First report of infection by *Debaryomyces* spp. in *Myotis velifer* (cave myotis) in Mexico

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Abstract

Interest in the study of mycotic diseases in bats has increased after the identification of bats affected by white-nose syndrome in the northern United States. In a temperate forest of the community of San Pedro Yolox, Ixtlán in the Sierra Madre de Oaxaca, Mexico, we collected bats of various species, including 13 specimens of *Myotis velifer* that showed lesions in the plagio- and uro-patagium. Clinical exploration, histopathological studies and molecular analysis were carried out to determine the causal agent of the lesions present in these individuals. It was determined that the cause was the pathogenic fungus *Debaryomyces* spp. The present study represents the first report of fungal infection in bats in southern Mexico.

Bats, dermatomycosis, diseases, mammals, mycosis, pathogens

The fungal agent *Pseudogymnoascus destructans* responsible for bat population declines in the northern United States has increased the interest in the study of bat fungal diseases. White-nose syndrome (WNS) is associated with the growth of *P. destructans* hyphae on the ears, nose, metatarsals, and other furless skin areas of body in insectivorous bat species during the period of hibernation. Therefore, *P. destructans* causes destruction of the apocrine and sebaceous glands, hair follicles and other dermal tissues (Cryan et al. 2010). While the wings play a fundamental role in gas exchange (contributing up to 10% of the total), wing membrane damage caused by WNS impacts the gas exchange, mobility and hydration status of infected bats. In addition to the fungus *P. destructans*, other pathogenic fungi have been discovered, such as *Trichophyton redellii* and *Debaryomyces* spp., responsible for similar but less common skin diseases (Blehert et al. 2009).

Trichophyton redellii is characterized by the development of lesions similar to WNS, with the difference that it does not fluoresce under a UV lamp and that the characteristic growth of fungal structures is in the snout, while *Debaryomyces* spp. was reported in a case with lesions similar to those of WNS but without describing the status of the infected specimen in greater detail (Lorch et al. 2015). Mexico is notable for the presence of 139 bat species distributed throughout its territory (Ramírez-Pulido et al. 2014). However, there is no published report on a fungal dermatological monitoring of this group of mammals.

During our study on the ecological aspects of a community of bats in the Sierra Norte de Oaxaca, we collected some individuals of *Myotis velifer* with damaged wings. For this reason, we decided to investigate the causal agent of the lesions present in these individuals.

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Materials and Methods

In February 2017, in the temperate forest located in the community of San Pedro Yolox, Ixtlán, in the Sierra Madre de Oaxaca, Mexico, a bat of the species *M. velifer* was collected. The specimen was subjected to the Wood lamp test, a battery-operated lamp from the LUX pro brand was used with 32 UV LEDs with a wavelength of 385–390 nm. The signology similar to WNS was the presence of "spots" in the patagium and uropatagium as well as the presence of fungal structures on histology (Turner et al. 2014). It was decided to euthanize the specimen using isoflurane overdose as described by Barnard (2009). Two tissue samples were collected from the plagiopatagium (1 cm^2), one placed in 10% formaldehyde and the other stored at −20 °C for molecular analysis. The wing membrane was stained with haematoxylin and eosin.

Genomic DNA was isolated from the collected tissue using the commercial kit DNeasy Blood & Tissue (Qiagen, Bodenseeallee, Stockach, Germany) following the manufacturer's instructions. The fungal ITS region was amplified with the primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and 5.8S_fungi (5'-CAAGAGATCCGTTGTTGAAAGTT-3'; Young et al. 2014), and sequenced using the 454 pyrosequencer (454 LifeScience, Roche Diagnostics, Branford, Pennsylvania, USA). The contigs obtained were assembled using the Bioinformatics software Newbler version 2.5.3 (Roche Diagnostic) and Consed version 23 (http://www.phrap.org/consed/consed.html). The sequences were arranged according to the number of readings obtained (Table 1) and compared with the information contained in the NCBI database through the BLAST tool (https://blast.ncbi.nlm.nih.gov/).

Subsequently, 12 individuals of *M. velifer* with similar patterns of wing lesions were captured and a clinical review was carried out. For the review, two 1 cm^2 samples of plagiopatagium were obtained from each bat with a scalpel for histopathological and molecular studies. In addition, an inspection of the wings was carried out according to the wing damage index proposed by Reichard and Kunz (2009). The specimens were captured with the permission of the Government of Mexico through La Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT), number FAUT-0037.

To confirm the presence of *Debaryomyces* spp., a pair of specific primers was designed (DEB-18S-FW: 5'-CAAGAACTTTTGCTTTGGTCT-3' and DEB-18S-RW: 5'-GCACTATCCAGTACCACTCAT-3') to amplify a 384-bp fragment from the ITS region of *Debaryomyces* spp. The fragment amplification reaction was carried out using the Accuzyme DNA polymerase kit (Bioline, Humber Road, London, UK) following the manufacturer's instructions.The reaction mixture included 1 µl of extracted genomic DNA (50 ng/µl), 1 µl (10 µMol) of each primer (DEB-18S-FW and DEB-18S-RW), 5µl Accuzyme reaction mixture (1 U DNA polymerase, $MgCl_2$, deoxynucleotides), and nuclease-free water to make up a final volume of 10 µl. A T100 thermocycler (BioRad) was used, which was programmed according to the following amplification conditions: Initial denaturation 5 min at 95 °C followed by 35 cycles of 95 °C for 30 s, 53 °C for 1 min, 72 °C for 1 min, and finally an extension of 72 °C for 10 min. The products obtained were subjected to electrophoresis in 1% agarose gels stained with ethidium bromide (GeminiScientific, Sunnyvale, CA, USA), including a molecular weight marker of 100 pb Hyperladder (Bioline, Humber Road, London, UK).

Table 2. Sequences used for the genus *Debaryomyces* and other genera to generate the dendrogram.

Species	NCBI code
Debaryomyces hansenii	KY103235.1
Debaryomyces fabryi	NR_138186.1
Debaryomyces maramus	KY103271.1
Debaryomyces vindobonensis	NR_138218.1
Debaryomyces coudertii	NR_138161.1
Debaryomyces nepalensis	NR_130651.1
Debaryomyces psychrosporus	HM769277.1
Debaryomyces renaii	NR_111609.1
Debaryomyces castellii	NR_111306.1
Debaryomyces udenii	NR_077068.1
Debaryomyces prosopidis	NR_077067.1
Debaryomyces vietnamensis	KY103297.1
Debaryomyces udenii	KY103296.1
Debaryomyces singareniensis	KY103290.1
Trichophyton spp.	MH021152.1
Malassezia vespertilionis	NR_157486.1
Aspergillus spp.	KT221871.1
Candida spp.	KX944465.1
Pseudogymnoascus destructans	NR_111838.1

To carry out the phylogenetic analysis of the causal agent, the amplification of a positive sample was carried out by PCR in a volume of 100 µl. The PCR product was verified on 1% agarose gel and gel-purified using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) following the manufacturer's instructions.The purified product was quantified on a Nanodrop 2000 (Thermo) nanospectrophotometer and sequenced in a DNA sequencer with 16 capillary 3130×l (Applied Biosystems, Foster City, CA, USA).

A dendrogram was generated by Sanger sequencing from the previously obtained sequence. The sequence was aligned with ITS sequences reported in the GenBank (https://www.ncbi.nlm.nih.gov/) for the genus *Debaryomyces*, as well as for other genera of mycotic agents reported as bat pathogens (Table 2). The construction of the dendrogram and bootstrap analysis (1000 repetitions) were performed through the bioinformatic program Mega v5.05 (Tamura et al. 2011).

Table 1. Fungal microbiome obtained by the technique of massive sequencing (fungal diversity): The first column corresponds to the species identified for each sequence, the second column to the percentage identity in relation to the NCBI databank, the third column to the number of readings obtained through massive sequencing, the fourth column belongs to code of the NCBI sequence with which the obtanined sequence was compared and the final column corresponds to the percentage of the total readings of the massive sequencing.

Species	Percent identity	Reads number	NCBI code	Percent reads
Debaryomyces maramus	98	17738	KM091320.1	89.40524194
Beauveria bassiana	100	364	KY806126.1	1.834677419
Pleurotus dryinus	99	342	KY962461.1	1.723790323
Malassezia dermatis	92	287	KY104083.1	1.446572581
Leptosphaerulina chartarum	100	108	KM979510.1	0.544354839
Curvularia aeria	98	84	MF101868.1	0.423387097
Cladosporium tenuissimun	100	75	KY781763.1	0.378024194
Alternia solani	99	71	KT384228.1	0.357862903
Nigrospora sphaerica	100	52	KX688172.1	0.262096774
L042880-122	99	45	GU053988.1	0.226814516
Cora leslactuca	100	41	KY772646.1	0.206653226
Oxyporus populinus	99	36	KJ140633.1	0.181451613
Ganoderma spp.	99	35	KF605667.1	0.17641129
Pyrigemmula aurantiaca	89	35	HM241692.1	0.17641129
Microascus hollandicus	99	32	KX923869.1	0.161290323
Hongo endofito 6303	100	32	KR016373.1	0.161290323
Trichurus spiralis	93	29	LN850977.1	0.146169355
Phialemonium inflatum	99	24	KY305080.1	0.120967742
Camarosporula persooniae	93	24	JF770449.1	0.120967742
109A77714	95	22	JX389420.1	0.110887097
Paraconiothyrium brasiliense	97	19	KM100720.1	0.095766129
Phialophora intermedia	96	18	JQ766431.1	0.090725806
Acalium albonigrescens	97	18	NR146258.1	0.090725806
Microascus hyalinus	97	18	KX923871.1	0.090725806
Marasmiceae spp.	96	17	JF691144.1	0.085685484
Punctularia subhepatica	99	17	KP814559.1	0.085685484
Aspergilius jensenii	100	17	LN898704.1	0.085685484
Ganoderma australe	99	16	kU569545.1	0.080645161
44- 2966	95	16	FJ60971.1	0.080645161
Hypomyces aurantius	100	15	AB591044.1	0.075604839
Filobasidium chernovii	99	14	KY514746.1	0.070564516
Cephalotrichiella penicillata	83	13	KJ869166.1	0.065524194
Microascus cirrosus	97	13	LN850782.1	0.065524194
Ilyonectria spp.	100	12	KT270187.1	0.060483871
Penicilium citreosulfuratum	98	11	KY786079.1	0.055443548
Hortaea werneckii	100	11	KU882134.1	0.055443548
Colletotrichum gloesporioides	93	9	JX669447.1	0.045362903
Microascus senegalensis	98	9	KX923932.1	0.045362903
Wallemia muriae	97	9	KX911860.1	0.045362903
Coprinopsis narcotica	91	8	FM163180.1	0.040322581
Neogymnmyces demonbreunii	97	8	JN038187.1	0.040322581
Phlebiopsis spp.	84	7	KJ832027.1	0.035282258
Coprinellus radians	97	6	KU761146.1	0.030241935
Rhodontura bacarum	99	6	KY104725.1	0.030241935
Steccherinum spp.	96	5	KM279619.1	0.025201613
Hyphodontia apacheriensis	98	5	KX857797.1	0.025201613
Passalora pseudotithoniae	100	4	NR137608.1	0.02016129
Penicilium angulare	98	4	NR121272.1	0.02016129
Aspergillus caninus	97	4	LC230093.1	0.02016129
Chaetosphaeria myriocarpa	94	4	JF340253.1	0.02016129
Cladophialophora chaetospira	93	3	EU137333.1	0.015120968
Oidiodendron spp.	94	3	JX270625.1	0.015120968
Exophiala angulospora	86	3	KR909169.1	0.015120968
Didymosphaeria spp.	92	2	KX965731.1	0.010080645
OTU F324	89	2	MF976438.1	0.010080645
Phialocephala dimorphospora	99	2	KX881592.1	0.010080645
Steccherinum albofibrillosum	95	2	KP401770.1	0.010080645
Chaetosphaeria spp.	100	2	AY618225.1	0.010080645
Hymenochaete spp.	100	2	KU975490.1	0.010080645
Chaetomium globosum	96	2	MF682409.1	0.010080645
Exophiala halophilia	100	1	NR111628.1	0.005040323

Results

The first bat of the *M. velifer* species we collected had ulcerative lesions of a round and irregular shape in the dactylo-, plagio-, and uropatagium (Plate III, Fig. 1). The specimen was fluorescence-negative; on microscopic examination, the stratum corneum was thickened by parallel sheets of anucleated keratin (hyperkeratosis). Within some hair follicles, oval amphophilic hyphae of 6–9 nm in diameter were observed (Plate III, Fig. 2).

The group of 12 bats were PCR-positive for the *Debaryomyces* genus. Histopathological analysis of all bats in the second group revealed severe lesions, such as periadnexal pyogranulomatous dermatitis, the presence of intralesional conidia and hyperkeratosis, orthokeratosis with the presence of hyphae in the form of clusters (Plate III, Fig. 2). Nine specimens presented an inflammatory infiltrate composed of lymphocytes and plasma cells below the dermis and around the cutaneous annexes. For some fungal pathogens, the normal reaction of a healthy individual to their colonization may be mediated by lymphocytes and cytokines (Romani 2004).

The phylogenetic analysis revealed that the sequence obtained from the amplification with the primers DEB-18S-RW and DEB-18S-RW was grouped in a cluster with the ITS sequences of *Debaryomyces* reported in the GenBank, clearly separating it from the rest of the genera of pathogenic fungi that affect bats. In addition, the sequence obtained shared 100% identity with *D. castelli, D. prosopidis, D. renaii, D. psychrosporus, D. vindobonensis, D. maramus, D. fabryi, D. hansenii* and *D. courdetii* (Plate IV, Fig. 3). The sequence with the highest number of readings (17738) showed a 100% similarity with the ITS region of *Debaryomyces* spp. (KM091320.1).

Discussion

The genus *Debaryomyces* is characterized by presence in a great variety of soils; some species of this genus have already been identified as human pathogens. *Debaryomyces hansenii* has been the most studied of these, being related to the contamination of intravenous catheters (Desnos-Oliver et al. 2008), causing bone infections. *Debaryomyces kloeckeri* has been isolated in abscesses and in urinary and dermatological infections, and finally *D. emphysematosis* has been isolated from patients with bronchitis (Wong et al. 1982).

The lesions observed in specimens such as periadnexal pyogranulomatous dermatitis, orthokeratosis hyperkeratosis, the presence of conidia and of an inflammatory infiltrate and plasma cells in the cutaneous adnexa can have a severe impact on the mobility of bats. Therefore, these lesions should be considered in the differential diagnosis of bat skin diseases such as white-nose syndrome. *Debaryomyces* spp. had been reported in only one other individual of *M. velifer* in Texas, United States (Lorch et al. 2015), and this is the first time that its presence in bats in a neotropical region has been recorded.

The implications of these findings can be severe for the conservation of Mexican bats. Though there are no demographic studies on the site, the concern is that other bats species could become infected by sharing shelters with sick individuals. To which is added the mobility and migration that different bat species can have, allowing the fungus to disperse. Although massive deaths due to fungal infections have not been reported in Mexico, the lesions shown on the wing membranes could have a severe impact on the mobility, hydration, thermoregulation and gas exchange of affected individuals. *Debaryomyces* spp. have not been evaluated at the level of epidemiological behaviour, so it is necessary to evaluate the populations infected with this pathogen to document the impact it may have on neotropical bat populations.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Fig. 1. *Myotis velifer* with ulcerative lesions, some of round shape and others of irregular form in dactylo-, plagio- and uropatagium.

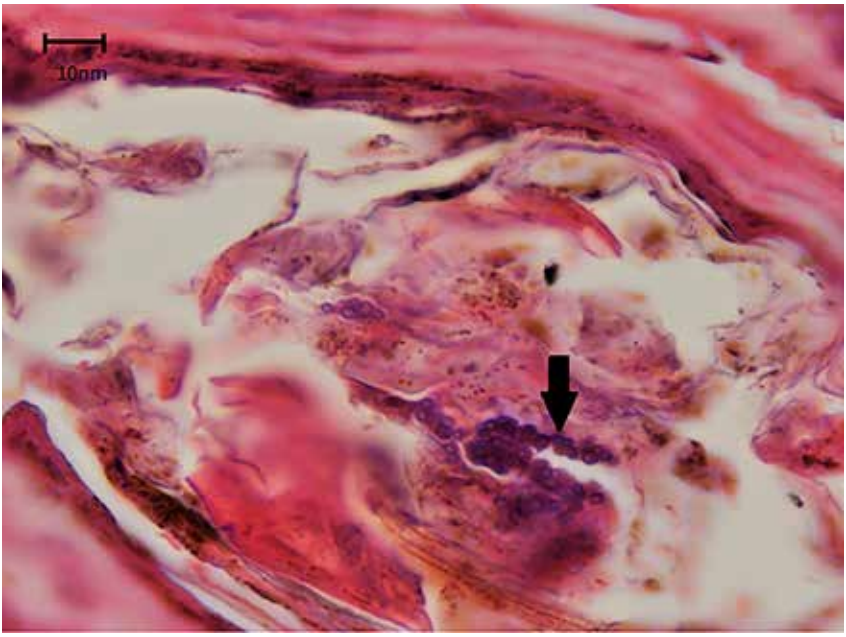

Fig. 2. The increased thickness of stratum corneum contains parallel sheets of anucleated keratin (hyperkeratosis); the presence of oval amphiphilic hyphae measuring 6–9 nm is observed within some hair follicles.

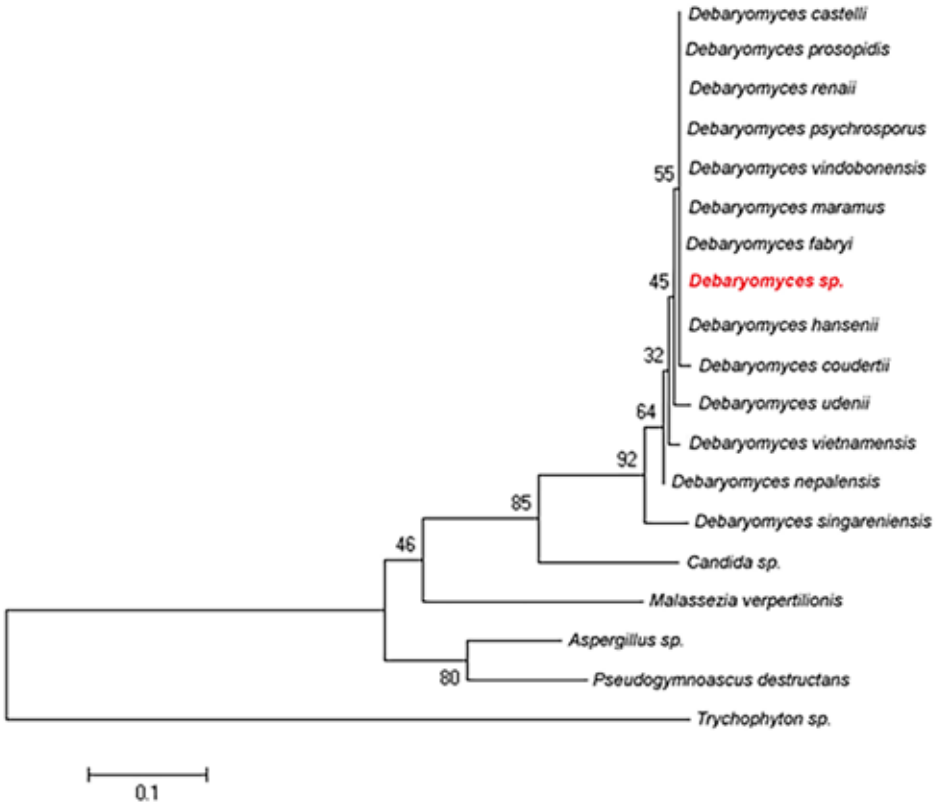

Fig. 3. Phylogenetic analysis: the referred sequence is compared to ITS databank sequences of the genus *Debaryomyces* and the mycotic agents reported in bats; the sequence is grouped in a clade with nine species of the genus *Debaryomyces*.