**Extremus adstrictus** from a dolomite wall in Poland: the first report outside Mallorca

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**Article info**
Received: 13 Oct. 2020
Revision received: 8 Nov. 2020
Accepted: 18 Nov. 2020
Published: 29 Dec. 2020

**Associate Editor**
Marcin Piątek

**Abstract.** Most species belonging to the Extremaceae family are rock-inhabiting fungi (RIF), which have a deteriorative potential towards colonized substrate. *Extremus adstrictus* originally isolated from limestone formations in Mallorca is reported from a dolomite wall in Poland. It is the first non-Spanish documented occurrence of this species. Identification of the strain is supported by morphological and molecular analyses. Sequences of uncharacterized fungal cultures and environmental data are analyzed in order to verify probable distribution of *Extremus adstrictus*.

**Key words:** Extremaceae, rock-inhabiting fungi, ITS, LSU, phylogeny

**Introduction**

Rock-inhabiting fungi (RIF) are a group of a poikilotolerant fungi (Gorbushina & Krumbein 2000) with geographical distribution being nearly as wide as rock itself (Friedmann 1982; Staley et al. 1982; Ruibal et al. 2005, 2008; Selbmann et al. 2005, 2008, 2014; Sert et al. 2007; Onofri et al. 2014; Egidi et al. 2014; Su et al. 2015; Isola et al. 2016; Brewer & Fierer 2018; Owczarek-Kościelniak et al. 2020; Sun et al. 2020). RIF, forced to withstand harsh environmental conditions, have developed a number of adaptations needed for survival. These small, slow growing fungi are able to reside under various climatic conditions, including ambient temperatures, high solar irradiation, osmotic stress, low water availability and limited nutrient source (Sterflinger & Krumbein 1995; Selbmann et al. 2005; Dadachova & Casadevall 2008; Onofri et al. 2012; Tesei et al. 2012; Isola et al. 2013; Zakharova et al. 2013).

The family Extremaceae, where most of the described species are RIF, was introduced in 2014 (Quaedvlieg et al. 2014) as a result of resolving a clade formerly known as Teratosphaeriaceae II. Currently, Extremaceae accommodates the following genera: Extremus, Petrophila, Saxophila, Staninwardia, Pseudoramichloridium, Vermiconidia (Wijayawardene et al. 2018), Castanedospora, Paradevriesia (Hongsanan et al. 2020; Wijayawardene et al. 2020) and Neohortaea (Delgado et al. 2018). All type species of these genera, except Staninwardia, are sequenced. Most of the species of the Extremaceae family were isolated from rock samples from sites located in Mallorca and Antarctica. The genera Staninwardia, Pseudoramichloridium, Castanedospora and Neohortaea originate from plant, soil and lignite material.

The genus Staninwardia was first introduced with *Staninwardia breviuscula* from *Eucalyptus* leaves (Sutton 1971). The second *Staninwardia* species discovered, *S. suttoni*, was isolated from *Eucalyptus robusta* in Australia (Summerell et al. 2006) and remains the only sequenced representative of the genus. The genus *Pseudoramichloridium* was first introduced in 2009 (Cheewangkoon et al. 2009) when *Pseudoramichloridium henryi* was isolated from *Corymbia henryi*. Simultaneously, originally described in 2007 as *Ramichloridium brasiliatinum*, an isolate from forest soil, was recombined and introduced as a second representative of the *Pseudoramichloridium* genus. The third species of the genus, *Pseudoramichloridium xinjiangense*, was isolated from soil and described in 2017 (Jiang et al. 2017), but was not sequenced. The genus *Castanedospora* includes a single species, *Castanedospora pachyanthicola*, originating from dead leaves of *Pachyanthus poirettii* and *Sabal palmetto* in Cuba and the USA (Delgado et al. 2018). The genus *Neohortaea* accommodates a single species, *Neohortaea acidophila*, isolated from lignite (Hölker et al. 2004; Quaedvlieg et al. 2014).

The genera *Petrophila* and *Saxophila* are each represented by a single species – *Petrophila incerta* and *Saxophila tyrrenica*, respectively, isolated from stone and a stone monument located in the Mediterranean (Egidi et al. 2014; Isola et al. 2016; Crous et al. 2019). *Vermiconidia* (Crous et al. 2019), originally published as a *Vermiconia* (Egidi et al. 2014) includes four species, *Vermiconidia antarctica* isolated only from Antarctica,
V. calcicola found at various sites in Italy, V. flagrans reported from the Mediterranean and V. foris originating from Italian Alps. All described species and strains of Vermiconidida were isolated from stone substrates. Similarly, the two described species of the genus Extremus are a rock-inhabiting fungi with E. antarcticus isolated from McMurdo Valleys in Antarctica and E. adstrictus from limestone formations in Mallorca (Ruibal et al. 2005; Quaedvlieg et al. 2014; Crous et al. 2019).

The genus Paradevriesia was introduced by Crous et al. (2019) and originally transferred to a new family, Paradevriesiaceae. Paradevriesia is comprised of Paradevriesia compacta from rocks, P. americana from air and P. pseudoamericana from Malus domestica fruit (Crous et al. 2019). The family Paradevriesiaceae is now regarded as a synonym of Extremaceae (Hongsanan et al. 2020; Wijayawardene et al. 2020).

In this work, the strain isolation of Extremus adstrictus from a second location, a dolomitic wall in the center of Kraków, Poland, is reported. Morphological and molecular characteristics of this new specimen are provided.

**Materials and methods**

Located in southern Poland, the city of Kraków is the second largest city in the country. The climate of Kraków is moderately humid continental with cold winters and warm to hot summers (Grøntoft 2017). Small fragments of a dolomite retaining wall situated in the center of Kraków, Poland (Fig. 1) were steriley collected in May 2018 and transferred to small tubes. In laboratory conditions, wall fragments were crushed in a mortar under sterile conditions and scattered on malt extract agar (MEA) medium as inoculum as described in Owczarek-Kościelniak et al. (2020). After 12 weeks of growth on MEA medium at 15°C, colonies were used for morphological description and molecular analyses. The isolated strain was deposited in the culture collection of the Westerdijk Fungal Biodiversity Institute (CBS) and as a dried voucher specimen in fungal collection of the W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków (KRAM F).

Culture characteristics were studied on MEA medium. Measurements and photographs of the colonies were taken using 6 month old cultures. Micromorphological
observations were made on 3 month old and 6 month old cultures. Slides were mounted with Shear’s medium and observed with a Nikon Eclipse 80i light microscope at a magnification of 1000X. The microscopic structures were measured and photographed using NIS-Elements BR 3.0 imaging software.

DNA extraction was performed following the Gerrits van den Ende and de Hoog (1999) protocol optimized by Owczarek-Kościelniak and Sterflinger (2018). The PCR reactions were performed in a 25 μl volume containing 1X buffer (with MgCl2), 200 μM dNTP, 5 pmol forward and backward primers and 0.05 U Taq polymerase (Sigma Aldrich). Primers selected for the reaction were ITS-1F (5’-CTT GGT CAT TTA GAG GAA GTA A-3’) (Gardes & Bruns 1993) and ITS-4 (5’-TCC TTC GCT TAT TGA TAT GC-3’) (White et al. 1990) for the ITS1–5.8S–ITS4 rDNA (ITS) region, and NL1 (5’-GCA TATCAA TAG TAA CTG GAT TAT GC-3’) (Gardes & Bruns 1993) for the D1-D2 domains of the 28S rDNA (LSU) gene. ITS amplification

Table 1. Fungal sequences used in phylogenetic reconstruction. Sequences generated in this study are shown in bold. T – type strain; ET – ex-type strain.

| Species                  | Strain      | Source         | Country       | NCBI accession number |
|--------------------------|-------------|----------------|---------------|-----------------------|
| Castanedospora pachyanthicola | HKUCC 10835 | –              | China         | –                     |
| Castanedospora pachyanthicola | CBS 140347T | Sabal palmetto | USA           | MH036004              |
| Cladosporium fusiforme     | CBS 119414T | hypersaline water | Slovenia     | NR_119608              |
| Cladosporium iridis       | CBS 138.40ET | Iris sp.       | Netherlands   | –                     |
| Extremus adstrictus        | CBS 118292 = TRN96ET | rock sample | Spain         | –                     |
| Extremus adstrictus        | W3          | rock sample    | Poland        | MW206772              |
| Extremus antarcticus      | CCFEE 5312  | rock sample    | Antarctica    | KF309979              |
| Extremus antarcticus      | CBS 136104 = CCFEE 5207 | rock sample | Antarctica    | –                     |
| Extremus antarcticus      | CBS 136103T = CCFEE 451 | rock sample | Antarctica    | NR_138389              |
| Extremus sp.              | CBS 119436 = CCFEE 5177 | rock sample | Antarctica    | –                     |
| Extremus sp.              | CCFEE 5551  | –              | –             | –                     |
| Extremus sp.              | CBS 118300 = TRN137 | rock sample | Spain         | –                     |
| Neohortaea acidophila     | CBS 113389  | –              | Germany       | GU214636              |
| Paradevriesia americana   | CPC 117726ET | air sample    | USA           | NR_159866              |
| Paradevriesia compacta    | CBS 118294 = TRN111ET | rock sample | Spain         | –                     |
| Paradevriesia pseudomexicana | CPC 16174T | Malus domestica | Germany      | GU570527               |
| Petrophila incerta        | CBS 118287 = TRN77 | rock sample | Spain         | –                     |
| Petrophila incerta        | CBS 118305 = TRN62 | rock sample | Spain         | –                     |
| Petrophila incerta        | CBS 118608 = TRN139ET | rock sample | Spain         | –                     |
| Polychaetum citri         | CBS 116435  | Citrus aurantium | Iran        | GU214649               |
| Pseudolamarichloridium brasiliannum | CBS 283.92ET | forest soil  | Brazil        | EU041797               |
| Pseudolamarichloridium henryi | CBS 124775 = CPC 13121ET | Corymbia henryi | Australia    | –                     |
| Saxophila tyrhenica       | TRN66       | rock sample    | Spain         | AY559337               |
| Saxophila tyrhenica       | CBS 139725 = CCFEE 5935T | rock sample | Italy         | NR_145013              |
| Staninwardia suttonii     | CBS 120061 = CPC 13055ET | Eucalyptus robusta | Australia | NR_137111              |
| Toxicocladosporium protecum | CBS 126499 = CPC 15254ET | Protea burchelli | South Africa | NR_152321              |
| Toxicocladosporium irritans | CBS 128777 = CPC 18471ET | Phaeoconoma prolifer | South Africa | MH865133               |
| Toxicocladosporium rubrigenum | CBS 124158 = CPC 15375ET | Eucalyptus calamudensis | Madagascar | NR_152319              |
| Vermiconidium antarctica  | CBS 136108 = CCFEE 5489 | rock sample | Antarctica    | KF309983               |
| Vermiconidium antarctica  | CBS 136107 = CCFEE 5488ET | rock sample | Antarctica    | –                     |
| Vermiconidium calcicola   | CCFEE 5905  | rock sample    | Vatican       | KP791756               |
| Vermiconidium calcicola   | CCFEE 5898  | rock sample    | Vatican       | KP791757               |
| Vermiconidium calcicola   | CCFEE 5714  | rock sample    | Italy         | KP791760               |
| Vermiconidium calcicola   | CBS 140080 = CCFEE 5770 | rock sample | Italy         | NR_132888              |
| Vermiconidium flagrans    | CCFEE 5922  | rock sample    | Vatican       | KP791753               |
| Vermiconidium flagrans    | CBS 118283 = TRN124 | rock sample | Spain         | AY559359               |
| Vermiconidium foris       | CBS 136106T = CCFEE 5459 | rock sample | Italy         | KP309981               |
| Verrucocladosporium dirinae | CBS 112794T | Dirina massiliensis | UK          | NR_152317              |
was performed in a touchdown-PCR according to Owczarek-Kościelniak and Sterflinger (2018). LSU PCR reaction was conducted using the following steps: initial denaturation at 95°C for 2 min, 35 cycles in the following order 95°C – 35 s, 56°C – 1 min and 72°C – 1 min, and the final elongation in 72°C for 10 min. Exo-BAP kit (Eurox, Poland) was used for enzymatic purification of amplicons.

Bidirectional sequencing was performed at Macrogen Europe B.V. (Amsterdam, The Netherlands). Reads were assembled and trimmed in Geneious Prime® 2020.0.4. Generated sequences were deposited at the NCBI’s GenBank nucleotide database (Table 1).

BLASTn query was performed in order to verify fungus identity and to find sequences of the closest relatives. Sequences showing high similarity with newly generated sequences, as well as sequences of other related species were downloaded from GenBank and aligned with the MAFFT algorithm (Katoh et al. 2005) as implemented in Geneious Prime® 2020.0.4. In Geneious Prime® 2020.0.4, a dataset of three concatenated loci, ITS, LSU and RPB2, was prepared. Polychaeton citri CBS 116435 was used as an outgroup. The best partitioning model was determined separately for each loci by PartitionFinder 2.1.1 (Lanfear et al. 2016). Analyses were performed for each loci and for the concatenated datablock at the CIPRES Science Gateway (Millet et al. 2010) using maximum likelihood (ML) analyses using RAxML (Stamatakis 2014) with 1,000 bootstrap replicates and the Bayesian Inference (BI) using MrBayes (Ronquist et al. 2012) in two concurrent runs of four chains for 2,000,000 generations. Final phylogenetic trees were prepared with FigTree 1.4.3. Estimates of the average evolutionary divergence over sequence pairs within groups were calculated in MEGA X (Kumar et al. 2018).

Results and discussion

The MEA cultures of *Extremus adstrictus* W3 strain from Kraków were mostly consistent with the original type strain description (Fig. 2). Colonies on MEA grew slowly, reaching up to 12.5 mm in diameter after 2 months of growth. Colonies were oval, compact, black with distinct margins and embedded in the medium, reverse was black. Hyphae were septate, sparsely branched, pale brown to brown, and 1.0–4.5 μm wide. Conidia were intercalary, one-septate, brown, several in chains, rarely single, and 6.0–9.5 × 2.5–4.5 μm. Chlamydospores were produced singly and were brown, globoid to ovoid, growing intercalary or apically, one to two, rarely three-celled, and 6.0–11.0 × 5.0–10.0 μm. Colony diameter formed by the isolate from Poland was smaller than colony diameter of type strain of *E. adstrictus*. Furthermore, chlamydospores

Figure 2. *Extremus adstrictus* W3 on MEA. A – colony morphology; B – hyphae and conidia in chain (arrow); C – dark-walled hyphae and conidia; D – hyphae, conidia and two and three-celled chlamydospores (arrows). Scale bars: A = 1 cm; B–D = 10 μm. Photographs: A – M. Owczarek-Kościelniak; B–D – M. Piątek.
were not reported in the original description of the species (Egidi et al. 2014).

Successfully amplified ITS and LSU loci from new Extremus adstrictus W3 strain were 514 bp and 575 bp for ITS and LSU, respectively. BLASTn searches confirmed a close affinity of the analyzed sequences to the sequences of the type strain of Extremus adstrictus, showing 98.27% (8 bp difference) and 100% identity for the ITS and LSU loci, respectively. Overall, 38 ITS sequences from the Extremaceae were downloaded from the GenBank. Extremus adstrictus and E. antarcticus similarity was checked in a distance matrix of the aligned ITS datablock. Type strains of these species were 95.09% identical. The number of Extremaceae sequences used in phylogenetic tree reconstruction was reduced to 31 by the elimination of the sequences which similarity to Extremus adstrictus was lower than 95.09%. Using MegaX, the average evolutionary divergence over sequence pairs within Extremus adstrictus group was calculated using the Tajima-Nei model. The rate variation among sites was modeled with a gamma distribution. The divergence within the group was 0.02, whereas in the Petrophila and Saxophila group it was 0.06 and 0.00, respectively.

The reconstructed phylogenetic tree of concatenated ITS, LSU and RPB2 datablocks (Fig. 3) confirms affinity of the isolate from Kraków to the genus Extremus and the identification of the Polish strain W3 as Extremus adstrictus is well supported. Several sequences of uncharacterized cultures and environmental sequences showed affinity to sequences of Extremus adstrictus obtained from type culture and the strain W3 from Kraków that is presented in supplementary files (Fig. S1, Table S1). Considering morphological characteristics of the type strain and isolate W3 from Kraków, it seems probable that most of the unclassified cultures also represent Extremus adstrictus.

The sequence from culture TRN80 from limestone and the sequence from an uncultured Devriesia clone 10S50C15 (from soil) form an unsupported clade between Extremus adstrictus and E. antarcticus (Fig. S1). The sequence from the alpine soil, fungal sp. MKOTU91, also has an inconclusive position, visible here as basal to the previous sequences. The specific affinities of these three sequences are unresolved.

Among analyzed sequences related to Extremus adstrictus, only one sequence, from uncultured fungus clone 4248_135 closest to isolate from Kraków, does not originate from the stone material, but from irrigation water from a pond in Lithuania (Marčiulynas et al. 2020). All uncharacterized cultures from the TRN collection originate from limestone material in Spain, TRN433 from the Central Mountain System and the remainder from Mallorca (Ruibal et al. 2005, 2008). Interestingly, all stone isolates of Extremus adstrictus are from substrates rich in calcium from limestone and dolomite in Spain and Poland.

Extremus species are classified as rock-inhabiting fungi. RIF are considered potentially harmful towards inhabited substrate. Not many RIF were reported from a temperate climate of the Central Europe, thus the discovery of Extremus adstrictus is noteworthy. Among other RIF reported from a temperate climate zone in Europe are Knufia marmoricola, Neocatenulostroma germanicum, Constantinomyces oldenburgensis (Sterflinger & Piñar...
Acknowledgements

This study was supported by the statutory funds of the W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków.

Supplementary electronic material

Figure S1. Maximum likelihood consensus tree of a concatenated ITS-LSU-RPB2 loci of Extremaceae. Numbers above branches indicate bootstrap support values (ML) and posterior probabilities (BI).

Table S1. Additional fungal ITS sequences used in phylogenetic reconstruction. T – type strain; eT – ex-type strain. Download file

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