Documentation of Hungarian *Xerocomellus (Boletaceae)* species by molecular methods

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Introduction

The diversity of species in the *Xerocomellus* genus is still quite unclarified. This is partly due to the high morphological variability within the species and in certain cases, the lack of a reliable species concept.
In order to partially clarify all this, we examined and identified the eclectic Hungarian species of the *Xerocomellus* genus - mainly from the year of 2018 to 2022. - based on ITS sequences.

Materials and Methods

In the course of the terrain mushroom gathering, we collected fresh fungal specimens of the *Xerocomellus* species from different regions of Hungary (mainly the areas of the Vértes and Gerecse) - with a special focus on the *X. porosporus* taxon - in the fungarium. The species were determined by analyzing the macromorphological features as well as the habitats, then we identified these results by molecular methods. We preferred specimens with atypical cuticle (color and morphology).

Molecular methods

We obtained DNA samples out of the dried fungi's cap tissues with Direct PCR method (Thermo Scientific Phire Plant Direct PCR Master Mix), also, in one case we gained DNA out of the fungus with DNA extraction (NucleoSpin Plant II, Mini kit for DNA from plants; Thermo Scientific DreamTaq Green PCR Master Mix).
Subsequently, the earned DNA were amplified with a fungal specific primer pair (ITS1F – CTT GGT CAT TTA GAG GAA GTA A; ITS4 – TCC TCC GCT TAT TGA TAT GC) in a polymerase chain reaction.

The PCR products were electrophoresed on an agarose gel, stained with nucleic acid stain and then visualized by UV transilluminator.

The sequencing of the finished PCR products were performed in two ways.
1.) By a third party, after cleaning, with the Sanger method.
2.) With the third generation sequencing technology available to us: Oxford Nanopore Technologies – Flow Cell (R10 Version), chemistry: Native Barcoding Kit 24, multiplexing/.

The results of the Sanger sequencing were purified by using FinchTV and SnapGene Viewer.
The sequence data analysis gained by Oxford Nanopore Technologies and consensus file generation were performed with using Amplicon_sorter.

The sequences obtained this way were compared with the available sequences of GenBank and UNITE using BLAST.

Afterwards, we supplemented our ITS data with additional high-quality sequences available from GenBank and UNITE (Table 1.) and then we created Phylogenetic trees by using the phylogeny.fr portal /Maximum Likelihood tree, was made by using PhyML, the reliability of the internal branch was assessed by the aLRT test (SH-Like) (Figure 1.)/, together with the RaxmlGUI software /the Maximum Likelihood tree, was created with 1000 bootstrap replications in this case and the tree was visualized with the UGENE program (Figure 2.)/.
Hortiboletus rubellus was used as an outgroup in both cases.
Sequence alignments were performed by using SeaView and Geneious Prime programs (Figure 3.).

Results

The preliminary macromorphological determination of the collected *Xerocomellus* species were mostly accurate and this was confirmed by molecular methods too.
In cases where the appearance of the basidiomata weren't typical (mainly the cuticle's skin color, cracking or the lack of it, flesh color and discoloration in the cutting), a more thorough examination was proved to be undoubtedly useful.

It should definitely be mentioned that in the course of our work we were able to document the first Hungarian specimen of *Xerocomellus sarnarii* from the Gerecse as a curiosity (1 June 2019).

Further multigenic molecular examinations as well as new specimens from different habitats would also be needed in the case of the *Xerocomellus porosporus* taxon to clarify the extent of variability and if necessary, redefine the species boundary.

Table 1. The nrDNA ITS sequences of *Xerocomellus* species used in the phylogenetic analysis. Hungarian data is in bold.

No.	Species name	Country	ITS (Sequences ID)
1.	*Xerocomellus cisalpinus*	Italy	KT271743.1
2.	***Xerocomellus cisalpinus***	**Hungary**	**ITS-VGy-13-11-2018_1**
3.	*Xerocomellus cisalpinus*	Spain	MW376718.1
4.	*Xerocomellus cisalpinus*	Germany	MT006036.1
5.	*Xerocomellus cisalpinus*	Cyprus	MH011838.1
6.	*Xerocomellus cisalpinus*	Poland	MK028949.1
7.	*Xerocomellus redeuilhii*	Cyprus	MH011929.1
8.	*Xerocomellus redeuilhii*	Italy	MT594542.1
9.	*Xerocomellus redeuilhii*	Cyprus	MH011842.1
10.	*Xerocomellus ripariellus*	Turkey	MH472623.1
11.	*Xerocomellus ripariellus* (syntype)	Italy	KX905051.1
12.	*Xerocomellus ripariellus* (type material)	Italy	KX889920.1
13.	*Xerocomellus ripariellus*	Spain	MN685108.1
14.	*Xerocomellus ripariellus*	Argentina	MH930227.1
15.	*Xerocomellus ripariellus*		KU355482.1
16.	***Xerocomellus ripariellus***	**Hungary**	**ITS-VGy-20220407_01-V47_AL**
17.	***Xerocomellus chrysenteron***	**Hungary**	**ITS-VGy-20220407_01-V42_AL**
18.	*Xerocomellus chrysenteron*	Cyprus	MH011845.1
19.	*Xerocomellus chrysenteron*	Switzerland	AF402139.1
20.	*Xerocomellus chrysenteron*	Germany	HQ207693.1
21.	***Xerocomellus chrysenteron***	**Hungary**	**ITS-VGy-20220407_01-V43_AL**
22.	*Xerocomellus chrysenteron*	France	KX449432.1
23.	*Xerocomellus chrysenteron*	Italy	JF908799.1
24.	*Xerocomellus poederi* (holotype)		NR_155971.1
25.	*Xerocomellus poederi*		KU355478.1
26.	***Xerocomellus sarnarii***	**Hungary**	**ITS-VGy-01-06-2019_1**
27.	*Xerocomellus sarnarii*	Cyprus	MH011930.1
28.	*Xerocomellus sarnarii*	Cyprus	MH011837.1
29.	*Xerocomellus sarnarii*	France	MH011926.1
30.	*Xerocomellus sarnarii* (holotype)	Italy	NR_138006.1
31.	*Xerocomellus sarnarii*	Italy	KT271750.1
32	*Xerocomellus sarnarii*	Italy	KT271751.1
33.	*Xerocomellus sarnarii*	Italy	KT271745.1
34.	***Xerocomellus porosporus***	**Hungary**	**ITS-VGy-20220407_01-V44_AL**
35.	*Xerocomellus porosporus*		KU355481.1
36.	*Xerocomus porosporus* isolate	Poland	MK583529.1
37.	***Xerocomellus porosporus***	**Hungary**	**ITS-VGy-15-09-2018_1**
38.	***Xerocomellus porosporus***	**Hungary**	**ITS-VGy-20210810_01-VD4-V27**
39.	***Xerocomellus porosporus***	**Hungary**	**ITS-VGy-20210803_01-V14**
40.	*Xerocomus truncatus*	Italy	JF908796.1
41.	Uncultured ectomycorrhiza *Xerocomus* clone	Germany	EU700259.1
42.	*Xerocomellus porosporus*	Poland	KM085410.1
43.	*Boletus* sp.	Germany	KM576322.1
44.	Uncultured *Xerocomus*	Germany	FR750648.1
45.	*Xerocomus porosporus*		HM190086.1
46.	Uncultured fungus clone	Poland	MH834519.1
47.	Uncultured *Xerocomellus* clone	Spain	MW282454.1
48.	Uncultured *Xerocomus* clone	Germany	GQ219853.1
49.	Uncultured fungus	Switzerland	KX886198.1
50.	***Xerocomellus porosporus***	**Hungary**	**ITS-VGy-20220407_01-V46_AL**

Table 1. (continued)

No.	Species name	Country	ITS (Sequences ID)
51.	*Xerocomellus porosporus*	Italy	KT271744.1
52.	*Xerocomellus zelleri*	Canada	MF908480.1
53.	*Xerocomellus zelleri*	USA	KU144802.1
54.	*Xerocomellus zelleri*	Canada	MN954756.1
55.	*Boletus zelleri*	USA	AY750158.1
56.	*Xerocomellus pruinatus*	Luxembourg	MW603181.1
57.	*Xerocomus pruinatus*	Germany	LC011385.1
58.	*Boletus pruinatus*	Denmark	AJ889933.1
59.	*Xerocomus pruinatus* isolate	Germany	MW603181.1
60.	**Hortiboletus rubellus**	**Hungary**	**ITS-VGy-21-07-2018_1**

Figure 1. (Maximum likelihood analysis, PhyML, aLRT test)
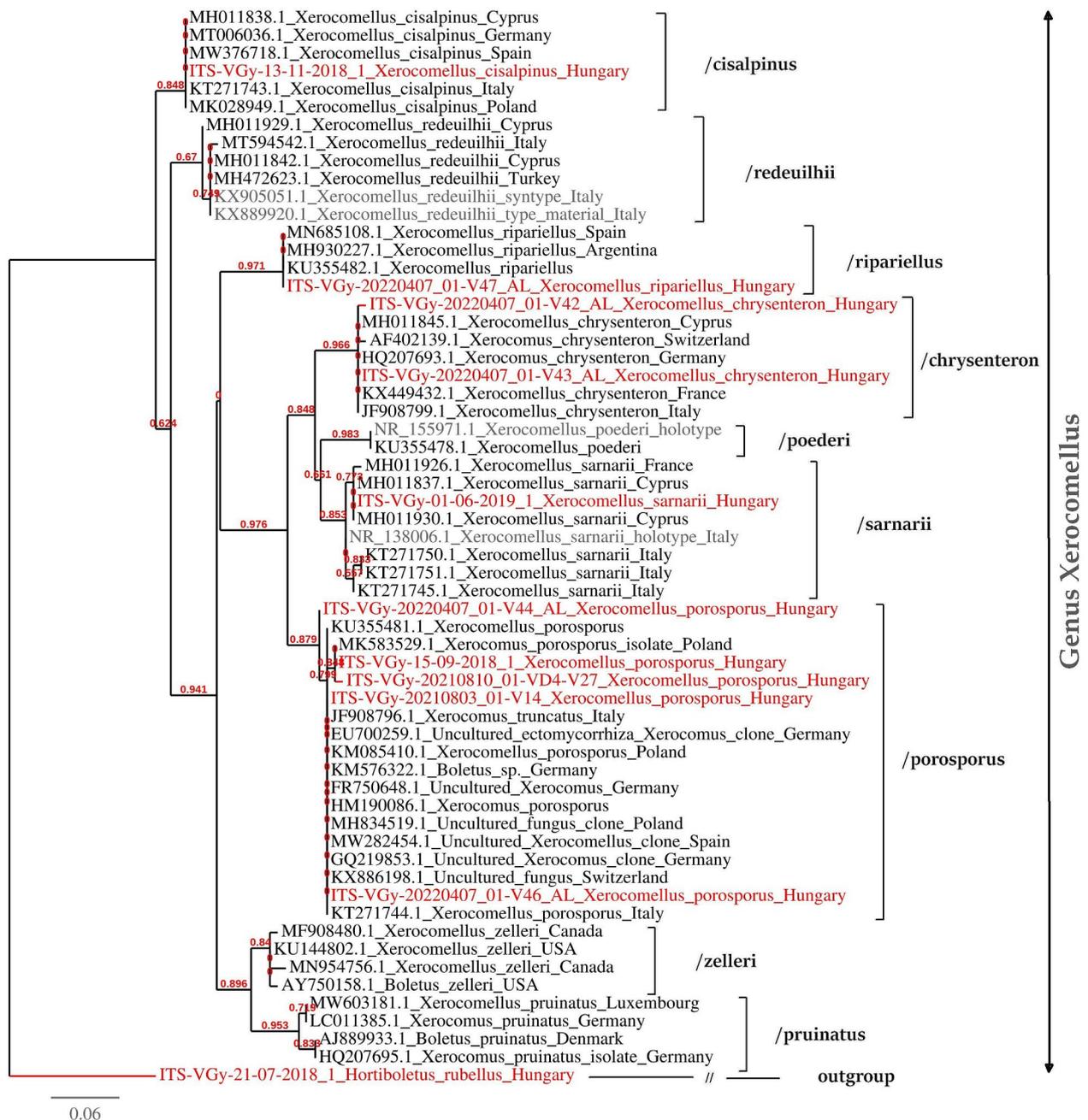
**Figure 2.** (Maximum likelihood analysis, RaxmlGUI, bootstrap: 1000)
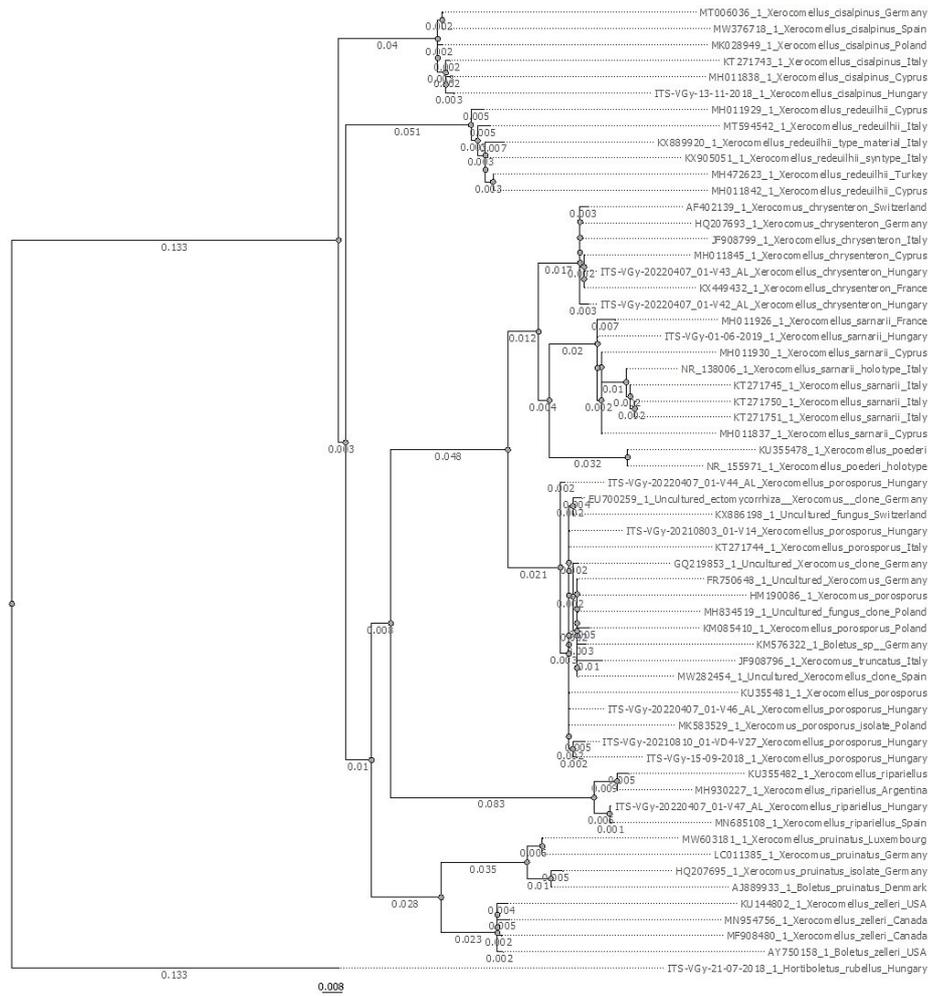
**Figure 3.** (Alignment View, Geneious Prime)
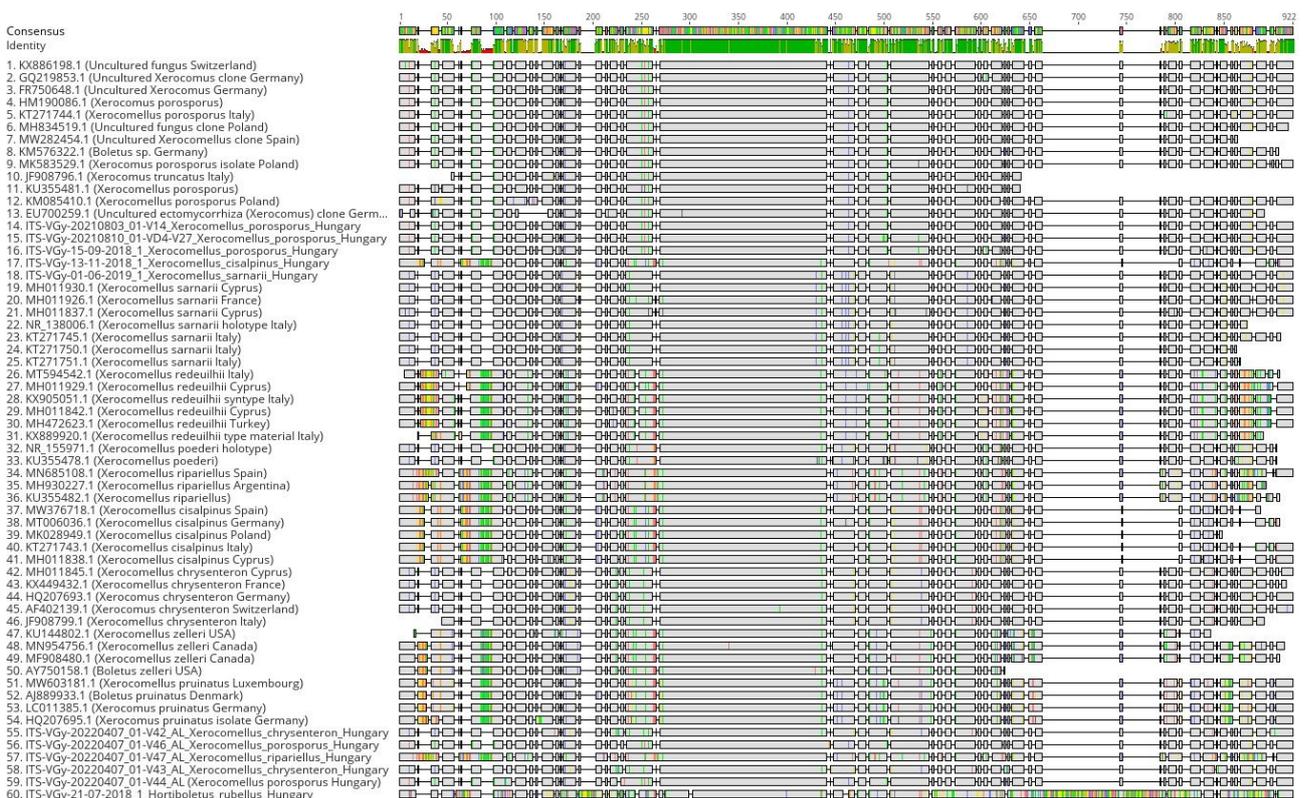
Figure 4. (Basidiomata of *Xerocomellus* species I.)

A: *X. cisalpinus* (ITS-VGy-13-11-2018_1); **B:** *X. ripariellus* (ITS-VGy-20220407_01-V47_AL, Photo: László Albert); **C:** *X. chrysenteron* (ITS-VGy-20220407_01-V42_AL, Photo: László Albert); **D:** *X. chrysenteron* (ITS-VGy-20220407_01-V43_AL, Photo: László Albert); **E-F:** *X. sarnarii* ITS-VGy-01-06-2019_1)

Figure 5. (Basidiomata of *Xerocomellus* species II.)

G: *X. porosporus* (ITS-VGy-20220407_01-V44_AL, Photo: László Albert); **H:** *X. porosporus* (ITS-VGy-15-09-2018_1); **I:** *X. porosporus* (ITS-VGy-20210810_01-VD4-V27; **J-K:** *X. porosporus* (ITS-VGy-20210803_01-V14; **L:** *X. porosporus* (ITS-VGy-20220407_01-V46_AL, Photo: László Albert)