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A new species of penzigoid *Xylaria* (Xylariaceae) from the cloud forest in eastern Mexico revealed through morphological and phylogenetic analyses

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Abstract

Xylaria subtropicalis, a species new to science, was collected from a montane cloud forest in eastern Mexico. It is described and illustrated, based on detailed macro- and microscopic morphological characteristics, habitat, and DNA sequence data. The distinctive morphological differences between *X. subtropicalis* and other *Xylaria* species include: the stromata being significantly erumpent, the size of the ascospores being $23\text{--}28 \times 13\text{--}15 \mu\text{m}$, and a straight germ slit as long as the spore. To determine if this was a novel species, ITS sequences were generated and compared with 2478 sequences available for the genus from GenBank using the software USEARCH. Additionally, a phylogenetic reconstruction was performed with Parsimony Ratchet, Maximum Likelihood, and Bayesian Inference, using 122 concatenated ITS sequences, and segments from the 28S rDNA and RPB2. Sequences of this novel species form a strongly supported lineage, clearly separated from other species of *Xylaria*. Phylogenetic relationships indicate that there have been numerous morphological transformations within the genus.

Keywords: Ascomycota, DNA sequences, Phylogeny, Taxonomy, Xylariales

Introduction

The montane cloud forest, known in Mexico as the “bosque mesófilo de montaña,” occupies about 1% of Mexican territory (Rzedowski 1978, 1996; Challenger 1998). This forest contains at least 10% of the vascular plants of the Mexican flora, including 762 species of trees (González-Espinosa et al. 2012). The fungal species in the cloud forest are diverse. Currently, a database of 6349 records representing 2962 fungal species from the Neotropical cloud forests has been assembled (Del Olmo-Ruiz et al. 2017). By 1993 in Mexico, 594 fungal species had been recorded from this ecosystem (Chacón and Medel 1993). Medel (2013) identified 107 species as ascomycetes, of which 69 were found in the cloud forests of Veracruz. For the genus *Xylaria* Hill ex Schrank, San Martín and Rogers (1995) and Medel (2007) listed 105 and 109 species, respectively, from Mexico. Veracruz contained 45 species (Medel et al. 2008), with approximately 25 species located in cloud forests.

Xylaria contains more than 300 species, which are primarily distributed around the world in tropical and subtropical regions, and is one of the genera with the greatest number of species within the family of Xylariaceae (Kirk et al. 2008). The genus *Xylaria* is characterized by its stromata that are usually erect, cylindrical, or clavate, sometimes sub-globose, and a height usually larger than the width. Perithecia are circumambiently embedded immediately beneath the surface, with asci having amyloid apical rings and pigmented ascospores usually with a straight, oblique, or spiral germ slit (San Martín and Rogers 1995; Ju et al. 2009). Some species of *Xylaria* are distinguished by their penzigoid stromata. Penzigoid species were once placed in *Penzigia* Sacc., but Ju and Rogers (2001) demonstrated that the type species of *Penzigia* (*P. cranioides* Sacc. & Paol.), belongs to *Xylaria*. There are more than 40 penzigoid *Xylaria* species worldwide (Ju et al. 2012), which are characterized by sessile stromata adhered to the substrates through a narrow central connective structure (Ju et al. 2012; Tapia et al. 2017). Some penzigoid *Xylaria* species known from Mexico include *X. albocinctoides* Y.M. Ju, H.M. Hsieh & J.D., *X. anisopleura* (Mont.) Fries, *X. berteri* (Mont.)

Cooke ex J.D. Rogers and Y.-M. Ju, *X. boergesenii* (Ferd. & Winge) P.F. Cannon, *X. discolor* (Berk. & Broome) Y.M. Ju, H.M. Hsieh, J.D. Rogers and Jaklitsch, *X. enteroleuca* (J.H. Mill.) P.M.D. Martin, *X. frustulosa* (Berk. & M.A. Curtis) Cooke, *X. xylarioides* (Speg.) Hladki & A.I. Romero (San Martín and Rogers 1995; Tapia et al. 2017), and *Penzigia conostoma* (Mont.) J.H. Mill, an invalidly published name recorded by Chacón and Guzmán (1983) from Veracruz.

Morphological identification of *Xylaria* species is difficult since some characters are often in conflict or missing, such as cultural features, anamorphs, exceptional variations in the color, size, and sometimes shape of stromata (Rogers 1984, 1985; Tang et al. 2009). Consequently, DNA sequence data are frequently incorporated into taxonomic studies to elucidate phylogenetic relationships. Ribosomal regions such as nuclear 28S rDNA, ITS (ITS1-5.8S-ITS2), and protein-coding genes including RPB2 and β -tubulin have been used for molecular phylogenetic studies of the Xylariaceae (Lee et al. 2000; Tang et al. 2009; Hsieh et al. 2010; U'Ren et al. 2016). These gene regions have been widely used in mycology as fungal DNA barcodes to identify unknown specimens or cultures (Stielow et al. 2015). In the present study, a new species of *Xylaria* from a cloud forest in central Veracruz, Mexico, is described based on morphological characters and phylogenetic analyses using sequence data.

Materials and Methods

Sample collection

Between 2011 and 2015, specimens of *Xylaria* with penzigoid stromata were collected from wood bark in a patch of a preserved montane cloud forest. The study area is in west Xalapa Veracruz, Mexico and had the following sampling sites: the “Santuario del Bosque de Niebla”, the Botanical Garden “Francisco Javier Clavijero”, and the “Molino de San Roque”, each separated from the others by approximately seven kilometers. Elevation at the study area varied between 1300 and 1460 m. The climate is mild and humid during the

entire year, with an annual temperature between 16 and 18°C, and total annual precipitation between 1517 and 1650 mm. Dominant canopy tree species have temperate affinities and include species of *Carpinus*, *Clethra*, and *Quercus*; whereas, understory trees and shrubs have tropical affinities (Muñiz-Castro et al. 2006; Toledo-Aceves et al. 2014).

Morphological studies

Freshly collected stromata were sectioned and mounted in water or 5% KOH prior to microscopic examination of structures. Melzer's iodine reagent was used to stain the ascus apical ring. Voucher specimens were deposited at the Herbarium of the Instituto de Ecología, A.C. (XAL). Photographs of the stromata, ascospores, and asci were used to prepare a detailed description of the species.

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from ten specimens of *Xylaria* from the cloud forest (Table 1), using the DNeasy plant and fungi mini kit (Qiagen, Valencia, California, USA). The DNA samples were amplified with specific primers and cycle conditions for ITS, partial 28S, rDNA, and gene coding for the second largest subunit of RNA polymerase II (RPB2) (Table 2). PCR reactions were carried out in a thermocycler Eppendorf Mastercycler pro S (Hamburg, Germany) in a 25 µL mix containing approximately 25–50 ng of extracted DNA, 5 µL of PCR buffer 5×, 0.2 mM dNTPs, 1.6 µM of both the forward and reverse primers, 2 mM MgCl₂, 0.2 U of Go Taq flexi DNA polymerase (Promega, Madison, Wisconsin, USA), and distilled water. The amplified DNA was purified prior to sequencing with the GenElute Gel extraction kit (Sigma-Aldrich, St. Louis, Missouri, USA), and sequenced using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. The PCR products were cleaned with an isopropanol precipitation and processed on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, California, USA). The resulting sequences

were edited with the BioEdit software 7.1.3.0 (Hall 1999), and initially blasted in GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) with blastn to assess genetic affinities.

Comparison of ITS sequences among Xylaria species

To evaluate the potential novelty of newly collected specimens of *Xylaria*, ITS sequences generated in this study were compared with 2478 available sequences of this genus downloaded from GenBank (retrieved March 2018). From these sequences, a database containing 2136 sequences was constructed by removing all identical or incomplete sequences, plus any sequences from other fungi. USEARCH software v10.0.240 was employed (Edgar 2010), with the 'usearch_global' option at a 90 and 95% identity threshold for identifying the sequences that could well align with our sequences. Within this database, the 'cluster_fast' command set at a 0.9 clustering threshold was used to assemble similar sequences into clusters through multiple alignments.

Phylogenetic analyses

To determine the identity of ten collections of a novel *Xylaria* species, three matrices were assembled (A, B, C) with sequences downloaded from GenBank and the newly collected specimens of *Xylaria*. Matrices A and B contained 122 concatenated sequences of ITS (ITS1-5.8S-ITS2), 28S, and RPB2; however, for matrix B the highly variable ITS1 region was excluded to avoid poorly aligned regions. These matrices contained sequences primarily from studies conducted by Hsieh et al. (2010) and U'Ren et al. (2016), and included multi-gene sequence data for most *Xylaria* species. Nonetheless, several terminals from the 28S were missing in their matrices; therefore, the third matrix C was constructed with 339 sequences available in GenBank, containing both ITS and 28S (including those from U'Ren et al. 2016). Alignment of the sequence data was performed using the MAFFT software v7.244, which allows for the detection of homologous segments through the iterative refinement of an initial alignment (Katoh and Standley 2013).

Analyses of matrices A and B were performed with three methods of phylogenetic reconstruction: Parsimony ratchet (PR), Maximum Likelihood (ML), and Bayesian Phylogenetic Inference (BPI). Analyses with matrix C were conducted with PR and BPI. PR was conducted using NONA (Goloboff 1994; Nixon 1999), with 2000 iterations, holding 10 trees per iteration, with a 10% of randomly constrained nodes and gaps recorded as missing data. Statistical branch support was determined through the means of a jackknife (JK) analysis, running 1000 replicates with 10 searches per replica with MULTMAX in effect. Before the analyses with ML and BPI, we used the program PartitionFinder 2 (Lanfear et al. 2017) for selecting the best model of sequence evolution for the analyses. The commands ‘unlinked’ for branch length and ‘all’ for the model were used. Metrics for comparing partitioning schemes were the Bayesian information criterion (BIC) and the command ‘all’ for the search option on the five partition schemes: ITS, 28S, and one for each codon position of the RPB2. ML was performed using RAXML at Trex-online (<http://www.trex.uqam.ca/index.php?action=raxml>), with the default rapid hill-climbing algorithm and the GTRGAMMAI substitution model selected after analysis with PartitionFinder 2. Support values for ML were calculated simultaneously by conducting 1000 rapid bootstrap (BS) replicates, with 10 BS searches per replicate to obtain better ML trees for each (Stamatakis 2006; Boc et al. 2012). The BPI analyses were conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003; Altekar et al. 2004), and the model selected by PartitionFinder 2 consisted of a general time-reversible model for each partition scheme (rates = invgamma, nst = 6), with unlinked parameters and variable rates for all partitions. The analyses were performed using two independent 3 to 5 million generation runs, with four chains (one cold and three hot) each, until an average standard deviation of split frequencies of 0.01 or less was reached. The trees were sampled every 100th generation along with discarded initial samples applying a “burn-in” value of 25%, before calculating the majority consensus tree and posterior probabilities (PP) for clades. *Poronia*

pileiformis (Berk.) Fr., *Podosordaria muli* J.D. Rogers, Y.M. Ju & F. San Martin, and *Podosordaria mexicana* Ellis & Holw. were used as the outgroup taxa (U'Ren et al. 2016) for rooting the tree. The trees were subsequently edited using Adobe Illustrator 16.0.0 (Adobe Systems, San Jose, CA, USA). The alignments and resulting trees are deposited in TreeBASE (TreeBASE accession: S21630). GenBank accession numbers with species names used in the analyses are included in each cladogram.

Results

Comparison of ITS sequences

Analysis of the 2136 sequences from the ITS region with the 'usearch_global' algorithm showed that *Xylaria primorskensis* Y.M. Ju, H.M. Hsieh, Lar.N. Vassiljeva and Akulov (JX160062), and *Xylaria longipes* Nitschke (AY909016.1) at 95% and 90% thresholds, respectively, had the highest identity with the novel species of *Xylaria*. Analysis with the 'cluster_fast' algorithm at a 0.9 clustering threshold generated 218 clusters. All newly collected specimens clustered within a group containing 21 additional sequences, in which seven corresponded to *X. bambusicola* Y.M. Ju and J.D. Rogers (MF435163, KF381074, GU300088, EF026123, KU940160, JX256820, JX256818), one to *X. grammica* (Mont.) Mont. (KF312440), one to *X. cornu-damae* (Schwein.) Berk. (AF163031), and 12 to unidentified *Xylaria* spp. (KJ572198, KJ572199, KF430809, KJ958374, AB495009, AB512330, KF227849, MF045812, KF227839, AB512331, DQ322133, KT004562). Most of these 21 species were collected as endophytes in different plant species and countries. Unfortunately, no 28S or RPB2 sequences of *X. primorskensis* were available in GenBank for phylogenetic analyses. However, a Fast-Minimum Evolution tree implemented in Blast of available ITS sequences showed that *X. longipes* and *X. primorskensis* are sister taxa, and together form a sister group to our new taxon.

Phylogenetic analyses

After the alignment process, matrix A consisted of 122 sequences and 3888 characters (1413 for the ITS, 1357 for the 28S, and 1118 for the RPB2), with 905 parsimony-informative sites. Matrix B had 3016 characters (541 for the ITS, 1357 for the 28S, and 1118 for the RPB2), and 754 parsimony-informative, and matrix C included 339 sequences with 2159 characters (1574 for the ITS, and 585 for the 28S), and 533 parsimony-informative sites. Phylogenetic analyses of matrix A showed that sequence data of all specimens collected in the cloud forest in central Veracruz, formed a monophyletic clade with the highest possible support values (BS=100, JK=100, PP=1.0; Fig. 1). Matrix B had a PP value of 0.99, and matrix C a JK value of 99 (topologies not shown). Our analyses recovered most clades obtained by U'Ren et al. (2016) and revealed that the novel species is nested within a small clade inside the “*Xylaria* PO clade” (U'Ren et al. 2016). *Xylaria longipes* (isolate CBS 148.73) was the sister taxon to the novel species with high support values (BS=89, JK=98, PP=0.98). *Xylaria allantoidea* (Berk.) Fr. (isolate 94042903) is a sister to this clade, with support values of BS=98, JK=100, and PP=1.0; and sister to these taxa was one small clade comprising two specimens of *Xylaria regalis* Cooke (isolates 920 and 92072001); one specimen of *Xylaria digitata* L. Grev. (isolate 919) and one of *Xylaria acuta* Peck (isolate AFTOL-ID 63). This last relationship; however, is uncertain due to low support values (BS=63, JK=65, PP=0.94). Analyses with matrix B recovered the same relationships among these species, but analyses with matrix C showed slight differences. Sister taxa to the novel species consisted of an unresolved moderately supported (JK=83, PP=0.95) clade, containing the same specimen of *Xylaria longipes* (CBS 148.73, KU683868), a different specimen of *Xylaria allantoidea* (voucher MEL 2382670, KP013033), and *Xylaria polymorpha* (Pers.) Grev. (strain 305713, AB512310).

Taxonomy

Xylaria subtropicalis S. Chacón and F. Tapia, *sp. nov.* (Figs. 2A–F, 3G, H)

MYCOBANK NO: MB 822922

TYPE:— MEXICO. Veracruz: Xalapa, Francisco Javier Clavijero Botanical Garden, 2.5 km old road Xalapa-Coatepec, 19° 30' 41" N, 96° 56' 36" W, elevation 1320 m, 3 July 2015, S. Chacón, D. A. Aguilar and F. Tapia 8154 (holotype XAL).

GENBANK:—ITS = MG013556; 28S = MG013566; RPB2 = MG013547.

ETYMOLOGY:—The name reflects the climate and vegetation of the region where this species was first observed.

DIAGNOSIS:—Characterized by stromata erumpent through the bark of dead wood, penzigoid, cracked into minute beige to pale brown scales when young. Ascospores $23\text{--}28 \times 13\text{--}15 \mu\text{m}$, ellipsoid, nearly equilateral, with a straight germ slit as long as the spore.

DESCRIPTION:—Stromata erumpent through the bark of dead wood, irregularly discoid to elongate, 0.4–1.7 cm diameter $\times$ 0.1–0.4 cm thick, flattened to pulvinate, then slightly sunken in the center, with conspicuous perithecial mounds; cracked into minute beige to pale brown scales that disappear with age; remaining carbonaceous surface, beige to brown; attached to substrate through a narrow, central connective, mostly multiperitheciate (up to 60 perithecia per stroma), with white and soft inner tissue. Perithecia globose to subglobose, 0.4–1.2 mm in diameter. Ostioles black, papillate, 0.1 mm broad, slightly raised. Asci with eight ascospores arranged in an uniseriate manner, hyaline, cylindrical, long-stipitate, $150\text{--}170 \times 12\text{--}14 \mu\text{m}$ with the spore-bearing part being 100–130 μm long, with an inverted, hat-shaped, amyloid, apical ring of $3.5\text{--}4 \times 5\text{--}6 \mu\text{m}$. Ascospores of $23\text{--}26$ (–28) $\times 13\text{--}15 \mu\text{m}$, dark brown to blackish brown, ellipsoid, nearly equilateral, with narrowly rounded ends sometimes pinched, with a straight germ slit spore-length.

HABITAT:—Gregarious on fallen twigs of *Quercus* sp.

DISTRIBUTION:—Eastern Mexico, in a montane cloud forest, 1300–1460 m.

OTHER SPECIMENS EXAMINED:—Mexico. Veracruz State, municipality of Xalapa, Cerro Las Garzas, annexed to Molino de San Roque Ecological Park, 19° 33' 12" N, 96° 56' 17" W, elevation 1476 m, 28 August 2014, Chacón and F. Tapia 7793; Francisco Javier Clavijero

Botanical Garden, 2.5 km old road Xalapa-Coatepec, 19° 30' 48" N, 96° 56' 24" W, elevation 1350 m, 2 September 2011, S. Chacón and E. Utrera 6332; 8 May 2014, F. Tapia and S. Chacón 3043; 24 June 2014, F. Tapia 3096; 30 June 2014, F. Tapia and L. M. López-Guzmán 3104; 3 July 2015, F. Tapia, S. Chacón and D. A. Aguilar 3343; INECOL Cloud Forest Sanctuary, 2.5 km old road Xalapa-Coatepec, 19° 30' 41" N, 96° 56' 36" W, elevation 1310 m, 14 October 2010, S. Chacón and E. Utrera 6090; 19 June 2013, S. Chacón and F. Tapia 2856; 29 April 2014, F. Tapia and S. Chacón 3031; 30 July 2014, S. Chacón, F. Tapia and L. M. López-Guzmán 7679; 17 September 2014, S. Chacón and F. Tapia 7896.

COMMENTARY:—*Xylaria subtropicalis* is recognized in the field by its penzigoid stromata erumpent through the bark of twigs of *Quercus* spp., the discoid form of the stromata that are slightly sunken in the center, and by the cracked surface that presents small scales from beige to pale brown in young specimens. Differences between *X. subtropicalis* and other *Xylaria* species are: The stromata being significantly erumpent, the size of the ascospores $23\text{--}28 \times 13\text{--}15 \mu\text{m}$, and a straight germ slit as long as the spore. There are other penzigoid *Xylaria* species with a resemblance to *X. subtropicalis*, such as *X. albocinctoides*, *X. boergesenii*, *X. anisopleura*, *X. berteri*, and *X. discolor*. *Xylaria albocinctoides* differs by having smaller stromata with a thin soft crust, $0.5\text{--}2 \text{ mm diameter} \times 0.5\text{--}0.8 \text{ mm thick}$, $\leq 10 \text{ mm}$ long when confluent, and ascospores of $18\text{--}23 \times 10\text{--}12 \mu\text{m}$, but with a straight germ slit that is similar to *X. subtropicalis*. *Xylaria boergesenii* also has a smaller stroma than *X. subtropicalis*, $0.5\text{--}3 \text{ mm diameter} \times 0.5\text{--}1 \text{ mm thickness}$, and ascospores measure of $19.5\text{--}26 \times 7\text{--}8.5 \mu\text{m}$, but with a spiral to sigmoid germ slit. *Xylaria anisopleura* has subglobose to elongated or irregular and often moriform stromata and ascospores that measure $20\text{--}30 \times 7.5\text{--}9.5 \mu\text{m}$, with a spiral or oblique germ slit that is shorter than the spore; whereas, *X. berteri* and *X. discolor* have a flattened or convex stroma, similar to the *X. subtropicalis*, but with ascospores less than $15 \mu\text{m}$ long (San Martín-Gonzalez and Rogers 1989; Ju et al. 2012; Tapia et al. 2017).

Discussion

The species of *Xylaria* are difficult to identify due to their diverse and often convergent morphology. However, by combining morphological and molecular characteristics it is possible to recognize a new species. Our study focused on morphological and sequence data for a reliable characterization of ten collections of a novel *Xylaria* species. Our analyses convincingly proved that these new collections from a cloud forest correspond to a novel species, called *Xylaria subtropicalis*. Specimens consistently formed a strongly supported monophyletic group, independent from all other available sequence data from other species of *Xylaria*.

It is interesting to note that *Xylaria subtropicalis* and other penzigoid species (e.g., *X. areolata* (Berk. & M.A. Curtis) Y.M. Ju and J.D. Rogers, *X. berteri*, *X. cranioides*, *X. discolor*, and *X. frustulosa*) are not grouped together, but scattered on different branches as was observed by U'Ren et al. (2016), despite *X. berteri* and *X. discolor* having a morphological resemblance to *X. subtropicalis*. Both specimens of *X. discolor* included in the analysis were monophyletic having *X. lechatii* Y.M. Ju, H.M. Hsieh, J.D. Rogers & Fournier, another penzigoid species, as a sister taxon. Specimens of *Xylaria berteri* were also grouped with another penzigoid species (*X. crozonensis* P. Leroy & Mornand); however, their relationship was paraphyletic.

According to the phylogenetic analyses, the sister taxa of the novel species are *X. longipes* and *X. allantoidea* while the Fast-Minimum Evolution tree showed that *X. longipes* and *X. primorskensis* are sister taxa, and together form a sister group to the novel species. These species considerably differ from *X. subtropicalis* by stipitate fruiting bodies, the form of fertile parts, and spore size, indicating that there have been numerous morphological transformations within the genus (Dennis 1956; Rogers 1984; San Martín-Gonzalez and Rogers 1989; Ju and Rogers 1999; Rogers et al. 2008). The only feature that *X. primorskensis* and *X. subtropicalis* appear to share is a long straight ascospore germ-slit (Ju et

al. 2009). There are other factors that contribute to the discrepancies between the morphology and the molecular data. Studies performed at different levels within the family Xylariaceae have identified that disagreements between morphology-based classifications and molecular phylogenies could also be attributed to the lack of molecular data from many species including, type specimens, misidentified specimens and/or sequences in public databases, and the need for additional phylogenetic molecular markers. Therefore, our phylogeny, which is similar to many other phylogenies (Peršoh et al. 2009; Tang et al. 2009; Hsieh et al. 2010; Daranagama et al. 2015; Senanayake et al. 2015; U'Ren et al. 2016), reiterates the need for a thorough revision within the family.

Xylaria subtropicalis was collected on twigs of *Quercus* spp., which are found throughout the northern hemisphere down to the Equator in Asia, North America, Europe, and Africa. On the American continent, species of *Quercus* occur from Canada to Colombia (Nixon 2006). To our knowledge, *X. subtropicalis* is the first report of an erumpent species growing through the twig bark of oak trees. The wide range of natural distribution of the genus *Quercus* could also suggest that *X. subtropicalis* has a more extensive distribution. The novel species of Xylariaceae that was collected in a patch of cloud forest appears to be only a small fraction of the species of this family in Mexico. Consequently, further collections and sequence data are required for a better comprehension of its diversity in tropical countries.

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Table 1. Collection numbers, localities, and GenBank accession numbers of *Xylaria subtropicalis*.

Collection ID	Voucher	Locality	GenBank accession number		
			ITS	28S	RPB2
X1	S. Chacón, D.A. Aguilar and F. Tapia 8154 (holotype)	Jardín Botánico FJC	MG013556	MG013566	MG013547
X3	S. Chacón, F. Tapia and M.L. López-Guzmán 7669	Santuario Bosque de Niebla	MG013559	MG013574	—
X4	F. Tapia and S. Chacón 3031	Santuario Bosque de Niebla	MG013564	MG013569	MG013552
X5	S. Chacón and F. Tapia 7793	Molino San Roque	MG013560	MG013572	MG013548
X6	F. Tapia 3096	Santuario Bosque de Niebla	MG013561	MG013573	MG013549
X7	S. Chacón and F. Tapia 2856	Santuario Bosque de Niebla	MG013565	MG013575	MG013550
X8	Tapia F. and S. Chacón 3043	Jardín Botánico FJC	MG013562	MG013571	MG013551
X9	S. Chacón and E. Utrera 6332	Jardín Botánico FJC	MG013563	MG013568	MG013553
X11	F. Tapia, S. Chacón and D.A. Aguilar 3343	Jardín Botánico FJC	MG013557	MG013567	MG013555
X12	F. Tapia and L.M. López-Guzmán 3104	Jardín Botánico FJC	MG013558	MG013570	MG013554

— = no sequence data

FJC = Francisco Javier Clavijero

Table 2. Primer sequences and PCR conditions applied for each gene employed to amplify DNA from specimens of the new species *Xylaria subtropicalis*.

Gene	Primer name	Reference	Sequence (5'-3')	Cycle conditions annealing/extension
ITS	ITS1	White et al. 1990	TCCGTAGGTGAACCTGCGG	55 C, 30 s
	ITS4		TCCTCCGCTTATTGATATGC	72 C, 1 min
28S	LR0R	Vilgalys and Hester 1990	ACCCGCTGAACTTAAGC	51 C, 45 s
	LR5		TCCTGAGGGAAACTTCG	72 C, 1 min
	bRPB2-6F		TGGGGYATGGNTTGYCCYGC	60 C, 1 min
RPB2	bRPB2-7.1R	Matheny 2005	CCCATRGCYTGYTTMCCCAT	72 C, 2 min
	RPB2-980F	Reeb et al. 2004	TGYCCIGCIGARACICCHGARGG	52 C, 1 min
	fRPB2-7cR	Liu et al. 1999	CCCATRGCTTGYTTRCCCAT	72 C, 2 min

Fig. 1. Maximum likelihood phylogram based on concatenated sequence data showing positions of ten collections of *Xylaria subtropicalis* and close relatives. Supporting values above 70% for at least two metrics (BS/JK/PP) are shown at the nodes. GenBank accession numbers for ITS, 28S and RPB2 are included following taxon names. NA = not available.

Fig. 2. Macro- and microscopic morphology of *Xylaria subtropicalis*. A, B: Stromata on wood; arrows indicate young stromata. C: Stromata surface with ostioles (dark spots) and remnants of pale brown scales. D: Asci with ascospores as seen by light microscopy. E: Mature and immature ascospores; arrow points to the tip of an ascus with an apical, inverted hat-shape ring. F: Ascospore; arrow indicates the straight germline as long as the spore. Bars: A = 5 mm; B, C = 3 mm; D = 23 μm ; E = 11 μm ; F = 8.5 μm . Photographed by S. Chacón.

Fig. 3. Ascospores as seen by scanning electron microscopy. Bars: G = 5 μm ; H = 20 μm . Photographed by T. Laez.

167x187mm (600 x 600 DPI)

169x63mm (600 x 600 DPI)