesis that that this group of sapwood endophytes represents a new monophyletic lineage of ascomycetous fungi within the superclass 'Leotiomyceta' (*sensu* Spatafora et al., 2006). The results from the phylogenetic analyses also suggested that this lineage should be designated as a new class, which we herein designate as the Xylonomycetes (type species, *Xylona heveae*).

As in many biodiversity surveys, the first attempt to identify this group of strains was by comparing their ITS sequences against the GenBank nucleotide data base. None of our strains matched closely with sequences already in GenBank (none showed more than 92% in similarity and $\geqslant$99% coverage), and highest top matches belonged to members of the Lecanoromycetes (Table S2). However, our phylogenetic analyses using multiple loci clearly demonstrate that the new lineage is not a member of the Lecanoromycetes. Interestingly, BLAST results did not include high quality matches to sequences obtained in environmental surveys of any kind (i.e., soil, wood

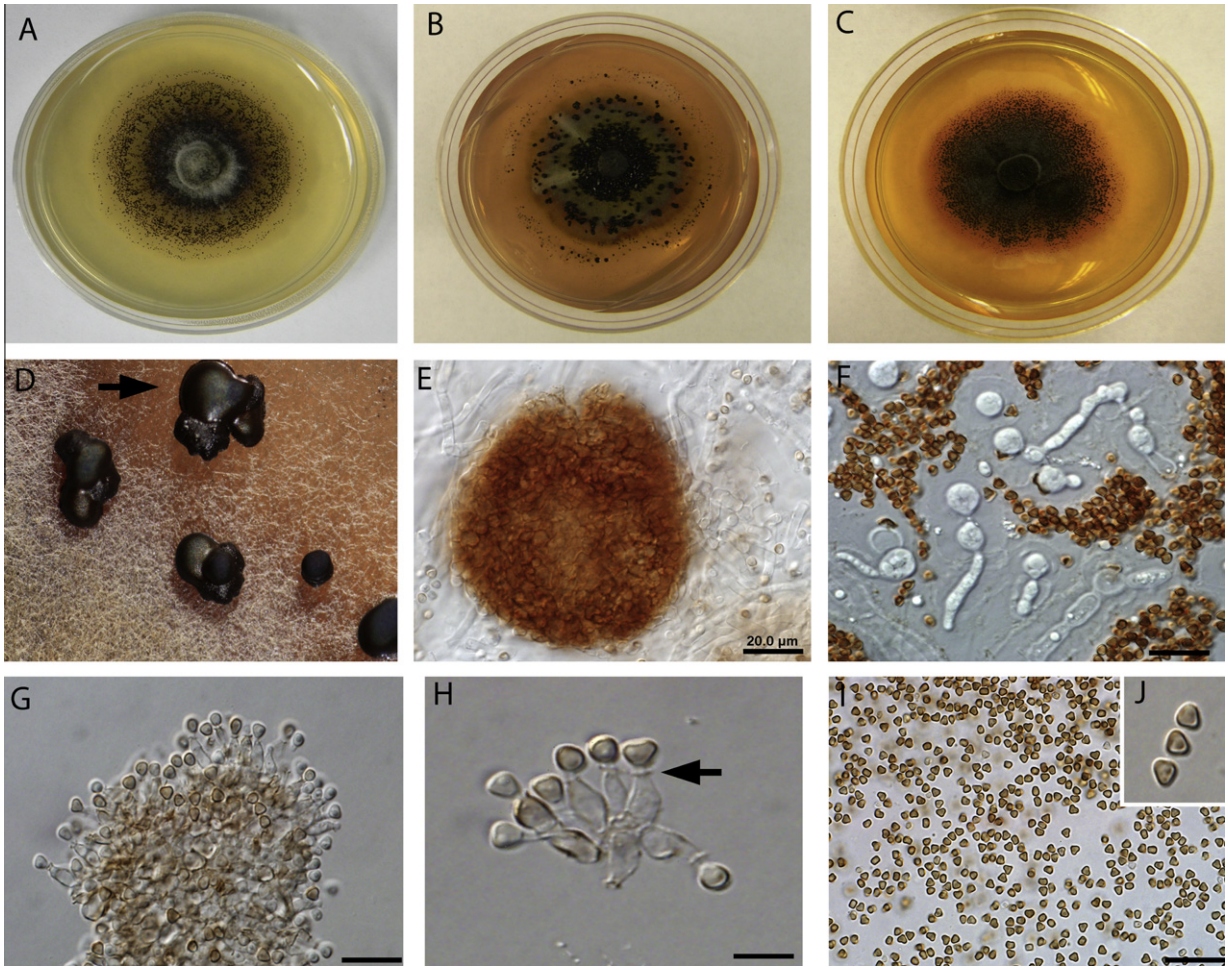

Fig. 3. *Xylona heveae* (type material). (A) Two-week old culture on MEA with profuse pycnidia, note the vinaceous pigmentation of the agar at the margin of the colony. (B–C) Four-week old culture on MEA and PDA respectively, showing mature pycnidia. (D) Opened pycnidia showing conidia mass (arrow). (E) Young pycnidium. (F) Chlamydospores. (G–H) Conidiogenous cell with conidia attached. (I–J) Conidia, note the truncated base and the guttulate feature. Scale bars: 20 µm.

decaying fungi, foliar endophytes), leading us to believe that members of this group had not been detected previously. We further compared our sequences against a database of 14,800 unpublished ITS sequences including leaf endophytes from boreal, temperate, tropical, and arid regions; endolichenic fungi from different biogeographic zones; coral and reef associated fungi; clone libraries from endophytes inhabiting tropical grasses; and tropical seed-associated fungi (Arnold, *unpubl. data*). No sequence in this database showed $\geqslant$95% similarity to the endophyte sequences obtained here.

The phylogenetic relationships of the main clades of Ascomycota (Fig. 1) was similar to the results reported by James et al. (2006) and Schoch et al. (2009), among others (e.g., Lutzoni et al., 2004; Spatafora et al., 2006). As in these studies, we found significant support for the superclass 'Leotiomyceta' as a group encompassing Arthoniomycetes, Dothideomycetes, Eurotiomycetes, Geoglossomycetes, Lecanoromycetes, Leotiomycetes, Lichinomycetes and Sordariomycetes. We also found strong support for a sister relationships between Arthoniomycetes and Dothideomycetes and between Sordariomycetes and Leotiomycetes (Fig. 1). With the 5-locus 107-taxon data set the sister relationship of the Lichinomycetes with the Geoglossomycetes was well supported by bootstrap values and posterior probabilities. The same is true for the sister relationship of the Lecanoromycetes with the Lichinomycetes + Geoglossomycetes clade. As in previous studies, the backbone of the 'Leotiomyceta' was never completely resolved with high confidence, even with the addition of protein-coding genes to ribosomal RNA-coding genes (Weir and Blackwell, 2001; Schoch et al., 2009).

We found strong support for phylogenetic placement of the putatively new lineage within 'Leotiomyceta', but outside all known classes within this superclass (Figs. 1 and 2). When we used the data set composed of 6 loci from 107 taxa, we obtained strong support (BS: 72, PP: 1; Fig. 1) for this new lineage being sister to the Lecanoromycetes–Lichinomycetes–Geoglossomycetes clade. However, bootstrap support of this relationship decreased when only 5 loci were used to infer the phylogeny (BS: 60, PP: 1; Fig. 1).

As for many groups of fungi, this new lineage can only be described based on its anamorphic state in culture (e.g., Rojas et al., 2008; Chaverri et al., 2011). To investigate whether it has been reported in the past, but has not been sequenced yet (such that its sequence would not be available in public databases; Bidartondo et al., 2009; Brock et al., 2009), we examined the most comprehensive works involving "coelomycetous" fungi (the artificial group that includes all anamorphic fungi with sporulation occurring within conidiomata such as pycnidia and acervuli). We reviewed the following papers: Morgan-Jones et al. (1972), Nag Raj (1978, 1980, 1993), Nag Raj et al., 1989, and Sutton (1980) and our reading suggests that this anamorph has not been described previously. As for many fungi described on the basis of growth *in vitro*, morphological features observed under laboratory conditions should be taken with caution because these might be different when fungi are growing under natural conditions (Pelletier and Aubé, 1970).

Most of the morphological characters we observed (see results) are shared by several classes within the 'Leotiomyceta' (e.g., conidiomata with pycnidial anamorph, found in several classes; a non-ostiolate pycnidium with enteroblastic phialidic conidiogenesis, resembling anamorphs of Sordariomycetes and Dothideomycetes). However, the shape of the conidia produced by this new lineage appears to be one distinctive character. From the literature (Sutton, 1980; Crous et al., 2007; Crous et al., 2009), we found that only *Readeriella* (Capnodiales, Dothideomycetes) produces conidia with similar morphology. This new lineage has other similarities with *Readeriella* (e.g., in their conidiogenous cells); however, that genus produces dark mycelia and ostiolate pycnidia. Furthermore, ITS sequences for *Readeriella* are represented in the NCBI database, but that genus did not appear in the top 100 matches when the sequences from the new class were submitted to the query. The type species of *Readeriella (R. mirabilis)* and the type strain from *X. heveae* (TC161) have an ITS sequence similarity of 71% (S.E 0.03%).

From visual examination of the 107 taxa–6 locus alignment, we found few molecular characters that distinguishing the new species (herein named *Xylona heveae*) from the rest of the strains. We define distinctive characters as characters with character states that are present in all the strains belonging to the new species but are not shared with the members of the other classes. Within the mitSSU alignment, we found only one distinctive character (character 140). We found more distinctive characters within our *RPB*1 and *RPB*2 alignments. Based on *RPB*1 we found four codons that were only present in the new species strains, but only one (characters 274–276) codifies for a distinctive amino acid (aspartic acid). In the case of *RPB*2, we found that the new species had three distinctive codons and, as in the case of *RPB*1, only one (characters 919–921) codifies for a distinctive amino acid (threonine). We found no distinctive characters within our alignments of nucSSU, nucLSU, and 5.8S sequences.

Fourteen of the focal strains were found as sapwood endophytes, and one was isolated from an asymptomatic leaflet. Foliar endophytes (Class 3, sensu Rodriguez et al., 2009) typically are horizontally transmitted and can enter the leaves through the stomata or directly by piercing the leaf's cuticle (Arnold and Herre, 2003; Mejia et al., 2008). However, the mode of transmission of Xylonomycetes is not yet known. Endophytes occur frequently in sapwood (Verma et al., 2007; Thomas et al., 2008; Giordano et al., 2009; Hanada et al., 2010; Gazis and Chaverri, 2010; Parfitt et al., 2010; Tayung and Jha, 2010), and may colonize via lenticels or wounds in adults (Pearce, 1996). Alternatively, they may translocate to living sapwood through the vascular system of the host (Bailey et al., 2008; Bailey et al., 2009). Vertical transmission also cannot be ruled out based on the present study. Further work that evaluates infections in propagules of *Hevea* and careful studies of tissue preference will provide insight into the life cycle of the newly found lineage, including where and when in nature these fungi reproduce.

Isolates from the proposed class were isolated primarily from rubber plantations in San Martin (Tocache, central Peru), but also were found in wild populations of rubber trees in Loreto (ACTS, North East Peru) and Madre de Dios (Los Amigos, South East Peru). These sites differed in management, land use history, elevation, and other characteristics (Table S6), suggesting that Xylonomycetes may occur over a wide range of conditions. To our knowledge, Xylonomycetes have not been isolated from any other species in the region (e.g., *Theobroma* spp.; Evans et al., 2003; Rubini et al., 2005; Thomas et al., 2008; Hanada et al., 2010; *Cecropia* spp., Arnold, *unpubl. data*). Thus strong conclusions about host breadth are premature in the absence of further sampling of sapwood and other tissues in co-occurring plants.

In terms of abundance, these fungi appear to be rare, in comparison to other rubber sapwood-endophytic species such as species of *Trichoderma* (Gazis and Chaverri 2010; Gazis et al., 2011; Gazis and Chaverri, in preparation). For instance, at Los Amigos we obtained 247 isolates from 270 tissue samples, but only two isolates belonged to the new lineage. In comparison, 47 *Trichoderma* isolates were recovered. Similarly, at ACTS we obtained 189 isolates from 252 tissue samples, but only two isolates belonged to the new lineage (vs. 23 *Trichoderma* isolates). However, at the Tocache rubber plantations, where we obtained 253 isolates from 270 tissue samples, 11 belonged to the new lineage vs. only 6 *Trichoderma* isolates. One important factor to consider is that the isolates from this study were obtained using culture-based methods. Strains that have rapid growth can mask the presence of slow growers (Arnold et al., 2007; Hyde and Soytong, 2008). Therefore, methods that do not depend on culturing, such as direct PCR from leaves and sapwood, will enhance our understanding of the ecology of this lineage.

Recent surveys of phylogenetically diverse fungal endophytes have shown their ability to degrade cellulose and lignin (e.g., Maria et al., 2005; Oses et al., 2006; Dai et al., 2010; Promputtha et al., 2010). A study involving five angiosperm and coniferous host species revealed growth on CMC-medium by 89% of foliar endophytes ($N = 35$ species representing Sordariomycetes, Dothideomycetes, and Pezizomycetes), and measurable cellulase activity by 90% of the foliar endophyte species that grew on that medium (Orozco and Arnold, in preparation). Among xylem-inhabiting endophytes from the same host species, 100% of species grew on indulin medium, and measurable ligninase activity was detected in 67% of those species ($N = 24$ species; Orozco and Arnold, in preparation). In contrast we found that none of the isolates belonging to the new lineage grew on CMC or indulin media, suggesting that these strains do not have the enzymes necessary to breakdown lignin and cellulose (ligninase and cellulase, respectively). The latter might indicate that this novel group of ascomycetous fungi lives only within living hosts, and perhaps is transmitted in a manner that does not require external colonization or the enzymatic activity associated with saprotrophy or pathogenicity.

5. Taxonomy of the new lineage

Xylonomycetes R. Gazis & P. Chaverri, class. nov.
MycoBank accession number MB800327.
Etymology: from Greek "xylon" = wood; referring to the preferred substrate of the new lineage; Greek "mykes" = fungus.
Type genus: *Xylona* R. Gazis & P. Chaverri.
Diagnosis: Phylogenetically placed among Pezizomycotina, within 'Leotiomyceta' (Schoch et al., 2009), but outside all known classes of fungi.
Gazis et al. Molecular Phylogenetics and Evolution x:x, 20xx (Fig. 1).
Phylogenetic notes: Strongly supported as a separate class within the Pezizomycotina (BS: 100%; PP: 1) and contained by the superclass 'Leotiomyceta' (BS: 100%; PP: 1) sensu Schoch et al. (2009); based on 6 loci phylogeny (nucSSU, nucLSU, mitSSU, 5.8S, *RBP*1, and *RPB*2).
Xylonomycetales R. Gazis & P. Chaverri, ord. nov.
MycoBank accession number MB800328.
Type Genus: *Xylona* R. Gazis & P. Chaverri.
Xylonomycetaceae R. Gazis & P. Chaverri, fam. nov.
MycoBank accession number MB800329
Type Genus: *Xylona* R. Gazis & P. Chaverri.
Xylona R. Gazis & P. Chaverri, gen. nov.
MycoBank accession number MB800330.
Etymology: From Greek Xylona – "from the forest".
Type species: *Xylona heveae* R. Gazis & P. Chaverri.
Description: Conidiomata pycnidial, astromatic; pycnidial wall composed of thin-walled cells forming tissue textura angularis;

ostiole absent. Conidiophores absent. Conidiogenous cells enteroblastic, phialidic, discrete, hyaline, 1 collarette, smooth-walled. Conidia apically rounded with two lateral obtuse projections appearing heart-shaped, narrower and truncated at base, hyaline when young, turning dark brick when mature, aseptate.

Xylona heveae R. Gazis & P. Chaverri, sp. nov., Fig. 3 A–J.

MycoBank accession number MB800331.

Etymology: The epithet refers to the name of the host (*Hevea*) from which the type species was isolated.

Description: Colonies on PDA and MEA effuse, at first white, later grayish sepia to fuscous black. Mycelium mostly superficial composed of branching sometimes anastomosing hyphae; hyphae septate, hyaline when young, turning pale brown to brown with age, smooth. Conidiomata pycnidial, astromatic; immersed when young, fuscous black, subspherical, (110) 170 (250) ± 40 μm × (120) 160 (180) ± 30 μm, entirely closed, pycnidial wall composed of thin-walled cells forming tissue textura angularis; when mature, superficial, unilocular, sometimes fusing with other pycnidia. Chlamydospores rarely formed, spherical, mostly terminal, single, and hyaline. Conidial masses liberated by apical rupture of pycnidia, ostiole absent. Conidiophores absent. Conidiogenous cells lining inner wall of pycnidial cavity, enteroblastic, phialidic, discrete, hyaline, 1 collarette, smooth-walled, (5.1) 6.8 (8.9) ± 0.97 μm long, (1.9) 2.9 (3.6) ± 0.6 μm broad. Conidia abundantly produced, arising singly, apically rounded with two lateral obtuse projections appearing heart-shaped, narrower and truncated at base, hyaline when young, turning dark brick when mature, aseptate, thick-walled, smooth, guttulated, (2.4) 2.8 (3.5) ± 0.2 μm × (2.0) 2.9 (3.7) ± 0.3 μm. Colonies producing greater amounts of aerial mycelium when grown on PDA than on MEA; pycnidia produced earlier and in greater quantities when grown in MEA (after 2 weeks versus 3 weeks in PDA). After *ca.* 3 weeks, both types of agar acquired a vinaceous color diffusing pigment, especially at the margins of the growing colony. None of the structures reacted with 3% potassium hydroxide (KOH).

Diagnostic molecular characters: In comparison to the members of the classes included in this study and in relation to TreeBase alignment #12729, the new species *Xylona heveae* can be distinguished by: mitSSU: character 140, the new species *X. heveae* has a C while all the other members of the included classes have an A, G, or T. *RPB1*: characters 169–171 (GTT); characters 457–459 (AGT); character 997–999 (AGG); and characters 274–276 (GAT). The latter codes for the amino acid Aspartic acid unlike the other Ascomycota classes included in this study. *RPB2*: characters 616–618 (ATC); characters 1036–1038 (AAG), and characters 919–921 (ACT). The latter codes for the amino acid Threonine unlike the other Ascomycota classes included in this study.

Ecology: The majority (14 of 15) of the isolates belonging to the newly described lineage were isolated from sapwood of living *Hevea* trees; one was recovered from a living, apparently healthy leaflet.

Known Distribution: Peru (Amazon basin).

Holotype: PERU. Dept. San Martin: Prov. Tocache, 8°11'33.69"S–76°32'35.86"W, altitude 520 m, endophytic in living sapwood of cultivated *H. brasiliensis*, collector R. Gazis, 09 June 2010 (HOLOTYPE DRY CULTURE BPI 884084; EX TYPE TC161 = CBS132557).

Additional cultures examined: PERU. Dept. San Martin: Prov. Tocache, 8°11'51.50"S–76°30'50.61"W, altitude 503 m., endophytic on living sapwood of cultivated *H. brasiliensis*, collector R. Gazis, 08 June 2010 (TC47, CBS132561); 8°11'33.69"S–76°32'35.86"W, altitude 520 m., endophytic on living sapwood of cultivated *H. brasiliensis*, collector R. Gazis, 09 June 2010 (TC137, CBS132558); 8°12'3.84"S–76°30'38.22"W, altitude 481 m, endophytic on living sapwood of cultivated *H. brasiliensis*, collector R. Gazis, 10 June 2010 (TC269, CBS132556).

Notes: Xylona heveae is distinct in having heart-shaped melanized conidia and in presenting an endophytic habit.

6. Conclusions

The recent discovery of the phylum-rank group Cryptomycota (Jones et al., 2011) emphasizes the need for further studies that help reduce the gaps in our knowledge of fungal diversity and stress the importance of elucidating the "missing fungi" for informing the fungal tree of life. The discovery of new fungal lineages can contribute not only to a better understanding of the phylogenetic history, evolution of morphological characters, and diversification of lifestyles of fungi, but also can shed light about the dynamics and ecology of the communities with which they interact. This study explored an understudied substrate (sapwood of tropical trees) and discovered a unique and previously unknown class of Pezizomycotina that could not have been identified using methods that are currently in place for culture-free surveys of fungal biodiversity. Together our results reveal the importance of pairing traditional and modern methods in studies of fungal biology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.06.019.

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