RESEARCH NOTE

**Xylariales: First results of mycological exploration in the Sangay and Llanganates National Parks, Ecuador [version 2; referees: 2 approved with reservations]**

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**Abstract**

Fungal samples were collected in the Sangay (SP) and Llanganates (LP) National Parks in Ecuador. Sequences of the internal transcribed spacer regions (ITS1-5.8S-ITS2) of the ribosomal DNA of the samples were analyzed. Taxonomic identification of fungi of the order Xylariales was done through phylogenetic analysis using a Maximum Likelihood method. All analyzed collections presented here belong to the genus *Xylaria*, of these eight belong to PL and two to SP. Four samples were not identified at the species level, suggesting it could be a new species. This data contributes with base information on the biodiversity of the Parks, necessary to design and implement measures for the conservation of fungi in Ecuador.

**Keywords**

Diversity, ITS, Llanganates, National Park, Sangay, Xylaria
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Competing interests: No competing interests were disclosed.

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According to the referee’s suggestions we have made the following changes in the manuscript:

- The abstract, introduction and methods were re-written for ease of comprehension.
- The results are explained objectively.
- The table and figure legends are also modified for ease of comprehension of the content.
- In addition, we rephrased 2 sentences in Conclusions.
- We have removed one of the authors from the second version of the article as an error was made when she was included in the moment of submitting the first version of the article. Paula Salazar is a valuable person for the Universidad Politécnica Salesiana and she has collaborated in several projects, but unfortunately she did not collaborate in this research done in association with Pontificia Universidad Católica.

See referee reports

Introduction

Sangay (SP) and Llanganates (LP) National Parks in Ecuador are considered as high priority conservation units in the Tropical Andes, due to their high biodiversity and high endemism. However, their mycological diversity is still unknown. This study aims to contribute to the conservation of fungi, showing the results of their diversity, based on molecular taxonomy, by analyzing the ITS (internal transcribed spacer) regions. ITS is the accepted fungal barcode marker for fungi. For this, the DNA sequence of specimens of exploratory fungal collections were analyzed within the aforementioned parks. Here we present results exclusively for the Xylariales order, other fungal orders were also collected but are not shown here.

Methods

Sequencing and molecular identification

Sample collection was carried out during the months of January and February 2015. The fruiting bodies collected were deposited in the QCAM Fungarium (Catholic University Mycology Collection, Quito). Table 1 displays the collection codes, as stored at the QCAM. The ITS1-5.8SITS2 region was amplified by PCR with primers ITS1F (5'-CTTGGTCATTTAGAGGAAATTT-3') and ITS4 (5'-TCCTCCGCTTTATTGATA TGC-3')6. The amplified fragments were sent for sequencing to Macrogen Inc. (Seoul, South Korea). The forward and reverse sequences obtained for each isolate were assembled in Geneious R8 (Biomatter Ltd. 2005–2012), and submitted to GenBank. Sequence data were analyzed by comparison with sequences available in GenBank. An assignment to the lower taxonomic level was made by direct homology of the consensus sequences with the search results in BLASTn (NCBI) optimized for highly similar sequences (megablast). Alignments that presented 100% coverage and at least 99% identity with a sequence

| QCAM Fungarium Code | GenBank Accession Number | Identification       | Sampling location | National Park | Altitude (m) |
|---------------------|--------------------------|----------------------|-------------------|---------------|--------------|
| QCAM4663            | MG768840                 | Xylaria enterogena    | Llangantes        | 1370          |
| QCAM4551            | MG768839                 | Xylaria enterogena    | Llangantes        | 1387          |
| QCAM4537            | MG768834                 | Xylaria fissilis      | Llangantes        | 1377          |
| QCAM4540            | MG768836                 | Xylaria schweinitzii  | Llangantes        | 1377          |
| QCAM4232A           | MG768832A                | Xylaria telfairii     | Sangay            | 2885          |
| QCAM4550            | MG768838                 | Xylaria sp. 1         | Llangantes        | 1387          |
| QCAM4666            | MG768841                 | Xylaria sp. 1         | Llangantes        | 1379          |
| QCAM4306A           | MG768833                 | Xylaria sp. 2         | Sangay            | 2885          |
| QCAM4545            | MG768837                 | Xylaria sp. 3         | Llangantes        | 1373          |
| QCAM4539            | MG768835                 | Xylaria sp. 4         | Llangantes        | 1377          |
previously reported in GenBank were considered. The results were compared with the previously made morphological identification at the QCAM Fungarium, to check the taxonomic designation.

Phylogenetic analysis
Sequence data were aligned with Geneious R8 and later manually adjusted with Mesquite version 3.04. Public sequences available in GenBank that corresponded to specimens that gave the greatest homology in BLASTn with the sequences of the collected specimens were included. Phylogenetic trees were constructed in Geneious R8 using the PhyML plugin for Maximum Likelihood (ML) with a custom substitution model (010230), determined by jModelTest 2.1.4, according to Corrected Akaike Information Criterion (AICc), and a bootstrap of 1000 replica was used.

Results
All the specimens analyzed were of the genus Xylaria. The eight specimens from Llanganates National Park were identified as X. enterogena, X. fissilis, X. schweinitzii, X. telfairii and three unidentified species. For the two samples from Sangay National Park, one was X. telfairi and the other was an unidentified species. Differences in the number of samples found at each park could be due to the sampling effort, and not necessarily to the richness of the Xylariales in the Parks. The unidentified species were different in each park. The analysis shows that there are no shared species of Xylaria at the two sampled sites (Table 1), this is important for conservation decision making. The phylogenetic relationships recovered from the analysis of the ITS sequences (Figure 1) shows two major groups. The first major group, composed by clades A and B, is well supported, it includes specimens from LP and PS. Clade A includes all X. enterogena specimens and Clade B includes all X. telfairii specimens, and Xylarya sp.1 specimens. It is possible that Xylarya sp.1 might belong to the X. telfairii group, but due to the differences among the sequences it is likely a different species. In the second major group, clade C is sister to clades D, E and F. This major group includes specimens from both LP and SP. Clade C includes all X. schweinitzii specimens. Clade D includes Xylaria sp. 2. The closest sequence to Xylaria sp. 2 from Ecuador was a previously reported collection also from Ecuador in a cloud forest in the province of Imbabura, that was also identified only at the genus level. Clade E shows Xylaria sp. 3, the closest sequence to this individual belongs to the same previously reported study, identified only at the genus level. Clade F includes Xylaria fissilis sequences from LP and one from Clade F also includes Xylaria sp. 4, an unidentified specimen. The number of nucleotide differences (SNPs) between the sample and the closest hit on the BLASTn search suggests that these specimens may belong to new species (Table 1). Additional loci and more detailed morphological analyses are needed to determine this. The genus Xylaria is probably the largest in the family Xylariaceae, with 35 estimated genera, but the real number remains unknown. Studies related to the biological diversity of this order in the

Figure 1. Maximum likelihood phylogenetic tree of specimens of the Xylariales order obtained in Sangay and Llangantes National Parks, based on sequences of the ITS (internal transcribed spacer) region.
National Parks of Ecuador are scarce, more systematic field studies would surely reveal a greater diversity of families, genera and species within the Xylariales in SP and LP, as well as other regions and protected areas of Ecuador, especially if we take into account the cosmopolitan distribution of Xylaria. In fact, new fungal species in SP, belonging to the Agaricales, have recently been described.

These entire unidentified specimens might represent new species. Additional loci and more detailed morphological analyses are needed to determine this. The genus Xylaria is probably the largest in the family Xylariaceae, with 35 estimated genera but the real number remains unknown. Studies in relation to the biological diversity of this order in the National Parks of Ecuador are scarce, more systematic field studies would surely reveal a greater diversity of families, genera and species within the Xylariales in SP and LP, as well as other regions and protected areas of Ecuador, especially if we take into account the cosmopolitan distribution of Xylaria. In fact, new fungal species in SP, belonging to the Agaricales, have recently been described.

Conclusions
The results obtained allow us to establish a baseline for the biological diversity of the Xylariales in SP and LP, an important step to the conservation of fungi. This is the main contribution of this study. We found four species of Xylaria: X. enterogena, X. telfairii, X. schweinitzii, and X. fissilis, and four potential new species based on ITS sequences. These species found in LP are different from those found in SP. However, there is much more to discover. A huge and complex task is pending. To advance our understanding of the Kingdom Fungi we must start by deciphering the diversity of species present in these sites.

Data availability
The sequencing data are available on the NCBI Genbank webpage:
Xylaria enterogena: https://www.ncbi.nlm.nih.gov/nuccore/MG768840
Xylaria fissilis: https://www.ncbi.nlm.nih.gov/nuccore/MG768839
Xylaria schweinitzii: https://www.ncbi.nlm.nih.gov/nuccore/MG768836
Xylaria telfairii: https://www.ncbi.nlm.nih.gov/nuccore/MG768832

Xylaria sp. 1: https://www.ncbi.nlm.nih.gov/nuccore/MG768838
Xylaria sp. 2: https://www.ncbi.nlm.nih.gov/nuccore/MG768841
Xylaria sp. 3: https://www.ncbi.nlm.nih.gov/nuccore/MG768833
Xylaria sp. 4: https://www.ncbi.nlm.nih.gov/nuccore/MG768835

Competing interests
No competing interests were disclosed.

Grant information
The Secretaría de Educación Superior, Ciencia, Tecnología e Innovación del Ecuador (SENESCYT), Arca de Noé Initiative and the QCAM Fungarium from “Pontificia Universidad Católica del Ecuador” supported the project.

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Version 1

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Introduction. Because the aim is to compare these two parks in Ecuador, it is important that the elevation ranges of each are included, as well as the distance between the two parks.

Methods, p. 3, last line seems to be an incomplete sentence. It ends with ‘BLASTn’ (NCBI) optimized for highly similar sequences (megablast), alignments that presented 100'
It is probably meant to say ‘overlapped 100%.'

Results: Text for Clade E does not match the phylogram. The text says that sp. 3 was related to another collection identified only at the genus level. However, the phylogram shows a well-supported clade (98% BS) comprised of sp. 3 and X. curta KP133352 ECU. I would expect X. curta to be reasonably common there. There is either an error in identification of KP133352 ECU or the text in results needs to be changed, and the abstract and first line of results and conclusions also need to be changed to include X. curta.

That also means that the unidentified species need to be renumbered in the text, table, and phylogram, with sp. 4 becoming sp. 3.

There are likely 3 undescribed species included in this study. The journal allows for photographs, and photos should be added to the manuscript. Even if photos were not taken of the fresh specimens, photos of the dried specimens can be used, and are very helpful. If photos of the asci, especially the ascus plug stained with iodine/Melzer’s reagent, and photos of the ascospores showing the germ slits, that would be very helpful also for future work. The ascospore length and width ranges, the shape and extent of the germ slit, and the shape and size of the ascus plug would be helpful additions.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes
If applicable, is the statistical analysis and its interpretation appropriate?  
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?  
Yes

Are the conclusions drawn adequately supported by the results?  
Yes

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Francisco J. Flores  
Carrer of Engineering Biotechnology, Universidad de las Fuerzas Armadas–ESPE, Sangolquí, Ecuador

The article "Xylariales: First results of mycological exploration in the Sangay and Llanganates National Park, Ecuador " provides internal transcribed spacer sequences from *Xylaria* isolates collected from Ecuadorian national parks. Information about fungal diversity in Ecuador is still scarce, which makes this manuscript relevant. Nevertheless, I have several concerns, mainly:

- Phylogenetic analysis using only the ITS sequence is very superficial, and, most likely, it won’t show an accurate representation of the real evolutionary relationships between isolates.
- There is no mention about the morphological characteristics of isolates. Is the morphology congruent with the genotype?

The manuscript needs major revision to improve clarity.

Several suggestions are made in the file linked below.  
https://f1000researchdata.s3.amazonaws.com/linked/196501.Bence_Matyas_rev.pdf

Is the work clearly and accurately presented and does it cite the current literature?  
Partly

Is the study design appropriate and is the work technically sound?  
Partly

Are sufficient details of methods and analysis provided to allow replication by others?  
Yes

If applicable, is the statistical analysis and its interpretation appropriate?  
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Phylogenetics

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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