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The naked-tailed armadillo *Cabassous centralis* (Miller 1899): a new host to *Paracoccidioides brasiliensis*. Molecular identification of the isolate

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The natural habitat of *Paracoccidioides brasiliensis* remains undefined but the repeated demonstration of infection by this fungus in the nine-banded armadillo *Dasypus novemcinctus* has opened interesting research avenues. We report here the isolation of this fungus from the spleen of a naked-tailed armadillo *Cabassous centralis* (Miller 1899) captured in a coffee farm localized in the Colombian endemic area for paracoccidioidomycosis. This particular isolate was identified by its dimorphism and also by comparison of the *PbGP43* gene and ribosomal internal transcribed spacer regions (ITS) with recognized *P. brasiliensis* strains. This finding extends the range of naturally acquired infections in mammals of the family Dasypodidae and confirms the existence of this human pathogen in areas where human paracoccidioidomycosis is known to occur.

Keywords armadillo, *Cabassous centralis*, molecular identification, *Paracoccidioides brasiliensis*

Introduction

Paracoccidioidomycosis is an important human mycosis restricted to certain countries of Latin America where it causes important morbidity and mortality, especially among male agriculturists [1]. The precise habitat of its aetiological agent, the dimorphic fungus *Paracoccidioides brasiliensis,* has remained undefined [2], but the repeated demonstration of infection by this fungus in the nine-banded armadillo *Dasypus novemcinctus* has opened the possibility of tracing *P. brasiliensis* habitats following its steps as, in contrast to humans, this mammal has restricted mobility [3].

The nine-banded armadillo has been amply recognized as a natural host to *P. brasiliensis* since its serendipitous finding by Naiff *et al*. (1986) in several armadillos captured in the virgin forests of Para, Brazil [4]. This finding was confirmed a few years later (1989) by the same group [5], as well as by Silva-Vergara *et al*. [6], Corredor *et al*. [7] and Bagagli *et al*. [8,9]. Published reports indicate that a total of 93 *D. novemcinctus* have been studied, mostly coming from Brazil and a few from Colombia. From these animals, 36 (38.7%) harboured *P. brasiliensis* in their internal organs (spleen, liver, mesenteric lymph nodes, lungs) as shown by histopathology, isolation in culture or mouse inoculation [2,9].

Variations in the frequency of *P. brasiliensis*-infected animals according to the site of capture have been recorded. Bagagli *et al.* noticed that most positive animals came from sites where riparian forest existed, water currents abounded and where man had disturbed the environment [9]. Nonetheless, it is difficult to determine the frequency of infected animals at a given site or to attempt comparisons with those found in other places, as the total number of animals killed has

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varied and no data are available concerning population numbers in a given region [2,3,8,9].

Dasypus novemcinctus also serves as host to other mycotic agents known to cause human diseases, such as *Sporothrix schenckii* [10], *Histoplasma capsulatum* [11] and *Coccidioides immitis* [12]. This is in contrast with the paucity of reports on the occurrence of fungal infections in armadillos belonging to other genus and/or species of the family Dasypodidae [13–15]. The present report describes the isolation of *P. brasiliensis* from the spleen of a naked-tailed armadillo (*Cabassous centralis,* Miller 1899) captured in a coffee plantation located within a highly endemic area for paracoccidioidomycosis in Colombia [16,17] and establishes its identity by molecular comparisons with recognized human and environmental isolates of the fungus.

Materials and methods

The site of capture, Santágueda county, municipality of Palestina, Department of Caldas, Manizales, Colombia, South America, is located at 05°0322.0″N, 75°2936.9″W, 1.330 m above sea level. The city of Manizales lies 404 km to the west of Bogotá, the capital of Colombia, while Santágueda is located 36 km to the west of Manizales. The terrain is hilly, has numerous old coffee trees intermingled with banana, yucca and bean plants; the native forestry has disappeared but spare arboreal vegetation common to this Andean region (*Corida alliodora, Trichantera gigantea, Cedrella* spp.) was observed. The mean temperature is 27°C. Two small watercourses ran through and bordered the coffee-planted area contributing to overall humidity (over 75%). The height of the coffee plants (approximately 1.7 m) and their proximity to each other kept the area under shade. Gross inspection revealed numerous armadillo burrows, some running deep into the colder (23–24°C) soil underneath. These ecological characteristics coincide with those described as significant for the *P. brasiliensis* habitat, such as altitude over 1000 m, rainfall over 2000 mm, humid forest configuration, according to Holdridge, and coffee crops in the area [16,17].

Permission was obtained from the local wildlife preservation authorities (Regional Autonomous Corporation of Caldas, Corpocaldas), and with the aid of a specialist hunter, a male armadillo was captured. It weighed 2 kg and measured 400 mm from head to tail; it had five large claws in the forefeet, with the middle one larger and sickle-shaped, its carapace was black and had 11 bands. The most notorious feature was the long tail (180 mm in length) with no plates ('clothed-tailed armadillo'). These features coincide with those described for the species *Cabassous centralis* [13,18–20], acknowledging that measure, weight and number of dorsal bands may vary slightly [13,18–21].

Immediately after capture, the animal was injected intramuscularly with a neuroleptoanesthesic combination (100 mg ketamine; Park-Davies, Ecuador; 20 mg xylazine, Bayer, Brazil; in 2 ml distilled water) and killed by prolonged anaesthesia. After thorough cleansing, autopsy was performed and samples from liver, spleen, lungs and mesenteric lymph nodes were taken for culture. These specimens were suspended in sterile phosphate buffered saline and sent by courier under refrigeration to the reference laboratory (Corporación para Investigaciones Biológicas, Medellín), reaching their destination approximately 6 h after shipping. Simultaneously, samples were fixed in buffered formalin and used for histopathological examination. Upon receipt, biopsies were washed twice with sterile water in a fresh recipient and transferred to a dry, sterile plate where they were minced with fine scissors into small (1–3 mm) pieces according to Bagagli [8]. Approximately 80 of these tissue bits were then placed – making an incision into the agar – in four Mycosel and four Sabouraud glucose agar plates (BBL; Beckton Dickinson Microbiology Systems, USA) until the whole organs had been cultured. The plates were incubated at 21–24°C in the dark for 8–10 weeks. When growth became apparent, microscopic preparations were done and the mycelial growth subcultured to Sabouraud glucose agar (BBL) with thiamine and asparagine (both at 0.1%) and incubated either at room temperature or at 36°C to facilitate the expression of dimorphism. Once growth began to appear, microscopic preparations were done searching for the presence of the characteristic propagules. This isolate received the code number CIB44197.

The fixed tissues were processed in the Pathology Laboratory of the School of Veterinary Medicine and Zootechnology, Manizales, using standard (haematoxylin and eosin) and special fungal (silver methenamine) stains.

For molecular characterization of the isolate, DNA was extracted from the yeast culture using the van Burik's glass beads protocol [22]. Exon 2 of the *PbGP43* gene was amplified by PCR as described previously [23], using the primers 5′-TCATCTCACG-TCGCATCTCACATT-3′ (sense) and 5′-GGCTCCTC-AAAGTCTGCCATGAGGAAG-3′ (antisense), which extended from nucleotide 733 to nucleotide 1213. Universal primers ITS1 5′-TCCGTAGGTGAACCT-GCGG-3′ and ITS4 5′-TCCTCCGCTTATTGA-

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TATGC-3′ were used to generate a 620-bp PCR product. Both strands of the amplicons were sequenced using standard automatic sequencing methods [26], at Dr John W. Taylor's laboratory, Department of Plant and Microbial Biology, University of California, Berkeley, USA or at MACROGEN Inc. (http://www.macrogen.com).

Various *P. brasiliensis* isolates were sequenced that were obtained from *D. novemcinctus* (CIB 40392) [6], *Cabassous centralis* (present report CIB44197), two from Colombian patients with chronic paracoccidioidomycosis, namely, P196 (unreported) and ATCC 60855 [24], as well as isolate U1 from penguin faeces [25]. The *PbGP43* gene (exon 2) and the ribosomal ITS regions sequenced from the above isolates were deposited in the GenBank (http://www.ncbi.nlm.nih.gov/) and their accession numbers can be seen in Tables 1 and 2. Three sequences corresponding to Brazilian and Venezuelan patients already deposited in the GenBank were also compared [26,27] (Tables 1, 2).

The sequences were processed with the program Sequence Navigator (Applied Biosystem, Foster City, CA, USA). We aligned the sequences using ClustalW from the Swiss EMBnet node server (www.ch.embnet.org) and evaluated their homology by calculating the mean pairwise alignment score. When aligning for the additional *P. brasiliensis* sequences stored in the GenBank we used the accession numbers indicated in Tables 1, 2.

Results

Six weeks after inoculation, one of the spleen-seeded cultures showed a delicate white tuft emerging from a tissue fragment; this growth was transferred to tubes with the solid media already described and incubated at the temperatures indicated. Further study of the mycelial showed thin septated mycelia with intercallary chlamydospores (not shown); the colonies were tan, adherent to the agar and with short aerial mycelia. After 6 days at 36°C, the corresponding cultures presented a pasty growth that microscopically revealed round to ovoid yeast cells, some exhibiting multiple buds (Fig. 1). The demonstration of dimorphism confirmed isolation of *P. brasiliensis* from the spleen of *C. centralis.* Cultures from lungs, liver and mesen-

Table 1 Distribution of the nucleotide polymorphisms in the *PbGP43* nuclear gene loci of *Paracoccidioides brasiliensis*

	Nucleotide position	716	751	763	779	830	856	872	874	964	981	1082	1086	1157	1166	1206	1244
Isolate designation	Accession number																
CIB44197	AY619000	A	G	C	G	C	A	A	C	A	C	C	C	A	T	C	G
CIB40392	AY619001	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
P196	AY626376	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ATCC 60855	AY626378	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
U1	AY626377	*	*	*	T	*	*	*	A	G	*	*	*	*	*	G	C
Pb2	AY005408	G	A	G	*	T	G	T	*	*	T	G	T	G	G	G	C
Pb4	AY005406	G	A	G	*	T	G	T	*	*	T	A	T	G	G	G	C
Pb8	AY005413	G	*	*	T	*	*	*	A	G	*	*	*	*	*	G	C

Numbers indicate positions in the reference sequences; asterisks indicate nucleotides identical to the *Paracoccidioides brasiliensis*/CIB44197 sequence [23].

Table 2 Distribution of nucleotide polymorphisms in the ITS sequence of *Paracoccidioides brasiliensis*

	Nucleotide position	113	156	384	518
Isolates designation	Accession number				
CIB44197	AY618999	G	G	C	A
CIB40392	AY631234	*	*	*	*
P196	AY631236	*	*	*	*
ATCC 60855	AY631237	*	*	*	*
U1	AY631235	*	*	*	*
Pb2	AY374338	*	T	*	*
Pb4	AY374336	A	T	T	G
Pb8	AY374337	*	T	*	G

Numbers indicate positions in the reference sequences; asterisks indicate nucleotides identical to the *Paracoccidioides brasiliensis*/CIB44197 sequence [26].

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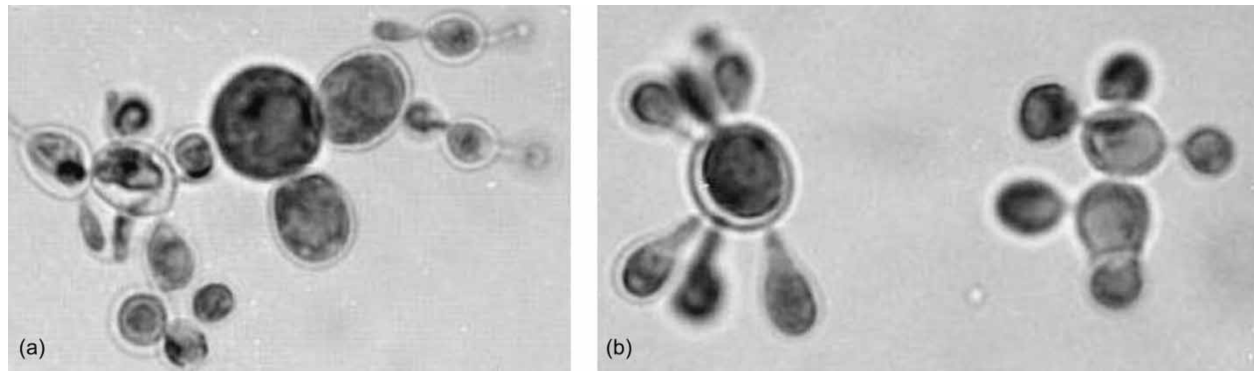

Fig. 1 (a) Lactophenol cotton blue preparation from a *Paracoccidioides brasiliensis* colony at 36°C corresponding to the CIB44197 isolate from the spleen of *Cabassous centralis*. Note the shape of the yeast cells, their double-contoured wall and their varying size (×20). (b) Note numerous buds emerging from the mother cell (×40).

teric lymph nodes produced no fungal growth despite prolonged (12 weeks) incubation. Only one plate with spleen tissue gave rise to *P. brasiliensis*. Histopathological observations of the organs taken at autopsy did not reveal macroscopic abnormalities or microscopic signs of inflammation, abscesses formation or presence of granulomas.

The molecular studies revealed that the CIB44197 isolate from *C. centralis* presented a high degree of homology with the remaining *P. brasiliensis* isolates studied, both in the *PbGP43* and the ITS sequences; this was especially true with the Colombian isolates, all of which proved to be identical. The non-Colombian isolates revealed 16 polymorphic sites (Tables 1, 2). The Clustal W analysis indicated a 98.2% mean pairwise alignment score for the *PbGP43* and 99.1% for the ITS region.

Discussion

This is the first report of *P. brasiliensis* infection in the naked-tailed armadillo *C. centralis*, a mammal that is rarely described as host to other pathogenic microorganisms, with the exception of certain parasites (*Trypanosoma cruzi* [13,14] and *Toxoplasma* [13,15].

Attempts to isolate *P. brasiliensis* from different armadillo species have been made in Brazil (e.g. *D. kapplari* [5] and *D. septemcinctus*), as well as from a new genus *Euphractus* species *sexcinctus* [28]; however, they did not yield positive cultures. It seems possible that the frequency of fungal infections in armadillos may vary with the genus and/or species, with *D. novemcincuts* being the preferred host.

C. centralis belongs to the taxon Cingulata and is one of the species of the family Dasypodidae (class Mammalian, order Xenarthra) [13,18–20]. Together with *D. novemcinctus,* a recognized host to *P. brasiliensis* [8,9], these armadillos are inhabitants of the Americas. *C. centralis* is found from Mexico to Southern Belize, going through Guatemala, Honduras, Nicaragua, Costa Rica, Panama, Colombia and up to Northern Venezuela; it is also present in Paraguay and Northern Argentina [13,19,20]. In Colombia it is distributed towards the central and eastern Andean regions within an ample altitude range of 0–1800 m above sea level [21]. Personal observations made by one of us (G.G.C.) indicate that *C. centralis* is often found in the coffee-growing regions of the Department of Caldas, Colombia. Nonetheless, the wildlife preservation authorities exert a strict control on the capture of this and other armadillo species, as they are considered endangered. Consequently, we have killed only one *C. centralis* (this study) and four *D. novemcinctus* in Colombia [7]. The isolation of *P. brasiliensis* in this one *C. centralis* might suggest high incidence in this species. It would be desirable to undertake other studies in these animals aimed at determining the frequency of infection with *P. brasiliensis* and by doing so find the fungal microniche [2].

Our finding expands the range of naturally acquired *P. brasiliensis* infections in armadillos. Both *D. novemcinctus* and *C. centralis* inhabit rather humid regions along riverbeds, in forests and fields [13,18–20], where most of the ecological conditions favourable to *P. brasiliensis* habitat can be found (high humidity, altitudes over 1000 m, presence of humid tropical forests and coffee growing) [16,17]. Bagagli *et al.* emphasized the disturbance of the native riparian forestry as an important condition favouring armadillo infection [9] and this type of damage was indeed noticed in the area studied here.

These burrowing animals live underground in ecosystems protected from temperature and humidity variations [13,18–20]. Furthermore, their low-body

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temperature and weak immune system [13,18,29], plus their longevity, render the armadillos more susceptible to infection by human pathogenic fungi [3,13,18,29].

The presence of the *PbGP43* gene in the CIB44197 isolate obtained from *C. centralis* indicates that it corresponds to *P. brasiliensis*. Molecular identity of the present isolate with other *P. brasiliensis* isolates brings into focus the highly preserved genetic constitution of this gene, as observed in the original analysis of *PbGP43* polymorphism [27] and in preliminary phylogenetic analysis of multiple *P. brasiliensis* isolates [30]. Both the 1206 and 1244 positions shown in Table 1 are characteristic of the Colombian isolates. Present sequence analysis of the *PbGP43* gene and ITS region revealed the presence of little polymorphism among the chosen isolates, which included clinical and environmental isolates from Colombia, Brazil, Venezuela and Antarctica. Furthermore, the sequences from the CIB44197 isolate were identical to the remaining isolates from Colombia, a fact reinforced by the high mean pairwise alignment score (98.2%) for the *PbGP43* and (99.1%) for the ITS region.

The demonstration of *P. brasiliensis* infection in armadillos is considered a clue to the fungus natural habitat [2,8,9]. Although this has not proven to be so as yet, the recognition of another mammalian host to *P. brasiliensis* within the paracoccidioidomycosis endemic areas indicates that the fungus habitat should be there. Thus the search must continue until its whereabouts can be precisely determined [31].

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