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A preliminary phylogeographic study of *Flavopunctelia* and *Punctelia* inferred from rDNA ITS-sequences

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Abstract: A preliminary phylogeny of the genera *Flavopunctelia* and *Punctelia* is presented. Genus and species delimitations have been investigated using ITS rDNA-sequencing of populations from different continents. Current genus delimitations of *Flavopunctelia*, *Punctelia* and *Parmelia* are confirmed and the species status of recently resurrected *Punctelia ulophylla* is confirmed. The status of three cryptic species, *Flavopunctelia soredica*, *Punctelia perreticulata* and *P. stictica* is discussed. *Flavopunctelia borrerioides* and *Punctelia perreticulata* are reported from China for the first time.

Kokkuvõte: Esialgne ülevaade perekondade *Flavopunctelia* ja *Punctelia* fülogeograafiast rDNA ITS-sekventside põhjal.

Esitatakse perekondade *Flavopunctelia* ja *Punctelia* esialgne fülogeneesi rekonstruktsioon. Perekondade ja liikide eraldamist on uuritud erinevatelt kontinentidelt pärinevate populatsioonide ITS rDNA sekventside alusel. Senine perekondade *Flavopunctelia*, *Punctelia* ja *Parmelia* piiritlemine on leidnud kinnitust, samuti liigi *Punctelia ulophylla* staatus. Arutletakse kolme krüptilise liigi, *Flavopunctelia soredica*, *Punctelia perreticulata* ja *P. stictica* staatuse üle. Teatatakse liikide *Flavopunctelia borrerioides* ja *Punctelia perreticulata* esmasleidudest Hiinas.

INTRODUCTION

The genus *Punctelia* Krog was segregated from *Parmelia* Ach. on differences in pseudocyphellae ontogeny, secondary chemistry and phytogeography (Krog, 1982). The genus, originally including 22 species, was subdivided into two distinct subgenera: *Punctelia* subgenus *Punctelia*, characterized by unciform spermatia and atranorin as a a major cortical substance, and *Punctelia* subgenus *Flavopunctelia* Krog characterized by bifusiform spermatia and usnic acid as a major cortical substance. Spermatial shape has been considered to be of great importance in genus delimitations (Kärnefelt, 1998). Based on spermatial and additional chemical characters, *Flavopunctelia* (Krog) Hale was recognized as a separate genus composed of four species (Hale, 1984); two additional species have been discovered in *Flavopunctelia* (Elix & Adler, 1987; Kurokawa, 1999) as compared with 30 species which constitute *Punctelia* today (Crespo et al., 2004; Egan, 2003; Elix & Johnston, 1988; Galloway & Elix, 1994; Kurokawa, 1999; Sérusiaux, 1983, 1984; Wilhelm & Ladd, 1992). Both *Flavopunctelia* and *Punctelia* have a temperate to subtropical distribution and reach their highest diversity in South- and North-America and in Africa (Krog, 1982). DNA-investigations support that *Flavopunctelia* and *Punctelia* are sister groups and that *Parmelia* Ach. may be the sister group of the two genera (Blanco et al., 2004, Thell et al., 2004). Populations of *Flavopunctelia* and *Punctelia* species, of which some are represented by collections from different continents, are analysed here, together with some *Parmelia* species, using nuclear ITS rDNA-sequences. Genus and species delimitations are studied and discussed.

MATERIALS AND METHODS

The material was collected by the authors and collegues during recent travels, resulting in 20 new sequences from the ITS1-5.8S-ITS2 rDNA region that were submitted to the NCBI GenBank (http://www.ncbi.nlm.nih.gov, Table 1). Eleven sequences were downloaded from the

Table 1. Specimens used in the study, extraction numbers (LD), sample-IDs and GenBank accession numbers

Species	Extr.	Sample-ID	GenB. acc.
Flavoparmelia caperata	555	Estonia, Tartumaa, Ahunapalu,Thell 9906 (TUR)	AF451750*
Flavopunctelia borrerioides	1521	China, Yunnan Prov., 29 Oct 2002, van Herk (ABL)	AY773129
Flavopunctelia flaventior	1285	Germany, Bavaria, Dachau, Feuerer s. n. (HBG)	AF251420*
Flavopunctelia flaventior	1517	China, Yunnan Prov., Aptroot 56024 (ABL)	AY773126
Flavopunctelia flaventior	1520	China. Yunnan Prov., Aptroot 560101 (ABL)	AY773127
Flavopunctelia soredica	1518	U. S. A., New York, Aptroot 50612 (ABL)	AY773128
Parmelia ernstiae	858	Germany, Schleswig-Holstein, Feuerer & Thell (HBG)	AF410834*
Parmelia ernstiae	965	Sweden, Scania, Eslöv, Thell 0101 (HBG)	AF247007*
Parmelia saxatilis	518	Chile, Magallanes, Feuerer 29542 (HBG)	AF410672*
Parmelia saxatilis	534	Finland, Regio aboënsis, Ruissalo, Thell 9926 (TUR)	AF410835*
Parmelia submontana	-	Spain, Hoya Redonda (MAF 3729)	AY037000*
Parmotrema crinitum	1273	Yemen, Socotra, Schultz 14297c (HBG)	AY251442*
Punctelia borreri	945	Italy, Trentino-Alto Adige, Feuerer & Thell s. n. (HBG)	AY773113
Punctelia borreri	959	Italy, Abruzzo, Tretiach 34124 (HBG)	AF451769*
Punctelia borreri	960	Italy, Friuli-Venezia-Giulia, Gambera 34126 (HBG)	AY773114
Punctelia borreri	1338	Kenya, Kakamega Forest Nat. R., Killmann (priv. herb.)	AY773110
Punctelia borreri	1339	Kenya, Kakamega Forest Nat. R., Killmann (priv. herb.)	AY773111
Punctelia borreri	1506	China, Yunnan Prov., Aptroot 56028 (ABL)	AY773115
Punctelia perreticulata	1286	U. S. A., Missouri, Osage Co., Ladd 23798 (HBG)	AY773123
Punctelia perreticulata	1331	China, Yunnan Prov., Yunlong, Aptroot 56005 (ABL)	AY773124
Punctelia perreticulata	1505	China, Yunnan Prov., Aptroot 56094 (ABL)	AY773122
Punctelia stictica	1020	Venezuela, La Culata, 31 Oct 1995, Feuerer s. n. (HBG)	AY773125
Punctelia stictica	1340	Kenya, Kakamega Forest Nat. R., Killmann (priv. herb.)	AY773112
Punctelia stictica	1609	Peru, Pisac, 20 Sept 2003, Thell & Feuerer s. n. (HBG)	AY773119
Punctelia subpraesignis	1310	Argentina, B. Aires, Adler & Protomastro s. n (BAFC)	AY267010*
Punctelia subrudecta	944	Germany, Schleswig-Holstein, Feuerer & Thell (HBG)	AY773116
Punctelia subrudecta	958	Italy, Venezia-Giulia, Gambera 34128 (dupl. HBG)	AY773117
Punctelia subrudecta	1509	Germany, Eifel, Aptroot 55416 (ABL)	AY773118
Punctelia ulophylla	956	The Netherlands, Gelderland, Aptroot 44450 (ABL)	AY773120
Punctelia ulophylla	957	The Netherlands, Prov. Utrecht, Sipman 43579 (HBG)	AY251726*
Punctelia ulophylla	1507	Belgium, Liege, Aptroot 57873 (ABL).	AY773121

*Sequences downloaded from the GenBank

same GenBank, of which 10 have appeared in earlier publications (Adler et al., 2004; Molina et al., 2004; Thell et al., 2002, 2004). The laboratory work was performed at the Department of General Botany and Botanical Garden, University of Hamburg.

Minute fragments of the fresh collections were ground with sterile plastic pestels. Total

DNA was extracted using the DNEasy Plant Mini Kit from Qiagen as described in Thell et al. (2004). ITS standard primers, ITS 4 and ITS 5, were used (White et al., 1990)

Ready To Go PCR beads (in 0.2 ml tubes) from Pharmacia Biotech Inc. were dissolved in 11.8 µl distilled water, 0.35 µl of a 16µM concentration of each of the primers ITS5 and ITS4 (White et al., 1990). The ITS fragments were amplified with a Perkin-Elmer Gene Amp PCR System 9700 thermal cycler. 12.5 µl of the concentrated DNA extractions were added to the solution, resulting in final reaction volumes of ca. 25 µl. The PCR started with 2 minutes at 95°C, followed by a 30–35 cycle schedule using a denaturation temperature of 95°C for 1 min., an annealing temperature of 60°C for 1 min., and an extension temperature of 72°C for 1 min.

The PCR products were purified with QIAquick PCR purification kit, and diluted in 30 µl of the enclosed elution buffer. A 25 cycle sequencing PCR, with a denaturation temperature of 96° C for 10 seconds, an annealing temperature of 50° C for 5 seconds, and an extension time of 60° C for 4 minutes, was performed to amplify the DNA-fragments prior to the sequencing procedure. 12 µl deionized water including 30–90 ng of the purified PCR-product and 3.2 pmol of the primers ITS1LM (Myllys, 1999) and ITS4 were added to 8 µl BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq Polymerase FS from Perkin Elmer according to the accompanying protocol. The sequences were produced using an automatic sequencer, ABI Prism 377 from Perkin-Elmer.

The phylogenetic analyses of the manually aligned ITS sequences were done with PAUP version 4.0b (Swofford, 1998). Trees were searched by using the heuristic option, with TBR branch swapping, 1000 replicates of random addition sequence order, and branches collapsed if the maximum length is zero. Gaps in the alligned sequences were treated as missing characters. Bootstrap analyses with 1000 replicates were done, using the same settings as in the heuristic search. Bootstrap support values of 60 or above are marked in the cladogram above the branches (Fig. 1). Large surveys of Parmeliaceae phylogeny were consulted when selecting the outgroup (Crespo et al., 2001; Thell et al., 2004)

RESULTS AND DISCUSSION

Results from the phylogeny analysis

The aligned matrix was composed of 520 nucleotide long sequences, including the gaps. Of the 163 variable characters, 114 were parsimony informative. The phylogeny was based on parsimony analysis using PAUP 4.0b. The analysis resulted in 12 shortest trees of length 328 (RI = 0,834; CI = 0,668). Bootstrap consensus (Fig. I) was identical to strict consensus of the 12 most parsimonious trees, except for some branching orders of the *Punctelia* subclades (Fig. 1. I–V).

Genus and species delimitations

Present genus delimitations are supported in the analysis, where *Parmelia* and *Flavopunctelia* are strongly supported, with bootstrap values of 94 and 100 respectively. *Punctelia* has a more moderate support, 75, of the three ingroup genera, and the clade is divided into five subclades (Fig. 1, I–V). Four of these subclades, however, constitute single species, and two of these species constitute monophyletic clades, *P. subrudecta* (Nyl.) Krog and *P. ulophylla* (Ach.) van Herk & Aptroot (Fig. 1, subclades II–III), both having a bootstrap support value at least 99. *Flavopunctelia soredica* (Nyl.) Hale, *Punctelia perrecticulata* (Räs.) G. Wilh. & Ladd and *P. stictica* (Duby) Krog appear as cryptic species (Fig. 1, subclades IV–V). The bootstrap support for the largest subclade (Fig. 1, I) is rather strong, 75. At the adjacent node of the tree, however, an even stronger monophyletic clade is identified, composed of *P. borreri* (Sm.) Krog, the *P. perreticulata*-samples collected in China, *P. subpraesignis* (Nyl.) Krog. African *P. borreri* and the Chinese *P. perreticulata* are not supported as separate species by ITS-sequences (Wilhelm & Ladd, 1987).

The number of cryptic species have increased rapidly in recent years becuse they are revealed by DNA-techniques. How to taxonomically treat these morphologically and chemically more or less identical but genetically different species is currently under constant review. Cryptic species are most frequently discovered by ITS-sequences, but the results should preferrably be confirmed by a second or third gene, such as mitochondrial SSU (Crespo et al., 2001) or GAPDH (Myllys et al., 2002). On the contrary,

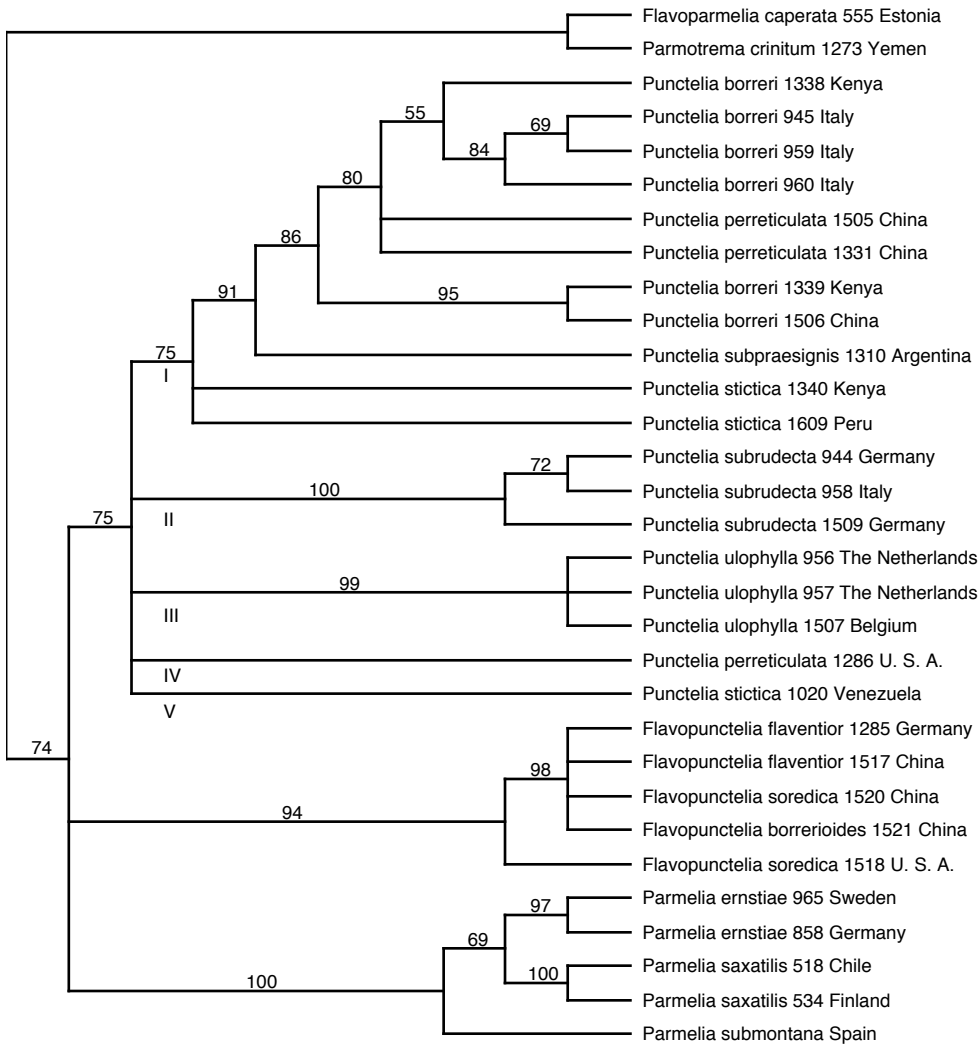

Fig. 1. Phylogeny of *Flavopunctelia, Parmelia* and *Punctelia* based on ITS rDNA-sequences. Bootstrap consensus, identical to strict consensus from 12 most parsimonious trees. Bootstrap support values of 60 or above are indicated above the branches, and the five subclades (I–V) of *Punctelia* below the branches.

ITS-sequences do not always separate formerly accepted morphological species (Grube & Kroken, 2000).

Punctelia ulophylla – a successfully resurrected taxon

Punctelia ulophylla was resurrected only recently (van Herk & Aptroot, 2000), based on detailed morphological observations and co-occurrence in Western Europe with *P. subrudecta.* Although the species was formerly described as a variety, it was rarely mentioned, and was not thought to merit much attention between the many other varieties and forms described in *Parmelia* s. lat. However, it has now been widely reported, mainly from Western Europe, but also from Northern Europe (Gauslaa, 2000) and Central Europe (Truong & Clerc, 2003). Crespo et al. (2004) confirmed its status as a separate species and even found it to be only distantly related to

P. subrudecta, which is confirmed here by our results. It is surprising how such a common and well-recognizable macrolichen has remained unnoticed for so many years. Even more surprising is the current reluctence of some authors to accept this fact, even including the unexplained citation of *P. ulophylla* as a synonym of *P. subrudecta* in Santesson et al. (2004).

Flavopunctelia borrerioides and *Punctelia perreticulata* – new to China

Among the material cited are two species which are new to China, namely *Flavopunctelia borrerioides* Kurok. and *Punctelia perreticulata.*

Flavopunctelia borrerioides was described by Kurokawa (1999) from Peru and Mexico and more recently has been reported from India by Divakar et al. (2003). It differs from the common *F. flaventior* (Stirton) Hale, with which it occurs, by the conspicuous rounded laminal pseudocyphellae developing into soredia, giving it the aspect of *Punctelia borreri* (hence the name). Our specimen from China and an additional one from South Africa (leg. C.M. van Herk, in ABL) suggest that this is a widespread subtropical species.

Punctelia perreticulata was recently reported (Aptroot, 2003) to be the most common and widespread sorediate *Punctelia* in North- and Central America (Egan & Aptroot, 2004). It is therefore not surprising that it is also present in Asia, as our specimen from China shows.

Notes on the cryptic species

Punctelia perreticulata is a species mainly distinguished by atranorin in the upper cortex and sorediate pseudocyphellae. The lower side is ivory to tan towards the centre and lecanoric acid in the medulla is the major secondary metabolite. It has been treated as a synonym of *P. subrudecta* by Krog (1982) and Nimis (1993), a view not supported in the cladogram, which contradicts a conspecific habit of the two species (Fig. 1). Alternatively, Whilhelm & Ladd (1987), who studied corticolous populations from the interior highlands of the USA, considered *P. perreticulata* to be a distinct species, distinguished by a strongly fovelate-reticulate upper surface compared with the occasionally somewhat ridged upper surface of *P. subrudecta*. Adler & Ahti (1996) also considered *P. perreticulata* as a distinct species, mainly differring from *P. subrudecta* in shape and length of spermatia: European studied specimens of *P. perreticulata* were all saxicolous, had a strongly foveolate upper surface, and 7 µm long, filiform spermatia. Specimens form Argentina were characterized by longer, 9–11 µm, spermatia, whereas those from the USA were mostly corticolous and supplied with an even upper surface (Adler & Ahti, 1996; Aptroot, 2003; Egan & Aptroot, 2004). The Chinese specimens of *P. perreticulata* are corticolous and have 9–12 µm long spermatia similar to the North-American material. Adler & Ahti (1996) concluded that *P. perreticulata* is a species with an unusually wide infraspecific geographic variation. On the contrary, Longán et al. (2000) prefer a narrow species concept for samples with long spermatia. The present analysis, comparing samples from China and the USA only, supports the view that several species may be accumulated in *P. perreticulata*, compared with the circumscription by Adler & Ahti (1996).

Punctelia stictica (Duby) Krog is characterized by a light to dark brown upper surface with secondarily sorediate pseudocyphellae, with granular to isidioid soredia, the underside brown to black towards the centre, with gyrophoric acid as the major medullary metabolite and long filiform spermatia (Adler, 1996). It is also a widely distributed, cosmopolitan species, reported from North- and South-America, Africa and Europe, growing mostly on rocks in very different climates and altitudes, but found also on soil and trunks (Adler, 1996). Both *P. perrecticulata* and *P. stictica* are very widely distributed and have wide ecological amplitudes. Apart from *P. perreticulata*, the cryptic habit of *P. stictica* is, however, not supported by any hitherto known morphological, chemical or anatomical traits, which, on the other hand, is the definition of being cryptic. Furthermore, the *P. stictica* samples do not group according to geographic origin (Fig. 1)

Finally, the *Flavopunctelia soredica* sample from the USA (New York) constitutes a sister group to the single *F. borrerioides* sample, the two *F. flaventior* samples and the *F. soredica* sample from China. Interestingly, in this preliminary study based on a single DNA-fragment, populations of the three possibly cryptic species correlate with DNA according to their geographical origins.

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