

# A new species of *Balansia* (*Clavicipitaceae*) associated with a cyperaceous plant in Brazil

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## Research Article

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# Abstract

Fungal species belonging to the genus *Balansia* (*Clavicipitaceae*) are well known as endophytic and epibiotic species commonly found on grasses or sedges. Among the 36 species of *Balansia* described worldwide, ten have been reported in Brazil. While most species of balansoid fungi were described on graminaceous plants, only four were characterized on cyperaceous hosts. To correctly identify the species of balansoid fungi associated with *Scleria bracteata* (*Cyperaceae*), specimens were collected in the state of Alagoas, Brazil, in 2014 and 2016. Nucleotide partial sequences of the second-largest subunit of RNA polymerase II (RPB2), translation elongation factor 1- $\alpha$  (TEF1), 18S subunit ribosomal DNA (SSU), 28S subunit ribosomal DNA (LSU), and internal transcribed spacers (ITS) were obtained from each balansoid specimen. Based on morphology and molecular data, the specimens were identified as a putative new species of *Balansia*, herein referred to as *Balansia scleriae* sp. nov.

## Introduction

*Balansia* Speg. (*Clavicipitaceae*) includes both endophytic and epibiotic species commonly found on grasses or sedges. *Balansia* species are, in general, characterized by capitate ascostromata that follow a well-defined ephelidial-stage (= *Ephelis*) in some instances, and sometimes arising within it (Diehl 1950). The genus was introduced by Spegazzini, with *Balansia claviceps* as the type species, based on a fungus developing on inflorescences of a graminaceous host showing a close affinity with *Claviceps* Tul., but distinguished from the latter for its conidial stage (Spegazzini 1885). Later Diehl (1950) monographed *Balansia* and related genera within the tribe *Balansiae*, and emended Spegazzini's concept of the genus, expanding the circumscription to include characteristics of the ephelidial-stage, an essential taxonomic trait to distinguish species.

Morphological characteristics such as stromata and ascomata morphology, symptomatology, features of asci, ascospores, together with conidial characteristics, geographic distribution, and the host affinities are traditionally used to differentiate *Balansia* species. Currently, species demarcation into this group is based on additional morphological characteristics combined with sequence and phylogenetic data (White et al. 2003). However, most nucleotide sequence information available in public databases such as GenBank-NCBI were obtained from species associated with plant hosts in the *Poaceae* botanical family (Kuldau et al. 1997, Reddy et al. 1998, White et al. 1997, 2000, Sung et al. 2007a, 2007b). Here, balansoid specimens infecting *Scleria bracteata* (*Cyperaceae*) in the state of Alagoas, Brazil, were morphologically and molecularly characterized as a novel *Balansia* species, herein referred to as *Balansia scleriae* sp. nov.

## Materials And Methods

### Sample collection and morphological characterization

Isolates of balansoid fungi associated with *Scleria bracteata* (*Cyperaceae*) were collected in the city of Maceio, Alagoas State, Brazil, in 2014 and 2016, and were deposited in the Mycological Collection of

Herbarium Universidade de Brasília (UB), Brasília, Federal District, Brazil. To morphologically characterize these specimens, the fungal structures were initially observed using a Leica 205C model stereomicroscope. Representative materials were sectioned using a Leica CM 1850 freezing microtome, yielding 20–30 µm thick sections that were placed on slides containing colorless lactoglycerol and visualized on a Leica DM 2500 microscope coupled to a Leica DFC 490 digital camera using Nomarski interference microscopy. Size estimations for the structural components were based on at least 30 measurements when possible. Comparisons with type descriptions and illustrations were carried out using the available literature.

### **DNA extraction, amplification, and sequencing**

Total DNA extraction was performed from material in the ascomata using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. PCR amplifications were performed on a DNA Engine (PTC-200) Peltier Thermal Cycler. Partial sequences were obtained from the nuc rDNA and two coding genes, second-largest subunit of RNA polymerase II (RPB2) and translation elongation factor 1- $\alpha$  (TEF1). The 18S subunit ribosomal DNA (SSU) was amplified with primers NS1 and NS4 (White et al. 1990) and the 28S subunit (LSU) and the internal transcribed spacers (nuc ITS1-5.8S-ITS2 = ITS) with primers V9G (de Hoog and Van den Ende 1998), LR5 (Vilgalys and Hester 1990), and ITS4 (White et al. 1990), while RPB2 was amplified with primers 5F2 (Sung et al. 2007) and 7cR (Liu et al. 1999), and TEF1 with primers EF1-938F and EF1-2218R (Rehner and Buckley 2005).

The PCR thermocycling conditions were: initial DNA denaturation at 94°C for 1 min 30 s; 35 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 53°C for 30 s, and extension at 72°C for 45 s, and a final extension step at 72°C for 5 min. The PCR products were analyzed on 1% agarose gel and purified using ExoSAP-IT® PCR Product Cleanup (Affymetrix Inc.). Then, the amplicons were directly Sanger sequenced at Macrogen Inc. (Seoul, South Korea; <http://www.macrogen.com>). The electropherograms were manually/visually evaluated, and ambiguous positions were clarified considering forward and reverse sequences. The contigs were individually assembled and annotated using GENEIOUS 9.0.5 (Kearse et al. 2012) and deposited in GenBank (<http://www.ncbi.nlm.nih.gov>).

### **Taxa sampling and alignment**

The phylogenetic relationship of the specimens reported here and other species into the genus was assessed using the ITS sequence data (Table 1) because it was the genomic regions from which more sequences were available. The nucleotide sequences were aligned using the E-INS-i method (in MAFFT 7.305; Katoh and Standley 2013) and manually adjusted in AliView (Larsson 2014). The ITS matrix was partitioned as ITS1-5.8S-ITS2, and *Claviceps purpurea* was selected as the outgroup. The sequences described in the present study, and sequences retrieved from GenBank, are shown in Table 1. The alignment was deposited in TreeBASE ([www.treebase.org](http://www.treebase.org)).

### **Phylogenetic analyses**

Maximum likelihood (ML) analysis was performed using RAxML 8.2.9 (Stamatakis 2014), starting with a randomized, stepwise addition parsimony tree under a GTR+G model. The branch support values were calculated using 1000 bootstrapping (BS) and replicated under the same model. Bayesian Inference (BI) was carried out using MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003), following 4x4 mode of the general time reversible (GTR) model for all partitions. Two independent chains were run, each one initiating from random trees and four simultaneous independent chains at  $10^6$  generations, with trees being sampled at every  $10^3$  generations. Four rate categories were used to approximate the gamma distribution. Average standard deviations of split frequencies (ASDSF) were used as a chain convergence criterion. Twenty-five percent of all sampled trees were discarded as burn-in, and the remaining 75% employed to estimate the Bayesian posterior probabilities (BPPs) for branches. Both MrBayes and RAxML were ran through the CIPRES Science Gateway 3.1 web portal (Miller et al. 2010).

## Results

The PCR amplification and sequencing of the regions SSU, LSU, ITS, TEF1- $\alpha$ , and RPB2 (GenBank Accession Nos. MK256221-MK256226, and MK249873-MK249875) yielded sequences of 990, 850, 400, 910, and 880 bp in length, respectively. The ITS nucleotide matrix contained 695 aligned sites, including gaps, with 268 variable sites of which 189 were parsimony informative (70% of variable sites). For each partition (ITS1, 5.8S, and ITS2) different evolutionary models were selected (Table 2). The nucleotide matrix and phylogenetic trees reconstructed in this study are available in TreeBASE (study number S23703). Although the SSU, LSU, TEF1- $\alpha$ , and RPB2 sequences were not used in the phylogenetic analyses, they were deposited in GenBank for future studies and identification purposes.

### Phylogenetic analyses

*Balansia* formed a monophyletic group in the BI reconstruction (0.95 Bayesian posterior probabilities – BPP). Both ML and BI analyses of the genus *Balansia* confirmed the genetic differences of the balansoid specimens on *S. bracteata* collected in Brazil from previously known *Balansia* species. Also, the new isolates were placed apart from *B. cyperi*, the only *Balansia* species that has been reported infecting cyperaceous hosts (Figure 1).

### Taxonomy

Morphological comparisons together with phylogenetic analyses using sequence data from nuclear ITS rDNA confirmed that the isolates described here are different from previously reported *Balansia* species, and therefore are classified as belonging to a new species, herein referred to as *Balansia scleriae* sp. nov.

***Balansia scleriae*** Guterres DC, Ramos-Sobrinho R, Pinho, Assunção IP, and Lima GSA, sp. nov. Fig. 2

Differs from *Balansia* spp. by having smaller asci and ascospores, and a thicker ascoma wall. Additionally, stroma of *B. scleriae* completely encloses the culms of the host at the internodes and is restricted to this tissue.

**Mycobank:** MB 829055

**Etymology:** referring to plant genus on which the fungus was found.

Stromata well-developed, thick (0.58–1.3 mm) black, carbonaceous, pulvinate, prosenchymatous-sclerotic, context brown, surface black, sessile, completely surrounding tissues in culms at internodes reaching up to 5 cm long and 0.6 mm thick. Ascomata perithecial, immersed in the stromata, context light brown, with punctiform ostioles, aggregated. Perithecia ovate to lageniform, perithecial walls well defined, 22–28 µm thick, composed of compressed stromatic cells; ostioles with filiform periphyses lining the short neck (30–42 × 32–54 µm); asci arising from the base of the perithecium, cylindrical (105–185 × 5.5–7.5 µm), with conspicuous slime-cap; ascospores hyaline, filiform (75–115 × 1.0–1.5 µm), with blunt ends, and pluri-septate at maturity with more than 5 septa; paraphyses filiform, deliquescent with development of the asci. Conidial stage absent.

**Material examined:** Brazil, Alagoas, Maceió, Universidade Federal de Alagoas, on culms of *Scleria bracteata*, 15 Jul 2014, R Ramos-Sobrinho (UB 23900 holotype). GenBank ITS = MK256225, SSU = MK256221, LSU = MK256223, TEF = MK249873, RPB2 = MK249875.

**Additional specimen examined:** Brazil, Alagoas, Maceió, Universidade Federal de Alagoas, on culms of *Scleria bracteata*, 2 Oct 2016, R Ramos-Sobrinho (CDUB 2231). GenBank ITS = MK256226, SSU = MK256222, LSU = MK256224, TEF = MK249874.

## Discussion

*Balansia* spp. are usually described as host specific or having a narrow host range. This feature still plays a major role in the taxonomy of the genus including species definition. Four species of *Balansia* have been previously described from Cyperaceae hosts, *Balansia carecis* Hosag. on *Carex filicina* in India (Hosagoudar 1994), *B. cyperacearum* (Berk. & M.A. Curtis) Diehl on *Cyperus ovularis*, *Cy. rotundus*, *Cy. strigosus*, and *Cyperus* sp. from the USA and Surinam, on *Cy. virens* from Venezuela and Brazil (Diehl 1950; Alfieri Jr. et al. 1984; Farr and Rossman 2021), and on *Scleria chinensis* (Zhuang 2001), and on an unidentified species of *Carex* (Teng 1996) both in China, *B. cyperi* Edgerton on *Cyperus rotundus*, on inflorescences of *Cy. virens* and *Cyperus* sp. in the USA (Edgerton 1919; Diehl 1950; Clay 1986; Leuchtman & Clay 1988; Farr and Rossman 2021), and *B. borealis* Tranzchel found on fruits of *Carex sparsiflora* in the former Soviet Union. *Balansia carecis*, *B. borealis* and *B. cyperi* are found only in inflorescences and spikelet of the hosts (Table 3).

Phylogenetically related species, *B. claviceps* and *B. granulosa* are found on *Panicum* sp., *Setaria palmicola*, and on an indeterminate graminaceous host. *Balansia claviceps* has stipitate stroma, arising from the hypothallus, ascoma wall narrower than *B. scleriae* sp. nov, and a known conidial stage. *Balansia granulosa*, first described as *Dothichlōe granulosa*, is not provided with a definite ascoma wall, has narrower perithecia, and longer ascospores.

*Balansia* species infecting culms and leaves are not rare in hosts of the family *Poaceae*, but they usually form a scythe shape stroma linked to the host tissue only at the base, which sometimes partly surrounds the culm or petioles. On the other hand, on sedges, it occurs less often, been only recorded for *B. cyperacearum*, which type on an unidentified species of *Cyperaceae* from Surinam is unique in infecting abaxial leaf surfaces, whereas all the other specimens occur on culms and leaf bases (Berkeley and Curtis 1853). Based on these differences, Diehl (1950) suggested that *B. cyperacearum* could be an assembly of two completely different species, an idea reinforced by White et al. (1997). The stromata of *B. scleriae*, described here, slightly resemble a specimen collected by Chardón in Venezuela in 1939 and treated by Diehl (1950) as *B. cyperacearum*.

Perithecial stroma morphology was also traditionally reinforced as a stable characteristic of taxonomic value. Diehl (1950) accommodated *Balansia* species within the subgenera *Eubalansia* and *Dothichloë*, the former characterized by the possession of flattened ascomatal stromata that develop on leaves or culms of grasses, while the latter included species with pulvinate or stipitate ascomatal stromata. Among the species infecting sedges, *B. cyperi* shows spherical to subspherical perithecial stromata, and *B. cyperacearum* has effuse and flattened stromata. *Balansia scleriae* seems to be an intermediate between those two species, with perithecia immersed in coalescent cushion-like stromata with punctiform ostioles (Figure 2 B–C).

*Balansia* species on *Cyperaceae* usually cause dwarfness, witch's broom, or alter the bloom of the host. *Balansia cyperi* causes sterility, dwarfness and foliage deformation, characterized by terminal bracts swollen, while *B. cyperacearum* causes sterility. Plants infected by *B. carecis* show stunted growth reduced to half of their normal size and a characteristic incense candle-like inflorescence due to the colonization by the fungus and abundant production of stroma (Hosagoudar 1994). *Balansia borealis* is known only for its type and its infection is restricted to fruits (Elenkin 1914). Although the stromatic development of *B. scleriae* sp. nov. along the internodes of *S. bracteata*, no apparent physiological symptoms were observed on this host.

The specimens of *Balansia* infecting *S. bracteata* were morphologically and genetically different from the four *Balansia* species known on *Cyperaceae* (Table 3). When compared to the two closest species, *Balansia cyperi* has larger ascospores and conidial stage, while *B. cyperacearum* is distinguished from the new species by smaller asci and ascoma wall. Further, *Balansia scleriae* can be morphologically differentiated from all currently recognized *Balansia* species by having very thick stroma, which completely encloses the culms of the host at the internodes and is restricted to this tissue.

## Declarations

### Data availability

The datasets generated for this study can be found in Genbank: MK249873- MK249875 and MK256221- MK256226; Treebase: S23703. The results obtained in this study are included in the contents of this report.

## **CRedit taxonomy**

Conceptualization: Debora Cervieri Guterres, Danilo Batista Pinho and Roberto Ramos-Sobrinho;

Specimen collections: Gaus S.A. Lima and Roberto Ramos-Sobrinho;

Identification of fungi: Danilo Batista Pinho and Roberto Ramos-Sobrinho;

Phylogenetic analysis: Debora Cervieri Guterres;

Deposition of specimens at Herbarium: Danilo Batista Pinho;

Laboratory resources and facilities: Danilo Batista Pinho;

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Writing—review and editing: Danilo B. Pinho, Iraildes P. Assunção and Gaus S.A. Lima;

Final proofreading: Roberto Ramos-Sobrinho;

Submission: Danilo Batista Pinho.

## **Contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Danilo Batista Pinho, Debora Cervieri Guterres, Iraildes P. Assunção, Gaus S.A. Lima, and Roberto Ramos-Sobrinho. The first draft of the manuscript was written by Debora Cervieri Guterres and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## **Ethics declarations**

## **Conflicts of interest**

The authors declare no conflict of interest.

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## Tables

**Table 1.** GenBank accession numbers of specimens included in this study. Sequences generated in this study are highlighted in bold.

Organism	Voucher	GenBank Accession #	Host*
<i>Aciculosporium take</i>	B1	EF363682	<i>Mao bamboo</i>
<i>Aciculosporium take</i>	B2	EF363683	<i>Mao bamboo</i>
<i>Aciculosporium take</i>	Okamezasa	AB086846	<i>Shibataea kumasaca</i>
<i>Balansia andropogonis</i>	CBS 214.81	U89370	<i>Cyrtococcum oxyphyllum</i>
<i>Balansia andropogonis</i>	CBS 501.70	U89371	<i>Cymbopogon roxburghii</i>
<i>Balansia andropogonis</i>	CBS 365.67	U89372	<i>Sorghum vulgare</i>
<i>Balansia claviceps</i>	1937	U89365	–
<i>Balansia claviceps</i>	1948	U89366	–
<i>Balansia cyperi</i>	CBS 77488	U89369	–
<i>Balansia cyperi</i>	–	DQ119112	–
<i>Balansia discoidea</i>	1958	U89373	<i>Pennisetum purpureum</i>
<i>Balansia discoidea</i>	1950	U89374	<i>Setaria paniculifera</i>
<i>Balansia gaduae</i>	–	U78054	<i>Panicum</i> sp.
<i>Balansia granulosa</i>	–	AF065613	<i>Lasiaci</i> ssp.
<i>Balansia henningsiana</i>	–	U57404	<i>Andropogon virginicus</i>
<i>Balansia henningsiana</i>	–	U78058	<i>Andropogon</i> sp.
<b><i>Balansia scleriae</i></b>	<b>UB 23900</b>	<b>MK256225</b>	<b><i>Scleria bracteata</i></b>
<b><i>Balansia scleriae</i></b>	<b>CDUB 2231</b>	<b>MK256226</b>	<b><i>Scleria bracteata</i></b>
<i>Balansia oblecta</i>	–	U57402	<i>Cenchrus echinatus</i>
<i>Balansia oblecta</i>	C_Schardl	DQ119113	–
<i>Balansia pilulaeformis</i>	–	AF065611	<i>Chasmanthium</i> sp.
<i>Balansia strangulans</i>	–	U57403	<i>Panicum</i> sp.
<i>Balansia strangulans</i>	–	U78055	<i>Panicum</i> sp.
<i>Claviceps grohii</i> (outgroup)	–	AJ133395	<i>Carex</i> sp.
<i>Claviceps purpurea</i> (outgroup)	T5	DQ119114	–

\*as indicated in data retrieved from GenBank.

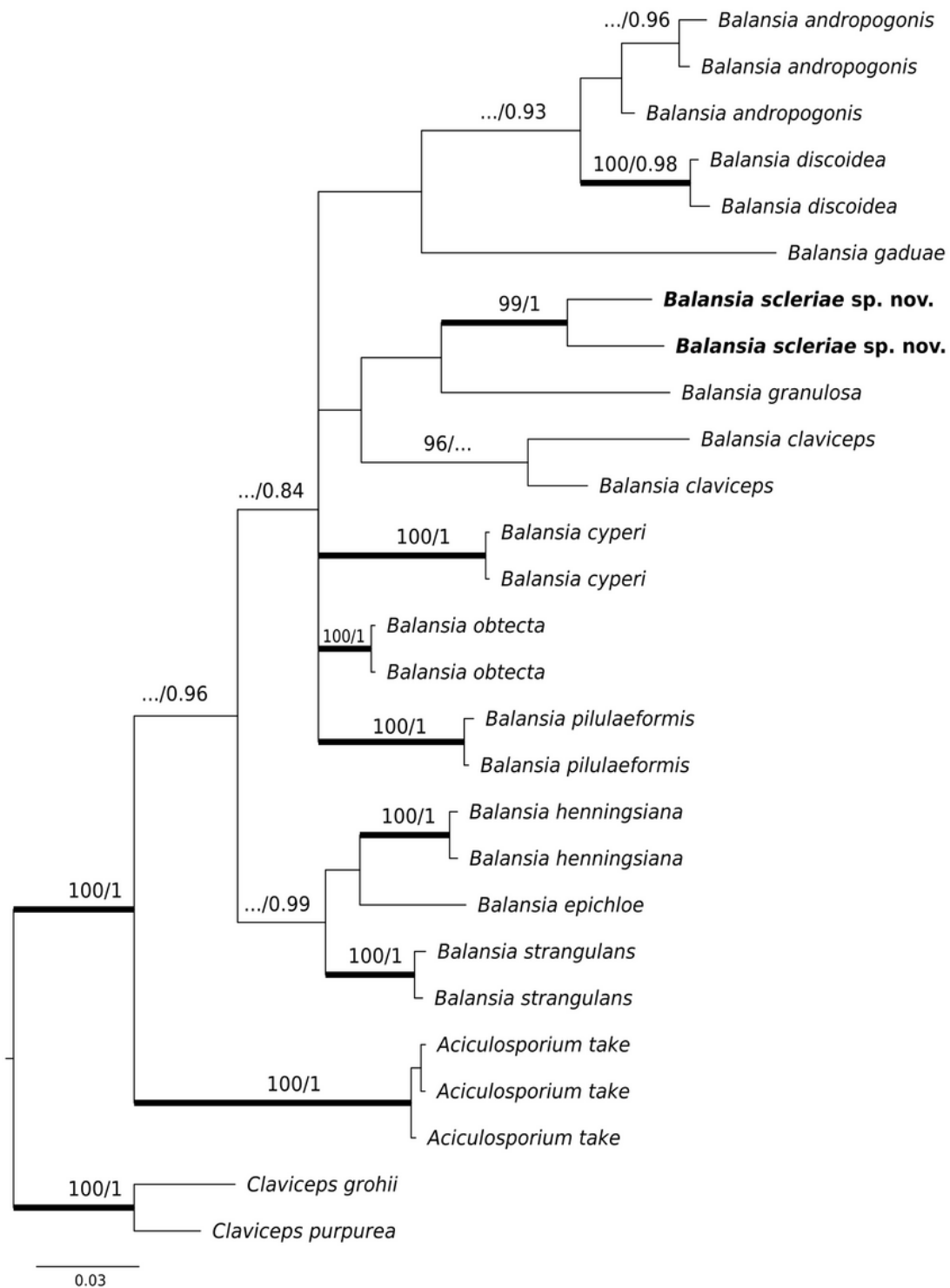
**Table 2.** Parameters and evolution models selected in phylogenetic analyses.

Parameter	Partition		
	ITS1	5.8S	ITS2
Evolutionary model	HKY+I	K80	TPM1uf+G
Likelihood	-1407,983	-259,170	-1481,625
Matrix length	250	140	303
Base frequencies	-	equal	-
Freq. A	0.1949	-	0.1398
Freq. C	0.3418	-	0.3781
Freq. G	0.2402	-	0.2892
Freq. T	0.2232	-	0.1930
Transition rates	Equal	equal	-
R (AC)	-	-	1,000
R (AG)	-	-	5,514
R (AT)	-	-	2,572
R (CG)	-	-	2,572
R (CT)	-	-	5,514
R (GT)	-	-	1,000
Prop. Invariable sites	0.4740	-	-
Gamma	-	-	0,457

**Table 3.** Morphological features of *Balansia species* on inflorescences, culms and leaves of cyperaceous hosts.

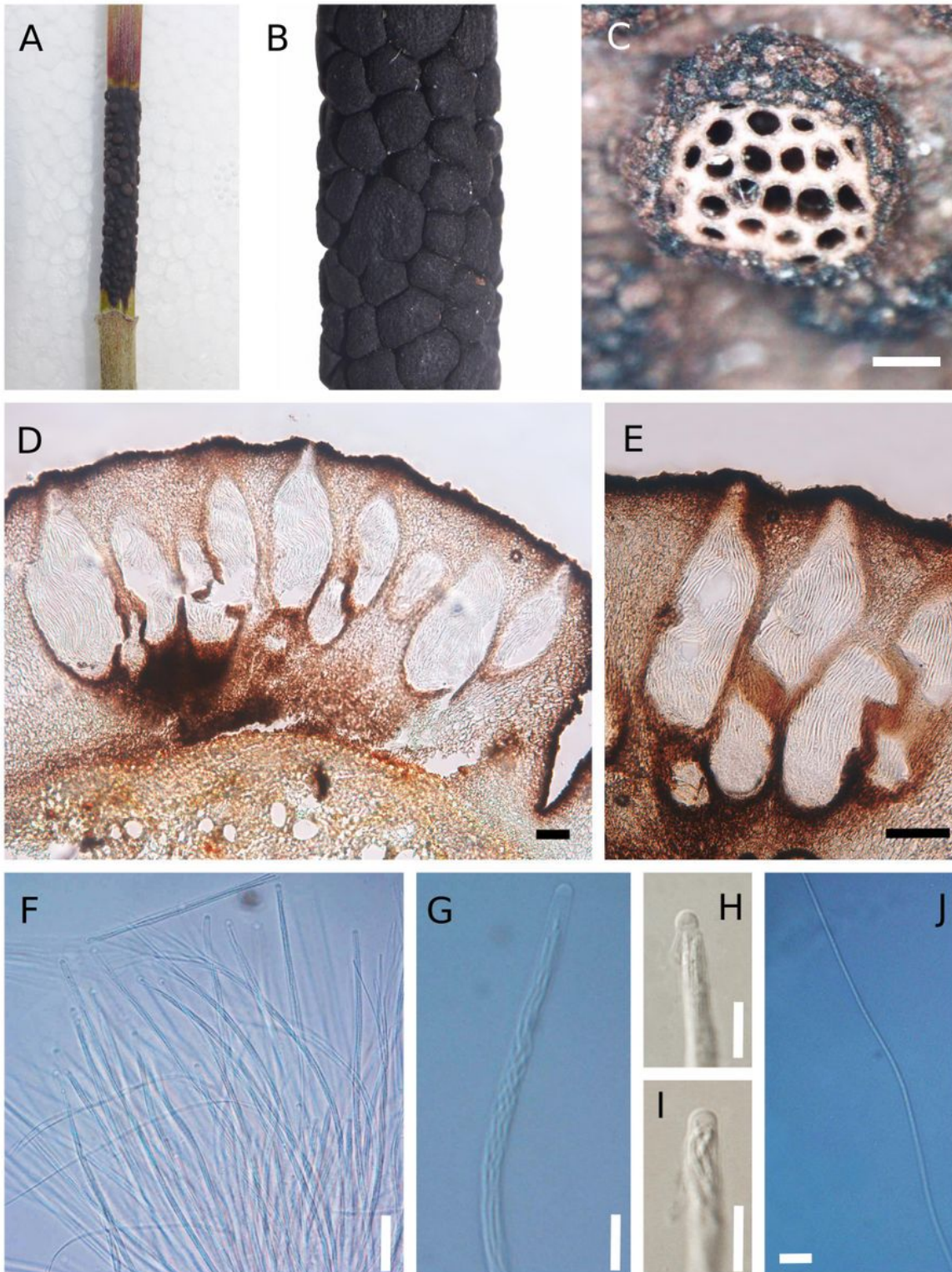
Species name	Ascoma ( $\mu\text{m}$ )	Ascoma wall ( $\mu\text{m}$ )	Asci ( $\mu\text{m}$ )	Ascospore ( $\mu\text{m}$ )	Conidial stage
<i>B. carecis</i>	257–315 × 114–172	–	139–223 × 3–5	62–77 × 1.5	absent
<i>B. claviceps</i>	200–260 × 120–140	10–15	106–195 × 5–6	100–180 × 1– 1.5	= <i>Ephelis</i>
<i>B. cyperacearum</i>	200–380 × 120–200	12–20	90–125 × 4–6  (ascigerous part 36–90 × 4–6)	36–100 × 1–1.5	absent
<i>B. cyperi</i>	360–600 × 120–200	16–25	160–225 × 6–9	100–200 × 1.5– 3	= <i>Ephelis</i>
<i>B. granulosa</i>	175–285 × 68–85	indefinite	120–165 × 4–5.4	nearly as long as the asci	absent
<i>B. scleriae</i> sp. nov.	230–435 × 95–177	22–28	105–185 × 5.5–7.5	75–115 × 1–1.5	absent

## Figures



**Figure 1**

Phylogenetic tree inferred from Bayesian Analysis of ITS1-5.8S-ITS2 sequences representative of *Balansia*. Values at the branches represent bootstrap support (BSS) and Bayesian posterior probability (BPP), respectively. Thickened branches denote BPP  $\geq 0.95$  and BSS  $\geq 90\%$ . The specimens reported in this study are highlighted in bold.



**Figure 2**

*Balansia scleriae* sp. nov. (Mycological Collection UB 23900) on culm of *Scleria bracteata*. A– Perithecial stroma of the fungus surrounds entirely the culm of the host at internodes. B– Close-up of stroma. C– Paradermal section of stroma showing ascomatal cavities. D– Section through stroma with perithecia. E– Ascus filled with filiform ascospores. F– Ascus tip. G– Ascospore – Bars = C– 200  $\mu\text{m}$ ; D– 50  $\mu\text{m}$ , E– G 10  $\mu\text{m}$ .