

***Sulzbacheromyces caatingae*: notes on its systematics, morphology and distribution based on ITS barcoding sequences**

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Abstract: *Sulzbacheromyces* is a recently erected genus in *Lepidostromatales*, differing from *Lepidostroma* in the crustose thallus. After the initial discovery of *S. caatingae*, the only species to be found in Brazil so far, a large quantity of additional data and ITS barcoding sequences for this taxon from a much broader geographical range and different habitats was collected. Phylogenetic analysis under a maximum likelihood framework demonstrated that all specimens are genetically uniform, showing no variation in their ITS, suggesting that *S. caatingae* has a wide ecological amplitude beyond the Brazilian Caatinga and Atlantic Forest biomes. Detailed descriptions and illustrations of the species are presented, including a map showing the distribution of *S. caatingae* in the Brazilian semi-arid region and the north-eastern Atlantic rainforest.

Key words: basidiolichens, ecological features, geographical distribution, *Lepidostromatales*, molecular systematics, taxonomy

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Introduction

Basidiolichens are very poorly understood organisms with many gaps in our knowledge of their systematics, morphology and species distribution (Oberwinkler 2012). Although less than 1% of all known lichens have a basidiomycete as mycobiont (Lawrey *et al.* 2007), their diversity is much higher than

previously assumed (Lücking *et al.* 2014b) and the best models to study the evolution of the lichen thallus can be found in basidiolichens (Dal-Forno *et al.* 2013). Most basidiolichens are concentrated in the family *Hygrophoraceae* (*Agaricales*, Agaricomycetidae), which includes a great variety of basidiome types, including agaricoid, cyphelloid, stereoid and corticioid (Lawrey *et al.* 2009; Dal-Forno *et al.* 2013; Lodge *et al.* 2014). Basidiolichens with clavarioid basidiomes are known from two orders, *Cantharellales* and *Lepidostromatales* (Nelsen *et al.* 2007; Ertz *et al.* 2008; Hodkinson *et al.* 2014).

Sulzbacheromyces B. P. Hodk. & Lücking is a recently erected genus in *Lepidostromatales*, differing from *Lepidostroma* Mägd. & S. Winkl., another basidiolichen genus with a tropical distribution (Mägdefrau & Winkler 1967), in the entirely crustose, undifferentiated thallus lacking cortex and medullary structures. Originally, *Sulzbacheromyces caatingae* was the only species recognized (Hodkinson *et al.* 2014), but a second species has recently been described from

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Japan under the name *Lepidostroma asianum* (Yanaga *et al.* 2015). For *Lepidostroma* s. str., four taxa are currently known: *L. calocerum* (G. W. Martin) Oberw. (including the type *L. terricolens* Mägd. & S. Winkl.), *L. rugaramae* (Eb. Fischer *et al.*) Ertz *et al.*, *L. vilgalysii* B. P. Hodk. and *L. winklerianum* B. P. Hodk. & Lücking (Oberwinkler 1984; Fischer *et al.* 2007; Ertz *et al.* 2008; Hodkinson *et al.* 2012, 2014). One species is accepted in the genus *Ertzia*, viz. *E. akagerae* (Hodkinson *et al.* 2014).

Although the number of basidiolichens was believed to be relatively small, recent efforts have led to an increase in the diversity of this group of organisms through the discovery of new species in several countries, especially in the Neotropics, such as Costa Rica, Ecuador and Brazil (Chaves *et al.* 2004; Nelsen *et al.* 2007; Sulzbacher *et al.* 2012; Yáñez *et al.* 2012; Dal-Forno *et al.* 2013; Lücking *et al.* 2013, 2014*a, b*; Hodkinson *et al.* 2014; Schmull *et al.* 2014).

Since the description of *Sulzbacheromyces caatingae* from north eastern Brazil (Sulzbacher *et al.* 2012 as *Lepidostroma*; Hodkinson *et al.* 2014), we have accumulated a large number of additional data and sequences for this species. Based on these data, we discuss here the morphological and ecological features of this species and its geographical distribution. We also point out mycobiont features that can help in separating *Sulzbacheromyces* from the unrelated but morphologically similar genus *Multiclavula* R. H. Petersen in the *Cantharellales*.

Materials and Methods

Morphological studies

Colour codes follow Korneup & Wancker (1978). Presentation of basidiospore data follows the methodology proposed by Tulloss *et al.* (1992), slightly modified by Wartchow (2012) and Wartchow *et al.* (2012). Measurements and statistics are based on 20 spores. Abbreviations include L(W) = average basidiospore length (width), Q = the length : width ratio range as determined from all measured basidiospores, and Q = the Q value averaged from all basidiospores

measured. For basidiospore shape, we follow Bas (1969): globose ($Q = 1.00\text{--}1.05$), subglobose ($Q = 1.05\text{--}1.15$), broadly ellipsoid ($Q = 1.15\text{--}1.30$), ellipsoid ($Q = 1.30\text{--}1.60$), elongate ($Q = 1.60\text{--}2.00$), cylindrical ($Q = 2.00\text{--}3.00$) and bacilliform ($Q > 3.00$). Collections studied are deposited in JPB, UFRN-Fungos and F (Thiers 2013).

DNA extraction, amplification and sequencing

New ITS sequences of additional samples of *Sulzbacheromyces caatingae* were generated for this study using the SIGMA REDExtract-N-Amp Plant PCR Kit (St. Louis, Missouri, SA) for DNA isolation following the manufacturer's instructions, except that 40 µl of extraction buffer and 40 µl dilution buffer were used. Primers for amplification were ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) for ITS. PCR reactions contained 5.0 µl R4775 SIGMA REDExtract-N-Amp™ PCR ReadyMix, 0.5 µl of each primer (10 µM), 2 µl genomic DNA extract and 2 µl distilled water, for a total of 10 µl. Thermal cycling parameters were: initial denaturation for 5 min at 94 °C, followed by 39 cycles of 30 s at 94 °C, 30 s at 48 °C, 1 min 30 s at 72 °C, and a final elongation for 5 min at 72 °C. PCR samples were visualized on a 1% ethidium bromide-stained agarose gel under UV light and bands were gel-extracted, heated at 70 °C for 5 min, cooled to 45 °C for 10 min, treated with 1 µl GELase (Epicentre Biotechnologies, Madison, WI, USA) and incubated at 45 °C for at least 24 h. The 10 µl cycle sequencing reactions consisted of 1.0–1.5 µl of BigDye v3.1 (Applied Biosystems, Foster City, California, USA), 2.5–3.0 µl of BigDye buffer, 6 µM primer, 0.75–2.00 µl gelased PCR product, and water. Samples were sequenced with PCR primers. The cycle sequencing conditions were as follows: 96 °C for 1 min, followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Samples were precipitated and sequenced using ABI Applied Biosystems 3730 DNA Analyzer (Foster City, California, USA), and sequences were assembled in DNASTAR SeqMan 4.03 and submitted to GenBank (Table 1).

Phylogenetic analysis

The newly generated ITS sequences for the new species were aligned with previously generated sequences of the same species downloaded from GenBank, and five sequences of *Lepidostroma calocerum* were used as outgroup (Table 1). Sequences were arranged into a multiple sequence alignment (MSA) using BIOEDIT 7.09 (Hall 1999) and automatically aligned with MAFFT 6.850b using the –auto option (Katoh & Toh 2005; Katoh *et al.* 2009). The final alignment was subjected to a maximum likelihood search using RAxML 7.2.6 (Stamatakis *et al.* 2005; Stamatakis 2006) using the GTR-gamma model, with parametric bootstrapping using 500 replicates.

TABLE 1. Specimens used in the study, with location, reference collection detail and GenBank accession numbers. Newly obtained sequences for this study are represented in bold font.

Taxon	Collection details	GenBank Accession Number (ITS)	Authors/year of publication
<i>Lepidostroma calocerum</i>	Colombia, Tisnes 1a, basidiome (F)	KT354926	This paper
<i>L. calocerum</i>	Colombia, Tisnes 1b, thallus (F)	KT354927	This paper
<i>L. calocerum</i>	Colombia, Lücking 35836a, basidiome (F)	KT354923	This paper
<i>L. calocerum</i>	Colombia, Lücking 35836b, thallus (F)	KT354925	This paper
<i>L. calocerum</i>	Colombia, Lücking 35836b, basidiome (F)	KT354924	This paper
<i>Sulzbacheromyces caatingae</i>	Brazil, Sulzbacher 237 (UFRN-Fungos 1479)	KC170320	Sulzbacher et al. (2012)
<i>S. caatingae</i>	Brazil, Sulzbacher 235 (UFRN-Fungos 1478)	KC170321	Sulzbacher et al. (2012)
<i>S. caatingae</i>	Brazil, Sulzbacher 235 (UFRN-Fungos 1478, type)	NR120240	Sulzbacher et al. (2012)
<i>S. caatingae</i>	Brazil, Sulzbacher 237 (UFRN-Fungos 1479, thallus)	KT354936	Sulzbacher et al. (2012)
<i>S. caatingae</i>	Brazil, Sulzbacher 235 (UFRN-Fungos 1478, isotype, thallus)	KT354934	Sulzbacher et al. (2012)
<i>S. caatingae</i>	Brazil, Ovrebo 5034 (JPB51318, UFRN-Fungos 2502, basidiome)	KT354928	This paper
<i>S. caatingae</i>	Brazil, Sulzbacher s. n. (UFRN-Fungos 2050, basidiome)	KT354929	This paper
<i>S. caatingae</i>	Brazil, Wartchow 58-2013 (UFRN-Fungos 2105, basidiome)	KT354932	This paper
<i>S. caatingae</i>	Brazil, Sulzbacher s. n. (UFRN-Fungos 2049, basidiome)	KT354930	This paper
<i>S. caatingae</i>	Brazil, Sulzbacher 237 (UFRN-Fungos 1479, basidiome)	KT354935	Sulzbacher et al. (2012)
<i>S. caatingae</i>	Brazil, Sousa 65 (UFRN-Fungos 2051, basidiome)	KT354931	This paper
<i>S. caatingae</i>	Brazil, Sulzbacher 235 (UFRN-Fungos 1478, isotype, basidiome)	KT354933	Sulzbacher et al. (2012)

Results and Discussion

Taxonomy

***Sulzbacheromyces caatingae* (Sulzbacher & Lücking) Hodkinson & Lücking**
Fung. Divers. 64: 176 (2014).

(Figs 1–5)

Thallus forming a thin green crust on the substratum (Fig. 3A), not further differentiated, containing a single-celled green alga (Fig. 5D).

Basidiomata clavarioid-caloceroid (Fig. 3B), 20–30 mm high, 1.0–1.5 mm diam., unbranched, terete or slightly flattened, straight and equal or sometimes slightly enlarged at mid-section then frequently narrowing downward near base; *surface* cracking circumferentially (similar to old carrot), otherwise glabrous; dull orange-pink (KW 6A4) overall, context dull yellow. *Basidia* 23–45 × 4–7 µm, clavate, thin walled, hyaline, 2–4 sterigmate, each up to 7 µm long (Figs 4B & 5C). *Basidioles* 25–40 × 5–6 µm slender clavate, abundant (Figs 4C & 5C). *Basidiospores* 5.0–8.0

(–8.5) × 3.5–4.5 µm, L = 6.5 µm, W = 3.9 µm, Q = 1.50–2.00(–2.28), Q = 1.67; inamyloid, hyaline, ellipsoid, elongate to sometimes cylindrical, sometimes slightly adaxially concave, smooth, thin walled; contents as one or two guttules, hilar appendix present (Fig. 4A). *Subhymenium* 2.0–4.5 µm in diam., compact hyphae, hyaline. *Tramal context* made of parallel (Fig. 5A), agglutinated hyphae 2.5–9.5 µm in diam., thin walled, yellowish in mass, individually hyaline, clampless; in some basidiomata (e.g. C. L. Ovrebo 5034), a crenulate surface is observed at the hymenium layer (Fig. 5B).

Ecology and distribution. The species is found on roadside soil banks and on termite nests, usually near edges of well-conserved forest; it is gregarious, with basidioma developing apparently after rainfall. The species is now known from the semi-arid Caatinga biome as well as from upland wet forest enclaves within the Caatinga ('brejo de altitude') and from the Atlantic rainforest (Fig. 1).

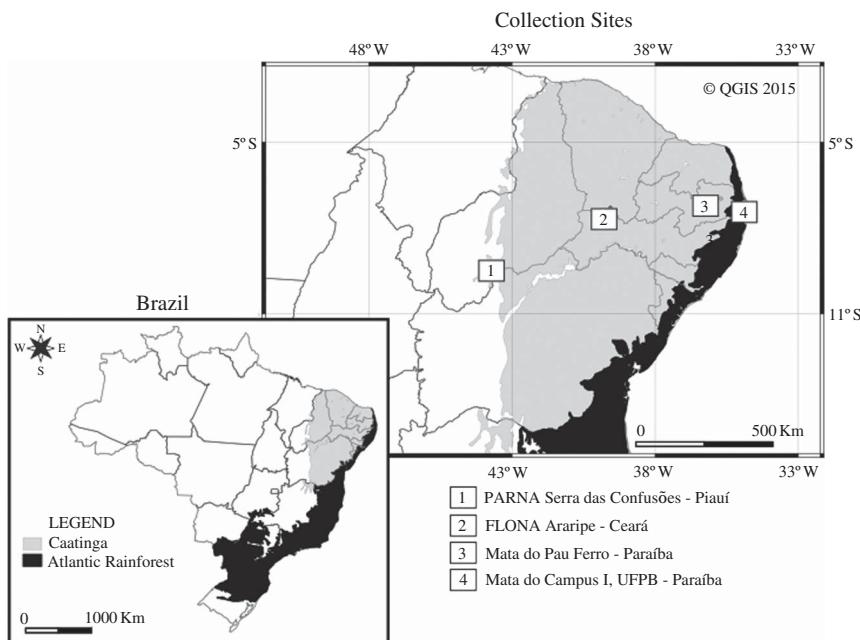


FIG. 1. Map showing the distribution of *Sulzbacheromyces caatingae* in the Brazilian semi-arid region and in the north-eastern Atlantic rainforest biome.

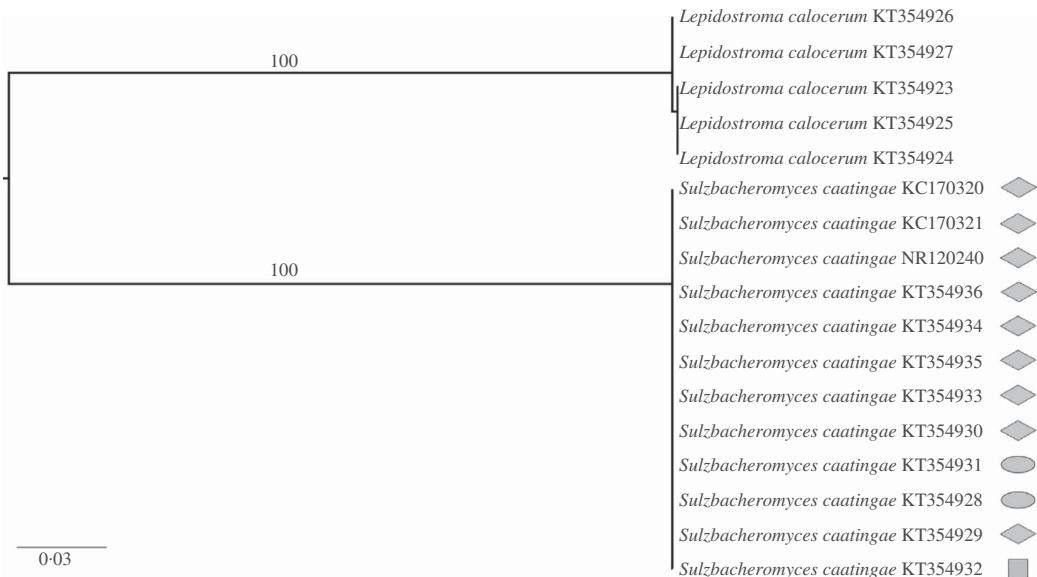


FIG. 2. Maximum-likelihood tree of the ITS alignment of selected Basidiomycota, focusing on *Sulzbacheromyces*, with GenBank accession numbers. Shapes indicate the vegetation sites: rhombus, Caatinga biome; ellipse, upland wet forest of the semi-arid zone; square, Atlantic rainforest biome.

Material examined. **Brazil:** Ceará: Crato, FLONA Chapa da Araripe, 07°17'23.14"S, 39°33'40.19"W, 3 v 2013, leg. M. A. Sulzbacher s. n. (UFRN-Fungos 2049).

Paraíba: Areia, Reserva Ecológica Mata do Pau-Ferro, 06°59'02"S, 35°44'64"W, 2012, C. L. Ovrebo 5034 (JPB 51318, UFRN-Fungos 2502, F); *ibid.*, 2013, Sousa JM65 (UFRN-fungos 2051); João Pessoa, Mata do Campus I da UFPB, 07°08'37"S, 34°50'73"W, 2013, leg. F. Wartchow 58-2013 (UFRN-Fungos 2105). Piauí: Parque Nacional Serra das Confusões, Caracol, Trilha da Andorinha 1, 09°13"S, 43°27"W, 2011, *Sulzbacher* 235 (UFRN-Fungos 1478, holotype; F, isotype); *ibid.*, 2011, *Sulzbacher* 237 (UFRN-Fungos 1479)

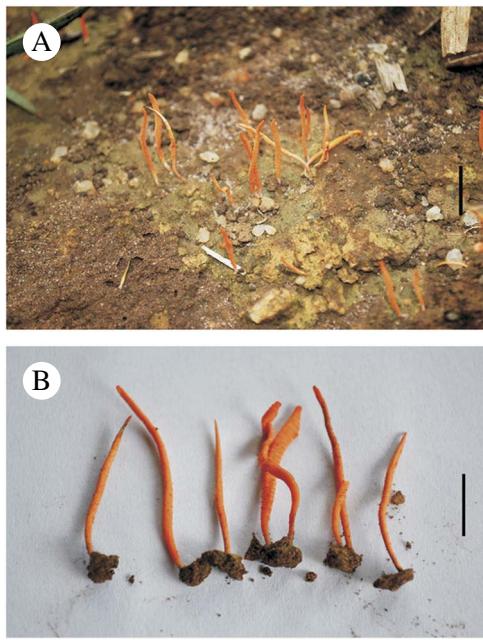


FIG. 3. *Sulzbacheromyces caatingae* (C. L. Ovrebo 5034). A, habit in situ; B, basidiomata. Photographs: C. L. Ovrebo. Scales: A & B = 10 mm. In colour online.

Notes on the vegetation types where *Sulzbacheromyces caatingae* has been found

The term ‘caatinga’, used for naming this taxon, usually refers to a large area in north-eastern Brazil where the climate is predominantly semi-arid (Prado 2003). However, this area presents several vegetation types with different floristic compositions (Sá *et al.* 2003; Leal *et al.* 2005), including thornbush and particular forest types. This has already been shown by Andrade-Lima (1981), who referred to this region as ‘caatingas’ in plural. While *Sulzbacheromyces caatingae* was originally described from the Caatinga biome (Sulzbacher *et al.* 2012, as *Lepidostroma*), the species was subsequently collected in several other localities partly belonging to different biomes:

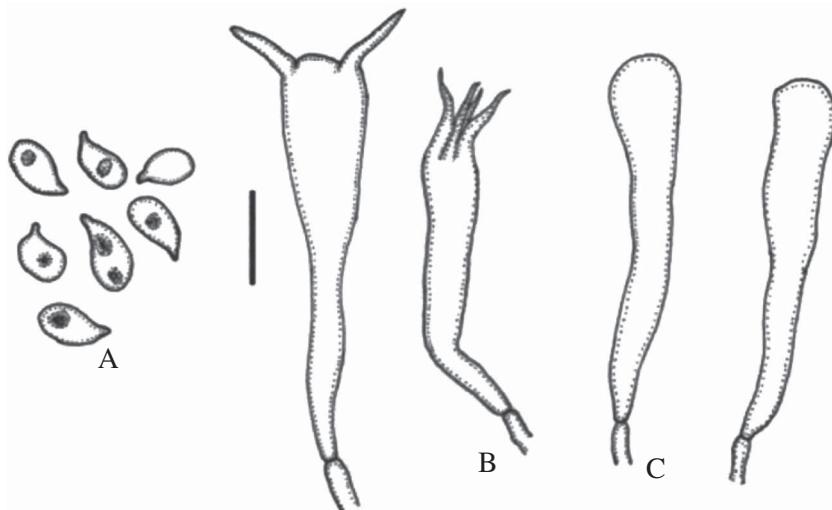


FIG. 4. *Sulzbacheromyces caatingae* (C. L. Ovrebo 5034). A, basidiospores; B, basidia and adjacent hyphae; C, basidioles and adjacent hyphae. Scale = 10 µm.

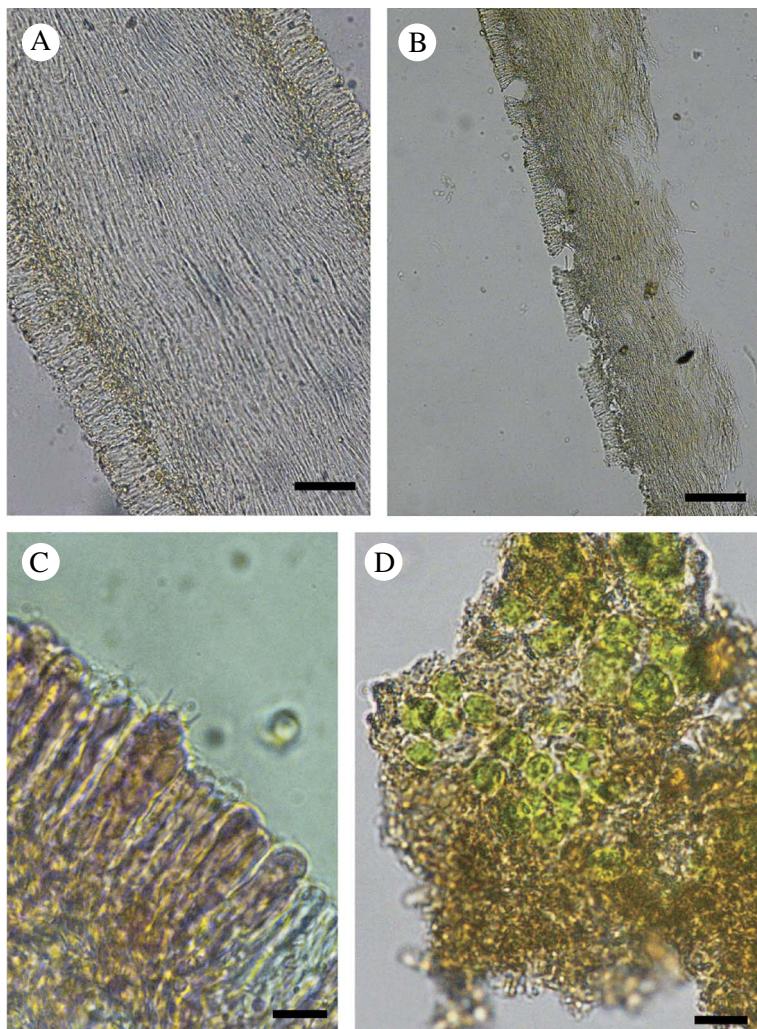


FIG. 5. *Sulzbacheromyces caatingae* (C. L. Ovrebo 5034). A, trama context; B, trama context showing a crenulate surface; C, basidia and basidioles; D, green algal cells from the thallus. Scales: A = 20 µm; B = 100 µm; C & D = 10 µm. In colour online.

- 1) "Parque Nacional da Serra das Confusões" (PARNA Serra das Confusões - 8°26'50"-8°54'23"S, and 42°19'47"-42°45'51"W) is located in the State of Piauí, North-east Brazil, representing the ecoregion Ibiapaba-Araripe complex, which covers an area c. 526.108 ha. The region is part of the Caatinga domain (IBAMA 2003), with a heterogeneous vegetation including caatinga-savanna (cerrado) transition zones (Ab'Sáber 1981), composed of shrubby and spiny trees (Santos *et al.* 2012). According to the Environmental Ministry in Brazil, this area is considered of extreme importance for biodiversity conservation in Brazil (Veloso *et al.* 2002).
- 2) The Araripe National Forest (FLONA Araripe - 07°11'42"-07°28'38"S and 39°13'28"-39°36'33"W) is located in the state of Ceará, North-east Brazil, ecoregion Ibiapaba-Araripe complex, on the Araripe Plateau (average height = 750 m), the southern tip of the Ceará state. The region covers an area c. 38.262 ha and is also part of the Caatinga domain, but comprises many phytophysiognomies, including upland wet forest enclaves (brejos de altitude), savanna (cerrado), savanna woodland (cerradão) and 'carrasco', also with ecotones between caatinga and savanna (Austregésilo-Filho *et al.* 2001). Field expeditions were conducted in 'carrasco' areas of the

- Caatinga. This particular and unique type of vegetation (Figueiredo 1986) occurs in a semi-arid region, comprising transitional vegetation between savanna and caatinga-savanna (Araújo & Martins 1999). According to the Environmental Ministry in Brazil, this area is also classified as important for biodiversity conservation (Velloso *et al.* 2002).
- 3) “Reserva Ecológica Estadual Mata do Pau Ferro” (Mata do Pau Ferro 06°58'12"S and 35°42'15"W): this natural reserve is located in the state of Paraíba, North-east Brazil, more specifically on the oriental humid slope of the Borborema Plateau. It covers an area c. 600 ha, with an altitude ranging between 400–600 m. The area represents a ‘brejo de altitude’ (upland wet forest enclave), humid ‘islands’ of Atlantic rainforest remnants which are isolated within the Caatinga biome (Barbosa *et al.* 2004; Tabarelli & Santos 2004; Andrade *et al.* 2006; Oliveira *et al.* 2006). According to the Environmental Ministry, this area is also considered of high importance for biodiversity conservation (Velloso *et al.* 2002). A recent inventory reported 309 angiosperm taxa, with Rubiaceae, Malvaceae, Asteraceae, Convolvulaceae, Solanaceae and Fabaceae being the most diverse (Barbosa *et al.* 2004).
- 4) “Mata do Campus I da UFPB” (7°08'S and 34°53'W): ‘Campus I’ of the Universidade Federal da Paraíba (UFPB) has 180 ha with c. 50% covered by Atlantic rainforest (Santos *et al.* 2011), and composed of 12 fragments from the same Atlantic forests (Silva *et al.* 2010). This area belonged to ‘Mata do Buraqueirinho’, until the 1970s when it was deforested for the construction of the UFPB (Barbosa 1996). The rainfall range is c. 1500–1700 mm and the humidity varies up to 80% between March and August (Barbosa 1996). The most abundant family is Rubiaceae, but Fabaceae, Lauraceae, Sapotaceae, Anacardiaceae, and Euphorbiaceae also occur.

Our additional collections of *S. caatingae* agree with the type material in the undifferentiated crustose thallus and the caloceroid orange basidiomes, which in some specimens show a circumferentially cracked surface that resembles an old carrot. Statistical measures give the basidiospore size as 5.0–8.0 (8.5) \times 3.5–4.5 μm , L = 6.5 μm , W = 3.9 μm , Q = 1.50–2.00 (2.28), Q = 1.67, with clampless basidia with two or four sterigmata. Sulzbacher *et al.* (2012) described the type with slightly deviating characters, so we initially thought our additional specimens corresponded to an undescribed taxon; for example, the basidiomes in the type are pale to reddish yellow to light orange and smooth, and the basidiospores are somewhat narrower, 6–9 \times 2.5–3.8 μm (est. Q > 2.00).

However, ITS analysis shows no base differences between all collections and suggests that they represent the same species (Fig. 2); the observed variation is then interpreted as age-related, since the basidiomes are ephemeral and grow quickly after rainfall.

Molecular studies show that the genera *Multiclavula* R. H. Petersen (*Cantharellales*) and *Sulzbacheromyces* belong to distantly related clades within the Agaricomycetidae (Ertz *et al.* 2008; Hodkinson *et al.* 2014), although they are morphologically very similar. One possible character to partially separate both genera is the number of sterigmata. *Multiclavula* generally has 4–8 sterigmata, as frequently observed in other taxa in *Cantharellales* (e.g., Petersen 1967; Moncalvo *et al.* 2006; Nelsen *et al.* 2007), while most other basidiomycetes, including *Lepidostromatales*, have (1–)2–4 sterigmata (Oberwinkler 1984; Fischer *et al.* 2007; Ertz *et al.* 2008; Hodkinson *et al.* 2012; Sulzbacher *et al.* 2012). According to Petersen (1967), most species of *Multiclavula* have 6-spored basidia, including, *M. mucida* (Fr.) R. H. Petersen, *M. coronilla* (G. W. Martin) R. H. Petersen, *M. hastula* (Corner) R. H. Petersen and the type species of this genus, *M. corynoides* (Peck) R. H. Petersen. However, *Multiclavula afflata* (Lagger) R. H. Petersen, *M. clara* (Berk. & M. A. Curtis) R. H. Petersen, *M. constans* (Corner) R. H. Petersen, *M. delicata* (Fr.) R. H. Petersen, *M. fossicola* (Corner) R. H. Petersen, *M. pogonati* (Coker) R. H. Petersen, *M. sharpii* R. H. Petersen and *M. vernalis* (Schwein.) R. H. Petersen have 4-sterigmate basidia (Petersen 1967) and some of these might represent *Lepidostromatales*, although *M. vernalis* has been confirmed in *Multiclavula* using sequence data.

Another possible way to distinguish *Sulzbacheromyces* from *Multiclavula* is by the mycological features of the thallus. The thallus granules in *Sulzbacheromyces* are ecorctate and composed of algal cells and fungal hyphae only, whereas species of *Multiclavula* usually form *Botrydina*-type granules with a paraplectenchymatous cortex (Oberwinkler 2012; Hodkinson *et al.* 2014). However, not all species of *Multiclavula* have been well documented regarding their thallus structure.

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