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Heterocyphelium leucampyx (*Arthoniales*, Ascomycota): another orphaned mazaediate lichen finds its way home

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Abstract: *Heterocyphelium* is a mazaediate genus containing a single species, *H. leucampyx*. The species was originally described from Cuba within the genus *Trachylia* (*Arthoniales*, *Arthoniaceae*) and later placed in various genera of the collective order *Caliciales* s. lat. For the past three decades, *Heterocyphelium* was considered an orphaned genus (*incertae sedis*) within the Ascomycota, since morphology alone could not resolve its systematic position. In this study, we added molecular data with the aim of resolving this uncertainty. Bayesian and maximum likelihood analyses of newly generated sequence data from the mitochondrial ribosomal RNA small subunit (mtSSU) and the RNA polymerase II second largest subunit gene (*RPB2*) provide clear evidence that *Heterocyphelium leucampyx* is nested within the order *Arthoniales*, in the family *Lecanographaceae*, sister to the genus *Alyxoria*. *Heterocyphelium* is a further example of parallel evolution of passive spore dispersal, prototunicate asci and the occurrence of a mazaedium in the Ascomycota, and another calicioid genus whose systematic placement could be eventually clarified by means of molecular data. *Heterocyphelium* is the fourth mazaediate genus in *Arthoniales*, in addition to *Sporostigma*, *Tylophorella* and *Tylophoron*.

Key words: *Alyxoria*, *Caliciales*, *Lecanographaceae*, mtSSU, phylogeny, *RPB2*

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Introduction

The mazaedium, a distinctive structure in which loose masses of ascospores accumulate in a layer covering the surface of the ascomata to be passively disseminated, was for a long time seen as the characteristic synapomorphy for the order *Caliciales* which was conceived as a natural or monophyletic group (e.g. Zahlbruckner 1926). For a long time in the premolecular era, classifications continued to regard the calicioid lichenized and non-lichenized fungi as a natural group included within a single order *Caliciales* (e.g. Poelt 1973), a view already questioned by Nannfeldt (1932) and later by Henssen & Jahns (1973). After analyzing morphological, chemical and ultrastructural traits with statistical and cladistic methods, Tibell (1984) suggested that this group was a highly polyphyletic assemblage of taxa which had evolved mazaedia and passive spore dispersal independently several times. Later, molecular phylogenetic studies have supported this view and shown that mazaediate fungi are spread over different classes within the Ascomycota (Prieto *et al.* 2013; Prieto & Wedin 2016). For instance, *Nadvornikia* (Harris 1990; Tibell 1996; Lumbsch *et al.* 2004) and *Schistophoron* (Tehler *et al.* 2009) were demonstrated to belong to *Graphidaceae* in the Ostropomycetes (Prieto *et al.* 2013; Rivas Plata *et al.* 2013),

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TABLE 1. *List of genera where* Heterocyphelium leucampyx *was successively placed together with their type species and current names.*

Genus	Type species	Current name
Trachylia Fries (1817)	*T. arthonioides* (Ach.) Fr. (basionym *Lecidea arthonioides* Ach.), designated by Fries (1822)	*Arthonia arthonioides* (Ach.) A.L. Sm.
Acolium (Ach.) Gray (1821)	*Calicium tympanellum* designated by Tibell (1984)	*Acolium inquinans* (Sm.) A. Massal.
Cyphelium Ach. (1815)	*Cyphelium tigillare* (Ach.) Ach.	*Calicium tigillare* (Ach.) Pers.
Heterocyphelium Vain. (1927)	*Heterocyphelium leucampyx* Vain.	*Heterocyphelium leucampyx* Vain.

Pyrgillus was placed within *Pyrenulales* in the Eurotiomycetes by Lumbsch *et al*. (2004), and *Tylophoron* belongs to the Arthoniomycetes, as shown by Lumbsch *et al*. (2009).

Historically, the name and systematic placement of *Heterocyphelium leucampyx* has undergone considerable changes (see Table 1). The species was first described from Cuba in *Trachylia* (Tuckerman 1862), a genus introduced by Fries & Sandberg (1817) to accommodate species characterized by circular, convex, rough and immarginate apothecia with spores 'spread in the margin'. The type species, *T. arthonioides* (Ach.) Fr. (basionym *Lecidea arthonioides* Ach.), was designated by Fries (1822) and later transferred to *Arthonia* as *A. arthonioides* (Ach.) A. L. Sm. (Smith 1911). Tuckerman (1888) transferred *T. leucampyx* to *Acolium* (Ach.) Gray, a genus in the *Caliciales* characterized by a crustose, flat, expanded, adnate, uniform thallus and apothecia that are cup-like, nearly sessile, cartilaginous and composed of a compact powdery mass forming a naked centre, the upper part flat or nearly globular (Gray 1821). However, the name *Acolium* had been used first by Acharius (1808) to create a subdivision of *Calicium* including three species (i.e. *Calicium turbinatum*, *C. stigonellum* and *C. timpanellum*), characterized by subsessile ascomata. *Acolium* has recently been resurrected to accommodate two species characterized notably by a dark excipulum that is strongly thickened at the base and by ornamented spores (Prieto & Wedin 2016). Before that, Zahlbruckner (1903) had transferred *Acolium leucampyx* to *Cyphelium*, another genus of *Caliciales*, and subsequently Vainio (1927) proposed the new genus *Heterocyphelium* in his treatment of the family *Coniocarpeae*, to accommodate species resembling *Cyphelium* but with 2-septate ascospores. Vainio did not, however, place the newly described genus within the *Coniocarpeae* or any other family. In the meantime, several other species have been considered conspecific with *Heterocyphelium leucampyx*. For example, Tibell (1996) synonymized *Tylophoron eckfeldtii*, described by Müller (1894) from Mexico, under *H. leucampyx*. *Tylophorum triloculare*, described by Müller from Australia (1893), was also added to the synonymy of *H. leucampyx* (Tibell 1987), considerably expanding the distribution range for the species. It is unclear in this respect whether Müller (1893) intended to describe a new genus different from *Tylophoron* (Nylander 1862), whether he deliberately changed the ending of the name, or whether he just produced an orthographic error. Again, in his monograph of the *Caliciales*, Tibell (1996) did not assign *Heterocyphelium* to any family. Therefore, the position of *H. leucampyx* has remained unresolved and it has not been included in any molecular phylogenetic study until now. Since Eriksson (1999) and until most recently Lumbsch & Huhndorf (2010), the genus has been listed under Ascomycota as *incertae sedis* in the *Outline of Ascomycota*.

In 2010, JLC collected material of *Heterocyphelium leucampyx* during fieldwork in Costa Rica which was sequenced by the third author (EG) and, based on blast results, preliminarily placed in *Arthoniales* without an assigned family. Comparison with DNA

sequence data obtained from a second specimen collected by the first author (DVDB) in Uganda in 2014 confirmed this result. The present study, therefore, aims to resolve the precise systematic position of *Heterocyphelium* within *Arthoniales* using additional molecular data in a phylogenetic framework.

Material and Methods

Morphological study

Macroscopic characters of the material were studied and measured using an Olympus SZ61 stereomicroscope and a Leica MS5 dissecting microscope. Macroscopic photographs were taken using a Keyence VHX-5000 digital microscope. Hand-cut preparations of ascomata were mounted in water or a solution of 5% potassium hydroxide, and for ascus structure in Lugol's iodine solution (1% I_2) without (I) or with KOH pretreatment (K/I) and studied using an Olympus CHR-TR45, an Olympus BX51 and a Zeiss Axioskop 2 compound microscope. For all measurements, the minimum and maximum values are given, all values rounded to the nearest multiple of 0·5 mm or 0·5 µm, followed by the number of measurements (*n*). Measurements refer to dimensions in water. Microscopic photographs were prepared using an Olympus BX51 microscope fitted with an Olympus UC 30 camera. Voucher specimens are deposited in the herbarium of the Botanic Garden Meise (BR), the Field Museum of Natural History (F), and the Instituto Nacional de Biodiversidad (INB).

Molecular techniques

Well-preserved and freshly collected specimens lacking any visible symptoms of fungal infection were used for DNA isolation. Hand-cut sections of the ascigerous areas of specimen '*Van den Broeck* 6326' from Uganda were used for direct PCR as described in Ertz *et al.* (2015). The lichen material was washed with a 1% KOH solution and then rinsed with water to remove remnants of pigments. The material was placed directly in microtubes with 0·2 ml of H_2O. Amplification reactions were prepared for a 50 µl final volume containing 5 µl 10× DreamTaq Buffer (Thermo Fisher Scientific, Waltham, MA), 1·25 µl of each of the 20 µM primers, 5 µl of 2·5 mg ml^{-1} bovine serum albumin (Thermo Fisher Scientific, Waltham, MA), 4 µl of 2·5 mM each dNTPs (Thermo Fisher Scientific, Waltham, MA), 1·25 U DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA) and the small fragments of lichen material. Specimen '*Chaves* 1758' from Costa Rica was prepared for extraction by carefully selecting portions of the hymenia beneath the mazaedia; extraction and PCR followed the protocol specified by Gaya *et al.* (2012). A targeted fragment of *c.* 0·8 kb of the mtSSU rDNA was amplified from both specimens using primers mrSSU1 and mrSSU3R (Zoller *et al.* 1999), and a fragment of *c.* 1 kb of the *RPB2* protein-coding gene was amplified from the Ugandan material using primers *fRPB2*-7cF and *fRPB2*-11aR (Liu *et al.* 1999). After examination by gel electrophoresis, the material from Uganda was purified and sequenced by Macrogen® using the same amplification primers. For the Costa Rican material, PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH). Sequencing was carried out in 10 µl reactions using: 1 µl primer, 1 µl purified PCR product, 0·75 µl Big Dye (Big Dye Terminator Cycle sequencing kit, ABI PRISM version 3.1; Perkin–Elmer, Applied Biosystems, Foster City, CA), 3·25 µl Big Dye buffer, and 4 µl double-distilled water. Automated reaction clean up and visualization was performed at the Duke Genome Sequencing & Analysis Core Facility of the Institute for Genome Sciences and Policies, as described in Gaya *et al.* (2012). Sequence fragments were subjected to BLAST searches for a first verification of their identities. They were assembled and edited with Geneious Pro 5.1.7 (Kearse *et al.* 2012).

Taxon selection and phylogenetic analyses

Five new sequences were obtained for this study and 110 additional sequences were retrieved from GenBank (Table 2). Two different taxon sets were used for the phylogenetic analyses: a set of 61 OTUs consisting of taxa representing all major clades currently accepted in the *Arthoniales* (Frisch *et al.* 2014) and for which at least the mtSSU was available, and a subset of seven specimens focusing on *Heterocyphelium* and its closest relatives with complete data (Table 2). For the first data set *Dothidea sambuci* was chosen as outgroup species and for the second data set, *Plectocarpon lichenum*. For the two data sets, the sequences were aligned using MAFFT v6.814b (Katoh *et al.* 2002) within Geneious Pro 5.1.7 and corrected for errors manually using Mesquite 3.04 (Maddison & Maddison 2015). Ambiguously aligned regions following Lutzoni *et al.* (2000) and introns were delimited manually and excluded from subsequent analyses. All new sequences were deposited in GenBank (Table 2) and the alignment data were deposited in TreeBASE (Accession number S20530).

To examine topological incongruence among data sets, Bayesian and maximum likelihood (ML) analyses were carried out on each of the single-locus data sets. We used MrBayes v.3.2.6 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003), with the same settings described below for the 61 sample data set, and RAxML v.7.2.7 (Stamatakis 2006) with 1000 replicates of ML bootstrapping (ML-BS) and the GTRGAMMA model. In both cases, analyses were run on the CIPRES web portal (Miller *et al.* 2010). All topological bipartitions were compared for the two loci. A conflict was assumed to be significant if two different relationships (one being monophyletic and the other being non-monophyletic) for the same set of taxa were both supported with PP values ≥95% and/or bootstrap values ≥70%

TABLE 2. *Specimens and their GenBank Accession numbers. Newly generated sequences are indicated by an asterisk; dash denotes missing data. Species in bold were used for the subset.*

		GenBank Acc. no.	
Species	Voucher	mtSSU	*RPB2*
Alyxoria bicolor	Rwanda; *Ertz* 8731 (BR)	EU704062	EU704026
A. mougeotii	Great Britain; LD:L10058	KJ851007	–
***A. ochrocheila* s. lat.**	Rwanda; *Ertz* 8624 (BR)	EU704071	EU704036
A. ochrocheila	Luxembourg; *Ertz* 7519 (BR)	EU704072	EU704035
***A. varia* 1**	Sweden; *Frisch* 11/Se1 (UPS)	KJ851006	KJ851147
***A. varia* 2**	Sweden; *Thor* 11/Se50 (UPS)	KF707642	KF707664
Arthonia anglica	Rwanda; *Ertz* 7775 (BR)	EU704049	EU704012
A. apatetica	Sweden; *Svensson* 2017 (UPS)	KJ850992	KJ851125
A. biatoricola	Japan; *Thor* 24350 (UPS)	KJ850990	KJ851149
A. calcarea	France; *Ertz* 7539 (BR)	EU704064	EU704028
A. didyma	Belgium; *Ertz* 7587 (BR)	EU704047	EU704010
A. lobariicola	Japan; *Frisch* 10/Jp124 (UPS)	KJ851002	KJ851128
A. mediella	Sweden; *Frisch* 11/Se22 (UPS)	KJ851014	KJ851133
A. physciidicola	Uganda; *Frisch* 11/Ug318 (UPS)	KF707646	KF707657
A. punctiformis	Sweden; *Thor* 21658 (UPS)	KJ850973	KJ851113
A. radiata	Belgium; *Ertz* s. n. (BR)	EU704048	EU704011
A. subfuscicola	Sweden; *Frisch* 11/Se15 (UPS)	KJ850972	KJ851111
Bryostigma muscigenum	Sweden; *Thor* 26206 (UPS)	KJ850991	KJ851124
Chiodecton natalense	Zambia; *Ertz* 6576 (BR)	EU704051	EU704014
Chrysothrix caesia	USA; *Amtoft* (AFTOL 775)	FJ469671	FJ469670
Combea mollusca	South Africa; *Tehler* 7725 (S)	AY571384	DQ987626
Coniocarpon cinnabarinum	Rwanda; *Ertz* 8730 (BR)	EU704046	EU704009
Crypthonia palaeotropica	Uganda; *Frisch* 11/Ug457 (UPS)	KJ850961	KJ851084
Cryptothecia subnidulans	Réunion; *v.d. Boom* 40613 (hb v.d. Boom)	KJ850952	KJ851087
Dendrographa decolorans	Sweden; *Frisch* 11/Se28 (UPS)	KJ851012	KJ851141
Dichosporidium brunnthaleri	Uganda; *Frisch* 11/Ug8 (UPS)	KJ851011	KJ524362
Dimidiographa longissima	Florida; *Ertz* 9155 (BR)	EU704069	EU704033
Dothidea sambuci	AFTOL-ID 274	AY544739	DQ522854
Enterographa crassa	France; *Ertz* 5041 (BR)	EU704056	EU704020
E. zonata	Belgium; *Vigneron* 104 (BR)	EU704081	EU704045
Erythrodecton granulatum	Gabon; *Ertz* 9908 (BR)	EU704058	EU704022
Felipes leucopellaeus	Sweden; *Frisch* 10/Se34 (UPS)	KJ850984	KJ851130
Fouragea filicina	Rwanda; *Ertz* 7994 (BR)	EU704067	EU704031
F. viridistellata	La Réunion; *Ertz* 4795 (BR)	EU704076	EU704040
Gyrographa gyrocarpa	Sweden; *Thor* 11/9 (UPS)	KJ851026	KJ851143
***Heterocyphelium leucampyx* 1**	Uganda; Van den Broeck 6326 (BR)	*KY360242	*KY360246
H. leucampyx 2	Costa Rica; *Chavez* 1758 (F, INB)	*KY360243	–
Inoderma byssaceum	Japan; *Thor* 25952 (UPS)	KJ850962	KJ851089
Lecanactis abietina	Belgium; *Ertz* 5068 (DUKE)	AY548813	AY552018
Lecanographa amylacea	Sweden; *Thor* 26176 (UPS)	KF707650	KF707659
L. atropunctata	Gabon; *Ertz* 9869 (BR)	*KY360244	HQ454688
L. farinosa	Canary Islands; *Ertz* 14053 (BR)	*KY360245	HQ454687
Myriostigma candidum	Gabon; *Ertz* 9260 (BR)	EU704052	EU704015
Nyungwea pallida	Uganda; *Frisch* 11/Ug24 (UPS)	KJ851023	KJ851145
Opegrapha brevis	Great Britain; LD:L10094	KJ851005	–
O. celtidicola	Portugal; *Diederich* 16053 (BR)	EU704066	EU704030
O. vermicellifera	Belgium; *Ertz* 7562 (BR)	EU704077	EU704041
O. vulgata	Belgium; *Ertz* 7564 (BR)	EU704080	EU704044
Pachnolepia pruinata	Sweden; *Frisch* 11/Se34 (UPS)	KJ850967	KJ851098
Phacographa glaucomaria	Sweden; *Frisch* 11/Se33 (UPS)	KJ851022	KJ851136
P. zwackhii	Sweden; *Frisch* 11/Se3 (UPS)	KJ851021	–
Plectocarpon lichenum	Sweden; *Thor* 26770 (UPS)	KJ850988	KJ851140
P. nephromeum	Sweden; *Nordin* 5813 (UPS)	KJ851004	KJ851139
Reichlingia leopoldii	Belgium; *Ertz* 13294 (BR)	JF830774	HQ454723
Simonyella variegata	AFTOL-ID 80	AY584631	DQ782861
Tylophoron galapagoense	Galapagos; *Ertz* 11794 (BR)	JF830777	–
T. hibernicum	Uganda; *Frisch* 11/Ug220 (UPS)	KJ850966	KJ851097
T. moderatum	DR Congo; *Ertz* 14504 (BR)	JF830780	–
T. stalactiticum	Canary Islands; *Ertz* 10880 (BR)	JF830781	–
Zwackhia sorediifera	Sweden; *Thor* 26210 (UPS)	KJ851024	KJ851142
Z. viridis	Luxembourg; *Ertz* 7619 (BR)	EU704078	EU704042

(Mason-Gamer & Kellogg 1996). Based on this criterion, no conflict was detected and therefore the mtSSU and *RPB2* data sets were concatenated.

Phylogenetic relationships and confidence were inferred on the combined data sets also using Bayes and maximum likelihood (ML) as optimization criteria. In both analyses, alignments were divided into four partitions (mtSSU, *RPB2*/1st, *RPB2*/2nd and *RPB2*/3rd positions). For the Bayesian analyses, best-fit evolutionary models for each partition were estimated using the Akaike Information Criterion (AIC) as implemented in jModelTest 2 (Darriba *et al.* 2012). For the data sets of 61 samples, the GTR+I+G model was selected for the mtSSU data set as well as for the *RPB2*/1st and *RPB2*/2nd codon positions, while the TVM+I+G model was selected for the *RPB2*/3rd position. For the subset of seven samples, the GTR+I+G model was selected for the mtSSU data set while the TIM2+G model was selected for the *RPB2*/1st, TIM3+G for the *RPB2*/2nd and the TPM3uf+I+G for the *RPB2*/3rd codon positions. Two parallel Bayesian MCMCMC runs were performed, each using four independent chains and 120 million generations for the 61 sample data set and 40 million generations for the 7 sample data set, sampling trees every 1000th generation in both cases. Tracer v.1.6.0 (Rambaut *et al.* 2013) was used to ensure that stationarity was reached by plotting the log-likelihood values of the sample points against generation time. Convergence between runs was also verified using the PSRF (Potential Scale Reduction Factor), where values were all equal to 1·000 or 1·001. A tree was generated from 180 002 post-burn-in trees out of 240 002 sampled for the 61 sample data set and from 60 002 post-burn-in trees out of 80 002 trees sampled for the seven sample subset for the two pairs of MCMCMC runs using the sumt option in MrBayes. Posterior probabilities (PP) were determined by calculating a majority-rule consensus tree. For the ML analyses, RAxML was used to estimate the most likely tree with 1000 replicates and a GTRGAMMA model of molecular evolution. Bootstrap proportions (ML-BS) were obtained from 1000 replicates of ML bootstrapping conducted with the same settings and program. Internodes with bootstrap proportions ≥70% and Bayesian posterior probabilities ≥95% were considered strongly supported. Internodes with a bootstrap value ≥70% and a posterior probability <0·95 were also interpreted as well supported (Alfaro *et al.* 2003; Lutzoni *et al.* 2004).

The combined two-loci data set of 61 samples consisted of 1347 unambiguously aligned sites, 525 for mtSSU and 822 for *RPB2*. The combined two-loci data subset of 7 samples consisted of 1666 unambiguously aligned sites, 790 for mtSSU and 876 for *RPB2*. Phylogenetic trees were visualized using FigTree v1.3.1 (Rambaut 2012). Since *RPB2* has been shown to produce aberrant topologies due to saturation of the third codon position (see Discussion below), we tested this potential effect by reanalysing the 61 sample data set with the third codon position excluded and using the same settings as above. In addition, we also used the smaller taxon set including only the close relatives of *Heterocyphelium leucampyx* (7 sample data set) to test the effect of exclusion of ambiguous regions in broad versus narrow alignments (Fig. 3).

Results

Taxonomy

Heterocyphelium leucampyx (Tuck.) Vain.

Acta Soc. Fauna Flora Fenn. **57:** 16 (1927). Basionym: *Trachylia leucampyx* Tuck., *Proceedings Am. Acad. Arts Sci.* **5:** 390 (1862).—*Acolium leucampyx* (Tuck.) Tuck., *Syn. N. Amer. Lich.* (*Boston*) **2:** 162 (1888).—*Cyphelium leucampyx* (Tuck.) Zahlbr., in Engler & Prantl, *Nat. Pflanzenfam., Teil I* (*Leipzig*) **1*:** 84 (1903); type: Cuba, Monte Verde, *Wright* s. n. (FH-Tuckerman!—holotype; FH, K, PC!, S!, UPS!—isotypes; Müller, Lich. Cub. 21).

Tylophorum triloculare Müll. Arg., *Hedwigia* **32:** 122 (1893); type: Australia, Queensland, ad cortices vetustos prope Brisbane, *Bailey* 1533 (G!—holotype).

Tylophoron eckfeldtii Müll. Arg., *Herb. Boissier* **2:** 89 (1894); type: Mexico, Jalisco, *Eckfeldt* s. n. (G!—holotype, with spore drawings and annotations).

(Fig. 1A–F)

Thallus corticolous, crustose, not endophloeodic, ecorticate, greenish grey to white, farinose, partly cracked and/or byssoid, hydrophobic. Prothallus visible as a black line when in contact with other lichens. *Photobiont* often inconspicuous, but according to Tibell (1996) it is *Trentepohlia*.

Ascomata first immersed in the thallus, becoming prominent to sessile, very variable in outline, rounded to lobate or lirellate, with or without a thalline, more or less byssoid margin, not or slightly constricted at the base, 0·10–0·35 × 0·16–0·55 mm ($n = 10$), hydrophobic. *Mazaedium* well developed, black, bordered by a white rim. *Excipulum* black, composed of brown, branched and anastomosing hyphae 2–3 µm thick. *Hamathecium* hyaline, composed of branched and anastomosing hyphae 1·5–2·0 µm thick, the apices not or very slightly swollen, without a dark cap, visible as a white rim between the mazaedium and the excipulum, I+ red, KI+ patchily pale blue, inspersed with rounded to angular, hyaline to orange crystals 0·5–2·5 × 1·0–3·0 µm, completely dissolving in K. *Asci* cylindrical, often curved, 23–26 × 3·5–4·5 µm ($n = 4$), with a single functional wall layer (prototunicate), disintegrating at an early stage, I−, KI−, without a K/I+ blue ring-like structure, wall 0·5–0·7 µm. *Ascospores* 2-septate, or rarely with 3–4 septa, 10·5–15·0 × 4·5–7·0 µm

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($n = 20$), hyaline, becoming dark brown, straight to slightly curved, young globose to ellipsoid or obovoid, uniseriately arranged in the asci, hyaline, without septa and round to angular, 8 per ascus, olivaceous in K. Mature spores distinctly constricted at septa and having a median cell much larger than the apical ones, without ornamentation, I−, KI−, a gelatinous sheet not observed, wall thick, dark brown. Septation of the spores starts with one extramedian septum.

Conidiomata not observed.

Chemistry (Costa Rican material tested with TLC). No secondary substances detected by TLC; thallus and ascomata K−, C−, KC−, P−.

Distribution and ecology. Heterocyphelium leucampyx grows on the bark of trees in tropical forests at 120–1200 m elevation. It is widespread in the tropics and known from the Neotropics (Bolivia, Brazil, Costa Rica, Cuba, Florida, Galapagos, Guatemala, Mexico, Venezuela) as well as tropical Africa (Ivory Coast, Uganda) and the eastern Palaeotropics (Australia, Bonin Islands, China, India, Thailand) (Tibell 1987, 1996, 2001; Harris 1990; Elix & McCarthy 1998; Aptroot & Sparrius 2013; Balaji & Hariharan 2013; Bungartz *et al*. 2013; Flakus *et al*. 2013).

Additional specimens examined. **China:** *Yunnan*: Xishuangbanna, 2 km from Menglun, Green Stone Park, 21°54'37"N, 101°16'51"E, UTM: 47QQE356246, 600 m, on tree trunk, 2002, *Aptroot* 57326 (BR).—**Costa Rica:** *Guanacaste*: Quebrada Azul, Tilarán (Arenal Conservation Area), 10°31'N, 84°59'W, 700–800 m, 2003, *Chaves* 1758 (F, INB).—**India:** *Tamil Nadu*: Salem District, Kolli Hills, 1000 m, 1994, *G. N. Harharan & M. S. S. Mohan* s. n. (BR).—**Uganda:** *Wakiso District*: Entebbe, Kisubi, Ziika Forest, 00°07'18·6"N, 032°31'34·4"E, 1141 m, on unidentified tree species, 2014, *Van den Broeck* 6326 (BR).

Phylogenetic analysis

The Bayesian tree obtained in the combined 2-locus analyses of 61 OTUs including the third codon position of *RPB2* is shown in Fig. 2. The clade on the left upper corner shows the different topology obtained from the same data set, but excluding the third position for the *RPB2* (Fig. 2). In both cases, statistical support from posterior probabilities and ML bootstrap replicates is indicated. Branches strongly supported by both analyses are highlighted with thicker lines. The topology obtained from the reduced data set is depicted in Fig. 3.

In the 2-locus tree of 61 OTUs based on 115 sequences, *Heterocyphelium leucampyx* is placed in the order *Arthoniales* in the family *Lecanographaceae*, nested within the genus *Alyxoria* with strong support (Fig. 2). In the analysis based on the combined 2-loci data set including only the first and second positions for the *RPB2*, *Heterocyphelium* is recovered as sister to *Alyxoria* with strong support (Fig. 2). In order to investigate whether *Heterocyphelium* is sister to *Alyxoria* or should be included in that genus, despite its strongly deviating morphology, we analyzed a subset of the original alignment including only *Heterocyphelium* and *Alyxoria*, with *Plectocarpon lichenum* as outgroup, since the three genera form a strongly supported monophyletic clade in both analyses of the larger data set. The analysis of the reduced set resulted in substantially more unambiguously aligned sites (1666 in the subset vs. 1347 in the complete data set) and in a strongly supported placement of *Heterocyphelium* as sister to *Alyxoria* (Fig. 3).

The backbone topology in our analysis differs somewhat from the topology presented in earlier studies, particularly Frisch *et al*. (2014). In that study, the *Bryostigma* clade was significantly recovered as sister to *Arthoniaceae*, while in the present manuscript this clade is strongly supported as sister to the other families of *Arthoniales* (i.e. *Chrysotrichaceae*, *Lecanographaceae*, *Opegraphaceae*, *Roccellaceae* and *Roccellographaceae*). In the 2014 study, *Roccellographaceae* appears sister only to *Roccellaceae*, whereas in the present manuscript *Roccellographaceae* is sister to both *Opegraphaceae* and *Roccellaceae*, but these conflicts lack support in both studies. The placement of *Dimidiographa longissima* is also not significantly supported in either of the two studies. These differences could be explained by the inclusion of a third locus (nuLSU) in Frisch *et al*. (2014) and by partially different

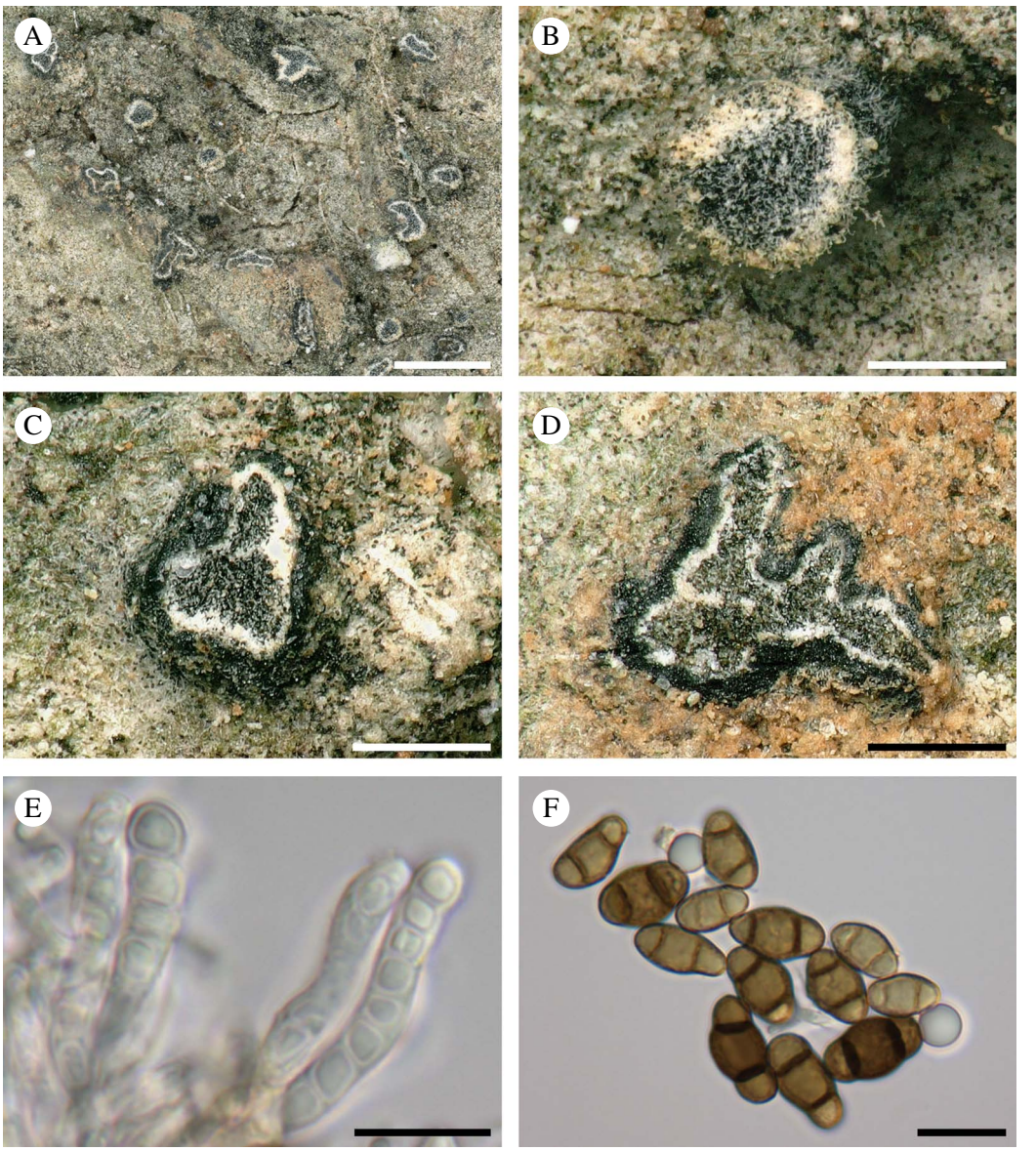

FIG. 1. *Heterocyphelium leucampyx*, showing the variability of the ascomata and the ascospores. A, thallus with ascomata; B, rounded ascoma with byssoid margin; C, elongate ascoma with black margin; D, lobed ascoma with black margin; E, asci with hyaline, young, unicellular ascospores; F, brown, mature, 2-septate ascospores. Scales: A = 1 mm; B–D = 0·25 mm; E & F = 10 µm. In colour online.

taxon sampling. However, while the relative position of families and family-level clades varied between the two studies, all families are likewise monophyletic and supported in both studies, including *Lecanographaceae*, and hence these differences do not affect the strongly supported placement of our target taxon, *Heterocyphelium*, within that family.

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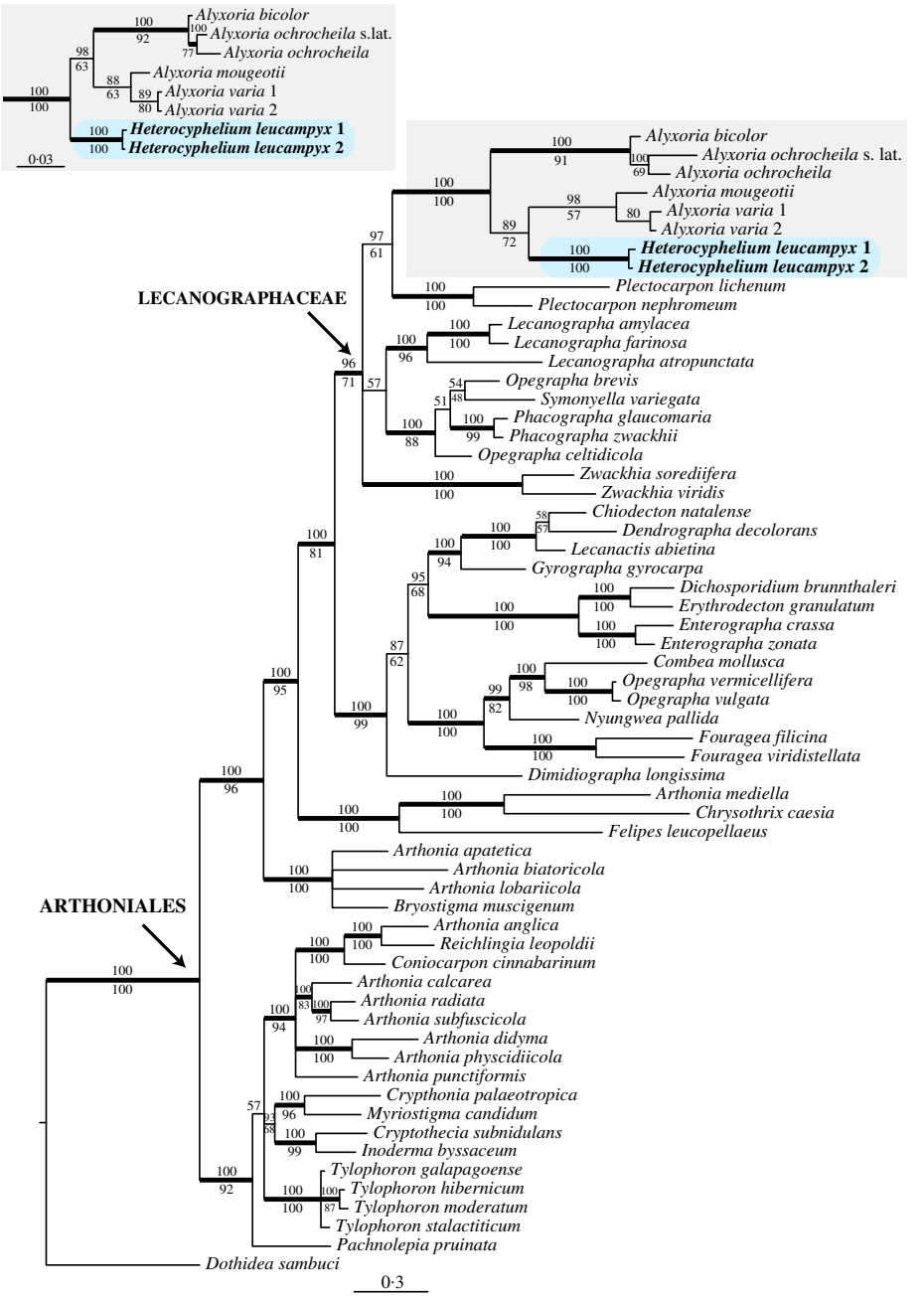

FIG. 2. Phylogenetic relationships among *Arthoniales* based on a data set of 61 samples of mtSSU and *RPB*2 sequences resulting from a Bayesian analysis. *Dothidea sambuci* was chosen as outgroup. Posterior probabilities ≥95 are shown above internal branches and maximum likelihood bootstrap values ≥70 obtained from a RAxML analysis are shown below internal branches. Internal branches, considered strongly supported by both analyses, are represented by thicker lines. *Heterocyphelium leucampyx* is in bold. The clade on the left upper corner shows the different topology obtained from the same data set of mtSSU and *RPB*2 sequences but excluding the third position for the *RPB*2.

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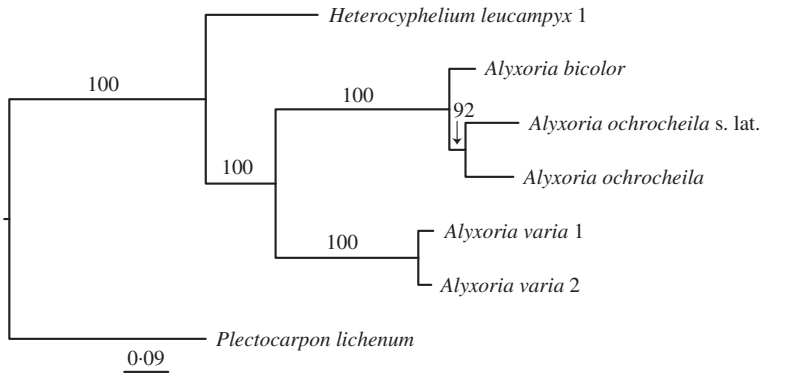

FIG. 3. Phylogenetic relationships based on a subset of seven samples of mtSSU and *RPB2* sequences of *Lecanographaceae* resulting from a Bayesian analysis. *Plectocarpon lichenum* was chosen as outgroup. Posterior probabilities (PP) are shown above internal branches. Internal branches with PP ≥95% are considered strongly supported.

Discussion

On the basis of morphology, *Heterocyphelium leucampyx* had initially been placed in the order *Arthoniales* (family *Arthoniaceae*, genus *Trachylia*), before it was included in the collective order *Caliciales* (successively within the genera *Acolium* and *Cyphelium*). After establishing a separate genus (*Heterocyphelium*) for this species, it remained tentatively in *Caliciales* but was eventually considered a genus *incertae sedis* within the Ascomycota, without a determined class, order or family (Eriksson 1999; Lumbsch & Huhndorf 2010). Our molecular analyses provide clear evidence that the species belongs in the *Arthoniales*, where it was already listed by Jaklitsch *et al.* (2016) in anticipation of the present study. However, unlike the only other mazaediate genus for which sequences are available in this order, *Tylophoron* (Lumbsch *et al.* 2009), *H. leucampyx* does not belong to the family *Arthoniaceae* but to the *Lecanographaceae* (Fig. 2). The latter is a recently described family that includes taxa characterized by a crustose, ecorticate thallus, a trentepohlioid photobiont, ascomata that are lirelliform to rounded, without a thalline margin, a well-developed dark brown excipulum, cylindrical to clavate, bitunicate asci, and hyaline, fusiform, distoseptate ascospores with a microcephalic ontogeny and a gelatinous sheath (Ertz & Tehler 2011; Frisch *et al.* 2014). The morphology of *H. leucampyx* deviates from all other *Lecanographaceae* by the mazaediate ascomata, asci with a single functional wall layer (prototunicate), disintegrating at an early stage, and dark brown ascospores lacking a gelatinous sheet.

In our combined analysis (Fig. 2), *Heterocyphelium leucampyx* appears clustered within the genus *Alyxoria*, suggesting that *Heterocyphelium* could be considered as a synonym of the latter. The genus *Alyxoria* was recently reinstated for a group of species previously placed in *Opegrapha* s. lat. characterized by an ascus of the '*Varia* type' and ascomata having an exposed, usually pruinose disc (Ertz & Tehler 2011). Although the ontogeny of the ascospores starting with one extramedian septum leading to a larger central cell in mature spores is unusual in *Heterocyphelium*, this seems also to be the case for some species of *Alyxoria*, where ascospores might have a larger central cell (e.g. *A. varia)*. This morphological trait could potentially explain the close relationship of both genera and needs further examination. On the other hand, *Heterocyphelium* differs from *Alyxoria* in the distinctly mazaediate ascomata. So far, only one case is known where a single genus includes mazaediate and non-mazaediate forms. This is the genus *Nadvornikia*, where recently two non-mazaediate species were

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added (Medeiros *et al.* 2017). Hence, including *Heterocyphelium* within *Alyxoria* would not be entirely out of the ordinary. However, when reanalyzing the data without the third codon position of the *RPB2* gene, *Heterocyphelium* was recovered as sister to the *Alyxoria* clade with significant support (Fig. 2). This sister relationship was again recovered with significant evidence in the analyses of the reduced data set including only *Alyxoria* and *Heterocyphelium*, with *Plectocarpon* as outgroup (Fig. 3), independently of whether the third codon position of the *RPB2* gene was removed or not. This phenomenon is due to homoplasy in the DNA data and the fact that alignments may include an imbalance between protein- and non-coding genes. Protein-coding genes are well alignable even between distantly related taxa and hence no columns are usually excluded due to potential alignment ambiguity, even if the third codon position tends to be saturated and might cause problems, as has been reported for *RPB2* (Reeb *et al.* 2004; Hansen *et al.* 2005; Dávalos & Perkins 2008; Breinholt & Kawahara 2013). In contrast, non-protein-coding genes result in alignment ambiguity, especially for saturated regions with a large proportion of indels which are then excluded. As a result, data sets combining both types of genes tend to produce aberrant topologies. This effect is nicely shown here: whereas the largest data set resolves *Heterocyphelium* as nested within *Alyxoria*, removing the third codon position of the *RPB2* (which is equivalent to the exclusion of ambiguously aligned columns in the non-coding mtSSU) places *Heterocyphelium* as sister to *Alyxoria*. This topology was then confirmed when analyzing a reduced taxon set that allowed the retention of over 300 additional columns in the mtSSU gene, which in the largest taxon set had to be excluded. We conclude that the complete set of nucleotide sites in both markers supports *Heterocyphelium* being sister to *Alyxoria*; however, this effect can only be obtained when looking at a sufficiently small clade of closely related taxa that allows most alignment columns to be retained. With a broader taxon set, columns that contain phylogenetic signal for the correct placement of *Heterocyphelium* in the mtSSU gene needed to be excluded due to alignment ambiguity, whereas the likely saturated third codon position of the *RPB2* partition remains to be included, leading to an aberrant topology.

The strategy employed here to examine the precise topology of a terminal clade by greatly reducing the data set to the smallest clade of interest, allowing the inclusion of much more data, is therefore recommended when terminals in relatively large-scale analyses lack resolution power and exhibit unexpected topologies.

Based on these phylogenetic results, we maintain *Heterocyphelium* as a genus distinct from *Alyxoria*, in accordance with the main morphological traits such as the production of mazaediate ascomata in *Heterocyphelium*. The strongly deviating morphology in *Heterocyphelium* compared to all other members of the family *Lecanographaceae* might be another example of the apparently strong selection pressure on passive ascospore dispersal in certain lineages, a phenomenon also observed in other families such as *Arthoniaceae*, *Caliciaceae*, *Graphidaceae*, and *Pyrenulaceae* (Wedin *et al.* 2000; Lumbsch *et al.* 2004, 2009; Tehler *et al.* 2009). Mazaedia or similar structures occur in many distantly related ascomycete lineages, and structurally different types of fruiting bodies can develop a mazaedium, for example stalked, immersed, or sessile apothecia, as well as perithecium-like, lirellate and stroma-like ascomata (Prieto *et al.* 2013). This suggests some positive evolutionary constraint on this type of fruiting body (Prieto *et al.* 2013) which is, however, not yet well understood (Lumbsch *et al.* 2004). A remarkably similar phenomenon can be found in gasteroid fungi in the Basidiomycota (Krüger *et al.* 2001; Binder & Bresinsky 2002; Matheny *et al.* 2006; Wilson *et al.* 2011). Three other genera currently placed in the *Arthoniales* develop mazaediate ascomata: *Sporostigma, Tylophorella* and *Tylophoron*. However, we cannot draw further conclusions about those genera since molecular data are available only for the *Tylophoron*. Lumbsch *et al.* (2009) placed *Tylophoron* Nyl. ex Stiz.,

previously thought to be related to pyrenocarpous lichens, in the *Arthoniaceae*, a placement that has since been confirmed and refined (Ertz *et al.* 2011; Frisch *et al.* 2014). The genus is morphologically similar to *Heterocyphelium leucampyx* in having sessile, well-delimited ascomata, a well-developed mazaedium, evanescent, cylindrical asci and transversally septate, dark brown ascospores. *Heterocyphelium leucampyx* differs in lacking secondary substances and ascospores that are predominately 2-septate with an enlarged median cell. *Sporostigma* is a monospecific genus containing the species *S. melaspora* (Tuck.) Grube. It was tentatively placed in the *Arthoniaceae* on the basis of ascomatal characters, in particular the lack of an exciple, the branched and anastomosing paraphysoids, as well as the shape of young asci (Grube 2001). *Tylophorella* (Müll. Arg.) Egea & Tibell is another monospecific mazaediate genus containing one species, *T. pyrenocarpoides* (Tibell 1996; synonym: *Tylophorella polyspora* Vain.). Based on morphological grounds, *Tylophorella* would be likely to belong to the Arthoniomycetes (Tibell 1984; Grube 2001); it differs from the other species discussed here by having oblong, initially multiseptate to submuriform, eventually disintegrating ascospores resembling those of *Opegraphaceae* and *Lecanographaceae* rather than *Arthoniaceae*.

Two other mazaediate tropical genera associated with *Trentepohlia* and traditionally included in *Caliciales* were also not assigned to any family by Tibell (1984, 1996): *Allophoron* and *Schistophoron*. Using molecular data, *Schistophoron* Stirt. has recently been placed in the subclass Ostropomycetidae, family *Graphidaceae* (Tehler *et al.* 2009; Lücking *et al.* 2013; Prieto *et al.* 2013; Rivas Plata *et al.* 2013). The type species, *S. tenue*, differs from *Heterocyphelium leucampyx* by the strongly sessile ascomata closely resembling those of the genus *Carbacanthographis*, the mazaedium forming a thin, dark slit and by the distinct chemistry (norstictic and stictic acids). Moreover, in *Schistophoron tenue* the asci are obclavate with biseriately arranged and, in part, overlapping ascospores. *Schistophoron* as currently delimited is heterogeneous; whereas *S. indicum* Kr. P. Singh & Swarnal. is closely related to the type species, both *S. variabile* Tibell and *S. aurantiacum* Aptroot & Sipman strongly deviate in morphology and chemistry and seem akin to *Arthoniales*. Finally, *Allophoron*, with the single species *A. farinosum* Nádv., is characterized by submuriform dark brown ascospores with 2–5 transverse and 0–4 longitudinal septa, and the absence of secondary substances. It shares the presence of sclerotinized hyphae in the hamathecium with *Heterocyphelium leucampyx*, which is easily distinguished from *Allophoron* by its 2-septate ascospores. Based on anatomical similarities with *Heterocyphelium* (Tibell 1996), we hypothesize again that *Allophoron* might most likely also be a member of *Arthoniales*. Therefore, *Arthoniales* could become the order with the highest number of mazaediate lineages, with two independent lineages (*Heterocyphelium*, *Tylophoron*) confirmed with molecular data to date and up to four potential additional lineages remaining to be tested (i.e. *Allophoron*, *Schistophoron* p.p., *Sporostigma*, *Tylophorella*). Unfortunately, these taxa are comparatively rare and more difficult to obtain for sequencing.

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