Heppia exalbescens: a new ecorticate species to the Brazilian Pantanal and Atlantic Forest

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ABSTRACT

Nowadays seven species of lichen-forming fungi belong to the genus *Heppia* Näegeli ex A. Massal. They are characterized by crustose thalli, squamulose or peltate; with or without cortex; *Scytonema* as photobiont; and prototunicate asci with eight spores. In this study, five specimens were collected in three different localities of mid-western Brazil, in Mato Grosso do Sul state. Sequences of nuITS, mtSSU, and nuSSU genomic regions were generated for all specimens. The nuSSU sequence was compared with other sequences available on GenBank and formed a separate lineage that grouped within the *Heppia* clade with high support value, named *Heppia exalbescens*. The new species has upper surface smooth to rugulose, and differs from *H. despreauxii* that has deep depressions on the upper surface. *Heppia exalbescens* is described as new species from the Atlantic Forest and *Pantanal*, highlighting the necessity of study the species from the Brazilian natural areas.

Key-words. cyanolichens, diversity, Mato Grosso do Sul

HEPPIA EXALBESCENS: UMA ESPÉCIE NOVA ECORTICADA PARA O PANTANAL BRASILEIRO E FLORESTA ATLANTICA

RESUMO

Atualmente sete espécies de fungos formadores de liquens pertencem ao gênero *Heppia* Näegeli ex A. Massal. Eles são caracterizados pela presença de talo crostoso, esquamuloso ou peltado; com ou sem córtex; *Scytonema* como fotobionte; e ascos protunicados com oito esporos. Neste estudo, cinco espécimes foram coletados em três diferentes localidades do Centro-Oeste do Brasil, no estado de Mato Grosso do Sul. Sequências de nuITS, mtSSU e nuSSU foram geradas para todos os espécimes. A sequência de nuSSU foi comparada com outras disponíveis no GenBank e formou uma linhagem separada que se agrupou com o clado de *Heppia* com alto valor de suporte, nomeada *Heppia exalbescens*. A espécie nova tem superfície superior lisa a rugulosa, e difere de *H. despreauxii* que possui profundas depressões na superfície superior. *Heppia exalbescens* é descrita como uma nova espécie da Floresta Atlântica e *Pantanal*, destacando a necessidade de estudar as espécies de áreas naturais brasileiras.

Palavras-chaves. cianoliquens, diversidade, Mato Grosso do Sul

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Introduction

Heppia adglutinata (Kremp.) Massal., *H. conchiloba* Zahlbr., *H. despreauxii* (Mont.) Tuck., *H. echinulata* Marton & Galun, *H. lutosa* (Ach.) Nyl., and *H. solorinoides* (Nyl.) Nyl. are lichen-forming fungi species characterized by crustose thalli, squamulose or peltate; with or without cortex; *Scytonema* as photobiont; prototunicate asci, with eight hyaline ascospores, simple to rarely one-septate (HENSSEN, 1994). These species were previously included within the *Heppiaceae* family, but a phylogenetic study revealed that they belong to the *Lichinaceae* (SCHULTZ; BÜDEL, 2003). After the family combination, only *Heppia arenacea* M. Schultz was described as new to science (SCHULTZ, 2005). The main characters of the seven species known to the genus are described in table 1, and the species are reported to Africa, Asia, Europe, North America and South America (HENSSEN, 1994; SCHULTZ; BÜDEL, 2003; CÁCERES et al., 2017)

In Brazil, *Heppia fuscata* Vain., *H. leptophylla* Vain. and *H. murorum* Vain. were already reported to the Rio de Janeiro state (VAINIO, 1890), but all species have numerous ascospores and currently do not belong to the *Heppia* genus.

Table 1. The known *Heppia* species and the characters used to differentiate species

Heppia species	Reference	Thallus	Size	Margin	Upper surface	Epinecral layer	Upper cortex	Medullary hyphae	Lower cortex	Spores
H. adglutinata	Henssen (1994), Massalongo (1854)	Squamulose or peltate lobes	Up to 6 mm diam.	Raised, entire or broken	Yellow-olive or brown	7–12 µm thick in aged lobes	Developed in part, 12–50 µm thick	Not reported	13–40 µm thick, 1–4 rows of enlarged cells	(15–)18–24(–30) × 6–10.5(–12) µm
H. arenacea	Schultz (2005)	Squamulose, resembling an areolate crust	0.6–2.5 mm wide	Lifted up from the substratum	Sand-colored to pale ochre	10–50 µm thick	20–35 µm thick, paraplectenchymatous	Absent, but with abundant soils particles incorporated in all parts of the thallus	Absent	17.4(±2.7) × 7.5 (±0.6) µm
H. conchiloba	Zahlbruckner (1902), Henssen (1994)	Peltate, concave lobes	Up to 8 mm diam.	Incurved or raised and easily broken	Greyish, pruinose	20–50 µm thick	8–50 µm thick	3.5–8.0(–12) µm thick, at base with globose and enlarged cells	Not reported	18–20 × 8–13 µm
H. despreauxii	Montagne (1940), Henssen (1994)	Peltate lobes	2–8 mm diam.	Downcurved or raised, entire or broken	Pale, with deep depressions	Up to 45 µm thick	25–50(–75) µm thick	3–8(–14.5) µm thick, at the base with globose	Not reported	(15–)17–29(–32) × (5–)8–10.5(–12) µm

								and enlarged cells		
H. echinulata	Marton & Galun (1974), Henssen (1994)	Squamu-lose	2.5(–5) mm long.	Mostly contorted	Black to brownish, warty, with spines 45–110 μm	Up to 35 μm thick	Irregular outline	Anticli-nally and reticulate-ly, ca. 5 μm thick	10–22 μm thick, 1–5 rows of cells	Not observed
H. exalbescens	**New species**	**Squamu-lose**	**0.5–3.5 μm**	**Plane to ascending**	**Smooth to rugulose, whitish pruina**	**2.5–5.0 μm thick, amorpho-us appea-rance**	**Absent with the aged, irregular outline**	**20–75 μm (2–10 cells) thick**	**Absent**	**15.0–17.5(–22.5) × 5.0–7.5(–10.0)**
H. lutosa	Acharius (1814), Henssen (1994)	Squamu-lose to granulose	Ca. 0.1–5 mm diam.	Not reported	Blackish	Not observed	Irregular outline	Hyphae 6–7 μm thick	Irregular outline	14–18(–26) × (4.5–)6–10.5 μm
H. solorinoides	Nylander (1869), Henssen (1994)	Peltate lobes	3–6 mm diam	Involute	Whitish warts	Whitish warts of the epinecral layer	20–60 μm thick	4.5–7.0 μm thick, with globose cells at the base	Not reported	14–21(–29) × 8–11(–13) μm

Nowadays, only *Heppia despreauxii* is reported to the Ceará, in the Caatinga biome (CÁCERES et al., 2017) and Mato Grosso do Sul states, in the Atlantic Forest (APTROOT; SPIELMANN, 2020).

The aim was to study the lichenized fungi diversity from Atlantic Forest and *Pantanal* areas, located in Mato Grosso do Sul state, mid-western Brazil. For that, we are describing a new species, the eighth species of *Heppia*, using molecular, morphological and anatomical data; and an identification key is provided to the genus.

Material & Methods

During field trips realized in 2018 and 2022, *Heppia* specimens were collected at three different points of Mato Grosso do Sul state: two in Bodoquena municipality, located in the Atlantic Forest (Santa Laura and Sol de Maio); and one in the Corumbá municipality, located in the Pantanal (São João) (Figure 1).
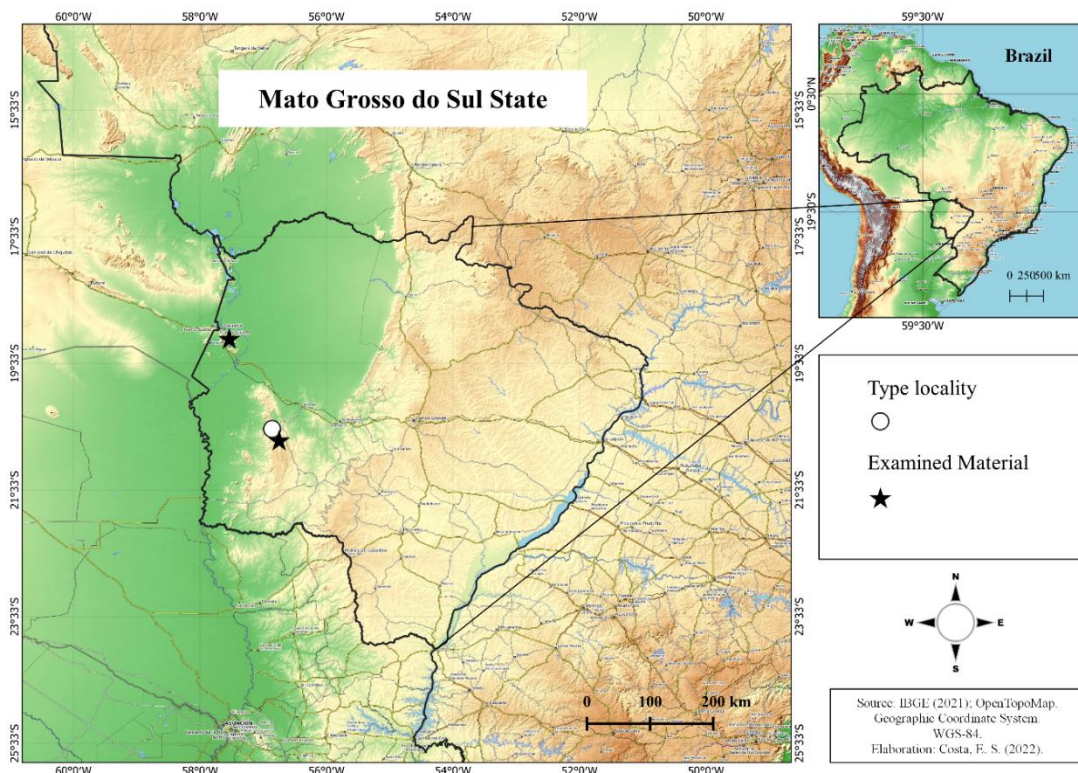

Figure 1. Collection points of *Heppia* specimens collected in the Mato Grosso do Sul state. Map built with QGIS Buenos Aires v. 3.26. (http://www.qgis.org).

The DNA was extracted from fresh specimens using the Wizard® Genomic DNA Purification Kit (Promega), following the manufacturer's protocol. The nuITS region and mtSSU region were amplified for all specimens, using ITS1F and ITS4 primers (GARDES; BRUNS, 1993; WHITE et al., 1990), and SSU1 and SSU3R (ZOLLER; SCHEIDEGGER; SPERISEN, 1999), respectively, using the same parameters described in Kitaura et al. (2018). Furthermore, the nuSSU region was amplified using NS1 (GARGAS; DEPRIEST, 1996) and 18L (HAMBY et al.; 1988) or nu-ssu-0072-5' and nu-ssu-1750-3' (GARGAS; DEPRIEST, 1996), according to Schultz and Büdel (2003).

The sequence dataset consisted of sequences of a *Lichinaceae* group established in Schultz and Büdel (2003*)*, with: *Anema nummularium, Ephebe brasiliensis, Heppia adglutinata, H. despreauxii, H. conchiloba, Pterygiopsis guyanensis, Pyrenopsis* sp., *Thyrea*

confusa and *T. pachyphylla.* The species *Lichina pygmaea* (GenBank accession number AF282909) and *Lichina confinis* (AF336897) were used as outgroups.

The alignment was performed in Geneious v9.1.2 with the MAFFT v7.308 algorithm (KATOH et al., 2002; KEARSE et al., 2012). The phylogenetic trees were estimated with the Bayesian (BA) and Maximum Likelihood (ML) approaches, using the MrBayes 3.2.7a (HUELSENBECK; RONQUIST, 2001; RONQUIST; HUELSENBECK, 2003) and RaxML 8.2.12 (STAMATAKIS, 2014) softwares, in the Cipres web portal (https://www.phylo.org/) (MILLER; PFEIFFER; SCHWARTZ, 2010). To the BA analysis, a chain length of 10,000,000 generations sampled every 1,000 steps were established and the 50% majority rule consensus tree was generated with a burn-in of 25%. The ML analysis was performed with 1,000 bootstrap replications. Both analyses were performed with the GTR+G nucleotide substitution and site heterogeneity model. FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) was used to check and edit the trees of both BA and ML analyses. The posterior probabilities above 95 (BA) and bootstrap values above 70 (ML) were considered significant for the phylogenetic relationship hypothesis.

The morphological and anatomical studies were made using an Olympus SZ40 stereomicroscope and Zeiss light microscopy. Pictures were made with a Cannon Rebel T3i camera coupled with the stereomicroscope.

Results

Five specimens of *Heppia* were collected in Bodoquena and Corumbá municipalities, respectively, in the Atlantic Forest and Pantanal biomes. Sequences of nuSSU, mtSSU and nuITS were generated, but not for all specimens (Table 2). The sequences of mtSSU and nuITS regions were generated to construct a genetic barcode library of tropical lichenized fungi that will be used in future studies, and they were not included in the phylogenetic analyses due to the absence of other sequences of *Heppia* available to comparison. On the other hand, the sequences of nuSSU were aligned and analyzed with one group of *Lichinaceae* established by Schultz and Büdel (2003), where *Heppia* were clustered, resulting in an alignment of 12 sequences and 1767 base pairs.

Table 2. Information of *Lichinaceae* used in the phylogenetic analysis. Lines in bold are sequences generated in the present study.

	nuITS	mtSSU	nuSSU
Anema nummularium	-	-	AF336888
Ephebe brasiliensis	-	-	AF336892
Heppia adglutinata	-	-	AF336893
H. despreauxii	-	-	AF336894
H. conchiloba	-	-	AF282908
H. exalbescens	**OQ0058812**	**-**	**OQ0058813**
H. exalbescens	**-**	**OQ0058814**	**-**
H. exalbescens	**OQ0058811**	**OQ0058815**	**-**
H. exalbescens	**OQ0058810**	**-**	**-**
Pterygiopsis guyanensis	-	-	AF282914
Pyrenopsis sp.	-	-	AF338236
Thyrea confusa	-	-	AF282915
Thyrea pachyphylla	-	-	AF336909

The phylogenetic relationships of the nuSSU region followed the found by Schultz and Büdel (2003), and the sequence of *H. exalbescens* grouped as a separate lineage with the other *Heppia* species, *H. conchiloba*, *H. despreauxii* and *H. adglutinata*, with high support values (Figure 2).
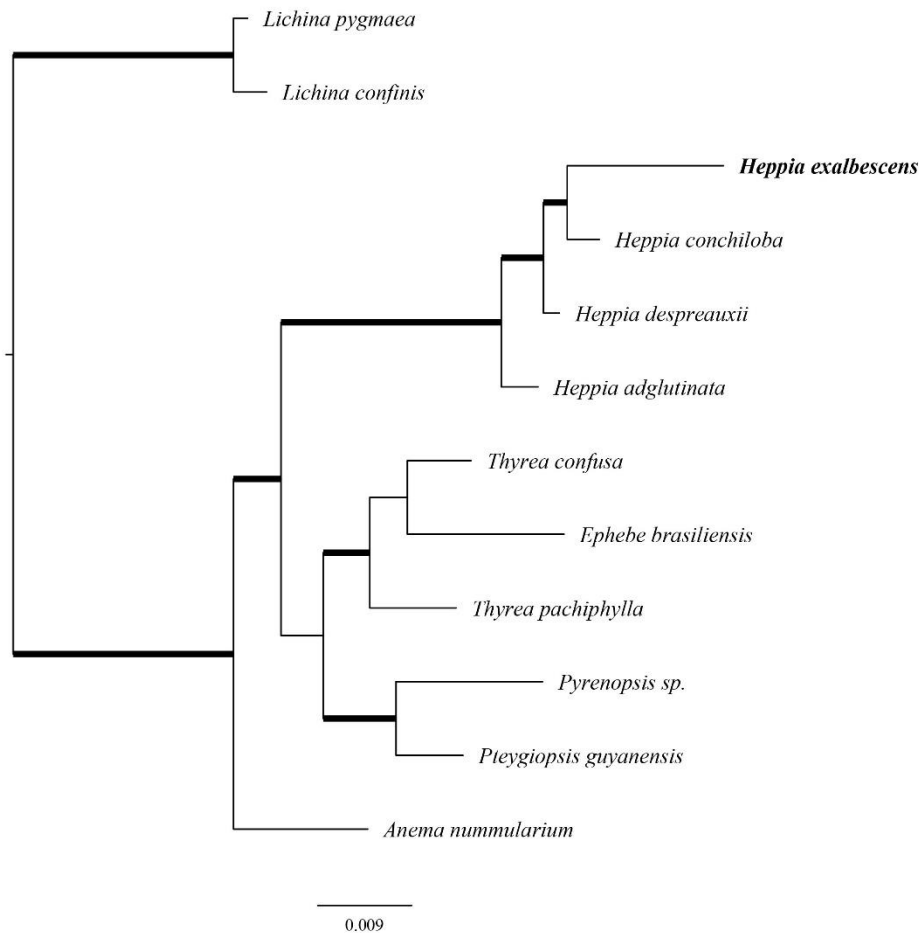

Figure 2. The phylogenetic tree resulted from the BA and ML approaches of the *Lichinaceae* nuSSU region. Ticker branches correspond to support values >90 and bootstrap values >75, considered significant to phylogenetic relationships hypothesis.

Heppia exalbescens **Kitaura & M.C. Scur, sp. nov**   **Figure. 3A-F**
MycoBank number. 846924

Etymology. The "*exalbescens*" epithet means to become white, which refers to the color greenish gray of the thallus that became whitish. The thallus is covered by the pruina with the aged, turning whitish.

Type. Brazil, Mato Grosso do Sul State, Bodoquena Municipality, Sol de Maio Farm, base of the stone outcrops, within the vegetation on the hill, terricolous, 20°35'33''S; 56°51'40''W 476 m alt., 05 Nov 2018. leg. M.J. Kitaura 4641 (holotype CGMS).

Description. Thallus squamulose, greenish gray, matt, opaque, green to blackish green under stereomicroscopy, polyphyllous, irregularly spread. Squamule 0.5–3.5 mm board, dispersed to overlapping, attached in points, adnate to ascending; upper surface smooth to rugulose at naked eye, rugulose and covered by pruina under 10× magnification. Apices of the squamule rounded, plane to ascending, smooth; lateral margin of squamule smooth, ascending, undulated. Pruina squamule-like or placodioid, 0.1 × 0.1 mm, laminal, amphithecia and lower margin when revolute. Lower side greenish to whitish, smooth under different magnifications. Isidia, lobules

and soredia absent. Thallus attached by medullar hyphae, on the substrate (organic material). Thallus 100–125 µm thick; upper cortex absent, with irregular outline; epinecral layer 2.5–5.0 µm thick, amorphous appearance; cyanobacteria abundant, green, simple or grouped in glomerulus, spherical, cell 5.0–10 µm diam.; gelatin sheath scarce, hyalin to yellowish near the upper surface; medullar region of closely aggregate hyphae, irregular arrangement, 20–75 µm (2–10 cells) thick, projecting (periclinal) columnar hyphae within the photobiont layer, 3–10 cells high; lower cortex absent, with irregular outline, attaching on the substrate by the most external cells of medullar region. Apothecia 0.7-3.0 mm diam., laminal, sessile, disc reddish; thalline margin whitish, pruinose, smooth. Amphithecia whitish, smooth, pruinose. Hymenia 100–150 µm thick; subhymenia 37.5–50 µm, hyaline; hypothecia 37.5–75 µm, paraplectenchymatous, hyaline, parahymenial tissue originating of hypothecia, proso- to euthyplectenchymatous tissue, 25 µm (4-6 cells) thick; thalline exciple 100 µm thick at the apices, 200–350 µm thick at the base; basal paraplectenchymatous tissue of the apothecia (lower cortex) up to 10 µm (2–4 cells) thick. Ascospores ellipsoid to fusiform, 15.0–17.5(– 22.5) × 5.0–7.5(–10.0) µm, usually immature, one to two-celled, acute apices, 8 per ascus (Figure 3F). Pycnidia immersed. Conidia bacilariform.

Examined material. Brazil, Mato Grosso do Sul State, Bodoquena municipality, Sol de Maio Farm, base of the stone outcrops, within the vegetation on the hill, terricolous, 20°35'33'' S; 56°51'40''W, -20.592528, -56.861139, 476 m alt., 05 Nov 2018. leg. M.J. Kitaura 4643. Idem, Santa Laura Farm, Morro dente de cão, on the top, saxicolous, 20°47'04.6"S 56°45'02.1"W, - 20.784611°, -56.750583°, 445 m alt., 07 Nov 2018. leg. M.J. Kitaura 4674. Idem, Corumbá municipality, São João Farm, 19°10'49''S, 57°32'19''W, 210 m elev., terricolous, 31 Jan 2021, leg. M.J. Kitaura & M.C. Scur 5291, 5303.

Diagnoses. *Heppia exalbenscens* is characterized by the squamulose thallus, polyphyllous, constituted by rounded squamules that are covered by pruina with the age, turning whitish (Figures 3A-B). The thallus is covered by the epinecral layer with the aged (Figure 3C-D); upper and lower cortices absent, with irregular outline; and 20–75 µm of the medullar hyphae.

The lower cortex was observed in the squamules with apothecia (Figure 3E), reported as basal paraplectenchymatous tissue of the apothecia, with up to 10 µm (2–4 cells) thick.

Discussion

The nuSSU sequence of *H. exalbescens* clustered with the sequences of *Heppia*, available on the GenBank with high support value. The sequences were generated for the study of systematic position of the lichen genus *Heppia*, and our analysis corroborated with previous study (SCHULTZ; BÜDEL, 2003).

On the other hand, the nuITS sequences of *H. exalbescens* showed only 20% similarity with *H. solorinoides* (GenBank accession number MZ391139, unpublished study) in analysis through the BLAST tool. The *H. solorinoides* has thallus with peltate lobes, 3–6 mm diam., irregularly orbicular, with involute margins, the upper cortex has 20–60 µm thick, and medullary hyphae are globose cells at the base, 4.5–7.0 µm thick (HENSSEN, 1994); whereas that *H. exalbescens* has squamulose thallus, 0.5–3.5 mm board, dispersed to overlapping, with margin plane to ascending, upper cortex with a simple layer of cells at the margin, ca. 10 µm thick, and amorphous appearance at the older parts, and medullar hyphae constituted by closely aggregate globose cells, irregular arrangement, 20–75 µm (2–10 cells) thick. Our sequences were generated from different specimens and localities, and are considered as good despite of the distance with *H. solorinoides,* that should be carefully revised. The nuITS sequences of *H. exalbescens* present 0.5% of divergence between the specimens from Bodoquena and Corumbá. Altought there are no sequences of *Heppia* nuITS region on the GenBank to perform a

phylogenetic analysis, the sequences generated in the present study will be a reference to future studies, especially to studies of the regional lichenized fungi.

Regarding the morphological analyses, *H. conchiloba* and *H. exalbescens* have an upper surface covered by pruina. However, *H. conchiloba* has peltate thallus, with upper cortex up to 50 µm thick and the epidermal layer with up to 50 µm thick; whereas that *H. exalbescens* is a squamulose species, ecorticate, with an irregular outline, and epinecral layer with 2.5–5.0 µm thick. In addition, *H. conchiloba* is reported to North America (HENSSEN, 1994).
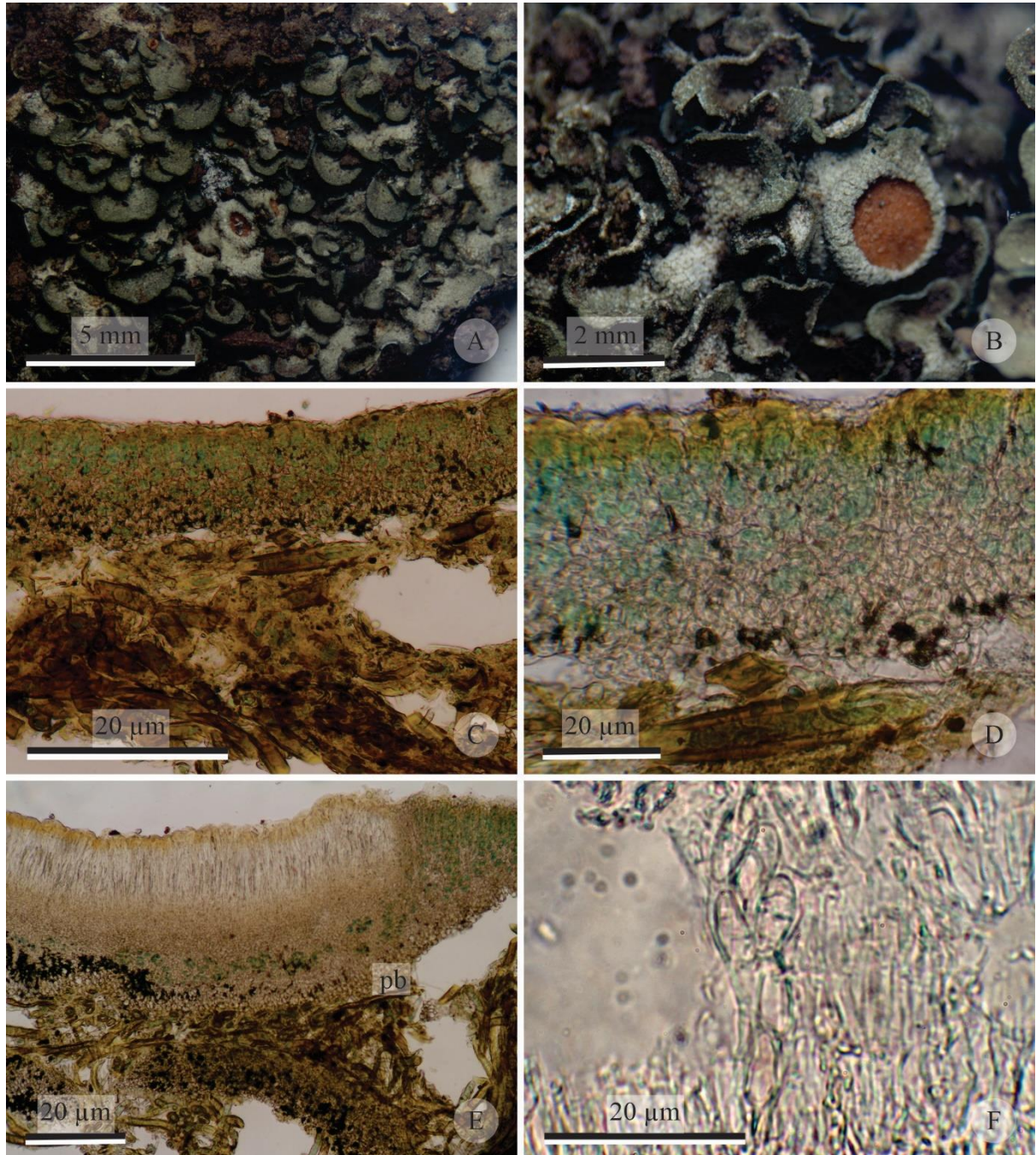

Figure 3. A-F. *Heppia exalbenscens*. A-B. Details of the surface. A. Holotype with greenish grey to whitish thallus. B. MJK 4643 specimen with pruina. C-D. Transversal section of the thallus C. Detail of the thallus attached on the substrate. D. Thallus with irregular outline. E.

Diametral section of the apothecia. pb = basal paraplectenchymatous tissue. F. Detail of the ascus with ascospores.

Heppia dexpreauxii, reported to Europe and Africa, has thallus with deep depression on the upper surface, and *H. echinulate*, Asia and Africa, has spines on the thallus (HENSSEN, 1994). Both species differ from the *H. exalbescens* that has upper surface smooth to rugulose, without depressions and ornaments.

Heppia arenacea (recorded from Asia), *H. conchiloba* (North America), *H. despreauxii* (South America)*,* and *H. solorinoides* (Asia and Europe), have thick upper cortex (HENSSEN, 1994; CÁCERES et al., 2017; SCHULTZ, 2005), differing of upper cortex of *H. exalbescens* that is ecorticate. The squamules of *H. exalbescens* are only covered by epinecral layer with the aged.

The lower cortex is reported to *H. adglutinata* and *H. echinulate*, but it was not observed in the *H. exalbescens* thallus. The lower cortex was observed in *H. exalbescens*, when the squamule has apothecia and described as basal paraplectenchymatous tissue of the apothecia.

The granulose and blackish thallus of *H. lutosa* differ from the other species, and an integrative study is still required. *Heppia antarctica* has hypothallus and biatorine apothecia (DODGE, 1948), which were not observed in other genus species and is unlikely to belong to *Heppia*.

Conclusion

The integrative studies have revealed great diversity of the lichens in the Mato Grosso do Sul state, including poorly studied taxa as the genus *Heppia*. Increasing the knowledge of lichen species diversity, we highlight the importance of conserving natural areas in highly impacted zones. Monocultures and livestock are expanding and shrinking natural areas of Pantanal, Cerrado and Atlantic Forest biomes. Only by knowing diversity will it be possible for decision-makers to create public policies.

Identification key

1a. Thallus ecorticate, with irregular outline — 2
1b. Thallus with upper surface and/or lower surface — 4
2a. Thallus with spines on the surface — *H. echinulata*
2b. Thallus without spines on the surface — 3
3a. Thallus squamulose, greenish grey, with pruina on the older part — *H. exalbescens*
3b. Thallus squamulose to granular, blackish, without pruina — *H. lutosa*
4a. Thallus with upper and lower cortex — *H. adglutinata*
4b. Thallus strictly with upper cortex — 5
5a. Thallus with deep depression on the upper surface — *H. despreauxii*
5b. Thallus without depression — 6
6a. Thallus with pruina — *H. conchiloba*
6b. Thallus without pruina — 7
7a. Thallus squamulose, resembling an areolate crust, sand-colored to pale ochre, and with soils particles incorporated in all parts of the thallus — *H. arenacea*
7b Thallus peltate, with whitish warts, and without soils particles incorporated in parts of the thallus — *H. solorinoides*

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