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# *Paraconiothyrium babiogorense* sp. nov., a new endophyte from fir club moss *Huperzia selago* (*Huperziaceae*)

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Abstract — *Paraconiothyrium* is a recently established genus within the order *Pleosporales*. Species from this genus are commonly associated with plants but can also be found in soil samples and be parasitic on fungi. Several isolates of a *Paraconiothyrium* sp. were obtained from *Huperzia selago* in Poland. Strains were characterized based on morphological characteristics and molecular data (SSU rDNA, ITS1, 5.8S rDNA, ITS2). Based on its unique morphology and DNA phylogeny, isolates were described as a new species: *Paraconiothyrium babiogorense*, which represents the first report of a *Paraconiothyrium* species from fir club moss. Comparison of characters with other *Paraconiothyrium* species is provided as well as a signature sequence for the new species.

Key words — *Paraphaeosphaeria*, *Coniothyrium*

## Introduction

*Huperzia selago* (≡ *Lycopodium selago* L.), fir club moss, is a member of the *Huperziaceae* (Rothmaler 1944, 1962). Both gametophytes and sporophytes of this plant are colonized by endophytic fungi (Higgins et al. 2007, Winther & Friedman 2008). Recently, some *Paraconiothyrium*-like, endophytic strains were isolated from healthy-looking propagules (e.g., bulbils, gemmae) of *Huperzia selago* collected in the Babia Góra National Park, Poland.

*Paraconiothyrium* comprises 9 species to date (Damm et al. 2008). The genus was established by Verkley (Verkley et al. 2004) to accommodate two species of *Coniothyrium*-like anamorphs, *Paraconiothyrium minitans* (W.A. Campb.) Verkley and *P. sporulosum* (W. Gams & Domsch) Verkley. The genus also comprised four newly described species: *Paraconiothyrium estuarinum*

Verkley & M. da Silva, *P. brasiliense* Verkley, *P. cyclothyrioides* Verkley, and *P. fungicola* Verkley & Wicklow. Damm et al. (2008) later described *P. variabile* Riccioni et al. and *P. africanum* Damm et al., and transferred *Microdiplodia hawaiiensis* Crous as *P. hawaiiense* (Cross) Damm et al. as indicated by ITS and SSU sequence analyses. *Paraconiothyrium* belongs to the order *Pleosporales*, class *Dothideomycetes* (Verkley et al. 2004, Damm et al. 2008). Based on SSU and ITS phylogeny, the teleomorph state of *Paraconiothyrium* belongs probably to the genus *Paraphaeosphaeria* s. str. (Câmara et al. 2001, Verkley et al. 2004).

*Paraconiothyrium* is characterized by presence of eustromatic, simple or complex, rarely pycnidial conidiomata. Conidiogenous cells are discrete or integrated, phialidic or sometimes percurrent. Conidia are aseptate or one-septate, thin-walled and generally smooth-walled, hyaline when liberated, later brown (Verkley et al. 2004).

Most *Paraconiothyrium* isolates to date have been obtained from woody plants (Damm et al. 2008). However, *P. minitans* and *P. fungicola* can also be fungicolous (Campbell 1947, Whipps & Gerlagh 1992, Verkley et al. 2004,), *P. sporulosum* was found in soil samples (Damm et al. 2008), and *P. estuarinum* was detected in an estuarine sediment (Verkley et al. 2004). Recently, *P. brasiliense* and *P. sporulosum* were determined as dominant endophytes of the mistletoe *Phoradendron perrottetii* (*Viscaceae*) from Brazil (Abreu et al. 2010). Damm et al. (2008) suggested that *Paraconiothyrium* was a commonly occurring fungal genus and that it was more often isolated from warm regions like South Africa, Italy, Brazil, Papua New Guinea, Hawaii (USA), and Turkey (Verkley et al. 2004, Crous & Groenewald 2006, Göre & Bucak 2007, Riccioni et al. 2007).

The aim of the present study was to evaluate the taxonomic position of *Paraconiothyrium*-like isolates from *H. selago* propagules collected from an alpine site in Babia Góra National Park, using morphological observations and analysis of ITS and SSU rDNA sequences.

## Materials & methods

### Isolates

Symptomless *Huperzia selago* propagules from nine different plant individuals were collected from one site in Babia Góra National Park (49°34'24"N 19°31'46"E), Beskidy Mountains, Poland. After transfer to the laboratory, the propagules were cleaned of remnants of soil and other plant leaves, rinsed in tap water with detergent, and then surface disinfected according to the protocols of Szypuła et al. (2005). In the first step propagules were subsequently rinsed for 1 min in the following solutions: 0.01 M hydrochloric acid (HCl), 5% sodium hypochlorite (NaOCl) and 2.4 mM citric acid. In the second step 70% ethyl alcohol ($C_2H_5OH$) for 1 min, 5% sodium hypochlorite (NaOCl) for 15 min, and 7% hydrogen peroxide ($H_2O_2$) for 10 min were used.

After surface sterilization propagules were plated onto potato-dextrose agar (2% PDA, Fluka® Analytical) and synthetic medium with yeast extract (BTL, Łódź, Poland)

containing 1 g $L^{-1}$ ammonium nitrate ($NH_4NO_3$), 1 g $L^{-1}$ ammonium sulfate [$(NH_4)_2SO_4$], 4 g $L^{-1}$ potassium phosphate dibasic ($K_2HPO_4$), 2 g $L^{-1}$ potassium phosphate monobasic ($KH_2PO_4$), 1 g $L^{-1}$ sodium chloride NaCl, 10 g $L^{-1}$ glucose,

1 g $L^{-1}$ yeast extract (Difco, USA), and 15 g $L^{-1}$Bacto™ Agar (Becton, Dickinson and Company, USA). The pH was adjusted to 4.55 prior to sterilization by using 10% citric acid. The sterilization was carried out in an autoclave at 121° C under increased pressure of 1 bar for 15 min. The cultures were incubated at room temperature (~17°C) for 4 weeks. Reference strains of isolated fungi are maintained in herbarium (WA17577, WA17612-WA17619) and culture collection (CBS128292).

**Morphology**

The strains were studied on 2% Potato Dextrose Agar (PDA, Fluka® Analytical). Petri dishes were incubated at room temperature for 2–4 weeks. To enhance sporulation, autoclaved fir club moss fragments were placed on 2% PDA medium. Growth characteristics were studied on PDA plates incubated in dark at room temperature. Sporulating structures were mounted in Lactophenol Mounting Medium (Amann's fluid; Stevens 1974) and observed using Nikon Eclipse 600 and Nikon SMZ800 microscopes. Digital images were recorded with Nikon DX 1200 and Canon EOS 450D. The structures were measured using Coolview v.1.6.0 software.

**DNA extraction & sequencing**

Total genomic DNA was extracted from the fresh mycelium grown on PDA plates using Genomic Mini AX PLANT Kit (DNA-Gdańsk, Gdynia, Poland). The internal transcribed spacer region (ITS; ca. 0.5 kb) and fragment of 18S rDNA (SSU rDNA; ca. 1.0 kb) were amplified by PCR. Forward primer ITS1-f and reverse primer ITS4 were used to amplify the ITS region (Gardens & Bruns 1993). Forward primers 402 (5': GCT ACC ACA TCC AAG GAA GGC), 1144 (5': GCC TGC GGC TTA ATT TGA CTC) and reverse primers 1308 (5': CTC GTT CGT TAA CGG AAT TAA), R (5': TGA TCC TTC TGC AGG TTC ACC) were used to amplify SSU rDNA. PCR protocols followed Korniłłowicz-Kowalska et al. (2006). Forward and reversed sequences were matched using BioEdit Sequence Alignment Editor v. 7.0.0 (Hall, 1999).

**Phylogenetic analysis**

Pairwise and global alignments of ITS region and SSU rDNA were performed in BioEdit Sequence Alignment Editor v. 7.0.0 (Hall, 1999). Phylogenetic trees were obtained from the data using maximum parsimony (MP), neighbor-joining (NJ) in PAUP* v. 4.0b10 (Swofford 2002) and Bayesian analysis (BA) in MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001). Tree robustness was evaluated by 1000 replicate bootstrap analysis. The Akaike Information Criterion (AIC) implemented in Modeltest 3.7 (Posada & Crandall 1998) was used to select the model that best fit each dataset. BLAST searches in GenBank with ITS region sequences were performed using the blastn algorithm. For phylogenetic analysis *Helminthosporium velutinum* Link (AF145704) and *Helminthosporium solani* Durieu & Mont. (AF163089) for the ITS dataset and *Peziza echinospora* P. Karst. (AF006309) for the 18S rDNA dataset were used as outgroups like in research by Damm et al. (2008). GenBank accession numbers used in these studies are indicated on the phylogenetic trees.

A species specific, signature DNA sequence in the Internal Transcribed Spacer 1 (ITS1) was determined basing on ITS region alignment. The accuracy of characteristic sequence identification was verified using the BLAST algorithm against the whole GenBank database.

## Results

### Morphological observations

Morphological characters are presented in Table 1, where they are compared with other closely related species. They are also presented in the taxonomic description.

### Phylogenetic analysis

The SSU region dataset contained 36 taxa and 933 characters (including gaps) and 92 characters were parsimony informative. After heuristic search, three most parsimonious SSU trees (length: 262 steps) were retained and the strict consensus tree was calculated using bootstrap 50% majority-rule. The ITS region alignment of 52 taxa contained 522 characters (including gaps), of which 179 characters were parsimony informative. After heuristic search, four most parsimonious ITS trees (length: 423 steps) were retained, and the strict consensus tree was calculated using bootstrap 50% majority-rule. The topologies of the ITS trees using MP and BA were similar (Figs 1 and 2). However, in NJ analysis *P. fungicola* was placed in outer position to all other *Paraconiothyrium* species, and *P. sporulosum*, *P. minitans,* and two *Paraphaeosphaeria* spp. formed a sister group separate from the to the remaining *Paraphaeosphaeria* spp. Although we did not resolve phylogenetic relationships within the *Paraphaeosphaeria/ Paraconiothyrium* clade, the main species groups were the same as in Verkley et al. (2004).

The genetic variability of ITS sequences between different strains of *P. babiogorense* was very low (less than 1%). These ITS sequences revealed the highest similarity to *Coniothyrium fuckelii* Sacc. (AY904055), *Paraconiothyrium* sp. (EU709779), *Coniothyrium wernsdorffiae* Laubert (AY904058), and *Coniothyrium* sp. (GU062312; e-value = 0.0; maximum identity = 95% all). The SSU sequence of our strain revealed the highest similarity to *Bipolaris sorokiniana* (Sacc.) Shoemaker (DQ337383), *Paraphaeosphaeria michotii* (Westend.) O.E. Erikss. (AF250817), *Paraphaeosphaeria* sp. (AB096264), and *Paraconiothyrium* sp. (AB303550; e-value = 0.0; maximum identity = 99% all).

The phylogenetic analysis of the SSU dataset confirmed that our isolates are placed in the *Paraconiothyrium/Paraphaeosphaeria* clade (Fig. 1) as defined by Verkley et al. (2004). The phylogenetic tree based on ITS dataset showed that strains from *H. selago* are placed on sister branch to two other *Paraconiothyrium* sp. from Polish club mosses (Fig. 2). However, without detailed information on morphology of these two other strains, we decided not

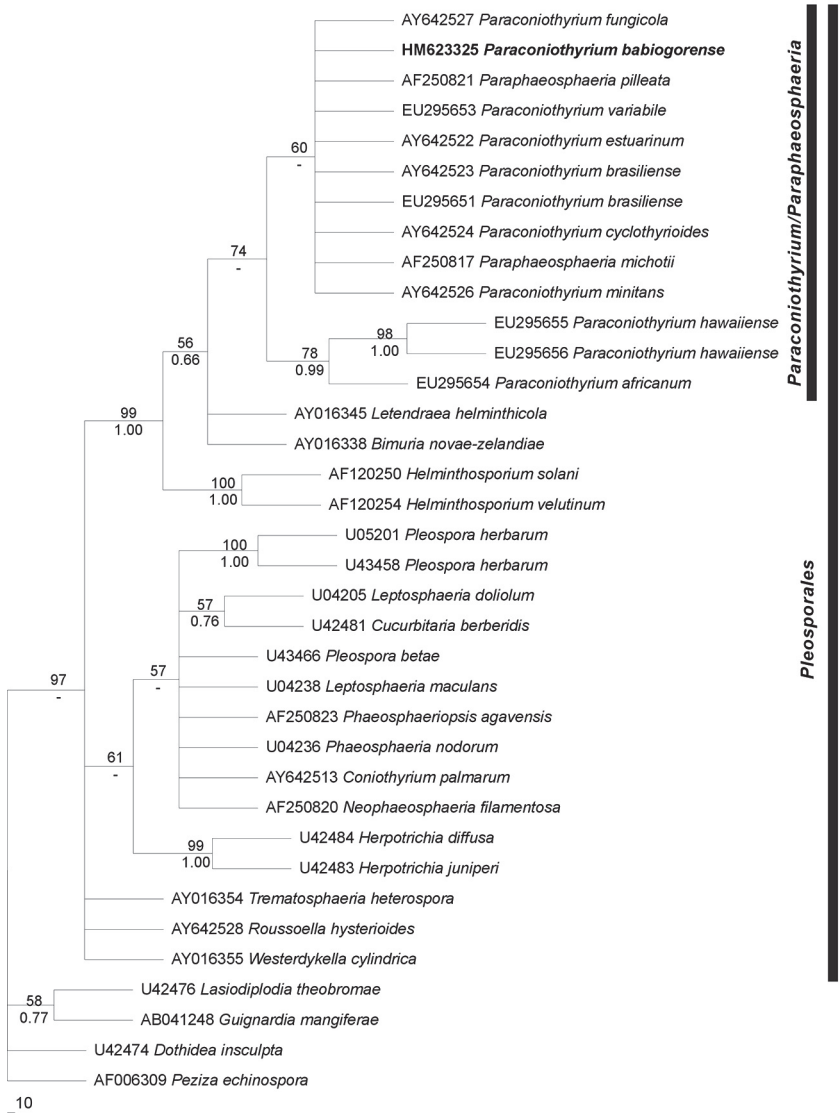


Fig. 1. Strict consensus tree obtained from partial SSU nrDNA sequences data of selected *Dothideomycetes* species. Numbers above branches indicate bootstrap support values inferred by maximum parsimony analysis (1000 replicates; length: 277 steps, CI: 0.661, RI: 0.796, RC: 0.526, HI: 0.339); numbers under branches indicate Bayesian posterior probability values. Only values higher than 50% are shown.

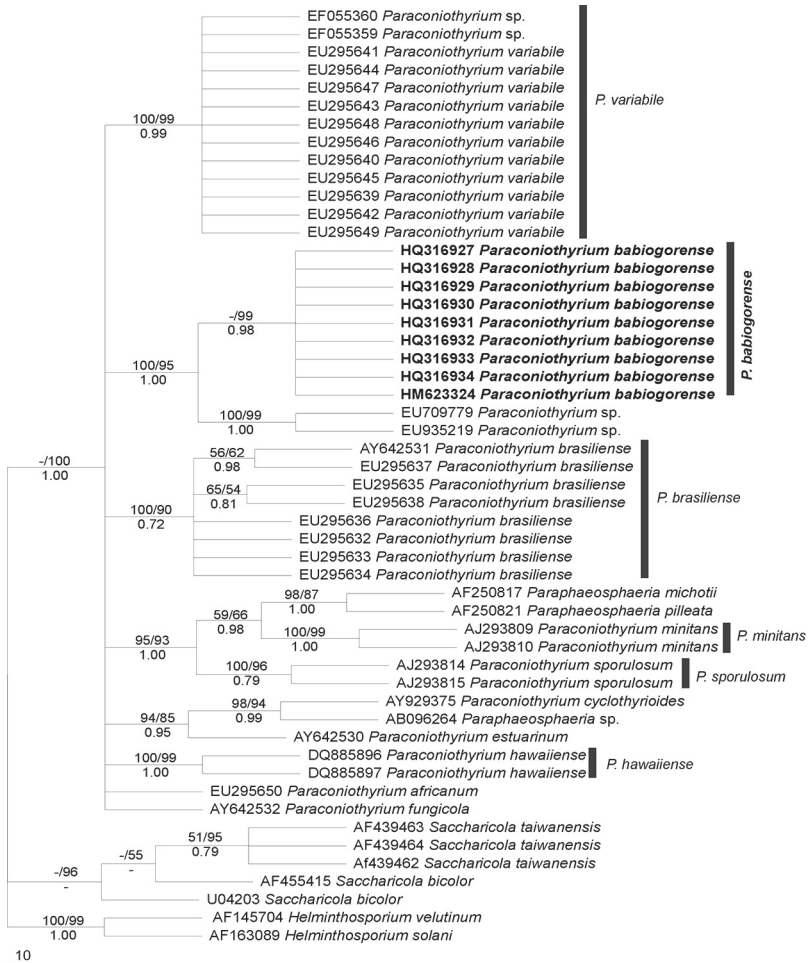


Fig. 2. Strict consensus tree obtained from ITS region sequences data of *Paraconiothyrium*/*Paraphaeosphaeria* species. Numbers above branches before slash indicate bootstrap support values inferred by neighbor-joining analysis (1000 replicates); numbers above branches after slash indicate bootstrap support values inferred by maximum parsimony analysis (1000 replicates; length: 423 steps, CI: 0.650, RI: 0.894, RC: 0.581, HI: 0.350); numbers under branches indicate Bayesian posterior probability values. Only values higher than 50% are shown.

to treat all of them as the same species. Taking into account the phylogenetic position of strains WA0000017577, WA0000017612-WA0000017619, their distinct morphological characters, and low ITS sequence similarity to other *Paraconiothyrium* representatives (less than 95%), we decided to describe them as a new, separate species, *Paraconiothyrium babiogorense*.

## Taxonomy

***Paraconiothyrium babiogorense*** Budziszewska, **sp. nov.** PLATE 1

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*Coloniae in PDA ad temp 17°C luteae, reverso brunneae; Conidiomata eustromatica, complexa, globosa (0.2–)0.3–0.5(–0.9) mm diametro; Cellulae conidiogenae discretae, phialidicae, ampulliformes, hyalinae vel pallide brunneae, 5–7(–9) × 3–5 µm; Conidia tempore liberationis hyalina, deinde luteo-fusca, cylindrica vel breviter cylindrica, glabra, aseptata, interdum uniseptata, guttulata, 1–2(–3) × (7–)8–9(–10) µm.*

TYPE: Poland, Babia Góra National Park, Carpathians Mountains (49°34'24"N, 19°31'46"E; WGS84 system) from healthy propagules of *H. selago*, 10 October 2009, *J. Budziszewska*; **holotype,** WA17577 (dried culture); **isotypes**, WA17612–WA17619 (dried cultures); **ex-holotype,** CBS128292 (lyophylised culture).

SIGNATURE SEQUENCE: 5' CCCCTGGGGGCGTGGGCGTCTCCCGGCGTCCTCTCTCCTG 3'

ETYMOLOGY: Named after the place of origin — the Babia Góra National Park, Poland.

Colonies on PDA reach 5 mm after 7 days and 25 mm after 28 days (~17°C, dark). Colonies are Colonial-Buff to Deep Colonial-Buff and reverse is Honey-Yellow to Isabella in color (Ridgway 1912). Conidiomata eustromatic, complex, mostly submerged in the agar, but also superficial on club moss fragments, globose to subglobose, dark brown to black, ostioles absent, (0.2–)0.3–0.5 (–0.9) mm diam. Conidiophores reduced to conidiogenous cells. Conidiogenous cell discrete, phialidic, ampulliform, hyaline to pale brown, 3–5 × 5–7(–9) µm. Conidia hyaline when liberated, later pale brown, cylindrical to short-cylindrical, rounded at both ends, aseptate, sometimes 1-septate, thin and smooth-walled, with 2–5 oil guttules, 1–2(–3) × (7–)8–9(–10) µm.

HOST: *Huperzia selago* (L.) Bernh. ex Schrank & Mart. (*Huperziaceae*)

DISTRIBUTION: Southern Poland.

NOTES: *P. babiogorense* is most similar to *Coniothyrium lycopodinum* Sacc. & Paol., described from *Lycopodium annotinum* L. However, *P. babiogorense* conidia are longer (8–9 µm) than those of *C. lycopodinum* (6 µm), and the two species were isolated from different hosts. The short-cylindrical conidia with oil guttules of *P. babiogorense* are also similar to those of *P. cyclothyrioides* (Verkley et al. 2004). However, *P. babiogorense* conidia are larger (1–2 × 8–9 µm) and have more (2–5) oil guttules than *P. cyclothyrioides.* The SSU and ITS phylogenies also support *P. babiogorense* as different from *P. cyclothyrioides* (FIGS. 1 & 2).

## Discussion

The signature sequence, low ITS sequence similarity to other *Paraconiothyrium* representatives (95%), distinct morphological characters, and monophyly support the *Huperzia selago* isolates as representing a new taxon, described above as *P. babiogorense.* The new species fits well in the concept of the

Table 1. Comparison of morphological characters of different *Paraconiothyrium* species.

| species | reference | conidioma type, diam. (mm) | ostiole | conidiogenous cell shape, size (µm) | Conidia | | |
|---|---|---|---|---|---|---|---|
| | | | | | # cells | polar guttules | size (µm) |
| ***P. babiogorense*** | **present study** | **eustromatic, 0.3–0.5** | **absent** | **ampulliform, 3–5 × 5–7** | **1-celled** | **2–5** | **1–2 × 8–9** |
| *P. sporulosum* | Sivanesan 1984 | pycnidial, ≤ 0.26 | ostiolate | ampulliform to globose | 1-celled | absent | 2.5–5 × 1.5–2.5 |
| *P. minitans* | Campbell 1947 | pycnidial, 0.15–0.6 | central | ovoid to ampulliform | 1-celled | absent | 4–7 × 3–4 |
| *P. brasiliense* | Verkley et al. 2004 | eustromatic, 0.5–2 | absent | ampulliform to globose, 4–6 × 3.5–5 | 1-celled | few | 3.2–4.6 × 2–3 |
| *P. variabile* | Damm et al. 2008 | pycnidial, 0.3–0.6 | 1–3 | variable in shape, 2.5–5 × 3–7 | 1-celled | sometimes 2 | 3–4 × 1–2 |
| *P. africanum* | Damm et al. 2008 | pycnidial, 0.1–0.6 | central | ampulliform to doliiform, 3–8 × 2–6 | mainly 2-celled | absent | 6.5–9.5 × 3–4 |
| *P. hawaiiense* | Crous & Groenewald 2006 | pycnidial, ≤ 0.5 | no data | ampulliform to subcylindrical, 5–15 × 6–7 | 2-celled | absent | 12–13 × 5 |
| *P. cyclothyrioides* | Verkley et al. 2004 | eustromatic, 0.3–1.2 | absent | ampulliform to subcylindrical, 4.5–8.0 × 2.5–4.0 | 1-celled | 1–2 | 3–4.8 × 1.2–1.6 |
| *P. fungicola* | Verkley et al. 2004 | eustromatic, 0.3–1 | papillate, oozes dark droplets | subglobose, or ampulliform, 5–7 × 3–5 | 1-celled | few | 4.4–6.2 × 3–3.7 |
| *P. estuarinum* | Verkley et al. 2004 | eustromatic, 0.2–0.5 | absent | ampulliform to subcylindrical, 4–6.5 × 2.5–3.5 | 1-celled | 1–2 | 3.2–4.2 × 1.4 –2 |

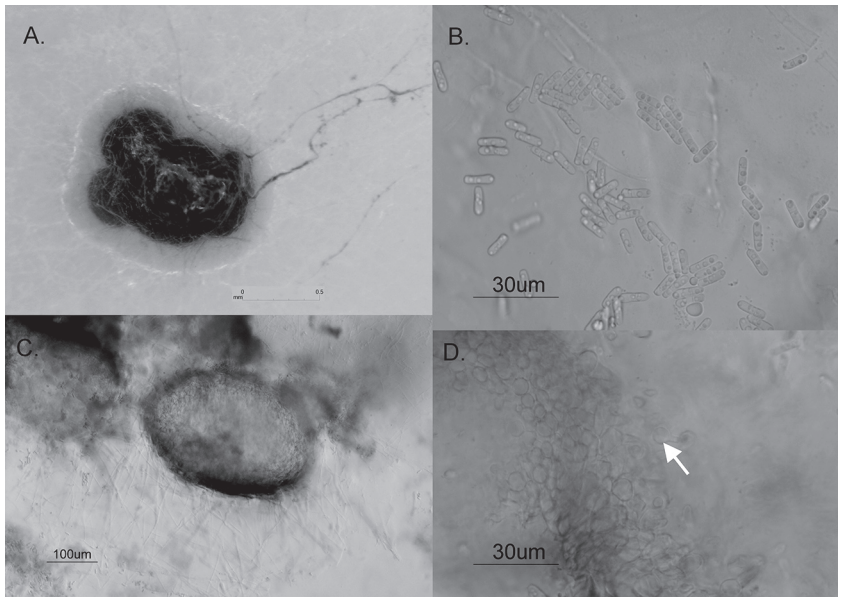


Plate 1. *Paraconiothyrium babiogorense*: a. complex conidiomata immersed in agar medium; b. conidia; c. longitudinal section of conidiomata; d. conidiomatal wall (arrow indicates phialidic cell).

genus by the presence of eustromatic, complex conidiomata, discrete, phialidic conidiogenous cells, and thin-, smooth-walled, hyaline (when liberated) to brownish spores (Verkley et al. 2004). DNA sequence data from *P. babiogorense* also support this species as representing *Paraconiothyrium*.

Fungal endophytes of *Huperzia selago* are poorly known. Budziszewska & Szypuła (2010) identified 13 endophytic species in *H. selago* shoots from different sites, of which only two were cited by Higgins et al. (2007), who also worked on this plant. Surprisingly, neither Higgins et al. (2007) nor Budziszewska & Szypuła (2010) isolated *Paraconiothyrium* species from fir club moss shoots when our study isolated *P. babiogorense* from the vegetative reproductive structures. However, the species could have been overlooked in previous research, as it is an extremely slow growing fungus that is easily overgrown by other, faster growing ones. Thus, the presence of a new *Paraconiothyrium* species in *H. selago* shoots should be re-examined.

Although *Paraconiothyrium* species are relatively common (Damm et al. 2008), this is the first report of one isolated from fir club moss. Interestingly, *Coniothyrium lycopodinum* (Saccardo 1892: 267–268), described from leaves of *Lycopodium annotinum* from a mountain forest in Siberia, is morphologically

similar to our *P. babiogorense* isolates, at least according to its diagnosis. Moreover, two *Paraconiothyrium* sp. isolates, which phylogenetic analysis indicate are the closest relatives of the isolates from *H. selago* propagules, were also isolated from the Polish club mosses *Diphasiastrum tristachyum* (Pursh) Holub (EU709779) and *Lycopodium clavatum* L. (EU935219), suggesting some degree of host specificity.

As *P. babiogorense* was isolated from healthy plant tissue, it is not known whether it could be pathogenic to the host. Interestingly, periodic formation of the specific vegetative propagules at the shoot apex is a unique feature of some *Huperzia* species (Øllgaard 1987, Gola 2008). Moreover, the populations differ in their strategies of balancing generative and vegetative reproduction with extensive propagule production in mountain locations in opposition to the moderate number of propagules produced by lowland plants (Gola 2008). All *P. babiogorense* strains were isolated from these vegetative reproductive structures in alpine *H. selago* plants, suggesting that it could be vertically transmitted.

This fact can also explain the low genetic variability in ITS sequences among *P. babiogorense* isolates. In fact, although we sampled nine plant ramets, we are unable to determine how many plant genets there were. Thus, all isolated fungal strains may actually be vertically transmitted clones of the same individual and hence should not be treated as different strains. More research on *P. babiogorense* and *H. selago* population structures and genetic variability on this site are needed to understand the biology of the fungus.

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