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Pyxine tiinae (Caliciaceae, Ascomycota), a new lichen species from high elevation in Peru

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Abstract: *Pyxine tiinae*, a new foliose lichen species, is described from an alpine locality in Peru. The new species is characterized by convex and radiating, grey lobes, a K– cortex and K– epihymenium, the presence of terpenes, and by comparatively long pycnoconidia. A phylogenetic analysis places *P. tiinae* as sibling to the genus *Culbersonia*, although the morphological characters do not fit with either this genus or with *Pyxine* as currently circumscribed.

Keywords: Caliciaceae, *Culbersonia*, Physciaceae, *Pyxine*

INTRODUCTION

The foliaceous lichen genera *Dirinaria* and *Pyxine* were for a long time treated in the family Physciaceae, together with superficially similar genera such as *Phaeophyscia* and *Physcia*. Recent phylogenetical studies have, however, shown that they are more closely related to crustose genera such *Buellia* and *Diplotomma*, and to mazaediate genera such as *Calicium*, all of which are now included in the family Caliciaceae (Miadlikowska et al., 2014; Prieto & Wedin, 2017). Also the genus *Culbersonia*, whose sole representative *C. nubila* (Moberg) Essl. was originally described as a *Pyxine* (Moberg, 1980; Esslinger, 2001), has been shown to belong to the Caliciaceae (Aptroot et al., 2019).

In 1981, during his studies of the Physciaceae of South America (mainly Peru), the senior author collected what appeared to be a new species of *Pyxine*. At the same time though, this species possessed a puzzling combination of morphological characters, leaving some doubts as to its correct generic placement. Here, we use this material as the basis for describing the new species *Pyxine tiinae* and also aim to evaluate the phylogenetic position of the new species using newly generated DNA data from the 1981 collection.

MATERIAL AND METHODS

Morphology and chemistry

Microscopical measurements were made under a light microscope on material mounted in water, using an oil-immersion, objective lens, with a precision of 0.5 µm for measurements of finer anatomical structures (e.g., ascospores and paraphyses). We performed HPTLC following the method described by Arup et al. (1993) using solvent systems C and G (Orange et al., 2010).

Taxon sampling

Both morphology and initial test analyses indicated that *Pyxine tiinae* is related to *Pyxine* and closely related genera in the Caliciaceae. To determine its phylogenetic position, we based our taxon sampling on the Caliciaceae phylogeny of Prieto & Wedin (2017), with some modifications. First, we reduced the number of taxa in sibling clades to the *Diplotomma*/*Dirinaria*/*Pyxine* clade (which was our main focus) as well as the taxa of the outgroup (Physciaceae). Second, we expanded the focus clade by including *Culbersonia* (Aptroot et al., 2019), a couple of additional representatives of *Dirinaria*, and by including most species of *Pyxine* of which at least mtSSU and ITS were available in GenBank. For the selected taxa, we downloaded sequences from GenBank from the mitochondrial ribosomal small subunit 12S, hereafter mtSSU; the nuclear rDNA ITS1-5.8-ITS2, hereafter ITS; the nuclear ribosomal large subunit 28S, hereafter LSU; the mini-chromosome maintenance complex 7, hereafter MCM7; the β-tubulin gene, hereafter β-tubulin; and the RNA polymerase II complex subunit, hereafter RPB2. Two terminals in our analysis, both nominally belonging to *Calicium viride*, had available sequences in GenBank that we did not use: for *Calicium viride* 1, the available MCM7 sequence (JX000153) was very different to other MCM7 sequences in our

alignment and BLAST-results also indicated that it was distant to other *Calicium* species; for *Calicium viride* 2, the available mtSSU sequence (AF356669) was likewise clearly deviant and BLAST-results indicated that its closest relatives were within the Lecideaceae. We hence viewed these sequences as unreliable and excluded them from the final analysis. GenBank IDs for downloaded and newly generated sequences are summarized in Table 1.

Table 1. Sequence data used for the phylogenetic analysis, with GenBank accession numbers and voucher information. Sequences newly generated for this study are in bold face.

Taxon	Voucher/source	mrSSU	ITS	LSU	MCM7	β-tubulin	RPB2
Acolium inquinans	Wedin 6352 (UPS), Wedin et al. 2002	AY143404	AY450583	AY453639	JX000161	KX529023	
Amandinea punctata 1	Wedin 2/3/96 (UPS), Wedin et al. 2002	AY143399	KX512899	AY340536	KX529025		
Amandinea punctata 2	Ertz 4647 (BR), Miadlikowska et al. 2006	DQ986874	HQ650627	DQ986756			DQ992435
Calicium salicinum	Tibell 22/10 1986 (CBS), Beimforde et al. 2014			KF157982			KF157998
Calicium tigillare	Prieto 3038 (S), Prieto et al. 2012	JX000123	JX000104	JX000088	JX000162	KX529002	
Calicium viride 1	Wedin 24/4 2000, Wiklund & Wedin 2003	AY584696	HQ650703	AY340538		KX529013	
Calicium viride 2	Søchting 7475 (DUKE), Lutzoni et al. 2001			AF356670			AY641031
Culbersonia nubila 1	Moberg 4488a (UPS L-082666, holotype)	**OQ866149**	**OQ866141**	**OQ866152**			
Culbersonia nubila 2	Maphangwa & Zedda (PRE), Aptroot et al. 2019		MH121318	MH121320			
Culbersonia nubila 3	Maphangwa (PRE), Aptroot et al. 2019		MH121317	MH121319			
Culbersonia nubila 4	v.d. Boom 54629 (herb. v.d. Boom)	**OQ866148**	**OQ866142**		**OQ867274**	**OQ877132**	**OQ867277**
Diplotomma alboatrum	Prieto 3034 (S), Prieto & Wedin 2017	KX512966	KX512924	KX512877	KX529043	KX529007	
Diplotomma venustum	Westberg 10–176 (S), Prieto & Wedin 2017	KX512968	KX512925			KX529005	
Dirinaria applanata 1	Seaward 109735 (S), Prieto & Wedin 2017	KX512990	KX512926	KX512856			
Dirinaria applanata 2	Lutzoni & Miadlikowska 03.24.03-13 (DUKE), Miadlikowska et al. 2006	DQ972983		DQ973035			DQ973098
Dirinaria confluens	Lötberg (UPS L-159685)	**OQ866146**	**OQ866144**		**OQ867271**		**OQ867275**
Dirinaria sp.	Thulin et al. (UPS L-056358)	**OQ866147**	**OQ866143**		**OQ867272**		
Phaeophyscia ciliata	Prieto (S), Prieto & Wedin 2017	KX512958	KX512929	KX512886	KX529051	KX529012	
Physcia aipolia 1	Wedin 6145 (UPS), Wedin et al. 2002	AY143406	KX512931	AY300857	KX529052	KX529021	
Physcia aipolia 2	Hillis 6-2-2002 (DUKE), Miadlikowska et al. 2006	DQ912290	DQ782836				DQ782862
Pseudothelomma occidentale	Fryday 10069 (MSC), Fryday et al. 2020		MT622500	MT611534			MT610735
Pseudothelomma ocellatum	Hermansson 18662 (UPS), Prieto & Wedin 2017	KX512952	KX512935	KX512891	KX529063	KX529020	

Taxon	Voucher/source	mrSSU	ITS	LSU	MCM7	β-tubulin	RPB2
Pyxine berteriana	Thiyagaraja (MFLU), Hyde et al. 2020	MN792788	MN792989				
Pyxine cocoes	Prieto (S), Prieto & Wedin 2017	KX512964	KX512936			KX529010	
Pyxine consocians	Wang et al. 15-49942 (KUN-L), Yang et al. 2019	KY751386	KY611879				
Pyxine endochrysina	Wang et al. 14-46439 (KUN-L), Yang et al. 2019	KY751395	KY611888				
Pyxine flavicans	Wang et al. 15-48196 (KUN-L), Yang et al. 2019	KY751391	KY611884				
Pyxine limbulata	Wang et al. 15-49117 (KUN-L), Yang et al. 2019	KY751392	KY611885				
Pyxine meissnerina	Wang et al. 12-34377 (KUN-L), Yang et al. 2019	KY751385	KY611878				
Pyxine minuta	Wang et al. 13-40695 (KUN-L), Yang et al. 2019	KY751379	KY611872				
Pyxine sorediata 1	Wetmore 91254 (S), Prieto & Wedin 2017	KX512973	KX512937	KX512870	KX529039	KX529001	
Pyxine sorediata 2	Lutzoni & Miadlikowska 07.02.03-18 (DUKE), Miadlikowska et al. 2006	DQ972984	JQ301697	DQ973036			DQ973071
Pyxine subcinerea	Amtoft 2060 (DUKE), Miadlikowska et al. 2006	DQ912292	HQ650705	DQ883802			DQ883758
Pyxine tiinae	Santesson et al. P13:47 (UPS L-129473, holotype)	**OQ866150**	**OQ866145**	**OQ866151**	**OQ867273**		**OQ867276**
Tholurna dissimilis 1	Wedin 6330 (UPS), Wedin et al. 2002	AY143407	AY143397	KX512893	KX529053	KX528992	
Tholurna dissimilis 2	Davydov et al. (O), Miadlikowska et al. 2006	DQ972974					DQ973086

DNA extraction and amplifications

We extracted DNA and used primers and PCR-protocols following the procedures described in Svensson & Fryday (2022). For LSU, we used the primers LR3 and LR5 (Vilgalys & Hester, 1990) in combination with LRLecF (Schneider et al., 2015), using the same PCR thermal profile as Svensson & Fryday used for LSU. Due to difficulties with obtaining markers for several of the taxa when using standard primers, we also designed the following new primers for mtSSU, MCM7, β-tubulin and RPB2, specific to the *Dirinaria*/*Pyxine*-clade: mtSSU-PyxF (5'-GAT GAA TGT CAT AGT ATA GA-3') and mtSSU-PyxR (5'- CCC ATY TCY TTB GTC AC-3'); MCM7-PyxF (5'-GAR TGT CCM TCK CCD GA-3') and MCM7-PyxR (5'-CCC ATY TCY TTB GTC AC-3'); BT-PyxF (5'-TAT GTK CCM CGT GCW GTT-3') and BT-PyxR (5'-RCG GCT CGT RAG RGG-3'); and RPB2-Pyx1F (5'-CCG RAC GCT KTT CAA CAA GC-3') and RPB2-Pyx1R (5'-GGT YTC YTC YTC HTC CGC ATC-3') with the internal primers RPB2-Pyx2F (5'-GGI GTB AAG TCD ACR ACC-3') and RPB2-Pyx2R (5'-CCA WSC CAK CCR AAM GTG-3'). For all these new primers, we used the VWR Red Taq Polymerase Master Mix (VWR International, Belgium) following the manufacturer's protocols. For mtSSU-PyxF and mtSSU-PyxR, we used the same PCR thermal profile as the one used for ITS by Svensson & Fryday (2022). We used MCM7-PyxF and MCM7-PyxR as nested primers after running a first PCR with the primers MCM7-709f and MCM7-1348r (Schmitt et al., 2009), in both cases with the same PCR thermal profile as was used for MCM7 by Svensson & Fryday (2022). Likewise, for the amplification of β-tubulin we used BT-PyxF and BT-PyxR as nested primers after running a first PCR with the primers BT3-LM and BT10-LM (Myllys et al., 2001). For these runs, we used the following thermal profile: an initial hold at 94 °C for 3 min; followed by 10 cycles of denaturization at 94 °C for 30 s, annealing at 65 °C for 45 s (decreasing 1 °C per cycle) and polymerization at 72 °C for 1 min

30 s; then 22 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min; and finally a hold at 72 °C for 10 min. For RPB2, we used RPB2-Pyx1F and RPB2-Pyx1R for a first PCR, and then used RPB2-Pyx2F and RPB2-Pyx2R for a nested PCR; in both cases with the same thermal profile as was used for β-tubulin. PCR products were subsequently purified with ExoCleanUp FAST (VWR International, Belgium).

Sequence alignment and partitioning schemes

For ITS, mtSSU and LSU, we estimated the alignment using PASTA 1.7 with default settings (Mirabab et al., 2015). For the three protein-coding genes MCM7, β-tubulin and RPB2, we estimated alignments with MAFFT (algorithm E-INS-i, Katoh et al., 2019). One intron (48 bp) was identified for *Culbersonia nubila* in the alignment of β-tubulin, and this was removed prior to analysis. The ends of all alignments were trimmed to minimize problems with missing data. We checked for gene incongruence by running a separate maximum likelihood analysis of each of the six alignments, using IQ-TREE 2.0.7 (Minh et al., 2020) with 2000 ultrafast bootstrap replicates (Hoang et al., 2018). The resulting phylogenetic trees were compared to identify possible conflicting, supported (UFBoot > 95%) clades. No such conflicts were identified, and the six alignments were thus concatenated into one.

We divided the concatenated alignment into 14 potential partitions (ITS1, 5.8S, ITS2, mtSSU, LSU, and the first, second and third codon position for each of the three protein-coding genes MCM7, β-tubulin, and RPB2), which we assessed with ModelFinder as implemented in IQ-TREE2 (Kalyaanamoorthy et al., 2017). We restricted the evaluated models to those available in MrBayes (Ronquist et al., 2012), used the Bayesian Information Criterion for model selection, and allowed for merging of partitions if this improved model fit. The best model fit was achieved when the 14 partitions were merged into five (with corresponding substitution model): (1) ITS1 + ITS2; GTR+F+I+G4 (GTR+I +G in MrBayes), (2) 5.8S + β-tubulin 1st and 2nd + MCM7 2nd + RPB2 2nd; SYM + I, (3) MCM7 3rd + β-tubulin 3rd + RPB2 3rd; HKY+F+G4 (HKY+G in MrBayes), (4) MCM7 1st + RPB2 1st + LSU; GTR+F+G4 (GTR+G in MrBayes), and (5) mtSSU; GTR+F+G4.

Phylogenetic analysis

The concatenated alignment with the estimated partition scheme and substitution models was analyzed both with MrBayes 3.2.7a (Ronquist et al., 2012) and with IQ-TREE 2.0.7 (Minh et al., 2020). For the Bayesian analysis, we used flat Dirichlet priors for the substitution rates and state frequencies, an exponential (1) distribution for the gamma shape parameter, a compound Dirichlet prior ($\alpha = 1$, $\beta = 0.1$) for branch lengths, uniform distributions for invariant sites and topology, set the temperature to 0.10 and the sample frequency to every 100th generation. We ran four Markov chain Monte Carlo (MCMC) chains each, three heated and one cold, set the fraction of trees to be discarded as burn-in to 25% and halted the analysis when convergence was reached, defined as an average standard deviation of split frequencies below 0.003. We considered a posterior probability of 0.95 or higher as indicating support. For the maximum likelihood analysis with IQ-TREE, we used the same partitioning scheme and models of molecular evolution as for the Bayesian analysis. We assessed branch support by running 500 nonparametric bootstrap replicates. We considered a bootstrap value of 75% as indicating support.

RESULTS

The final alignment had 36 terminals, 122 sequences and 7263 characters of which 1735 were parsimony-informative. As the Bayesian and maximum likelihood analyses resulted in the same topology, only the majority-rule consensus tree from the Bayesian analysis is shown in Figure 1, but bootstrap values >75% from the corresponding maximum likelihood analyses have been included.

The phylogenetic analysis recovered the same relationships within the Caliciaceae as those of earlier studies (Prieto & Wedin, 2017; Aptroot et al., 2019;). The phylogenetic position of *Pyxine tiinae* in a clade together with *Culbersonia* and *Pyxine* received high support (PP=1; BS=0.82). Its position in relation to *Culbersonia* and *Pyxine* was less certain however, as it appeared as sibling to *Culbersonia* with some support in the Bayesian analysis (PP=0.97) but not in the maximum likelihood analysis.

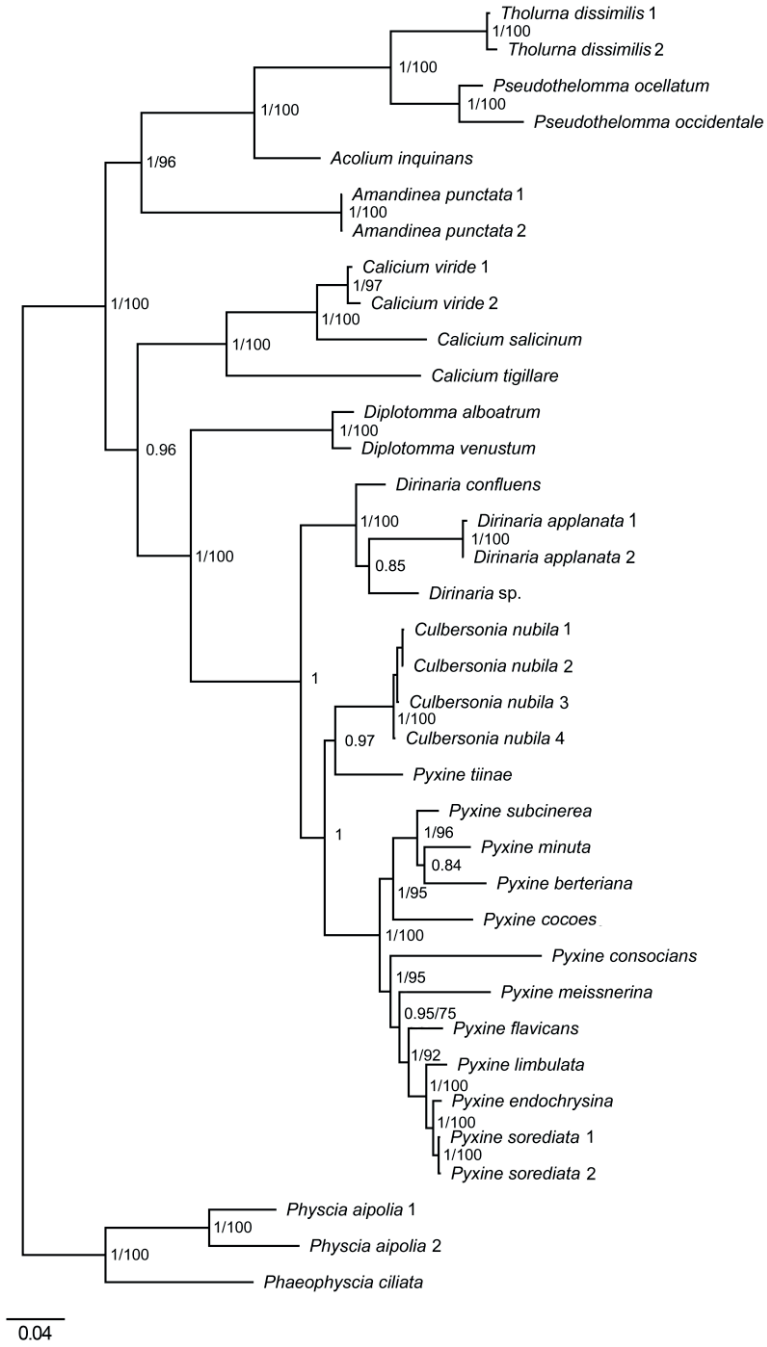

Fig. 1. Majority-rule consensus tree based on a Bayesian MCMC analysis of a concatenated, six marker (mrSSU, LSU, ITS, MCM7, β-tubulin and RPB2) data set, showing the phylogenetic position of *Pyxine tiinae* in the Caliciaceae. Branch support is given both as posterior probabilities and bootstrap support values, the latter from a corresponding maximum likelihood analysis. Only bootstrap values > 75% are shown.

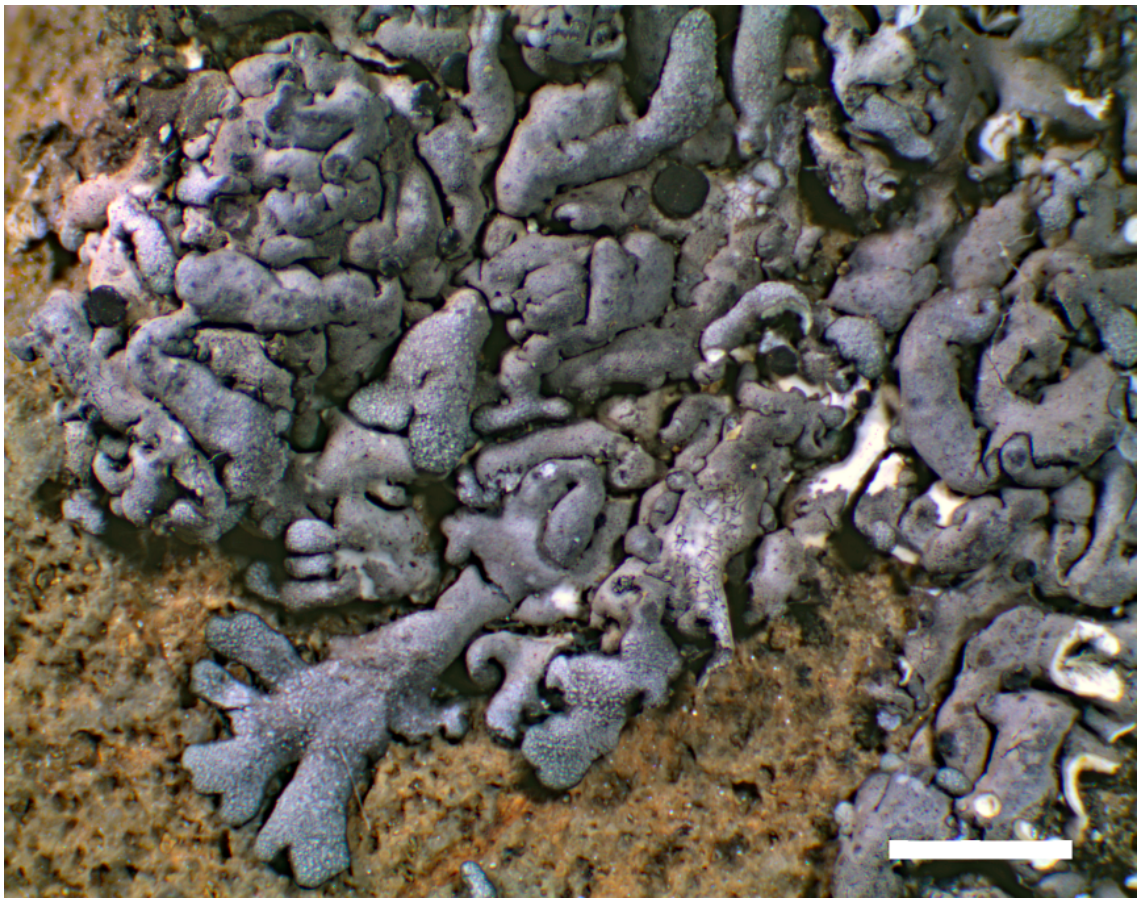

Fig. 2. *Pyxine tiinae*, UPS L-129473 (holotype). Scale=2mm.

TAXONOMY

PYXINE TIINAE Moberg & M. Svensson sp. nov. (Fig. 2)

MycoBank no: MB 848575

A foliose lichen with convex and radiating, grey lobes somewhat similar to *Pyxine sorediata* (Ach.) Mont. but differing in the colour of the medulla. Differs in chemistry from *Culbersonia nubila* by presence of terpenes and from *Culbersonia* and other species of *Pyxine* by the K– epihymenium.

Type: Peru. Junin Dept., Tarma Prov. c. 3 km (road distance) ESE of Acobamba, 11°22'S 75°41'W [WGS84 -11,36667 -75,68333], alt. 3000 m. On open rocks. 7 February 1981, R. & B. Santesson & R. Moberg P13:47 (UPS L-129473 – holotype; TU, M – isotypes).

Etymology – The new species is named in honour of Tiina Randlane on occasion of her 70th birthday.

Description – *Thallus* orbicular to irregular forming 2–5 cm large closely attached patches, lead grey to dark grey. *Lobes* ± radiating, convex, to 1 mm wide, widening and becoming darker and white pruinose at tips. *Medulla* white without pigment. *Lower side* black with few black, simple rhizines and with short black projections in between them. *Upper cortex* paraplectenchymatous; *lower cortex* prosoplectenchymatous with short, black projections. *Soralia and isidia* absent. *Apothecia* few, to 0.7 mm diam., sessile, black, epruinose or thinly white pruinose on the sides, at first concave with a prominent, non-thalline margin concolourous with the disc; disc later level

with margin (= *cocoes*-type sensu Kalb, 1987). *Epihymenium* brownish green, K–. Hymenium colourless, K– or sometimes K+ violet when the hypothecial pigment reaches the subhymenium. *Hypothecium* light brown, strongly K+ violet. *Paraphyses* simple or sometimes sparingly branched, often forked in their upper part, 1–2 µm broad, apically not thickened or thickened –5 µm broad, in the latter case sometimes with a pigment cap. *Ascospores* 8/ascus, brown and of *Physcia*-type, 12–20 × 6–9 µm. *Pycnidia* rare, immersed with dark upper part; pycnoconidia cylindrical, 4–6 × 1 µm.

Chemistry – Thallus K–, C–, Pd–, UV–. All reactions within apothecia or medulla negative, except for the strong K+ violet reaction in the lower part of the hymenium and in the hypothecium, which may be caused by an anthraquinone. Two unknown terpenes were detected in the thallus with HPTLC.

Ecology and distribution – The new species is known only from the type locality, growing on exposed rocks at high elevation in Peru. The study site is located in a relatively arid region of the Peruvian highlands, where the average annual rainfall ranges between 600 and 700 mm (Silva Vidal, 2005). A species of *Candelariella* and a few indeterminate crustose lichens grew together with *Pyxine tiinae*. Other species collected on exposed rocks or soil at the same locality included *Heterodermia albicans* (Pers.) Swinscow & Krog, *H. chilensis* (Kurok.) Swinscow & Krog, *H. speciosa* (Wulf.) Trevis., *Phaeophyscia hirsuta* (Mereschk.) Essl., *Physcia biziana* (A.Massal.) Zahlbr., *P. tribacia* (Ach.) Nyl., and *P. undulata* Moberg.

Notes – There is no comprehensive treatment of South-American *Pyxine* species but several papers have dealt with species from e.g., Argentina (e.g., Sarlej, 2019; Scurati, 1995), Brazil (Kalb, 1987; Malme, 1897; Jungbluth & Marcelli, 2011; Jungbluth et al., 2011; Aptroot et al., 2014), and Colombia (Aptroot, 1989), and the Guianas (Aptroot, 1987). Our material does not fit with any of those species, nor with any species of *Pyxine* from other parts of the world (e.g., Kalb, 2002; Moberg, 2004; Mongkolsuk et al., 2012; Nayaka et al., 2013; Aptroot et al., 2014) but display some similarity in habitus to *Pyxine sorediata* and *P. limbulata* Müll.Arg.. Many species of *Pyxine* contain terpenes, but they all differ from *P. tiinae* by their K– hypothecium, a K+ violet epihymenium and by the presence of a pigment in the medulla. *Culbersonia nubila* has a pale lower cortex in contrast to the black one in the new species; it further differs by having a K+ violet cortex and by the lack of any lichen substances detectable by TLC. When it comes to pycnoconidia, the new species is closer to *Culbersonia* (5–7 µm vs. 3–4 µm in *Pyxine*; Moberg, 1980; Esslinger, 2002). See Table 2 for an overview of characters.

The ascospores of *Pyxine* have been previously described as belonging to the *Dirinaria*-type (Kalb 1987) or referred to as 'mischoblastimorphic' (Kalb 2002). However, we did not observe the characteristic wall thickening at septa (best observable in K) that is typically associated with the *Dirinaria*-type. To determine if the ascospore type of *P. tiinae* consistently differs from that of other *Pyxine* species, further material of *P. tiinae* would be required for a comprehensive analysis.

Based on the morphological comparisons and the uncertain phylogenetic position of *Pyxine tiinae*, there are three alternatives for its generic placement: (1) to include it in *Culbersonia*, (2) to describe a new monotypic genus for it, and (3) to include it in *Pyxine*. The first alternative may seem as the most natural one given the phylogenetic tree (Fig. 1). We are, however, reluctant to include *P. tiinae* in *Culbersonia*. The latter monotypic genus is well defined by characters such as the pale lower side and the absence of secondary metabolites (Table 2).

Table 2. Overview of morphological and chemical characters of *Pyxine*, *Culbersonia* and *Pyxine tiinae*.

	Lower side	Upper cortex	hypothecium	epihymenium	chemistry	conidia
Pyxine	black	K– or K+ yellow	K–	K+ purple	atranorin, lichexanthone, norstictic acid, terpenes	3–4 µm
Culbersonia	pale	K+ purple	K+ purple	K+ purple	nil	5–6.5 µm
Pyxine tiinae	black	K–	K+ purple	K–	terpenes	4–6 µm

Including *P. tiinae* in *Culbersonia* would make the morphological distinction of this genus unclear in comparison to *Pyxine* (essentially reducing it to a difference in conidial length) and in addition, the sibling status of *C. nubila* and *P. tiinae* is only supported in the Bayesian analysis, in spite of a comparatively large data set comprising six markers. The second alternative would be to describe a new monotypic genus for *P. tiinae*. The aberrant characters of *P. tiinae*, which seem somehow intermediate between *Culbersonia* and *Pyxine* and has one unique feature (the K– epihymenium), could be viewed as speaking in favour of this solution. Still, we are reluctant to create another monotypic sibling genus to *Pyxine*. This is due, in part, to the uncertain position of the species and its possibly close relationship to *Culbersonia*, but also because many species of *Pyxine* have not been sequenced yet, leaving some doubt as to the optimal generic subdivision of the *Dirinaria*/*Pyxine* clade. We have thus settled for the conservative option of describing *P. tiinae* as a species of *Pyxine*, recognizing that additional data may eventually justify an inclusion of the species in *Culbersonia* or in a yet-to-be-described genus.

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