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New *Arthoniales* from Amapá (Amazonian North Brazil) show unexpected relationships

André APTROOT and Marcela Eugenia da Silva CÁCERES

Abstract: Recent field trips to Amapá and the adjacent states of Amazonas and Pará uncovered unknown lichen species with unexpected relationships. The following new species of *Arthoniales* are described, mainly from Amapá in North Brazil: *Neosergipea hyphosa*, a corticolous species with an olive-green thallus, central part verrucose, marginal zone of 5–10 mm wide consisting of procumbent branching rhizohyphae, surrounded by a pinkish brown hyphal prothallus *c.* 3 mm wide into which the rhizohyphae protrude, globose ascomata, *c.* 0·1 mm diam., arranged in stromatoid groups of 5–15, with hyaline walls, without ascospores; *Neosergipea septoconidiata*, a corticolous species with a bluish grey thallus and bright orange, mostly conical pycnidia with fluffy hyphal surface, hyaline conidia, 3–6-septate, filiform, 12–23 × 1·5–2·0 µm, with rounded ends; *Nyungwea pycnidiata*, from a termite nest on a tree, with abundant pycnidia that are whitish, papillate, smooth, *c.* 0·1–0·2 mm diam., *c.* 0·2–0·4 mm high, at the base covered by thallus, and hyaline conidia that are simple, ellipsoid, 2·5–4·0 × 1·5–2·0 µm; and *Opegrapha ramisorediata*, a corticolous species with a thallus with initially rounded soralia that soon cover the complete thallus with a layer of fine yellowish green branched soredia, and containing gyrophoric acid. Apothecia are not known from any of these species but sequencing elucidated their generic affiliation. The genera *Neosergipea* and *Nyungwea* were previously monotypic and *Nyungwea* was known only from Africa. *Nyungwea anguinella* (Nyl.) Aptroot comb. nov. is a further new species in the latter genus, and through these two extra species we now know about the morphology of the pycnidia and apothecia of *Nyungwea*, which were unknown in the type species (a sterile crust).

Key words: Amazonas, corticolous, lichen, *Neosergipea*, *Nyungwea*, *Opegrapha*, Pará

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Introduction

Although the study of lichens in the Amazon started almost 200 years ago with collections by Richard Spruce (1817–1893), the knowledge of this flora is still very scant today. Until 2012, less than 30 lichen species were known from each of the Amazonian states of Rondônia, Acre and Amapá.

In 2012, a project to survey lichens commenced in the primary rainforests of the Amazon basin, initially in Rondônia which is situated at the SW end of the Amazon. The results published so far (Aptroot & Cáceres 2013, 2014*a*, *b*, 2016; Cáceres *et al.* 2014*a*, *b*) show that the region may have one of the highest lichen diversities in the world. It is especially surprising that the majority of the species found were still undescribed. This includes species that were found in abundance in each region visited, the prime example being *Astrothelium eustomurale* Aptroot & Cáceres which was for many years the most common undescribed lichen known worldwide. Two subsequent trips to the state of Amapá at the mouth of the Amazon River on both sides of the equator (Cáceres & Aptroot 2016) and the heart of the Amazon near Manaus (Cáceres & Aptroot 2017) were organised.

It was found that the vast majority of the identified species were new to the state, even in Amazonas. There is clear overlap between the regions, however every region and every forest yielded undescribed species that have not been found anywhere else.

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Arthoniales comprises *c.* 25% of the huge lichen diversity in tropical rainforests (Aptroot & Cáceres 2014*b*). Here, we describe new species in several genera of *Arthoniales*, all from Amapá, but one species is already known from several other Amazonian states and these specimens are also cited here. Most species have been sequenced and their respective generic affinities were, in part, unexpected.

Material and Methods

Identification and descriptive work was carried out in Itabaiana, Universidade Federal de Sergipe, using a Leica EZ4 stereomicroscope and a Leica DM500 compound microscope, and in Soest using an Olympus SZX7 stereomicroscope and an Olympus BX50 compound microscope with interference contrast, connected to a Nikon Coolpix digital camera. Sections were mounted in water, in which all measurements were also taken. The specimens from this study are preserved in ISE and ABL. The chemistry of the type specimens was investigated by thin-layer chromatography (TLC) using solvent A (Orange *et al*. 2001).

Total DNA was extracted from dry specimens employing a modification of Murray & Thompson's (1980) protocol. A portion was blended with the aid of a micropestle in 600 µl CTAB buffer (CTAB 2%, NaCl 1·4 M, EDTA (pH 8·0) 20 mM, Tris-HCl (pH 8·0) 100 mM). The resulting mixture was incubated for 15 min at 65 °C. A similar volume of chloroform: isoamilalcohol (24:1) was added and carefully mixed with the samples until their emulsion. It was then centrifuged for 10 min at 13 000 **g** and the DNA in the supernatant was precipitated with isopropanol. After a new centrifugation of 15 min at the same speed, the pellet was washed in 70% cold ethanol, centrifuged again for 2 min and dried. It was finally resuspended in 200 µl of ddH_2O. PCR amplification was performed with the primers ITS1F and ITS4 (White *et al*. 1990; Gardes & Bruns 1993) for ITS. PCR reactions were performed under a program consisting of a hot start at 95 °C for 5 min, followed by 35 cycles at 94 °C, 54 °C and 72 °C (for 45, 30 and 45 s respectively) and a final 72 °C step for 10 min. PCR products were checked in 1% agarose gels and positive reactions were sequenced with primer ITS4. Chromatograms were checked searching for putative reading errors, and these were corrected.

Sequences selected for alignment came mainly from Ertz & Tehler (2011) and Frisch *et al*. (2015). Sequences were first aligned in MEGA 5.0 (Tamura *et al*. 2011) software with its ClustalW application and then corrected manually. Gblocks 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) was used to eliminate poorly aligned positions with the less stringent options available. The resulting alignment was loaded in PAUP* 4.0b10 (Swofford 2001) and subjected to MrModeltest 2.3 (Nylander 2004). The model GTR+Γ was selected and implemented in MrBayes 3.1 (Ronquist & Huelsenbeck 2003), where a Bayesian analysis was performed (two simultaneous runs, six chains, temperature set to 0·2, sampling every 100th generation) until convergence parameters were met after *c.* 4 960 000 generations, standard deviation having fallen below 0·01. Finally, a full search for the best-scoring maximum likelihood tree was performed in RAxML (Stamatakis 2006) using the standard search algorithm (2000 bootstrap replications). The significance threshold was set above 0·95 for posterior probability (PP) and 70% bootstrap proportion (BP).

Results

Neosergipea hyphosa Aptroot & M. Cáceres sp. nov.

MycoBank No.: MB 821291
GenBank No.: MF093744

Corticolous *Neosergipea* with an olive-green thallus, central part verrucose, marginal zone of 5–10 mm wide consisting of procumbent branching rhizohyphae, surrounded by a pinkish brown hyphal prothallus *c.* 3 mm wide in which the rhizohyphae protrude, globose ascomata *c.* 0·1 mm diam., arranged in stromatoid groups of 5–15, with hyaline walls, without ascospores.

Type: Brazil, Amapá, Macapá, Fazendinha, Ecotel garden, alt. *c.* 5 m, 0°03'N, 51°07'W, on tree bark in garden, 22 August 2015, *M. E. S. Cáceres & A. Aptroot* ISE 27595 (ISE—holotype; ABL—isotype).

(Fig. 1)

Thallus c. 0·1 mm thick, dull, not corticate, olive-green; central part verrucose, with upright hyphal tips, marginal zone 5–10 mm wide, olive-green with violet-grey hue, completely covered or even largely consisting of procumbent branching thin rhizohyphae, surrounded by a pinkish, pale to dark violet-brown (darkest at the outer edge) hyphal prothallus, *c.* 3 mm wide, into which the rhizohyphae protrude. *Medulla c.* 50–100 µm thick, whitish, strongly contrasting in colour with the thallus surface, without calcium oxalate crystals. *Hyphae* hyaline to pale violet-brown towards the margins, rather straight, branched and septate, 2–3 µm wide, minutely verruculose, without superficial crystals, all IKI− . *Algae* trentepohlioid, cells in branched filaments, *c.* 5–7 × 8·0–10·5 µm.

Ascomata globose, *c.* 0·1 mm diam., arranged in stromatoid groups of 5–15, with hyaline walls, without ascospores.

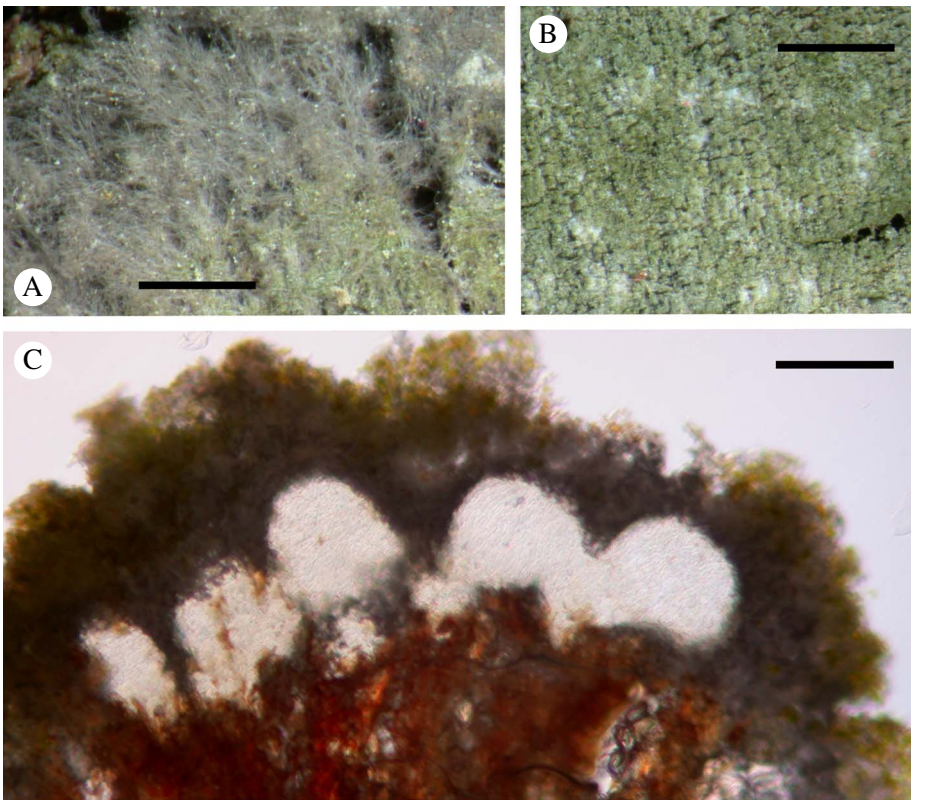

FIG. 1. *Neosergipea hyphosa* (isotype). A, thallus margin with rhizohyphae. B & C, central part of thallus with immature ascomata; B, surface view; C, section. Scales: A & B = 1 mm; C = 100 µm. In colour online.

Pycnidia not observed.

Chemistry. Thallus UV−, C−, P−, K−. TLC: no substances detected.

Etymology. Named after the hyphose thallus surface.

Ecology and distribution. On tree bark in garden; known only from Brazil.

Discussion. *Arthoniales* constitute around a quarter of the lichen diversity in tropical forests (Aptroot & Cáceres 2014*b*; Cáceres *et al*. 2014*b*). Many of these are not currently identifiable, partly because no revisions exist for major genera such as *Arthonia* Ach., *Arthothelium* A. Massal. and *Opegrapha* Ach., and partly because the specimens (and in most cases probably the species) do not produce ascomata, although conidia are frequently produced, often characteristic or in characteristic conidiomata, but these do not allow for a generic placement. The reinstatement of the largely sterile genus *Herpothallon* Tobler (Aptroot *et al*. 2009) marked the beginning of a new direction. With DNA sequencing, sterile specimens and species can now be linked to known genera; even new genera are being described for sterile *Arthoniales* (Frisch *et al*. 2015). We sequenced this characteristic specimen (Fig. 2) and it clustered close to the hitherto monotypic genus *Sergipea* M. Cáceres *et al*. (Aptroot *et al*. 2013), now renamed *Neosergipea* M. Cáceres *et al*. (Lücking *et al*. 2016) because a fossil dinoflagellate spore genus exists with the name *Sergipea*.The new species is described in the genus *Neosergipea*. There is also a fourth species in the genus *Neosergipea* which occurs

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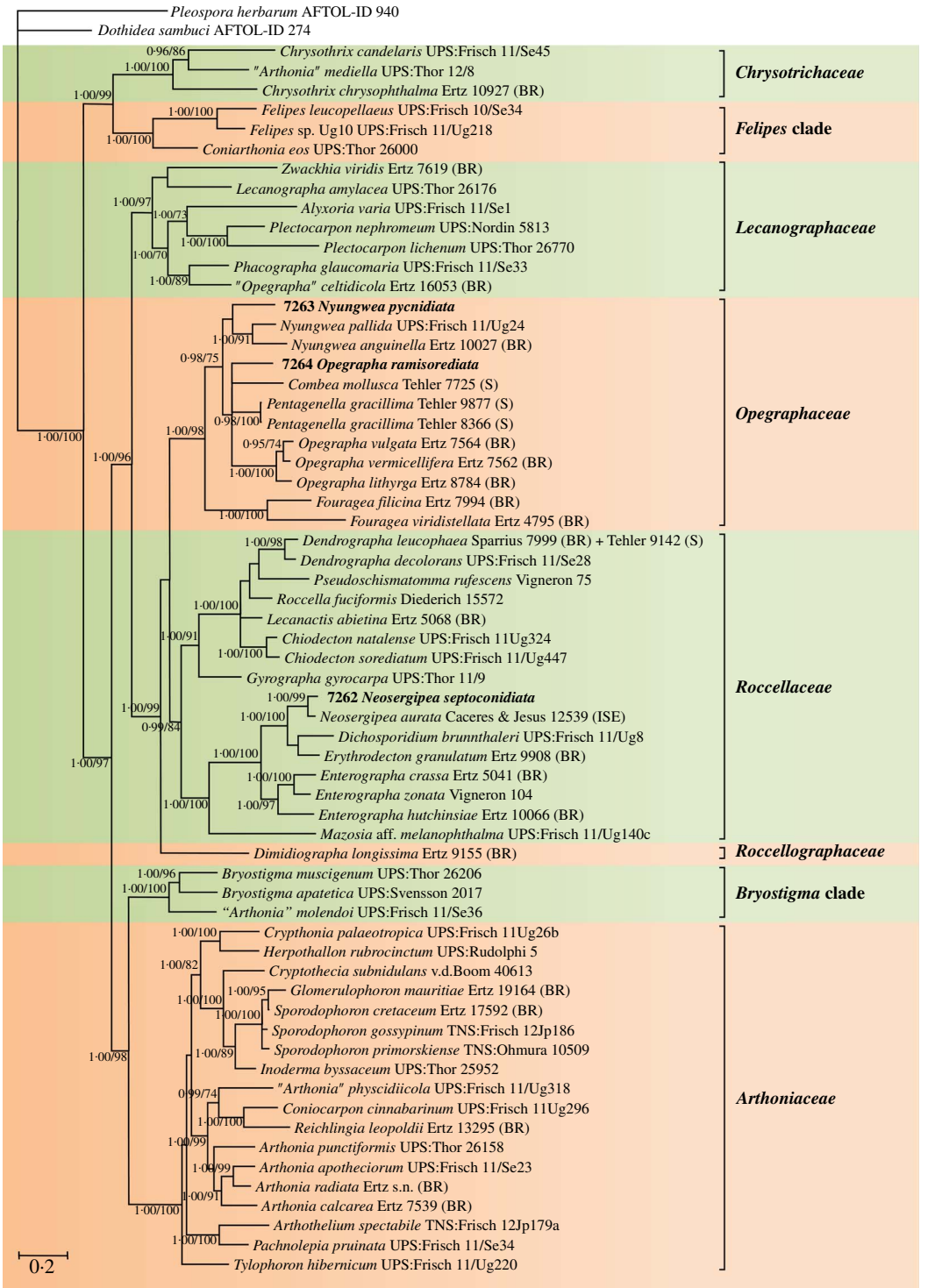

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in the restinga (coastal forests) in Santa Catarina. *Neosergipea hyphosa* has a very characteristic hyphal morphology; in addition, it lacks the anthraquinones and thus the orange or yellow colour of the other known species of *Neosergipea*.

Neosergipea septoconidiata Aptroot & M. Cáceres sp. nov.

MycoBank No.: MB 821292

Corticolous *Neosergipea* with a bluish grey thallus and bright orange, mostly conical pycnidia with a fluffy hyphal surface, hyaline conidia, 3–6-septate, filiform, 12–23 × 1·5–2·0 µm, with rounded ends.

Type: Brazil, Amapá, Mazagão, Reserva Extrativista Maracá, alt. *c.* 30 m, 0°01'N, 51°51'W, on tree bark in tall primary forest, 21 August 2015, *M. E. S. Cáceres & A. Aptroot* ISE 27567 (ISE—holotype; ABL—isotype).

(Fig. 3A–C)

Thallus less than 0·1 mm thick, dull, not corticate, bluish grey to olivaceous, usually surrounded by a *c.* 0·3 mm wide brown somewhat hyphal prothallus. *Medulla* not clearly distinguishable in colour, filled with large hyaline calcium oxalate crystals. *Hyphae* hyaline, rather curvy, branched and septate, 1–2 µm wide, smooth, without superficial crystals, all IKI−. *Algae* trentepohlioid, cells solitary or in unbranched crenulate filaments, *c.* 4–6 × 5–9 µm.

Ascomata not observed.

Pycnidia abundant, bright orange-yellow to orange, mostly conical, with fluffy hyphal surface, *c.* 0·2–0·4 mm diam., *c.* 0·2–0·3 mm high, with cirrhus of extruding conidia at the tip. *Conidia* hyaline, 3–6-septate, filiform, 12–23 × 1·5–2·0 µm, with rounded ends.

Chemistry. Thallus UV−, C−, P−, K−; pycnidia UV+ pink, K+ blood red. TLC: an anthraquinone, probably parietin.

Etymology. Named after the septate conidia.

Ecology and distribution. On tree bark in tall primary forest; known only from Brazil.

Discussion. This species shares the bright orange-yellow to orange colour of the type species of *Neosergipea* (Aptroot *et al*. 2013, as *Sergipea*) but it differs in the unique conidiomata with septate conidia of very different length but of constant width; see also discussion under the previous species. This species is common and widespread in the Amazon and we report it here also from the states of Amazonas and Pará. Ascomata have not been found; pycnidia are usually present but some younger specimens mainly show only young pycnidia. There is some variation in colour but not in chemistry.

Additional specimens examined. **Brazil:** *Pará*: Villa Nazaré, 85 km N of Dom Eliseu, 3°53'56"S, 48°05'44"W, on bark of tree, *c.* 120 m alt., 2016, *M. E. S. Cáceres & A. Aptroot* ISE 40141 (ISE; ABL); Fazenda Pantera, 85 km N of Dom Eliseu, 3°49'54"S, 48°03'37"W, on bark of tree, *c.* 120 m alt., 2016, *M. E. S. Cáceres & A. Aptroot* ISE 40354 (ISE; ABL). *Amazonas*: Manaus, Reserva Florestal Adolpho Ducke, along trails in vicinity of field station, alt. 80 m, 2°56'S, 59°57'W, on tree bark in primary rainforest, 2016, *M. E. S. Cáceres & A. Aptroot* ISE 28238, 28253, 28470 & 28548 (ISE; ABL).

Nyungwea pycnidiata Aptroot & M. Cáceres sp. nov.

MycoBank No.: MB 821293
GenBank No.: MF093746

Termitariicolous *Nyungwea* with abundant pycnidia that are whitish, papillate, smooth, *c.* 0·1–0·2 mm diam., *c.* 0·2–0·4 mm high, at the base covered by thallus; hyaline conidia, simple, ellipsoid, 2·5–4·0 × 1·5–2·0 µm.

Type: Brazil, Amapá, Macapá, Fazendinha, Ecotel garden, alt. *c.* 5 m, 0°03'N, 51°07'W, on termite nest on tree in garden, 22 August 2015, *M. E. S. Cáceres & A. Aptroot* ISE 27602 (ISE—holotype; ABL—isotype).

(Fig. 3D)

FIG. 2. Phylogenetic relationships within *Arthoniales* based on ITS sequences using Bayesian analysis highlighting the position of the three new species (in bold) described here: 7262 = *Neosergipea hyphosa*; 7263 = *Opegrapha ramisorediata*; 7264 = *Nyungwea pycnidiata*. Posterior probabilities (PP) ≥95 are shown to the left and Maximum Likelihood bootstrap values (BP) ≥70% are shown to the right at the nodes. *Pleospora herbarum* and *Dothidea sambuci* used as outgroups.

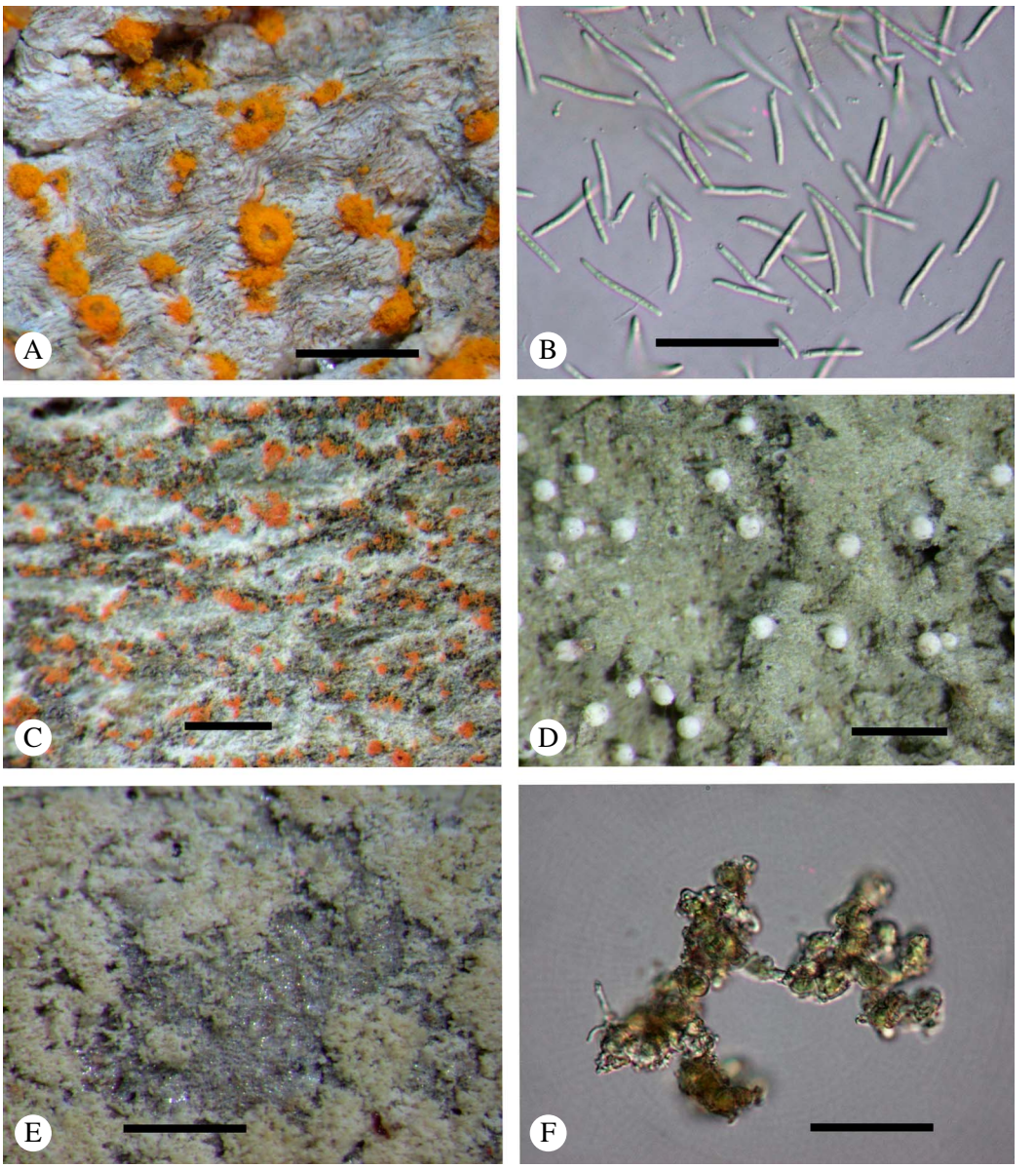

FIG. 3. A–C, *Neosergipea septoconidiata* (A & B, isotype; C, *Cáceres & Aptroot* 28238); A, habitus with pycnidia; B, conidia; C, juvenile thallus with immature pycnidia. D, *Nyungwea pycnidiata* (isotype), habitus with pycnidia. E & F, *Opegrapha ramisorediata* (isotype); E, habitus with soredia; F, soredia. Scales: A, C–E = 0·5 mm; B & F = 25 µm. In colour online.

Thallus less than 0·1 mm thick, dull, not corticate, greyish green, resembling clay, without prothallus. *Medulla* not clearly distinguishable in colour, without calcium oxalate crystals. *Hyphae* hyaline, rather brittle, branched and septate, 2–3 µm wide, rough from superficial lecanoric acid crystals, all IKI−. *Algae* trentepohlioid, mostly solitary, in a dense layer, *c.* 4–5 × 5–8 µm.

Ascomata not observed.

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Pycnidia abundant, whitish, papillate, smooth, *c.* 0·1–0·2 mm diam., *c.* 0·2–0·4 mm high, at the base covered by thallus. *Conidia* hyaline, simple, ellipsoid, 2·5–4·0 × 1·5–2·0 µm, with rounded ends.

Chemistry. Thallus UV−, C−, P−, K−; pycnidia C+ red. TLC: lecanoric acid.

Etymology. Named after the pycnidia.

Ecology and distribution. On a termite nest on tree in garden; known only from Brazil.

Discussion. With the advent of DNA sequencing, sterile specimens and species can be linked to known genera, and new genera are being described for sterile *Arthoniales* (Frisch *et al*. 2015). We sequenced this distinctive specimen (Fig. 2) and it clustered close to the monotypic genus *Nyungwea* Sérus. *et al*. (Sérusiaux *et al*. 2006), a genus known only from a sterile specimen of the type species collected in Africa. We therefore describe our new species in this genus. Following the recent realization that termite nests regularly yield essentially terricolous lichens that are very different from those in the surrounding rainforest (Aptroot & Cáceres 2014*a*), we will in future pay special attention to the termite nest habitat. This new species was abundant in the type location.

Opegrapha ramisorediata Aptroot & M. Cáceres sp. nov.

MycoBank No.: MB 821294
GenBank No.: MF093745

Corticolous *Opegrapha* having a thallus with initially rounded soralia that soon cover the complete thallus with a layer of fine yellowish green soredia, and containing gyrophoric acid.

Type: Brazil, Amapá, Macapá, Fazendinha, Ecotel garden, alt. *c.* 5 m, 0°03'N, 51°07'W, on bark on tree in garden, 22 August 2015, *M. E. S. Cáceres & A. Aptroot* ISE 27617 (ISE—holotype; ABL—isotype).

(Fig. 3E & F)

Thallus thin, pale greenish mauve, with initially rounded soralia of *c.* 0·2–0·4 mm diam. that soon cover the complete thallus with a layer of fine yellowish green soredia, surrounded by a thin brown hypothallus line. *Medulla* not clearly distinguishable in colour, without calcium oxalate crystals. *Soredia* very loosely built, more or less following the branching pattern of the *Trentepohlia* algal cells, *c.* 40–80 µm diam., individual branches hyaline, *c.* 10–15 µm wide, densely encrusted with hyaline crystals (probably of gyrophoric acid). *Hyphae c.* 2–3 µm wide, hyaline, densely encrusted by hyaline crystals (probably of gyrophoric acid), all IKI−. *Alga Trentepohlia*, hyphal to moniliform, cells 8–10 × 10–14 µm.

Ascomata and pycnidia not observed.

Chemistry. Thallus UV−, C+ red, P−, K−. TLC: gyrophoric acid.

Etymology. Named after the branched soredia.

Ecology and distribution. On tree bark in garden; known only from Brazil.

Discussion. This is a characteristic mauve, sterile sorediate crust. That it indeed belongs to or is at least close to the genus *Opegrapha*, which was inferred based on similarities with another sorediate *Opegrapha* species, was confirmed by sequencing (Fig. 2). So far only one tropical sorediate species has been described, viz. *O. salmonea* Ertz & Diederich from the Seychelles (Diederich *et al*. 2017), differing markedly from our species by the presence of norstictic acid instead of lecanoric acid. Phylogenetically our new species clusters with the clade in which the species of *Opegrapha* s. str. (Ertz & Tehler 2011) cluster. Two further small genera, viz. *Combea* De Not. and *Pentagenella* Darb., also cluster with *Opegrapha* s. str. but are kept separate for the moment because they are morphologically very distinct with their fruticose thallus and because specimen sampling in *Opegraphaceae* phylogeny is still preliminary. The description of this new crustose species in any of these fruticose genera is not a serious option, which leaves *Opegrapha* as the logical genus.

Discussion

Three of the species described above could be sequenced from their type specimens (Fig. 2). This made a generic assignment of some of the many non-fertile rainforest

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Arthoniales possible. Surprisingly, three of the new species are assigned to previously monotypic genera, viz., *Neosergipea* (already known from Brazil but outside the Amazon) and *Nyungwea* (which was known only from Africa). Both genera are named, now somewhat inappropriately, after the region where their type species was originally found.

The phylogenetic analysis (Fig. 2) also reveals that a further species clusters with the genus *Nyungwea*, viz. *Nyungwea anguinella* (Nyl.) Aptroot comb. nov.; MycoBank No: MB 821295; basionym: *Stigmatidium anguinellum* Nyl., *Annales Sci. Nat., Bot., sér. 4* **19:** 381 (1863). This characteristic and common pantropical species was for a long time known as *Enterographa anguinella* (Nyl.) Redinger but was recently placed, based on preliminary phylogenetic analyses, in *Opegrapha*, although it differs markedly from every species ever put in that genus by the absence of a carbonized excipulum and the pale reddish discs. It is therefore not surprising that it clusters elsewhere, although the placement in *Nyungwea* was not anticipated. Fortuitously, the morphology of the pycnidia and apothecia of *Nyungwea* (which are unknown in the type species, a sterile crust) are now known by means of these two further species.

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