

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

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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; Tessei 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 brasilianum*, an isolate from forest soil, was recombined and introduced as a second representative of the *Pseudoramichloridium* genus. The third species of the genus, *Pseudoramichloridium xinjangense*, 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 poiretii* 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 tyrrhenica*, 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,

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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 *Vermiconidia* 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 sterilely 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



Figure 1. Sampling site. A – view of the dolomitic wall in Kraków; B – black fungi colonization at the surface and in pits of a dolomite slate.

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 TCC GCT TAT TGA TAT GC-3') (White et al. 1990) for the ITS1–5.8S–ITS4 rDNA (ITS) region, and NL1 (5'-GCA TAT CAA TAA GCG GAG AA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (O'Donnell 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		
				ITS	LSU	RPB2
<i>Castanedospora pachyanthicola</i>	HKUCC 10835	–	China	–	DQ408557	–
<i>Castanedospora pachyanthicola</i>	CBS 140347T	<i>Sabal palmetto</i>	USA	MH036004	MH036005	–
<i>Cladosporium fusiforme</i>	CBS 119414T	hypersaline water	Slovenia	NR_119608	KJ564333	–
<i>Cladosporium iridis</i>	CBS 138.40ET	<i>Iris</i> sp.	Netherlands	NR_111271	EU167591	KT223022
<i>Extremus adstrictus</i>	CBS 118292 = TRN96ET	rock sample	Spain	NR_144954	KF310022	KF310103
<i>Extremus adstrictus</i>	W3	rock sample	Poland	MW206772	MW206771	–
<i>Extremus antarcticus</i>	CCFEE 5312	rock sample	Antarctica	KF309979	KF310020	KF310086
<i>Extremus antarcticus</i>	CBS 136104 = CCFEE 5207	rock sample	Antarctica	–	KF310021	–
<i>Extremus antarcticus</i>	CBS 136103T = CCFEE 451	rock sample	Antarctica	NR_138389	NG_059204	KF310085
<i>Extremus</i> sp.	CBS 119436 = CCFEE 5177	rock sample	Antarctica	–	KJ564326	–
<i>Extremus</i> sp.	CCFEE 5551	–	–	–	KC315879	–
<i>Extremus</i> sp.	CBS 118300 = TRN137	rock sample	Spain	–	GU323973	KF310098
<i>Neohortaea acidophila</i>	CBS 113389	–	Germany	GU214636	GU214428	GU357768
<i>Paradevriesia americana</i>	CBS 117726ET	air sample	USA	NR_159866	NG_059077	–
<i>Paradevriesia compacta</i>	CBS 118294 = TRN111ET	rock sample	Spain	NR_144955	NG_059089	KF310095
<i>Paradevriesia pseudoamericana</i>	CPC 16174T	<i>Malus domestica</i>	Germany	GU570527	GU570544	–
<i>Petrophila incerta</i>	CBS 118287 = TRN77	rock sample	Spain	AY559337	GU323963	KF310101
<i>Petrophila incerta</i>	CBS 118305 = TRN62	rock sample	Spain	AY559328	GU323961	KF310084
<i>Petrophila incerta</i>	CBS 118608 = TRN139bET	rock sample	Spain	NR_144956	KF310030	KF310091
<i>Polychaeton citri</i>	CBS 116435	<i>Citrus aurantium</i>	Iran	GU214649	GU214469	–
<i>Pseudoramichloridium brasilianum</i>	CBS 283.92ET	forrest soil	Brazil	EU041797	EU041854	–
<i>Pseudoramichloridium henryi</i>	CBS 124775 = CPC 13121ET	<i>Corymbia henryi</i>	Australia	NR_156571	NG_057832	KF902344
<i>Saxophila tyrrhenica</i>	TRN66	rock sample	Spain	AY559330	GU323962	–
<i>Saxophila tyrrhenica</i>	CBS 139725 = CCFEE 5935T	rock sample	Italy	NR_145013	NG_059571	–
<i>Staninwardia suttonii</i>	CBS 120061 = CPC 13055ET	<i>Eucalyptus robusta</i>	Australia	NR_137118	NG_058023	KF442591
<i>Toxicocladosporium protearum</i>	CBS 126499 = CPC 15254ET	<i>Protea burchellii</i>	South Africa	NR_152321	HQ599587	KJ564344
<i>Toxicocladosporium irritans</i>	CBS 128777 = CPC 18471ET	<i>Phaenocoma prolifera</i>	South Africa	MH865133	JF499868	KJ564345
<i>Toxicocladosporium rubrigenum</i>	CBS 124158 = CPC 15735ET	<i>Eucalyptus camaldulensis</i>	Madagascar	NR_152319	NG_057817	KJ564338
<i>Vermiconidia antarctica</i>	CBS 136108 = CCFEE 5489	rock sample	Antarctica	KF309983	KF310023	KF310090
<i>Vermiconidia antarctica</i>	CBS 136107 = CCFEE 5488ET	rock sample	Antarctica	NR_132888	KJ564324	KF310089
<i>Vermiconidia calcicola</i>	CCFEE 5905	rock sample	Vatican	KP791756	KR781043	KT247879
<i>Vermiconidia calcicola</i>	CCFEE 5898	rock sample	Vatican	KP791757	KR781044	KT247880
<i>Vermiconidia calcicola</i>	CCFEE 5714	rock sample	Italy	KP791760	KR781047	KT247883
<i>Vermiconidia calcicola</i>	CBS 140080 = CCFEE 5770	rock sample	Italy	NR_145012	KR781046	KT247882
<i>Vermiconidia flagrans</i>	CCFEE 5922	rock sample	Vatican	KP791753	KR781040	KT247876
<i>Vermiconidia flagrans</i>	CBS 118283 = TRN124	rock sample	Spain	AY559359	GU323971	KF310097
<i>Vermiconidia flagrans</i>	CBS 118296 = TRN114ET	rock sample	Spain	AY559354	KF310024	KF310096
<i>Vermiconidia foris</i>	CBS 136106T = CCFEE 5459	rock sample	Italy	KF309981	GU250390	KF310088
<i>Verrucocladosporium dirinae</i>	CBS 112794T	<i>Dirina massiliensis</i>	UK	NR_152317	EU040244	–

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 (Eurx, 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 (Kato 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. Chlamydo spores 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, chlamydo spores

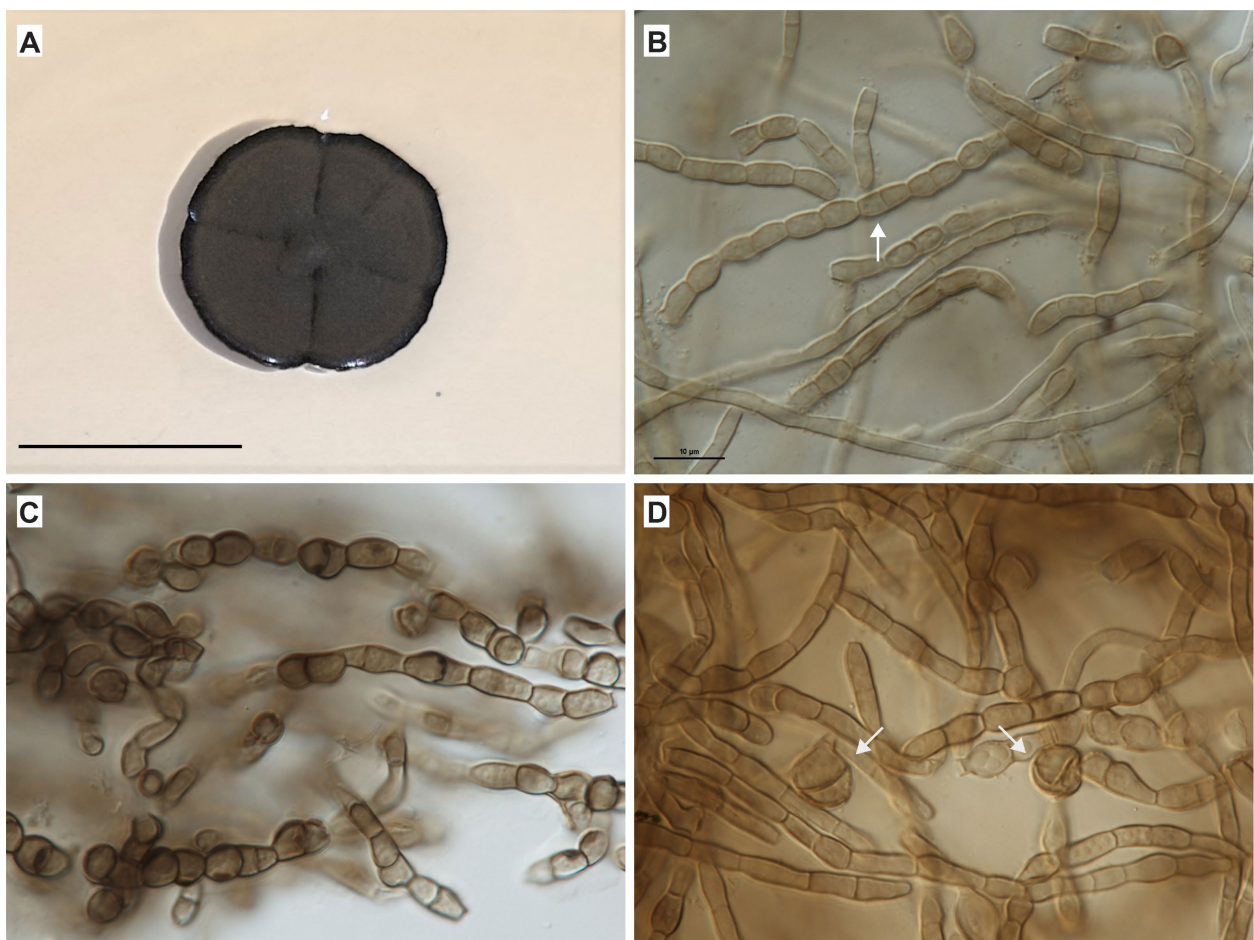


Figure 2. *Extremus adstrictus* W3 on MEA. A – colony morphology; B – hyphae and conidia in chain (arrow); C – dark-walled hyphae and conidia; D – hyphae, conidia and two and three-celled chlamydo spores (arrows). Scale bars: A = 1 cm; B–D = 10 µm. Photographs: A – M. Owczarek-Kościelniak; B–D – M. Piątek.

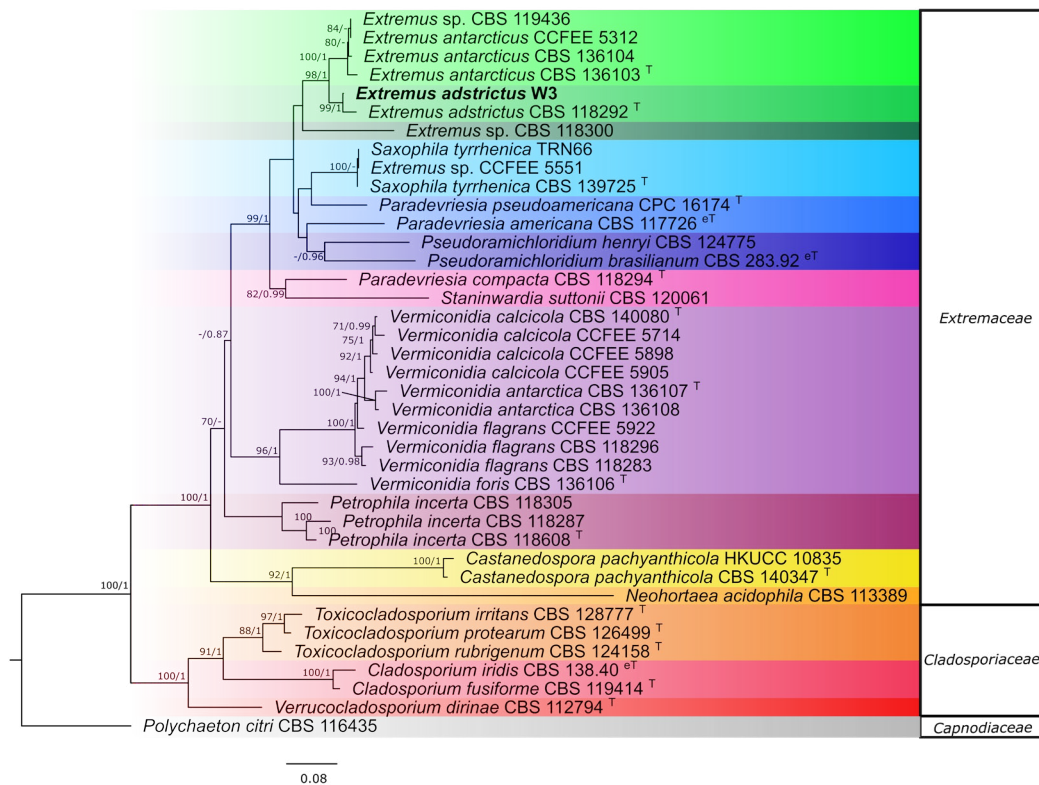


Figure 3. Maximum likelihood consensus tree of a concatenated LSU-ITS-*RPB2* loci. Numbers above branches indicate bootstrap support values (ML) and posterior probabilities (BI). The scale bar represents the expected changes per site. *Polychaeton citri* CBS 116435 was used as an outgroup. T – type strain; eT – ex-type strain.

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 *E. 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 the *E. adstrictus* was lower than 95.09%. Using Mega X, the average evolutionary divergence over sequence pairs within *E. 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 *E. 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 *E. 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 *E. adstrictus* is noteworthy. Among other RIF reported from a temperate climate zone in Europe are *Knufia marmoricola*, *Neocatenulostroma germanicum*, *Constantinomyces oldenburgensis* (Sterflinger & Piñar

2001; Owczarek-Kościelniak & Sterflinger 2018; Ruibal et al. 2018; Owczarek-Kościelniak et al. 2020). More black, slow growing fungi were reported from the Mediterranean area (Marvasi et al. 2012; Edigi et al. 2014; Isola et al. 2015; De Leo et al. 2019).

Although fungi do not raise significant interest, their role in stone biodeterioration is important and undeniable. Recent studies have proven that some RIF species possess corrosive properties (Isola et al. 2013; Breitenbach et al. 2018; Gerrits et al. 2020, 2021), thus awareness of their colonization on man-made structures and objects of cultural heritage needs to be raised and further studies should be conducted.

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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). The scale bar represents the expected changes per site. T – type strain; eT – ex-type strain. [Download file](#)

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

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