MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Species delimitation and phylogenetic analyses of the genus *Tricholomopsis* in Austria"

verfasst von / submitted by

Peter Sarkany BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien 2022 / Vienna 2022

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066832
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Botanik
Betreut von / Supervisor:	ao. Univ.-Prof. Mag. Dr. Irmgard Greilhuber
Mitbetreut von / Co- Supervisor:	Mag. Dr. Michael H. J. Barfuss

Content

Kurzfassung

Die Gattung *Tricholomopsis* in der Ordnung *Agaricales* ist eine taxonomische Gruppe, die einige gut umschriebene unterschiedliche Arten umfasst. Obwohl viele DNA-Barcodes für bestimmte Arten existieren, gibt es Unsicherheiten in mehreren Abstammungslinien.

Im Rahmen dieser Diplomarbeit wurden DNA-Sequenzen von *Tricholomopsis*-Herbarbelegen für Barcoding und phylogenetische Analyse sequenziert. Die ausgewählten DNA-Regionen waren die „Large-Subunit"-Region (LSU) und die „Internal-Transcribed-Spacer"-Region (ITS). Diese beiden Gensequenzen eignen sich aufgrund ihrer hohen Mutationsrate, und weil sie in vielen Kopien vorliegen, gut für die taxonomische Unterscheidung auf Artniveau und innerhalb der Arten mithilfe des Barcodings.

Die Ergebnisse zeigten, dass es einige gut etablierte Gruppen gibt, die korrekt unterschieden werden konnten, aber es gibt auch Unsicherheiten. Mithilfe des Barcodings konnten einerseits Fehlbestimmungen korrigiert, andererseits kryptische Arten aufgezeigt werden.

Insgesamt gut verfeinerte und überarbeitete Sequenzdaten zeigten eine deutliche Häufung der meisten Arten, und im Allgemeinen stimmten die analysierten Proben mit der Referenzliteratur überein.

Abstract

The genus *Tricholomopsis* in the order *Agaricales*, is a taxonomic group which compromises some well-circumscribed, distinct species. Although many DNA barcodes for certain species exist, there are uncertainties in multiple lineages.

In this master thesis, DNA regions of several *Tricholomopsis* herbarium specimens have been sequenced for barcoding and phylogenetic analysis. The chosen regions were the Large Subunit region (LSU) and the Internal Transcribed Spacer region (ITS). These two gene sequences are well suited for taxonomic differentiation at the species level and within species using barcoding because of their high mutation rate and because they are present in many copies.

The results showed, that there are certain well-established clusters, which could be correctly distinguished, but there are as well uncertainties. With the help of barcoding, it was possible to correct misidentifications on the one hand, and to identify cryptic species on the other.

Overall well refined and reworked molecular genetic sequence data showed distinct clustering of most species and in general analyzed samples have fallen in line with reference literature.

Acknowledgements

I would like to express my deepest appreciation to my supervisors Irmgard Greilhuber and Michael H. J. Barfuss, who have led me through the process of writing my master thesis and who have been supporting me to finally achieve my goal. All the competence that I could have established is their fruitful influence, which also motivated me to finish my thesis.

I especially want to thank Dominik Metschina for his kind, helpful personality, who withstood my daily dose of questions about the laboratory equipment and methods that I kept forgetting. With his reliable assistance and good humor every day at the laboratory was a joyful time.

I would also like to thank to my supportive relationship, who has been always on my side, even at some tiring and hard moments. Without him I would not have been able to overcome my challenges during the master studies.

Sincere thanks to my family and friends, who have been always supporting me with constructive comments, kind words and enthusiastic monologues, which kept me on track.

Finally, I want to thank once again Irmgard Greilhuber, who may have helped me deciding my faith, my path in life, which would be definitely mushrooms. Through her point of view, I immediately fell in love with fungi and since that exact one course about macromycetes lighted up my passion about the importance and need of these fascinating creatures.

Roland Eberwein and Herbert Pötz (KL, Klagenfurt) and Christoph Hahn (Munich) are thanked for loan of herbarium material.

Introduction

DNA sequencing is a modern technique in past few decades that has fundamentally changed the classification of species. Until 1977 taxonomic classification was traditionally based on morphological characteristics, but with modern, new techniques it has improved. Every organism has its own specific barcode as a fingerprint. For identification a DNA sequence region is needed, which has a reasonable mutation rate. Closely related organisms, such as individuals of the same species, show a very low variability within their ”fingerprint”-barcode. In general, the higher the difference between marker regions, the less related the organisms are assumed to be (Heather & Chain 2016).

With the application of modern sequencing methods for species definition, taxonomy has changed a lot. The definition of species has always been a problem and there exist a considerable number of species concepts. After inventing Sanger sequencing in 1977, there was a huge leap towards ensuring information and achieving a better than the classical morphological species concept. The Sanger method has been used also for already identified species. However, not all problems could be solved at the species level. Generally, the main definition described to consider organisms as species is, if they can produce fertile offspring, which is called the biological species concept. It can be more or less successfully applied in zoology, but is less suited for plants and fungi. It has some problematic perspectives, for example in interspecific hybridization, where a fertile offspring could still occur (Mallet 1995).

Further problems with the morphological species concept occur with cryptic species, which are morphologically identical, so that they cannot be separated by morphological discriminating characters (Bickford et al. 2007).

Especially in fungi taxonomy, classification appeared to be very hard. Because of the fact that in the kingdom of fungi, the cryptic species concept is more relevant. That means, many mushrooms are very hard to identify just by morphological characteristics. Although their persistent life stage as mycelium and fruiting bodies have sometimes distinct growth patterns, interspecific variation can often not so simply be distinguished from intraspecific variation (Talbot 1978). Therefore, molecular sequencing analysis is even more valuable in fungi for species and higher taxonomic levels.

The marker region generally agreed upon as barcoding region for fungi (Schoch et al. 2012) is the ITS (Internal Transcribed Spacer) and the LSU region within the rDNA (ribosomal DNA) operon. These two regions (ITS and LSU) are variable regions with quite high mutation rate, perfectly accurate to use it as molecular fingerprinting (Chambers et al. 2014). ITS region is also used in plant taxonomic identification, but for fungi it is even more relevant, because its variability suits inter- and intraspecific comparison greatly.

The genus *Tricholomopsis* belongs to the order *Agaricales* which is one the largest groups within the *Basidiomycetes*. Originally, three different species, namely *T. rutilans* (Schaeff.) Singer, *T. decora* (Fr.) Singer and *T. flammula* Métrod ex Holec were reported, also from Austria. Regarding to the phylogenetic classification and improvement of molecular sequencing techniques, studies have shed light on species that had not been classified before. For example, Holec et al. (2019) discovered *T. badinensis* J. Holec, M. Kolařík & V. Kunca in Slovakia, within very old, intact forests and *T. sulfureoides* (Peck) Singer in Poland, similarly old forests which has not been influenced by human activity (Holec et al. 2019).

Several further studies exist of other recently discovered species, such as: *T. osiliensis* Vauras, which is now considered to be a synonym of *T. sulfureoides*, *T. pteridicula* Olariaga, Laskibar & Holec (Olariaga et al. 2015), *T. scabra* J. A. Cooper, and *T. ornaticeps* (G. Stev.) E. Horak from New-Zealand (Cooper & Park 2016). These and other cases confirm that molecular genetic sequencing alongside with traditional identifying methods is important Worldwide. Index Fungorum (http://www.indexfungorum.org/) lists about 43 different *Tricholomopsis* species.

This master thesis focuses on the genus *Tricholomopsis* regarding to mainly Central European species. The aim was to observe, if are there any clustering features in the given samples on the phylogenetic tree level concluding from molecular genetic investigation, and if there are any cryptic species within in the genus *Tricholomopsis*. In case of separate clustering within species, additional microscopic investigations have been performed.

Morphological Characteristics

For a broader overlook to identify the different species of the genus *Tricholomopsis*, it is beneficial to shortly introduce the most abundant three species, which can be found in Central-Europe, especially in Austria. In the sense of already existing scientific articles (see below) a short description about the overall appearance could help to gain some knowledge of these three species.

***Tricholomopsis rutilans* (Schaeff.) Singer, Schweiz. Z. Pilzk. 17: 56 (1939)**

The species *Tricholomopsis rutilans* is one the most widespread species in Central-Europe. The fruiting bodies are quite large and fleshy when mature, the pileus is densely covered with red-violet fibrils to scales. The spore print is white.

It is a common mushroom throughout Europe, living saprobiontic on dead wood of conifers, in all types of forests, not sensitive for the soil composition. It is an edible species with an earthy flavor. (Gminder & Böhning 2020).

Fig. 1. *Tricholomopsis rutilans.*
(source:https://www.mykoweb.com/CAF/species/Tricholomopsis_rutilans.html)

***Tricholomopsis flammula* Métrod ex Holec, J. National Mus. (Prague), Nat. Hist. Ser. 178: 8 (2009)**

The fruiting body is much slender than that of *T. rutilans*, not fleshy, small to medium-sized, rarely a large pileus. The young pileus is densely covered with fine red-violet to purplish-brown fibrillose scales, which will eventually slowly vanish when mature. The stipe is more yellowish and rarely covered with purple fibrils (Krisai-Greilhuber & Voglmayr 2000, Holec & Kolarik 2012).

Tricholomoposis flammula is widespread in Central-Europe but rare. It grows on dead conifer wood (mainly *Picea* and *Abies*), sometimes on wood of deciduous trees (like *Fagus*). In the Czech Republic and Slovakia, it clearly prefers virgin forests, although records from habitats strongly influenced by human are known too (Holec & Kolarik 2012).

Fig. 2. *Tricholomopsis flammula.* (Czech Republic, Boubínský prales (PRM 899108). Photo J. Holec, Holec & Kolarik 2011)

***Tricholomopsis decora* (Fr.) Singer, Schweiz. Z. Pilzk. 17: 56 (1939)**

Diagnostic characteristics are a medium-sized fruiting body, with a yellow to orange ground color, a pileus covered with densely arranged, upraised, yellow-green to bronze, olive-brown scales.

This species is scattered to common throughout Europe, living on dead conifer wood, especially on old stumps and decaying fallen trunks, in moist and shady mixed coniferous forests, above all in the mountains. (Breitenbach & Kränzlin 1991, Ryman & Holmäsen 1992, Boekhout & Noordeloos 1999, Ludwig 2000, Roux 2006, Vesterholt 2008).

Fig. 3. *Tricholomopsis decora*

(https://upload.wikimedia.org/wikipedia/commons/9/9f/2012-10-03_Tricholomopsis_decora_%28Fr.%29_Singer_268487.jpg)

Materials and Methods

Fungal Material

The fungal material that has been used for this thesis were dried fruiting bodies of different species of *Tricholomopsis*. The samples were kept in dark, dry cardboard boxes or in plastic bags in the Fungarium of the University of Vienna (herbarium WU-MYC) and in the herbarium of Klagenfurt (KL), or the private collection of Christoph Hahn.

The material (Tab. 1) was collected from different parts of Germany and Austria, from different collectors. For comparison, additional sequences have been searched on the National Center for Biotechnology Information (NCBI) from all over Europe.

Tab. 1. List of *Tricholomopsis* material used with genbank/unite accession no. or herbarium voucher no. respectively, source and country of origin, sorted after accession no.

Accession no.	UNITE taxon name	Herbarium no.	Sequence source	Conutry of origin
FN554890	*T. decora*	PRM:882317	Holec & Kolarik (2011)	Czech Republic
FN554891(2)	*T. decora*	PRM:898238	Holec & Kolarik (2011)	Czech Republic
FN554893	*T. flammula*	PRM:899108	Holec & Kolarik (2011)	Czech Republic
FN554894	*T. flammula*	personal:Jan Holec:227/2008	Holec & Kolarik (2011)	Czech Republic
FN554895	*T. rutilans*	PRM:889120	Holec & Kolarik (2011)	Slovakia
FN554896	*T. flammula*	PRM:909608	Holec & Kolarik (2011)	Czech Republic
FN554897(2)	*T. flammula*	WU-MYC:0012087	Holec & Kolarik (2011)	Austria
HE649939	*T. flammula*	PRM:899162	Holec & Kolarik (2012)	Slovakia
HE649940	*T. flammula*	PRM:899180	Holec & Kolarik (2012)	Slovakia
HE649941	*T. flammula*	PRM:899190	Holec & Kolarik (2012)	Slovakia
HE649942	*T. decora*	PRM:899160	Holec & Kolarik (2012)	Slovakia
HE649943	*T. osiliensis*	PRM:899461	Holec & Kolarik (2012)	Estonia
HE649944	*T. osiliensis*	TU:101571	Holec & Kolarik (2012)	Estonia
HE649945	*T. osiliensis*	PRM:899184	Holec & Kolarik (2012)	Slovakia
HE649946	*T. rutilans*	PRM:899460	Holec & Kolarik (2012)	Czech Republic
HE652866	*T. flammula*	WU-MYC:0013075	Holec & Kolarik (2012)	Austria
JX029952	*Tricholomopsis* sp.	-	Baldrian & Vetrovsky (2013)	Czech Republic
KP058973	*T. flammula*	ARAN-Fungi 00322	Olariaga et al. (2015)	Spain
KP058975	*T. flammula*	S-F156625	Olariaga et al. (2015)	Sweden
KP058977	*T. rutilans*	UPS-F-646219	Olariaga et al. (2015)	Sweden
KP058979	*T. rutilans*	BIO11313	Olariaga et al. (2015)	Sweden
KP058981	*Tricholomopsis* sp.	TUB11582	Olariaga et al. (2015)	Germany
KP058982	*Tricholomopsis* sp.	ARAN-Fungi 00323	Olariaga et al. (2015)	Spain

Accession number	UNITE taxon name	Voucher-number	Sequence source	Conutry of origin
KP058984	*Tricholomopsis* sp.	UPS-F-646220	Olariaga et al. (2015)	Sweden
KP058986	*T. pteridicola*	ARAN-Fungi 00320	Olariaga et al. (2015)	Spain
KP058988	*T. pteridicola*	ARAN-Fungi 00121	Olariaga et al. (2015)	Spain
KP058992	*T. pteridicola*	ARAN-Fungi 00321	Olariaga et al. (2015)	France
KP058994(3)	*T. pteridicola*	ARAN-Fungi 00115	Olariaga et al. (2015)	Spain
KY435938	*T. rutilans*	-	Zifcakova (2017) direct submission	Czech Republic
LS992163	*T. badinensis*	PRM:946195	Kolarik (2018) direct submission	Slovakia
LS992164	*T. badinensis*	PRM:899423	Kolarik (2018) direct submission	Slovakia
LS992165	*T. badinensis*	PRM:946194	Kolarik (2018) direct submission	Slovakia
LT984726	*T. osiliensis*	PRM:946096	Kolarik (2018) direct submission	Poland
LT984727	*T. osiliensis*	PRM:946095	Kolarik (2018) direct submission	Poland
LT984728	*T. osiliensis*	PRM:945185	Kolarik (2018) direct submission	Poland
LT984729	*T. osiliensis*	BRNU DD160914-21	Kolarik (2018) direct submission	Poland
LT984730	*T. osiliensis*	PRM:944836	Kolarik (2018) direct submission	Poland
LT984731	*T. osiliensis*	BRNU DDBIA17/401	Kolarik (2018) direct submission	Poland
MK028524	*T. flammula*		Hofstetter et al. (2019)	Switzerland
MT117052	*Tricholomopsis* sp.	PC0125126	Larue (2020)	France
MT341465	*T. sulfureoides*	AF-124-2017	Fellin & Ferrari (2019)	Italy
MW215181	*Fungi*	-	Marciulyniene et al. (2021)	Lithuania

Accession number	UNITE taxon name	Voucher-number	Sequence source	Conutry of origin
UDB0108001(2)	*Tricholomopsis*	S454	soil sample	Estonia
UDB011101	*T. sulfureoides*		JV26540F (Specimen)	Estonia
UDB011443	*T. rutilans*		TUF106244(Specimen)	Estonia
UDB011477	*T. flammula*	TUF101684	herbarium specimen	Estonia
UDB011838	*T. decora*	TUF118267 (Specimen)	Saar 2011	Estonia
UDB015070(3)	*T. sulfureoides*		TUF101571 (Specimen)	Estonia
UDB015402	*T. flammula*		TUF118217 (Specimen)	Estonia
UDB015434(1)	*T. flammula*		TUF118261 (Specimen)	Estonia
UDB015437	*T. decora*	TUF106634 (Specimen)	Saar 2014	Estonia
UDB015633	*T. decora*	TUF106836 (Specimen)	Saar 2010	Estonia
UDB015728	*T. rutilans*		TUF106690 (Specimen)	Estonia
UDB017970	*T. sulfureoides*		TUF118547 (Specimen)	Estonia
UDB017971	*T. sulfureoides*		TUF118543 (Specimen)	Estonia
UDB019502	*T. sulfureoides*		TUF118828 (Specimen)	Estonia
UDB019610	*T. sulfureoides*		TUF118834 (Specimen)	Estonia
UDB0213069	*Tricholomopsis*	UPS-F-646219	sample	Estonia
UDB0276404	*Tricholomopsis*	UPS-F-646219	sample	Estonia
UDB031626	*T. decora*		H6012126 (Specimen)	Finland
UDB031733	*T. decora*		Fruitbody | TUF109242 (Specimen)	Finland
UDB031950	*T. rutilans*		H6012837 (Specimen)	Finland
UDB034601	*T. sulfureoides*		Fruitbody |TUF111521 (Specimen)	France
UDB036188	*T. flammula*		O-F76030 (Specimen)	Norway

Accession number	UNITE taxon name	Voucher-number	Sequence source	Conutry of origin
UDB036320	*T. rutilans*		O-F-260409 (Specimen)	Norway
UDB037020	*T. decora*		TROM_F_17037 (Specimen)	Norway
UDB037025	*T. rutilans*		TROM_F_17101 (Specimen)	Norway
UDB037268	*T. rutilans*		O-F-75739 (Specimen)	Norway
UDB037399	*T. decora*		O-F-75781 (Specimen)	Norway
UDB037840	*T. decora*		O-F-304980 (Specimen)	Norway
UDB037897	*T. rutilans*		O-F-248192 (Specimen)	Norway
UDB052975(1)	*Tricholomopsis* sp.	UPS-F-646219	sample	Estonia
UDB054996	*Tricholomopsis* sp.	UPS-F-646219	sample	Estonia
UDB056619	*Tricholomopsis* sp.	UPS-F-646219	sample	Estonia
UDB075618	*Tricholomopsis* sp.	UPS-F-646219	sample	Estonia
UDB0768856	*Tricholomopsis* sp.	UPS-F-646219	sample	Estonia
UDB347767	*Tricholomopsis* sp.	UPS-F-646219	sample	Estonia
TD1	*T. decora*	WU-MYC-0018748	Darcy 2019, unpubl.	Austria, Mariazell
TD2	*T. decora*	WU-MYC-0014364	Darcy 2019, unpubl.	Austria, Dornbirn
TD3	*T. decora*	WU-MYC-0010249	Darcy 2019, unpubl.	Austria, Jennersdorf
TR1	*T. rutilans*	WU-MYC-0007943	Darcy 2019, unpubl.	Austria, Langau
TR2	*T. rutilans*	WU-MYC-0021484	Darcy 2019, unpubl..	Austria, Langenlois
TR3	*T. rutilans*	WU-MYC-0036732	Darcy 2019, unpubl.	Austria, Drösing
TR4	*T. rutilans*	WU-MYC-0031785	Darcy 2019, unpubl.	Austria, Michelbach
TR5	*T. rutilans*	WU-MYC-0026265	Darcy 2019, unpubl.	Austria, Kleinpertholz
TF1	*T. flammula*	WU-MYC-0040233	Darcy 2019, unpubl.	Austria, Hintenberg
TF2	*T. flammula*	WU-MYC-0023870	Darcy 2019, unpubl.	Czech Republic, Sumava, Vala niva
TF3	*T. flammula*	Darcy 2019, unpubl.	Darcy 2019, unpubl.	Austria, Feldkirch

Accession number	UNITE taxon name	Voucher-number	Sequence source	Conutry of origin
TF4	*T. flammula*	WU-MYC-0012087	Darcy 2019, unpubl.	Austria, Gloggnitz
TF5	*T. flammula*	WU-MYC-0022535	Darcy 2019, unpubl.	Austria, Mitterbach
TF6	*T. flammula*	WU-MYC-0013075	Darcy 2019, unpubl.	Austria, Greith
3_WU-0039507	*T. rutilans*	WU-MYC-0039507	present work, 2020	Germany, Bayern
4_ Kogseder	*Tricholomopsis* sp.	WU-MYC-0046368	present work2020	Austria, Oberösterreich
5_20100154	*T. flammula*	WU-MYC-0046369	present work, 2020	Austria, Steiermark
6_BK-60323; 9052/1	*T. ornata*	KL-60323	present work, 2020	Austria, Kärnten
7_Goetis	*T. rutilans*	WU-MYC-0046366	present work, 2020	Austria, Voralberg
8_20170189	*T. flammula*	WU-MYC-0046367	present work, 2020	Austria, Steiermark
9_CH-2017090501	*T. rutilans*	WU-MYC-0046375	present work, 2020	Germany, Bayern
10_CH-2017090401	*T. rutilans*	WU-MYC-0046362	present work, 2020	Germany, Bayern
11_CH-2017090402	*T. rutilans*	WU-MYC-0046376	present work, 2020	Germany, Bayern
12_CH-2017090502	*T. rutilans*	WU-MYC-0046365	present work, 2020	Germany, Bayern
14_08/2018	*T. decora*	WU-MYC-0046363	present work, 2020	Austria, Tirol
15_CH-2017090403	*T. decora*	WU-MYC-0046302	present work, 2020	Germany, Bayern
16_7934/312	*T. cf. decora*	WU-MYC-46304	present work, 2020	Germany, Bayern
19_Inv. Nr.: 49465;1884	*T. rutilans*	KL-49465	present work, 2020	Austria, Kärnten
21_Inv. Nr.: 49405;2051	*T. rutilans*	KL-49405	present work, 2020	Austria, Kärnten
23_Inv. Nr.: 43535;9051/4	*T. decora*	KL-43535	present work, 2020	Austria, Kärnten
25_Inv. Nr.: 42871;9351/4	*T. decora*	KL-42871	present work, 2020	Austria, Kärnten
26	*T. badiensis*	WU-MYC-0044784	present work, 2022	Austria, Niederösterreich
27	*T. decora*	WU-MYC-0042135	present work, 2022	Austria, Niederösterreich

Accession number	UNITE taxon name	Voucher-number	Sequence source	Conutry of origin
28	*T. flammula*	WU-MYC-0041095	present work, 2022	Austria, Niederösterreich
29	*T. flammula*	WU-MYC-0042029	present work, 2022	Austria, Tirol
30	*T. rutilans*	WU-MYC-0000962	present work, 2022	Austria, Niederösterreich
31	*T. rutilans*	WU-MYC-0000963	present work, 2022	Austria, Niederösterreich
32	*Tricholomopsis sp.*	WU-MYC-0043261	present work, 2022	Austria, Niederösterreich
33	*T. rutilans*	WU-MYC-0023634	present work, 2022	Austria, Niederösterreich
34	*T. decora*	WU-MYC-0023731	present work, 2022	Austria, Niederösterreich
35	*T. decora*	WU-MYC-0008862	present work, 2022	Austria, Niederösterreich
36	*T. decora*	WU-MYC-0023055	present work, 2022	Austria, Niederösterreich
37	*T. decora*	WU-MYC-0023795	present work, 2022	Austria, Niederösterreich
38	*T. decora*	WU-MYC-0004983	present work, 2022	Austria, Niederösterreich
39	*T. rutilans*	WU-MYC-0042954	present work, 2022	Austria, Steiermark
40	*T. rutilans*	WU-MYC-0043941	present work, 2022	Austria, Oberösterreich
41	*T. flammula*	WU-MYC-0041978	present work, 2022	Austria, Steiermark
42	*T. flammula*	WU-MYC-0044777	present work, 2022	Austria, Niederösterreich
43	*T. decora*	WU-MYC-0005463	present work, 2022	Austria, Niederösterreich
44	*T. decora*	WU-MYC-0010006	present work, 2022	Austria, Niederösterreich
45	*T. decora*	WU-MYC-0034745	present work, 2022	Austria, Tirol
46	*T. flammula*	WU-MYC-0039507	present work, 2022	Germany, Bayern
47	*T. decora*	KL-50424,9453/3	present work, 2022	Austria, Kärnten
48	*T. rutilans*	KL-48360,1466	present work, 2022	Austria, Kärnten
49	*T. rutilans*	KL-48358,1707	present work, 2022	Austria, Kärnten
50	*T. rutilans*	KL-49664,1966	present work, 2022	Austria, Kärnten
51	*T. decora*	KL-49941,9451/1	present work, 2022	Austria, Kärnten
52	*T. rutilans*	KL-48359,1655	present work, 2022	Austria, Kärnten
53	*T. rutilans*	KL-48357,778	present work, 2022	Austria, Kärnten

Accession number	UNITE taxon name	Voucher-number	Sequence source	Conutry of origin
54	*T. rutilans*	KL-31455,9352/3	present work, 2022	Austria, Kärnten
55	*T. rutilans*	KL-48356,1599	present work, 2022	Austria, Kärnten
56	*T. rutilans*	KL-46842,9351/3	present work, 2022	Austria, Kärnten
57	*T. decora*	KL-43552,9451/1	present work, 2022	Austria, Kärnten

Sample Preparation

The material for the genetic study was taken from the cap and the hymenium layer of the dried fungi with tweezers; 10 to 15 mg were put into small Eppendorf-tubes with 5 glass beads.

After weighing out the material, liquid nitrogen (-196 ^{o}C) was used for deep cooling the samples. Then the tubes were taken into the grinder machine (QIAGEN, Tissuelyzer II) with the basic settings of: frequency: 30,0 1/s, time: 5 min. After homogenizing the material centrifugation was needed with Sigma 1-15 centrifuge with the settings of: rpm: 8000 × g, time: 30 sec.

DNA Extraction, PCR and Sequencing Reaction

For the DNA extraction the DNeasy Plant Minikit (QIAGEN) was used, following the manufactures instructions with minor modifications tested in-house. The QIAGEN protocol can be downloaded from www.qiagen.com. The modifications are shown in the protocol, in brackets (Appendix).

Further extractions were performed with a GeneJet Extraction Kit (ThermoFisher), with also some slight modifications, that have been tested in-house. (Appendix).

For isolating DNA from the samples of number 32, 48, 50, 51 and 57 a CTAB (cetyl-trimethylammonium-bromid) method (Doyle & Doyle 1987) was used in addition, due higher chance of getting a clean DNA product, since the first attempts with the commercial kits led to quite poor concentrations.

Nanodrop Measurements

To ensure that extractions were successful for further experiments, a Nanodrop ND-1000 Spectrophotometer (PeqLab) was used for assessing the quality and rough quantity of DNA. DNA has an absorbance of light at a certain wavelength (220-350 nm) and a proxy for DNA quantity can be assessed. The DNA light absorption has a maximum at 260 nm. A high absorbance therefore indicates high DNA concentrations. The ratio of absorption between 260-280 nm and also the shape of the curve and the appearance of several peaks, indicates contamination with other secondary metabolites than DNA. (Fig. 4).

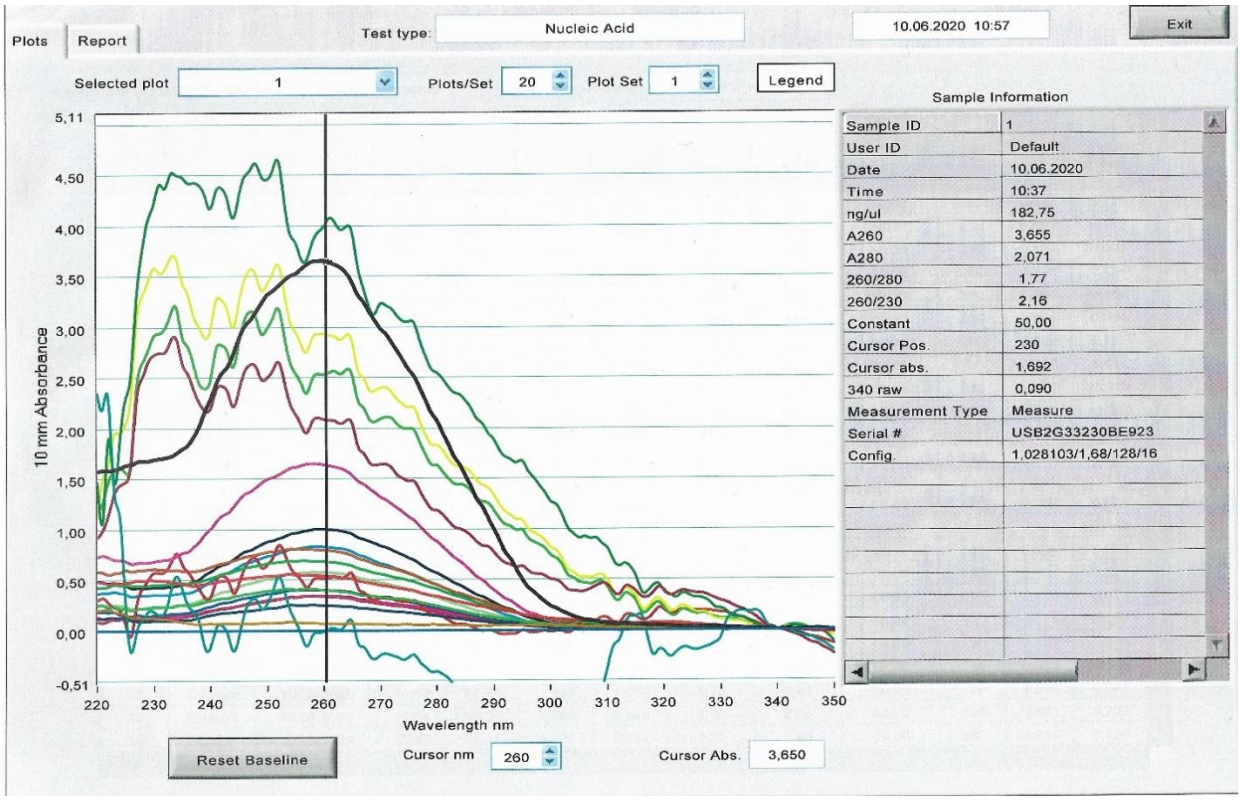
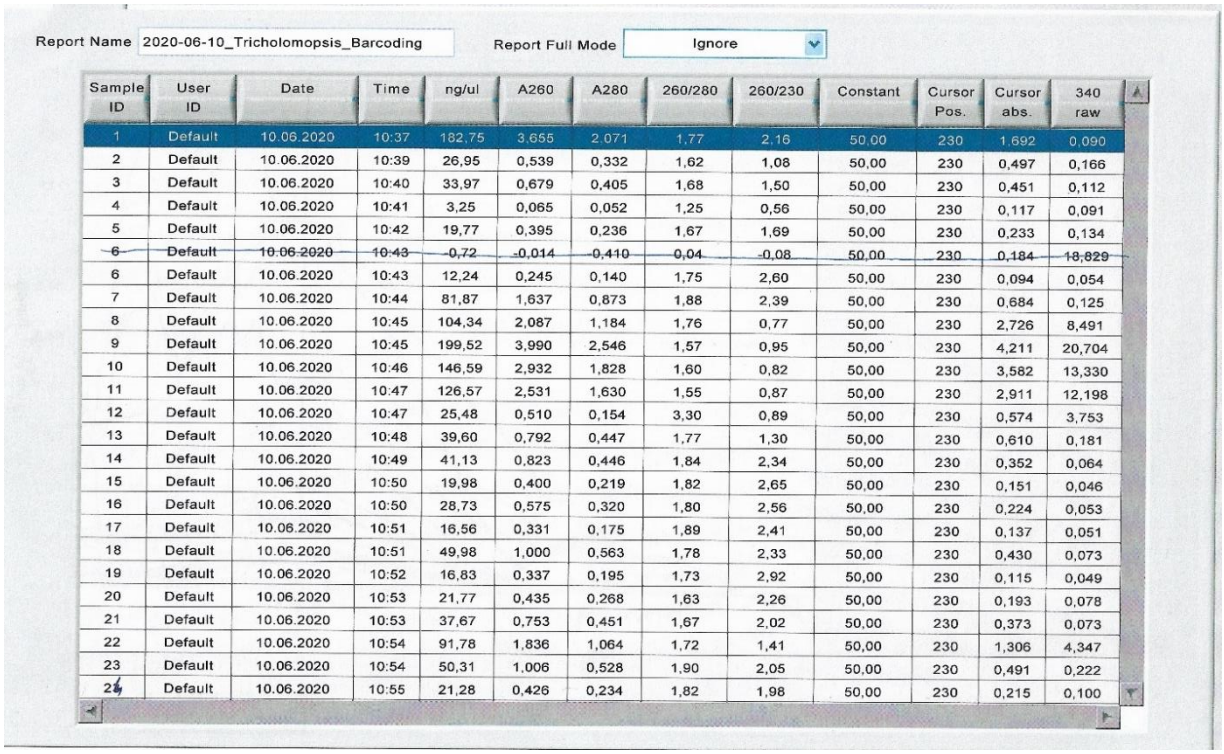


Fig. 4. Nanodrop software output graphically shows absorption spectra of all samples from 2020.



Report Name 2020-06-10_Tricholomopsis_Barcoding Report Full Mode Ignore

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
1	Default	10.06.2020	10:37	182,75	3,655	2,071	1,77	2,16	50,00	230	1,692	0,090
2	Default	10.06.2020	10:39	26,95	0,539	0,332	1,62	1,08	50,00	230	0,497	0,166
3	Default	10.06.2020	10:40	33,97	0,679	0,405	1,68	1,50	50,00	230	0,451	0,112
4	Default	10.06.2020	10:41	3,25	0,065	0,052	1,25	0,56	50,00	230	0,117	0,091
5	Default	10.06.2020	10:42	19,77	0,395	0,236	1,67	1,69	50,00	230	0,233	0,134
6	Default	10.06.2020	10:43	-0,72	-0,014	-0,410	0,04	-0,08	50,00	230	0,184	18,829
6	Default	10.06.2020	10:43	12,24	0,245	0,140	1,75	2,60	50,00	230	0,094	0,054
7	Default	10.06.2020	10:44	81,87	1,637	0,873	1,88	2,39	50,00	230	0,684	0,125
8	Default	10.06.2020	10:45	104,34	2,087	1,184	1,76	0,77	50,00	230	2,726	8,491
9	Default	10.06.2020	10:45	199,52	3,990	2,546	1,57	0,95	50,00	230	4,211	20,704
10	Default	10.06.2020	10:46	146,59	2,932	1,828	1,60	0,82	50,00	230	3,582	13,330
11	Default	10.06.2020	10:47	126,57	2,531	1,630	1,55	0,87	50,00	230	2,911	12,198
12	Default	10.06.2020	10:47	25,48	0,510	0,154	3,30	0,89	50,00	230	0,574	3,753
13	Default	10.06.2020	10:48	39,60	0,792	0,447	1,77	1,30	50,00	230	0,610	0,181
14	Default	10.06.2020	10:49	41,13	0,823	0,446	1,84	2,34	50,00	230	0,352	0,064
15	Default	10.06.2020	10:50	19,98	0,400	0,219	1,82	2,65	50,00	230	0,151	0,046
16	Default	10.06.2020	10:50	28,73	0,575	0,320	1,80	2,56	50,00	230	0,224	0,053
17	Default	10.06.2020	10:51	16,56	0,331	0,175	1,89	2,41	50,00	230	0,137	0,051
18	Default	10.06.2020	10:51	49,98	1,000	0,563	1,78	2,33	50,00	230	0,430	0,073
19	Default	10.06.2020	10:52	16,83	0,337	0,195	1,73	2,92	50,00	230	0,115	0,049
20	Default	10.06.2020	10:53	21,77	0,435	0,268	1,63	2,26	50,00	230	0,193	0,078
21	Default	10.06.2020	10:53	37,67	0,753	0,451	1,67	2,02	50,00	230	0,373	0,073
22	Default	10.06.2020	10:54	91,78	1,836	1,064	1,72	1,41	50,00	230	1,306	4,347
23	Default	10.06.2020	10:54	50,31	1,006	0,528	1,90	2,05	50,00	230	0,491	0,222
24	Default	10.06.2020	10:55	21,28	0,426	0,234	1,82	1,98	50,00	230	0,215	0,100

Fig. 5. Output suggests effective DNA extraction with values.

DNA Marker and Primer Selection

Usually for plant and fungal sequencing methods the most commonly used region for identification is the ITS region (Internal Transcribed Spacer) and especially in Fungi in addition the LSU (Large Subunit) region, which are both in the cell nuclei operon and really close to each other. Universal primers were used for this purpose, which were ITS4, ITS5, LR5 and LR0R (Tab. 2). The primers were previously diluted to 500 µM concentration.

Primer name		Direction	Sequence (5´-3´)	Reference
ITS5		Fwd	GGAAGTAAAAGTCGTAACAAGG	White et al. 1990
ITS4		Rev	TCCTCCGCTTATTGATATGC	White et al. 1990
LROR		Fwd	ACCCGCTGAACTTAAGC	Vilgalys & Hester 1990
LR5		Rev	TCCTGAGGGAAACTTCG	Vilgalys & Hester 1990

Tab. 2. Primer sequences from the region of ITS and LSU (ca. 700-800bp)

PCR Reagents

The PCR protocol has been developed and optimized in-house. The chemicals used for the reactions in the ITS region are the following (volumes are given for one single reaction):

1.1× ReddyMix PCR Master Mix (AB-0619/LD)	9.0 µl
Forward and reverse primers (500 µM)	2×0.5 µl
BSA (Bovine-Serum-Albumine)	0.1 µl
DNA template	1.0 µl

Total reaction volume is therefore **11.1** µl per tube. The tubes were shortly centrifuged after pipetting.

Alternatively, from the ReddyMix instead of 9,0 µl, a total amount of 10 µl has been used in the case of the LSU region. Which resulted in 12,1 µl of reaction volume.

PCR Machine Settings

PCRs were achieved using the GeneAmp PCR System 9700 (Life Technologies). The PCR conditions were the following in the case of ITS region.

1. Initial heating:	95 ^{o}C for 2 min
2. Denaturation step:	95 ^{o}C for 25 sec
3. Annealing step:	50 ^{o}C for 35 sec
4. Extension step:	72 ^{o}C for 50 sec
5. Final extension:	72 ^{o}C for 5 min

Step 2,3 and 4 were repeated for 40 cycles.

Since the LSU region contains different amount of nucleotide composition, also the PCR-program had to be adjusted. These minor changes were only applied in Step 4 (Extension), where instead of 50 sec, a total of 90 sec was the modified value.

Agarose Gel Electrophoresis

The success of PCR was verified with agarose gel electrophoresis. For this purpose, 0.45 g of agarose was diluted in 30 ml of TBE (Tris-borate-EDTA) buffer and 2 µl of GelRed dye. After measuring out the components the liquid was placed into the microwave to increase the solubility of the agarose powder. After heating it was necessary to re-add some distilled water to gain the original weight of the solution. After agarose gel solidification 1 µl of each sample was loaded into the pockets. A DNA sample ladder at the last pocket was added to track down the size of the PCR products. Results are shown in Figs. 6-7.

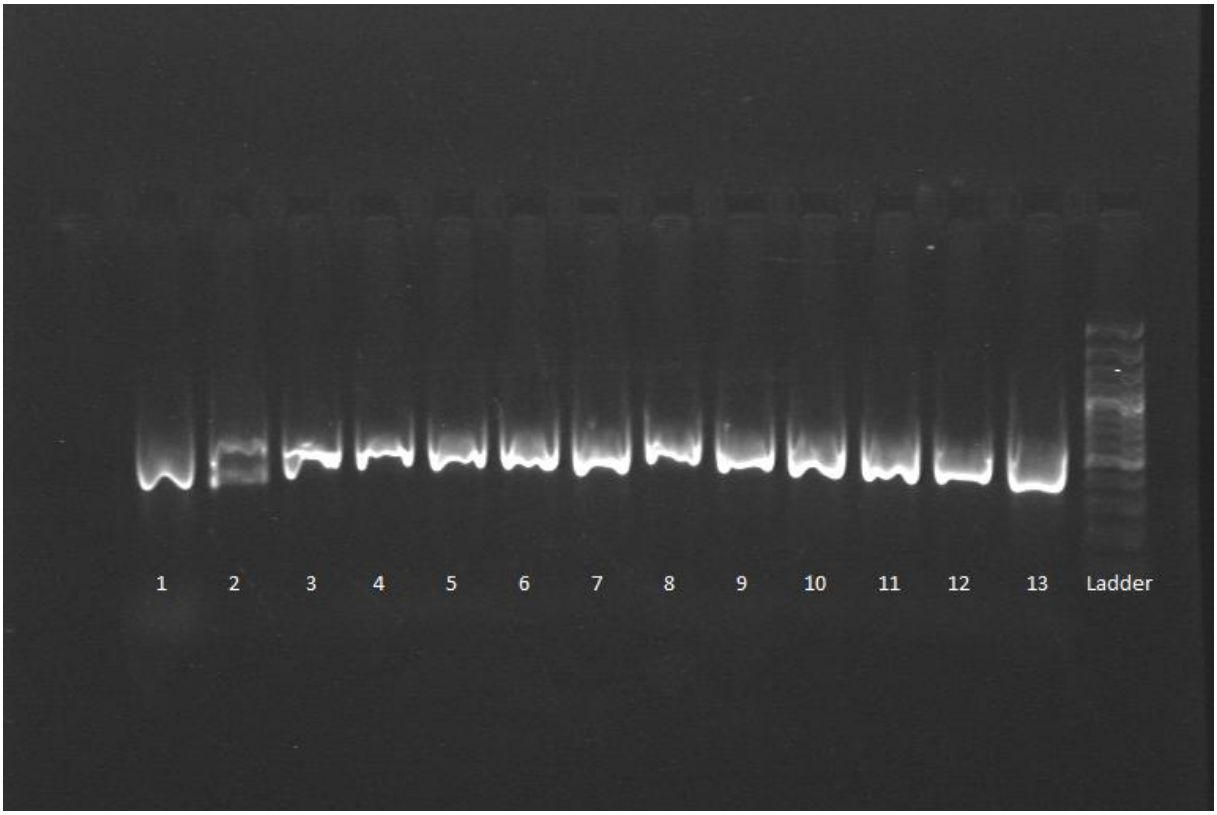


Fig. 6. Agarose gel electrophoresis samples from 1-13 and DNA sample ladder.

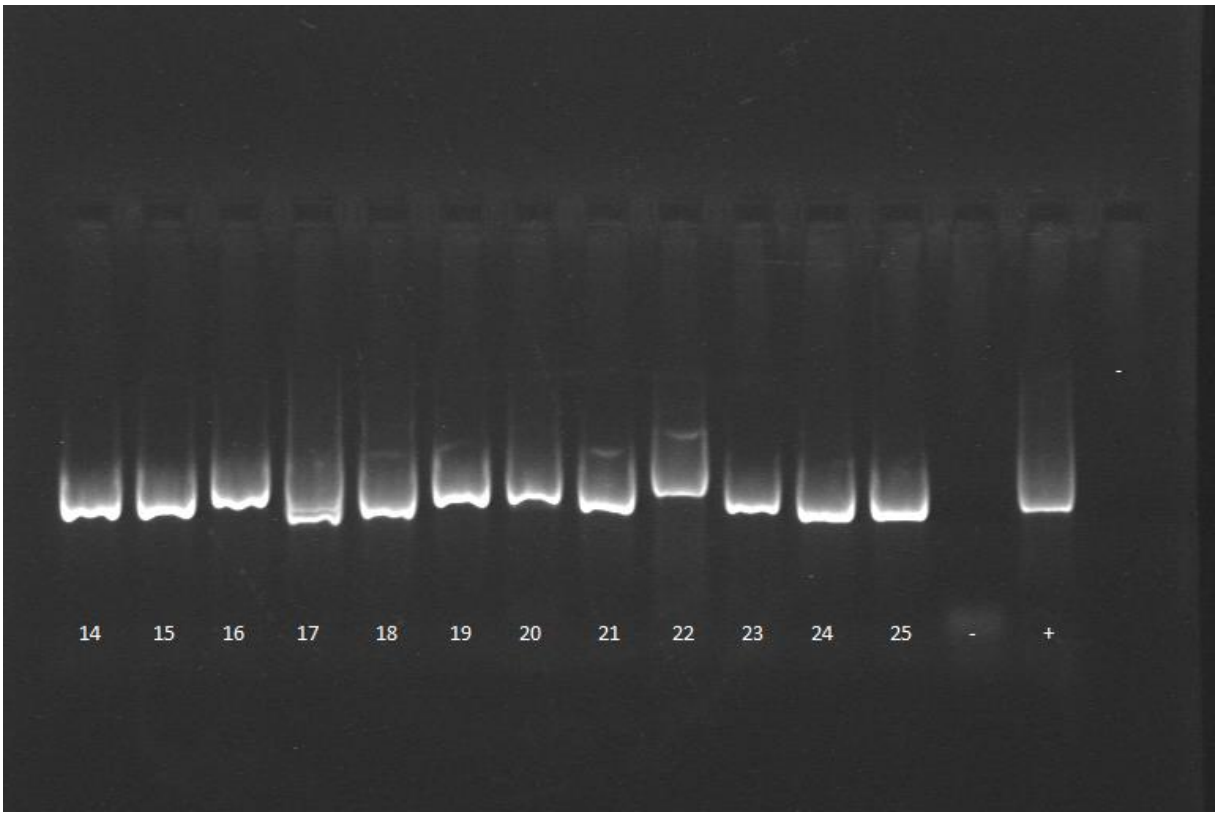


Fig. 7. Agarose gel electrophoresis samples from 14-25, plus a – and a + control.

Further electrophoresis pictures with the remaining samples are in the Appendix.

PCR Product Purification Step

For the purification of PCR products an enzyme mix was used, which (as it is shown in its name) is an enzymatic digestion of remaining PCR components, such as unused dNTPs and primers. The protocol for purification is:

1. 11.1-12.1 µl of PCR product
2. 1.0 µl of enzyme mix (master mix containing FastAP, the Fast Alkaline Phosphatase and Exonuclease I).

This combined solution was spinned down and incubated at 37°C for 30 min. Afterwards enzymes were denatured at 85°C for 15 min. The progress was conducted in the earlier mentioned PCR machine with a pre-set program.

Cycle Sequencing (Sanger Sequencing Reaction)

The conditions for the cycle sequencing reaction were (for one reaction only):

1. Trehalose (1M)	2 µl
2. Sequencing Buffer (5x)	1,8 µl
3. Primer (5 µM) (only one per reaction)	1 µl
4. H_2O (RNA-free, PCR grade water)	2,6 µl
5. BigDye Terminator v3.1 (2.5x)	0,6 µl
6. Cleaned PCR product (>20 ng)	2 µl

The end volume therefore for the cycle reaction concluded in 10 µl of solution. The PCR settings were the in house tested general PCR settings:

1. Initial hold	96°C for 1 min
2. Denaturation	96°C for 10 sec
3. Annealing	50°C for 5 sec
4. Extension	60°C for 3 min
5. Final hold	4°C until use

The number of cycles between Step 2 and Step 4 have been repeated 35 times.

BigDye terminator labelling is used for labelling and preparing the samples for Sanger Sequencing. This method uses both dNTPs (deoxynucleotide triphosphates) and ddNTPs (dideoxynucleotide phosphates). Each of the ddNTPs are tagged with different fluorescent dyes regarding to their nature of the four nucleotide types in DNA.

When the samples are taken back to the thermocycler for amplification, there will be randomly generated fragments with different lengths of DNA samples. On each different fragment a ddNTP will take place which will provide further information later in the sequencing. Each PCR product will be sequenced using the PCR primers.

The cleaning of the sequencing reactions with Sephadex and the operation of the 48-capilary Sequencer (AB 3730 DNA Analyzer, Applied Biosystems) was conducted by the laboratory manager of the Department of Botany and Biodiversity Research (Mag. Dr. Michael H. J. Barfuss), who was also a co-supervisor in this project.

The Cycle Sequencer detects the DNA of interest as different lengths of the DNA fragments with the ddNTPs on it, which flows in front of a detector through the capillars. Then the detector identifies which kind of ddNTPs are flowing in front of it, due to different wavelengths of light. Furthermore, the sequencer creates an electropherogramm, which can be recognized on the display of the computer. The programme will also generate the most supported sequence based on the electropherogram, which can be then manually edited.

Data Processing and Phylogenetic Analyses

Data Processing Program

The programs used to handle the amount of data were: Seqman, BioEdit, PAUP, MAFFT online version, ClustalX and Text editors for data management.

Assembling and Editing

The sequences obtained from the sequencer were assembled and edited with the program Seqman. In this software, raw sequences were manually edited by eliminating mistakes, such as unrecognizable sequence pattern (trimming) and controlling ”conflict-sites”, where the sequence has to be manually corrected (missing base from the other chain, or gap in the sequence, which can be corrected from the complement chain).

Additional Sequence Data

Unpublished sequences from an earlier study by Sean Darcy, were kindly supplied by Mag. Dr. Michael H. J. Barfuss. Darcy was also working with the genus *Tricholomopsis.* His results from the year 2019 were included in order to have a larger sample number to be able to draw meaningful conclusion.

Alignment

Alignments were conducted in BioEdit with ClustalW alignment settings, but it seemed more efficient to use MAFFT online alignment on the internet (https://mafft.cbrc.jp/alignment/server/). After aligning with MAFFT, ClustalX has been used to avoid the unnecessary gaps between the sequences.

Phylogenetic Analysis

For phylogenetic analysis investigation PAUP*4.0 Software (Swofford 2003) has been used. With the program three different analyses have been calculated. First, a distance tree analysis which generated a Neighbor-Joining Tree (NJ-Tree), which is just a comparative method where only one tree is generated. The algorithm simply creates a comparison between the sequences what is in the sequence matrix: the most similar sequences are belong to the same group.

The second analysis that have been carried out was the Maximum Parsimony (MP) analysis which is an analysis type where the algorithm searches for the shortest trees possible. For that, general settings have to be arranged: number of replicates was 1000 with no more than 10 best trees saved per replicate. Heuristic branch swapping algorithm was tree-bisection-reconnection (TBR). With this analysis several most-parsimonious trees have been generated and one has been randomly selected which is shown in the results section.

From these most-parsimonious trees a strict consensus tree has been generated in which only shows branches that are present in all trees. Furthermore, a majority-rule consensus tree has been made which displays branches that are present in at least 50% of the calculated most-parsimonious trees. Branches that are present in all trees (100%) are the same as in the strict consensus tree.

Finally, a bootstrap analysis has been conducted and a 50% bootstrap consensus tree generated in which one could observe how well relationships are supported (50-74% weakly supported, 75-89% moderately supported, 90-100% highly supported).

In all cases *Pluteus romellii* was used as the outgroup to root the trees.

Microscopic Observations

Additional observations have been made on a microscopic level of species characteristics, such as spore shape and size, as well as presence and shape of cystidia.

The microscopic research has been accomplished with a Zeiss Axio Imager.A1 at different magnifications (100×, 1000×, 4000×). Samples for microscopic analysis were prepared with 3% Ammonium-solution. The used material were dried herbarium specimens of the different *Tricholomopsis* species, which have been soaked in the ammonium solution for some minutes to regain shape. It was placed between a slide and a cover glass. For the highest magnification immersion-oil was used.

For the spore measurements the Axio Imager measurement program was used. At least 20 spores were observed, spore length and width were noted. From those parameters the mean, median, minimum, maximum, the standard deviation and the Quotient (length divided with width) were calculated.

Results and Discussion

Tricholomopsis rutilans

Observed microscopic characteristics: No cystidia were found during observation (neither cheilo- nor pleurocystidia). Cystidia are additional end cell variants in a fruiting body of a mushroom. They can be recognized quite easily and often have identification characteristics (Corner 1947).

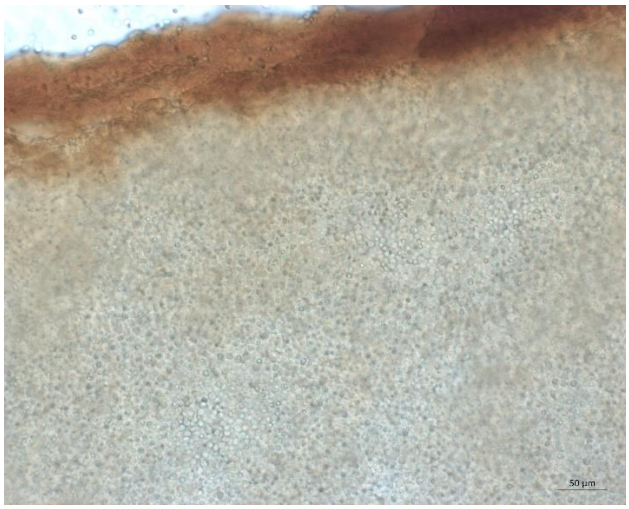


Fig. 8. Overall look of the surface of a *T. rutilans* hymenium part. As it is nothing oddly conspicuous, it indicates absence of cystidia on the surface. Specimen WU-MYC-0042954.

The spores are broadly ellipsoid to subglobose. The measurements were: Mean 7.0 × 5.0, median 6.9 × 5.0, length (6.2-)6.5-7.5(-8.2)., width (4.4-)4.5-5.4(-6.1), quotient (1.2-)1.3-1.6(-1.7) (n=21). The basidia are four-sterigmatic.

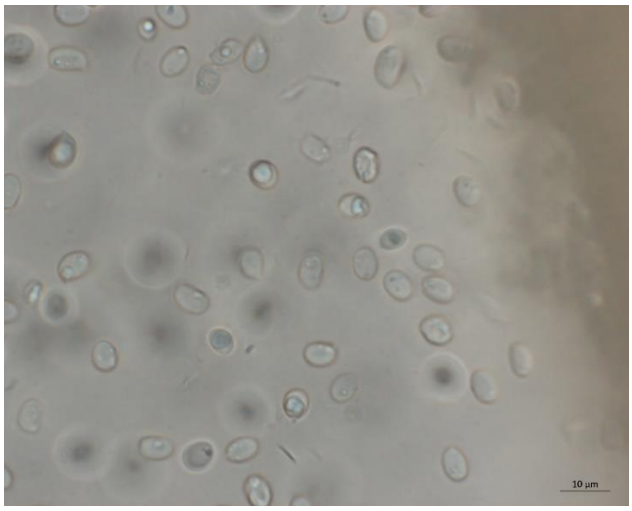


Fig. 9. Spores of *Tricholomopsis rutilans* under light microscope. Specimen WU-MYC-0042954.

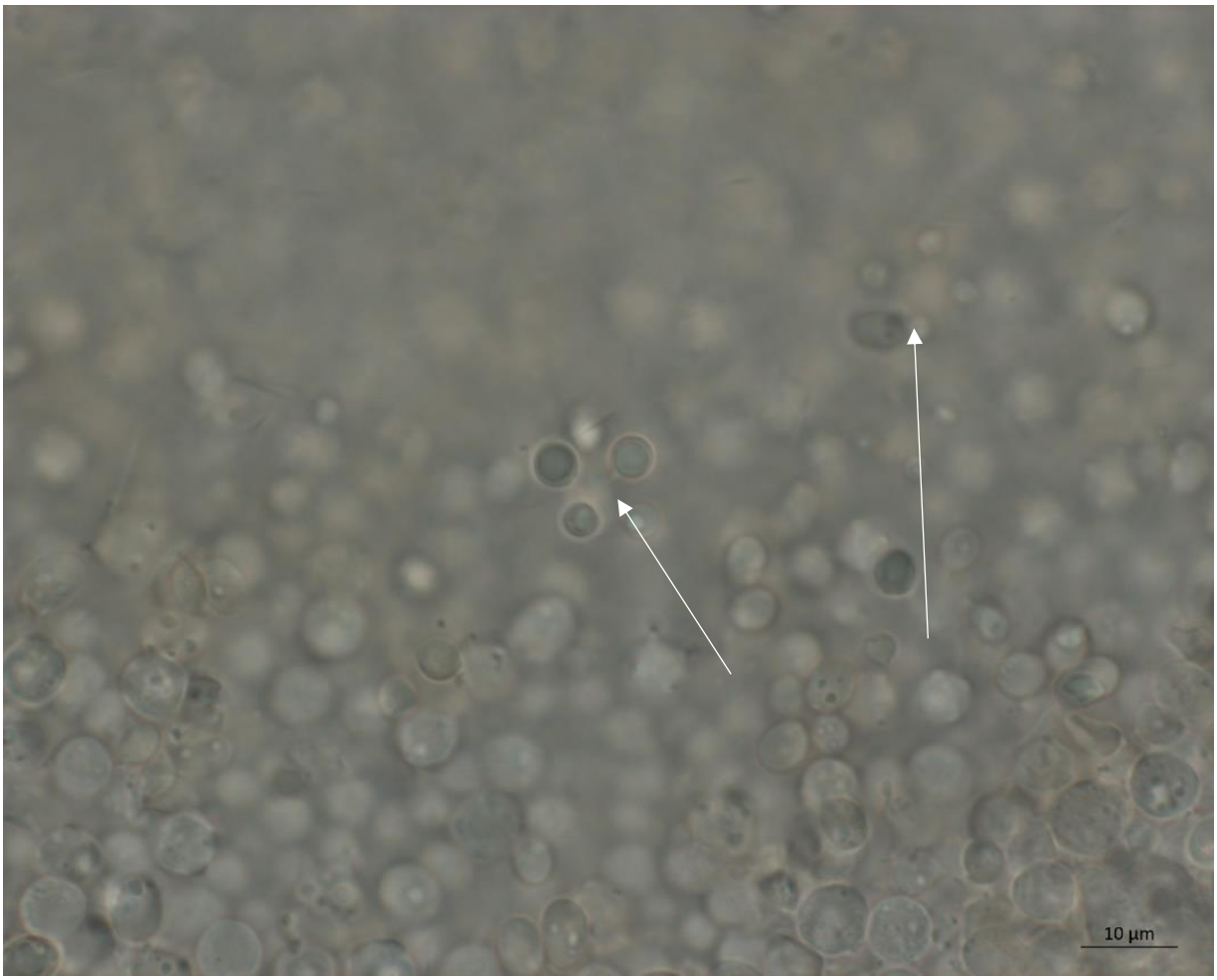


Fig. 10. Basidiospores attached to the basidia, resulting in a significant quadruple pattern in *T. rutilans* (indicated with white arrows). Specimen WU-MYC-0042954.

Tricholomopsis flammula

Observed microscopic characteristics: *Tricholomopsis flammula* has a significant abundance in pleuro- and cheilocystidia, in comparison with *T. rutilans*. Both cystidia types are pale with yellow refractive content. Basidia are 4-sterigmatic.

Spores are ellipsoid rather elongated, but variable in length (5.7-)5.9-6.9(-7.5) and width: (3.7-)4-4.9(-5.3). The quotient is: (1.1-)1.3-1.7(-1.9) (n=23).

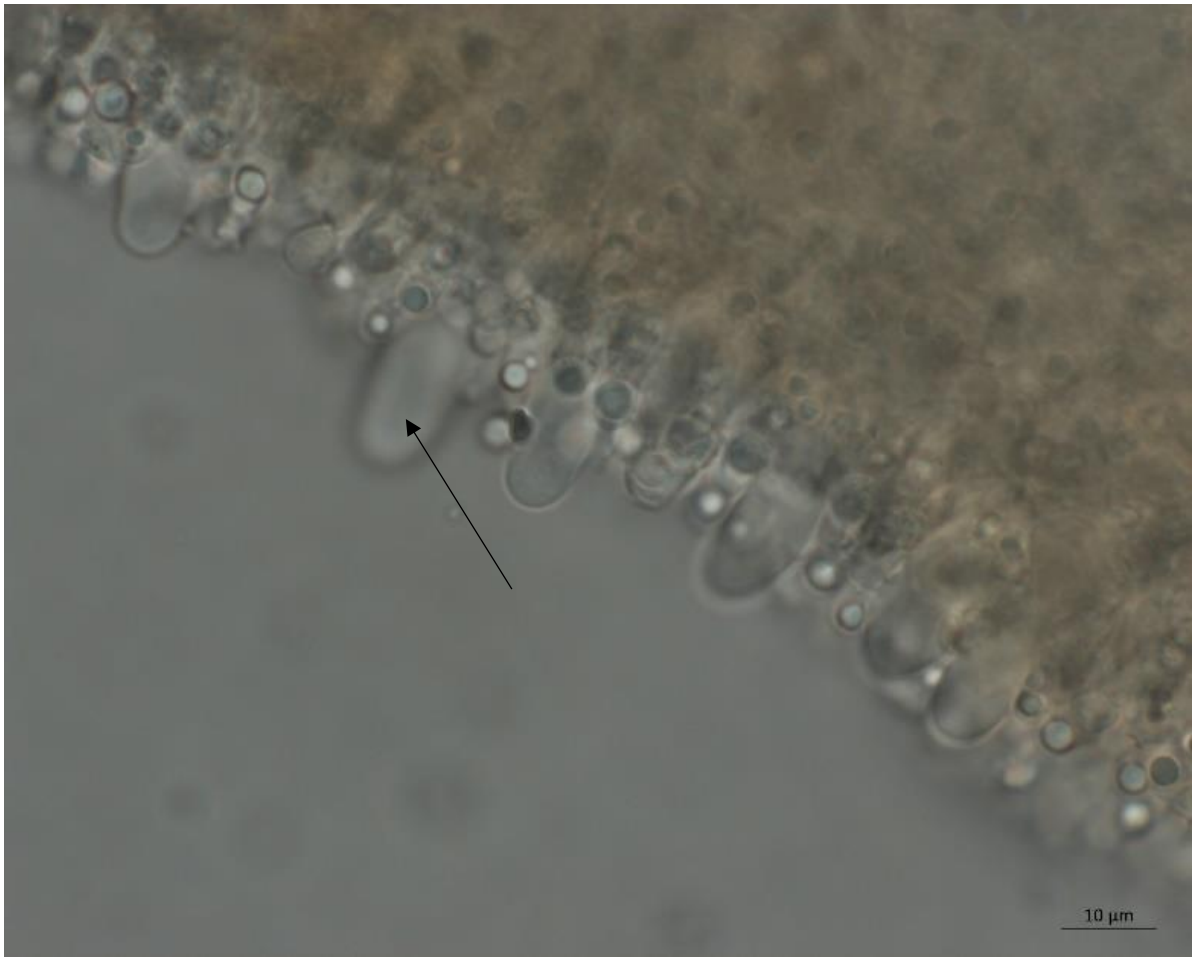


Fig. 11. Pleurocystidia on the side of the lamella of *Tricholomopsis flammula* (black arrow). Specimen KL-9351.

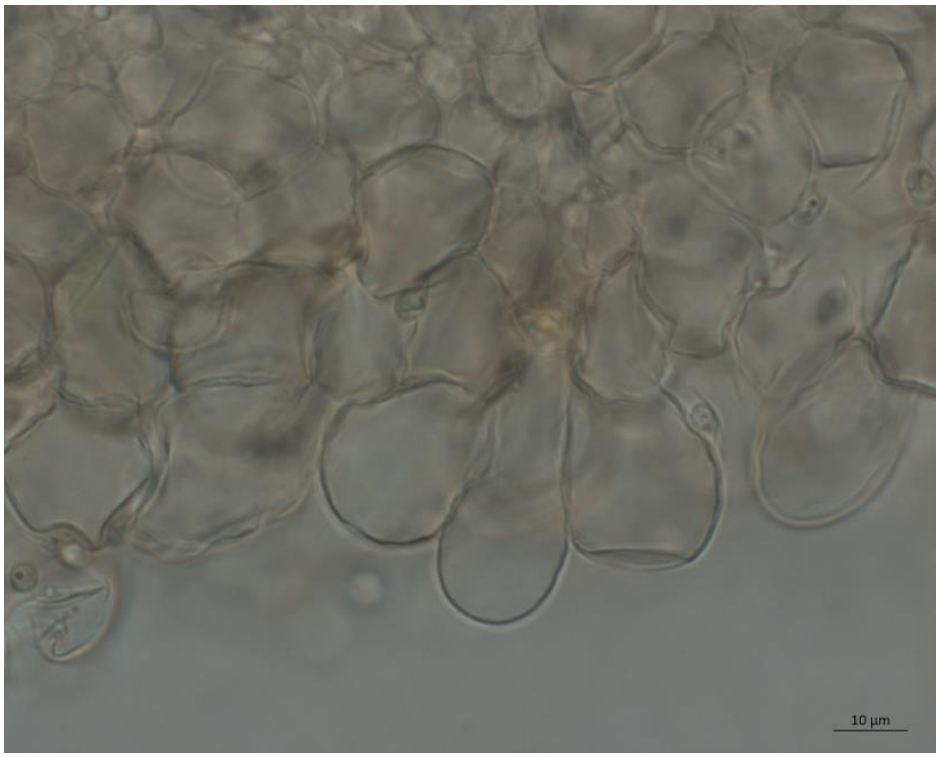


Fig. 12. Cheilocystidia at the edge of the lamella in *Tricholomopsis flammula*. Specimen KL-9351.

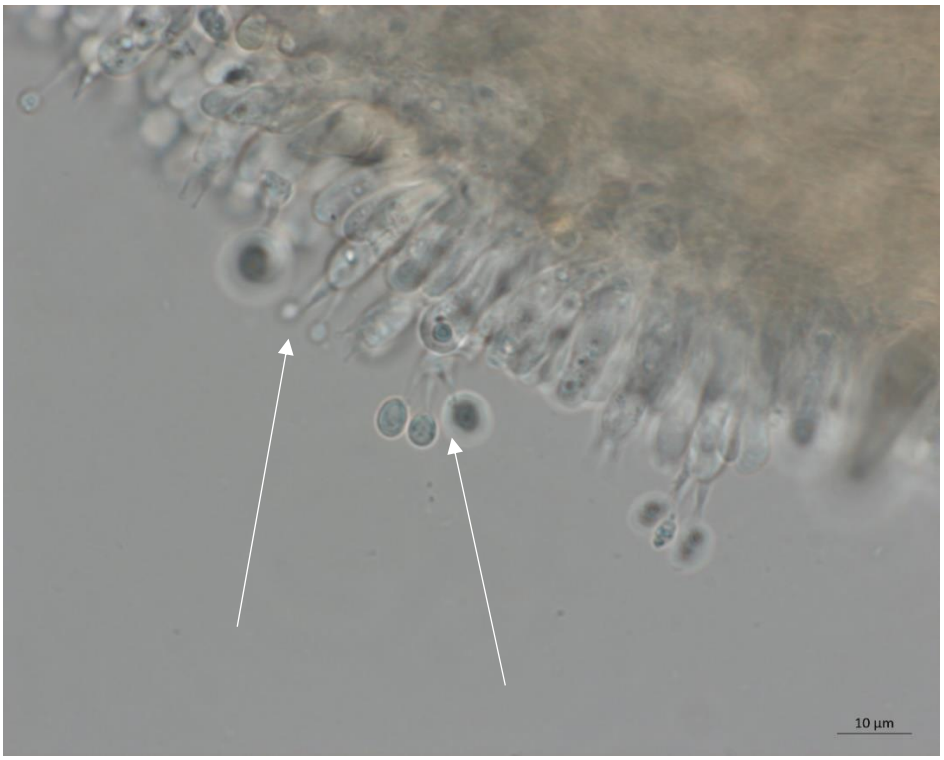


Fig. 13. *Tricholomopsis flammula*. Basidia with the four basidiospores connected with four sterigmata (white arrows). Specimen WU-MYC-0042029.

Tricholomopsis decora

Observed microscopic characteristics: Pleurocystidia are abundant, basidia 4-sterigmatic. The spores are variable in size and shape, broadly ellipsoid to ellipsoid, rarely obovoid or slightly phaseoliform in side view.

Spore length is (5.9-)6.1-7.1(-7.5), width (4.5-)4.6-5.4(-5.9) and quotient (1.1-)1.2-1.4(-1.6) (n=22).

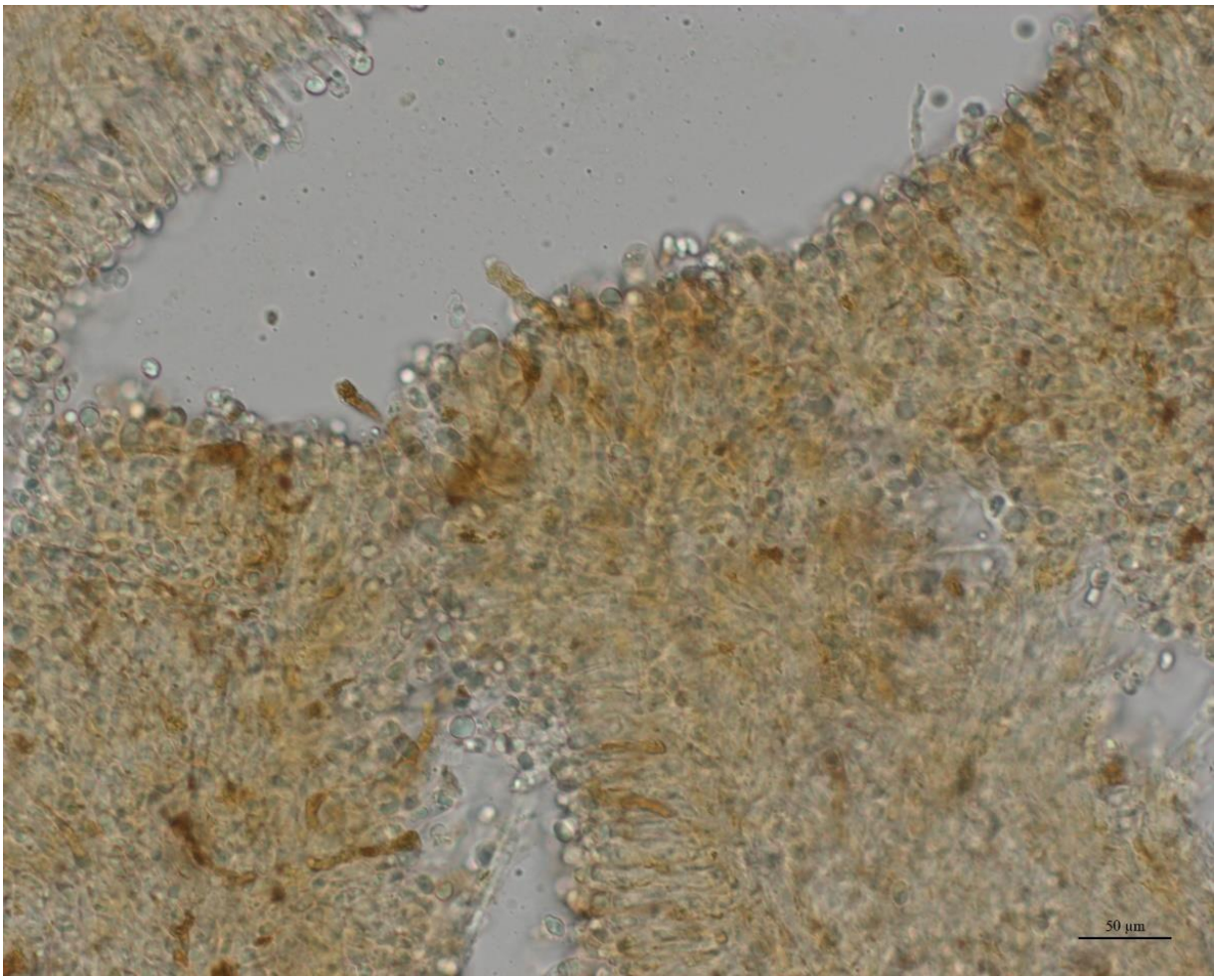


Fig. 14. Pleurocystidia of *Tricholomopsis decora* (dark-brown elongated patches). Specimen KL-50424.

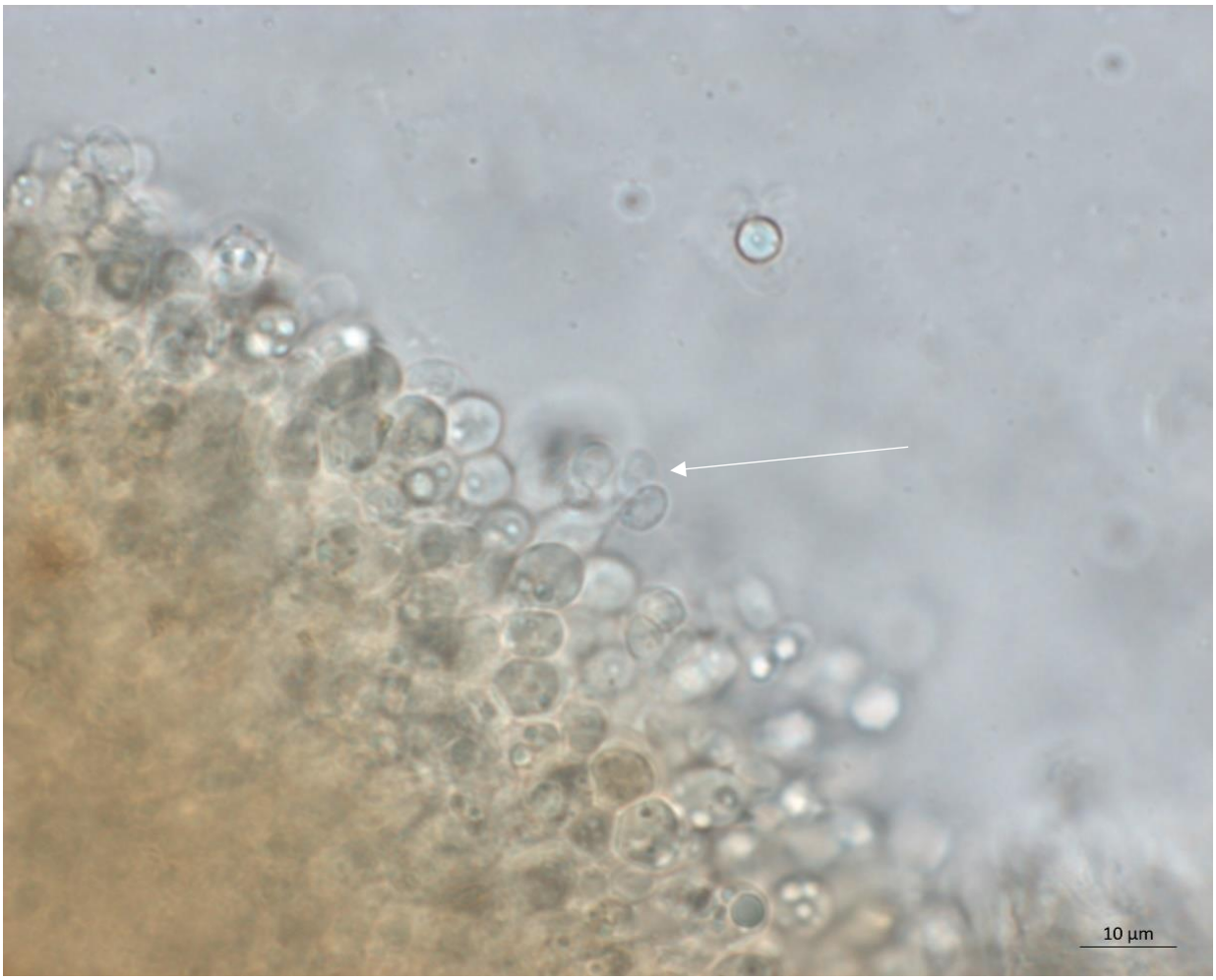


Fig. 15. Basidiospores (white arrow) attached to a basidium with four sterigmata in *T. decora*. Specimen KL-50424.

Phylogenetic Analysis

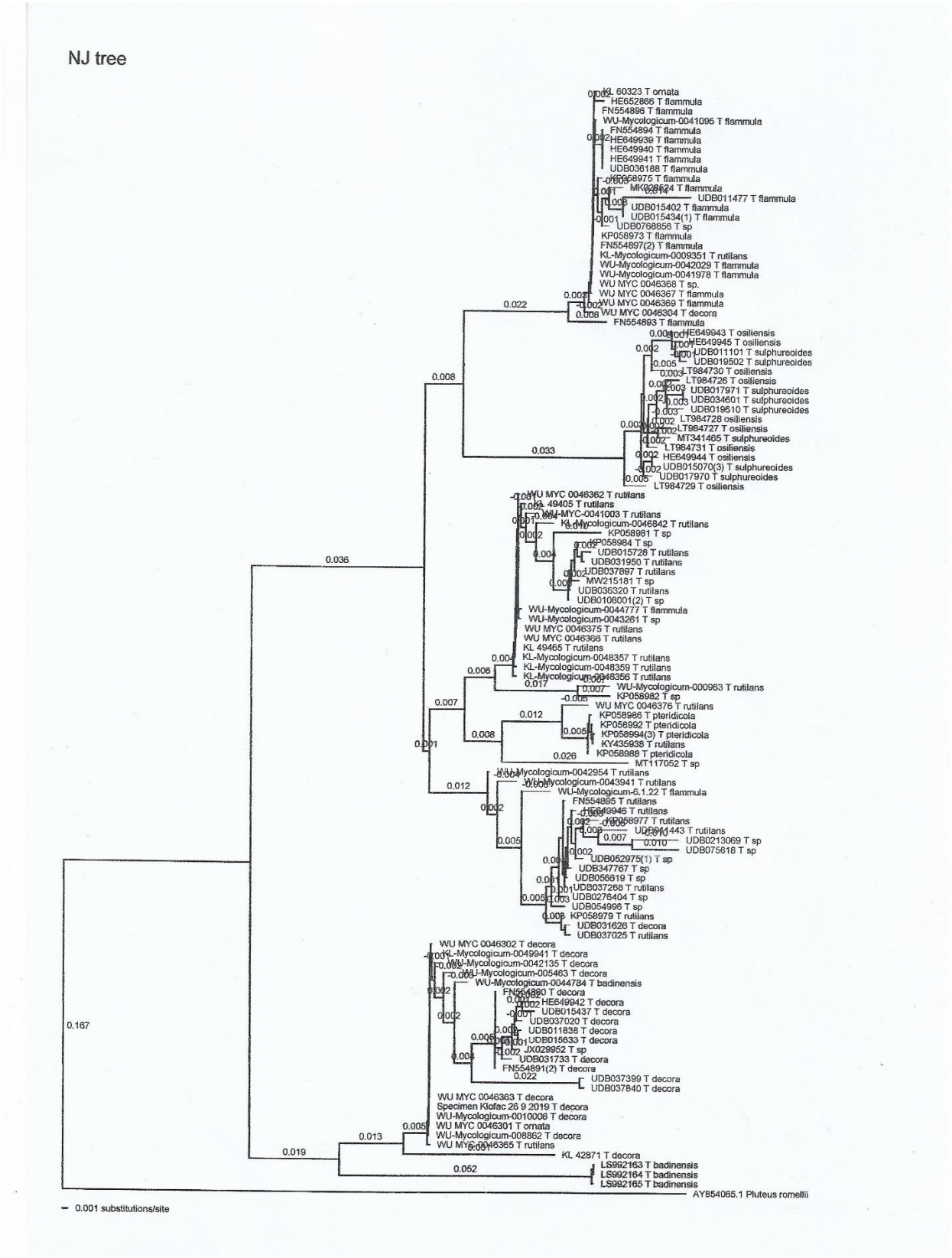


Fig. 16. Neighbor-joining tree generated with ITS and LSU markers, with substitutions per site indicated above branches.

In the Neighbor-joining tree from the LSU and ITS regions with the substitutions per site feature one can observe how far each of the groups previously mentioned is located to each other on the evolutionary level. The higher the number, the further the specimens are separated from each other. In that sense the highest value is between the outgroup species *Pluteus romellii* and the *Tricholomopsis* species (0,167).

Tricholomopsis flammula is forming a cluster with rather small substitutions per site with the *T. osiliensis*-*T. sulfureoides* and a high amount of substitutions per site with the two distinctly forming *T. rutilans* cryptic species.

Tricholomopsis pteridicola tends to interfere with quite small substitutions per site and in fact lies within *T. rutilans*.

Tricholomopsis decora and *T. badinensis* however group up together; a quite high value of substitutions per site (0,052) implies that they are different species and should be handled as it.

Fig. 17. Maximum Parsimony tree obtained from the genetic marker of LSU and ITS with number of character states changes indicated above branches.

The Maximum Parsimony analysis resulted in 8360 best trees (out of 10 000, regarding to the original settings), in which the score for the best tree was 479. Through Heuristic search the found total characters were 1636, from which 1354 characters were constant, 106 (7%) variable characters were parsimony-uninformative and 176 (12%) were parsimony-informative.

As previously mentioned the Maximum Parsimony analysis results in several alternatives of the best possible, shortest phylogenetic trees. For presentation on of that tree has been chosen to show. This phylogenetic tree generated from that analysis shows the possible changes (mutations) in between the species of the study. The higher the value is the further the clusters are from each other on the evolutionary scale. In that case only *T. rutilans, T. decora* and *T. flammula* are the most distant to one another.

Within the branches the previously mentioned features are occurring such as: *T. badinensis* branching from *T. decora*, and *T. sulfureoides* with *T. osiliensis* forming its own group branching from *T. rutilans*.

In comparison with the Neighbor-Joining method, the Maximum Parsimony analysis starts to show some meaningful difference such as *T. sulfureoides* with *T. osiliensis* rather have closer relation with *T. rutilans* than *T. flammula*.

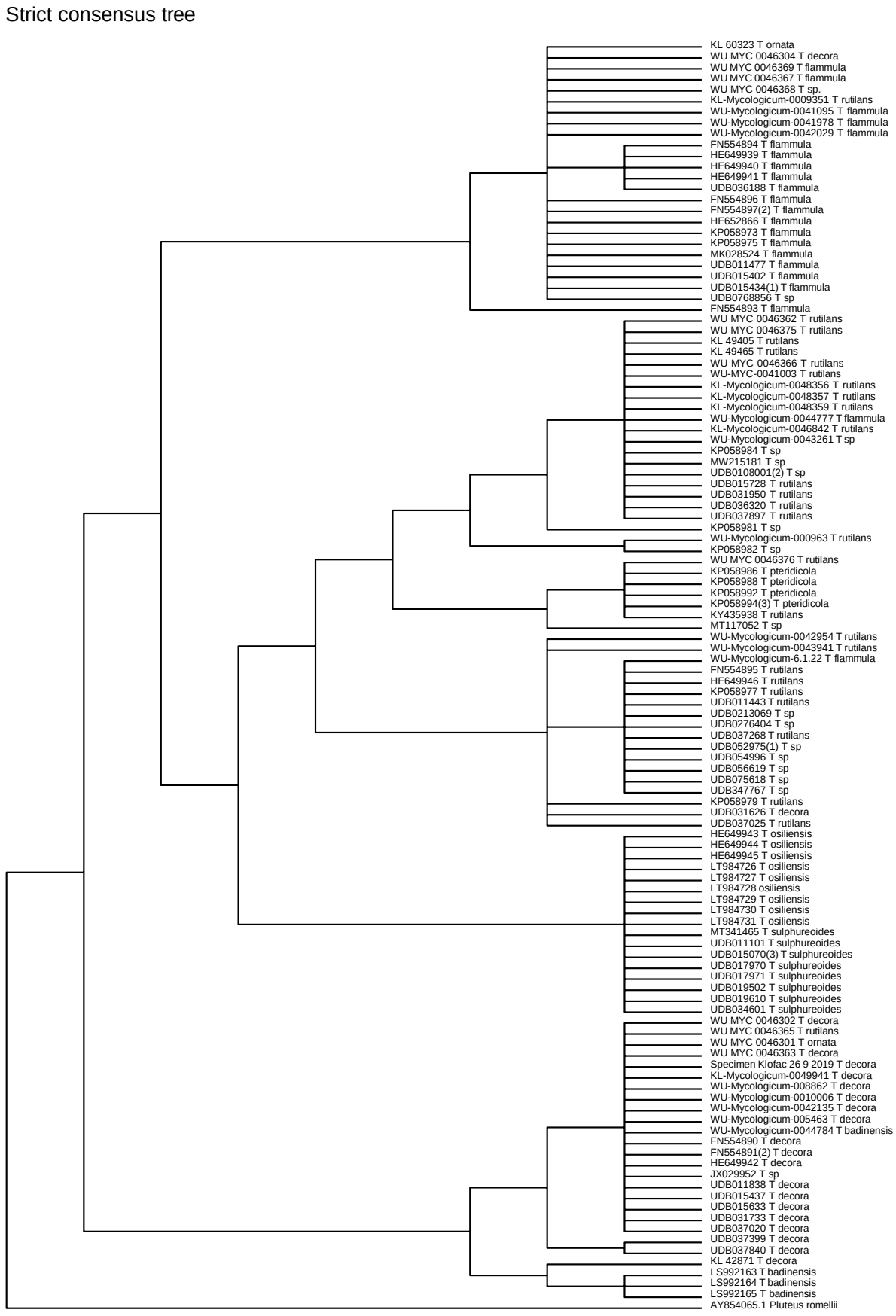


Fig. 18. Strict consensus phylogram of *Tricholomopsis* species from ITS and LSU sequence data.

When analyzing the strict consensus tree, it is quite conspicuous that several clusters of the samples were formed. These clusters are formed by different *Tricholomopsis* species, such as *T. decora, T. rutilans, T. flammula, T. badinensis, T. pteridicola, T. sulfureoides* incl. *T. osiliensis*.

What is worth noting is that there are two distinctly formed branches, each forming *T. rutilans* groups alongside with unidentified *T.* sp. samples.

Tricholomopsis sulfureoides and *T. osiliensis* are located under only one group and they are not branching within themselves. This confirms that these two species from the evolutionary aspect of view are closely related to each other or even the same. *Tricholomopsis osiliensis* was described by Vauras (2009). Vauras et al. (2012) already expressed the suspicion that the two species might be synonymous. Saar & Voitk (2015) studied these species from Newfoundland and Europe and revealed them finally as synonyms.

The group of *T. decora* is budding from the same branch as *T. badinensis*, which would indicate a close relation with the two species.

Finally, the outgroup, *Pluteus romellii*, is rooting the tree. This species is used in several other studies (e.g. Holec & Kolarik 2012, Saar & Voitk 2015) as outgroup and serves this purpose well.

Going through the samples individually there are some incongruences. Several specimens used in this research seem to be misidentified as they are appearing in other groups as they should be.

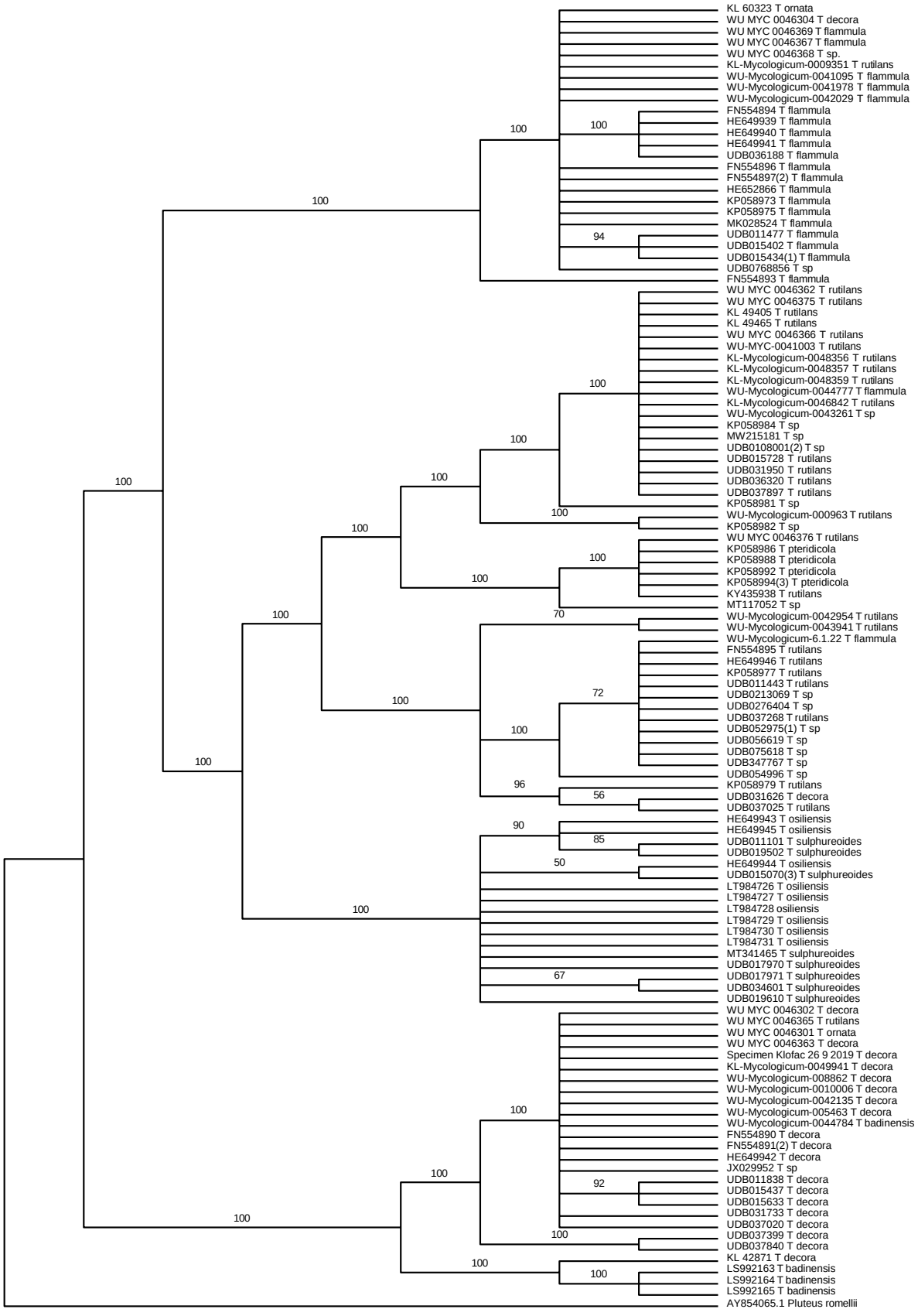


Fig. 19. Majority-rule consensus tree generated from the LSU and ITS regions with ”present branch” values (>50), indicated above branches.

In the majority-rule consensus tree there is yet another confirmation of the separate clades with value over 50. The different groups are again showing up with: two discrete *T. rutilans.* In this case *T. pteridicola* is in-between the two *T. rutilans* groups which proposes relation with the species.

Being synonyms *T. osiliensis* and *T. sulfureoides* are in the same clade.

Tricholomopsis flammula and *T. decora* are in distinct groups as well, but *T. badinensis* seems to show some relation with *T. decora* as it is branching from it with a value of 100, which means that in 100% it is present in all other generated trees as well.

All in all, the same specimens that were mentioned previously as possible misidentifications are also appearing in this scenario, which implies that those samples are indeed misidentified species.

Fig. 20. Bootstrap consensus tree generated from LSU and ITS regions with bootstrap values (>50) indicated above branches.

In the bootstrap consensus tree, one can observe seven distinct clusters that consist of *T. rutilans, T. decora, T. flammula, T. badinensis* from Slovakia and the Czech Republic, *T. osiliensis* and *T. sulfureoides* from the Baltics, these latter two are now considered as one species, another cluster of a probable cryptic *T. rutilans* species (samples with the accession number starting with ”UDB”) and *T. pteridicola*.

Regarding to the results shown in Fig. 20, there are some misidentified species, that should be mentioned. In cluster one, where *T. flammula* forms its own group with a bootstrap value of 100, there are five samples namely: KL-60323 (*T. ornata*), WU-MYC-0046304 (*T. decora*), WU-MYC-0046368 (*T.* sp.), KL-0009351 (*T. rutilans*) and UDB-0768856 (*T.* sp.). Considering the DNA sequence data and from that created tree, these five samples should belong to *T. flammula*.

The next cluster would be *T. rutilans* with a bootstrap value of 82. In this group again, there are six conspicuous sites where mismatches occur. These are: WU-MYC-0044777 (*T. flammula*), WU-MYC-0043261 (*T.* sp.), UDB0108001 (2), KP058984, KP058981 and KP058982 (all *T.* sp.). In this sense, the above-mentioned specimens should be *T. rutilans*.

Another group is from that of the *T. pteridicola*, which is associated with a fern during its life cycle (Olariaga et al 2015). The bootstrap support value is 70 at the first branching where the group forms, but in-between the species this value is always higher than 90. In that sense WU-MYC-0046376 (*T. rutilans*), KY435938 (*T. rutilans*) and MT117052 (*T.* sp.) could actually be *T. pteridicola* specimens. Since ferns are certainly not involved as substrate in all of these collections, the question arises whether the substrate spectrum of the species is actually larger, which Hahn (pers. comm.) also suspects.

Then another *T. rutilans* cluster which would be the cryptic specimens. In this group there are eight samples regarding to molecular genetic data. These would be the *T.* sp. ones (UDB0213069, UDB0276404, UDB054996, UDB056619, UDB075618, UDB347767 and UDB052975(1)), there is one from the recent study namely WU-Mycologicum-6.1.22 which was previously thought to be *T. flammula* and finally UDB031626 which was identified as *T. decora*. In this sense the above-mentioned specimens should belong to this cryptic *T. rutilans* agg. species.

Next cluster would be the *T. osiliensis* and *T. sulfureoides* one with 100 bootstrap value, all presenting itself in the same group regardless of the different nomenclature. As mentioned above, meanwhile it was already shown that these belong to only one species, which has to be called *T. sulfureoides.*

An interesting phenomenon starts at the last two clades as *T. badinensis* and *T. decora* seem to sit on the same branch, while it also disassembles to several smaller branches. The bootstrap value starts with a quite high number (98), but sometimes there is low support (52). For sure there is a decent group for *T. decora* where there are already two misidentifications, such as WU MYC 0046365 *T. rutilans* and WU MYC 0046301 *T. ornata*, the latter being a nomen dubium. A sample from the fungarium WU-Mycologicum-0044784 *T. badinensis* is possibly just a *T. decora* since it sits quite close to the distinct group with a support value of 100. JX029952 *T.* sp. should be definitely considered as *T. decora* as well.

Finally, the *T. badinensis* group looks a bit the same situation as previously have been shown at *T. osiliensis* and *T. sulfureoides*, because the samples are sitting on the same branch as *T. decora* and have a high support (100).

Conclusions

It is clearly visible that there are three main species clusters, namely *Tricholomopsis rutilans, T. flammula* and *T. decora*.

Olariaga et al. (2015) and others already discussed the cryptic species nature of the *T. rutilans* alliance and showed it to be a species complex consisting of about five lineages. They described one of these lineages as *T. pteridicola* and settled the species concept of *T. rutilans* ss. str. by chosing an epitype. Other lineages were not formally described because further morphological and genetical studies, such as the present thesis, are needed.

Also, there is an ongoing debate of *Tricholomopsis ornata* (KL-60323) because it is a poorly described species (no records agreeing with the original description by Fries 1838), which for the time being is considered as nomen dubium.

Tricholomopsis flammula, T. decora and *T. rutilans* whereas are considered as well-described species (complexes) regarding to the studies of Holec & Kolarik (2012) and they also appear in the presented phylogenetic tress as well distinguishable clusters.

Tricholomopsis sulfureoides and *T. osiliensis* although not really in focus in this study, conspicuously form in every generated phylogenetic models their own group with no specific branching, or substitutions per site within them. This implies that these two species are actually synonymous, as was elaborated in detail by Saar & Voitk (2015) and confirmed by Holec et al. (2019).

Tricholomopsis badinensis indeed forms its own group within *T. decora* as it appears in the study of Holec et al. (2019). The morphological characteristics are different from the original three species and it has until now a limited very local distribution in the virgin forest of Badínsky Prales.

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Appendix

DNeasy Plant Minikit Protocol (www.qiagen.com/resources/download). Amounts given in brackets and bold are the modifications of the original protocol used.

Perform all centrifugation steps at room temperature (15–25 °C). If necessary, re-dissolve any precipitates in Buffer AP1 and Buffer AW1 concentrates. Add ethanol to Buffer AW1 and Buffer AW2 concentrates. Preheat a water bath or heating block to 65 °C.

1. Disrupt samples (≤100 mg wet weight or ≤20 mg lyophilized tissue) using the TissueRuptor®, the TissueLyser II or a mortar and pestle.
2. Add 400 **[600]** µl Buffer AP1 and 4 µl RNase A. Vortex and incubate for 10 **[15]** min at 65 °C. Invert the tube 2-3 **[4-5]** times during incubation. After the process spin down the tubes for several seconds to collect all liquid at the bottom.

Note: Do not mix Buffer AP1 and RNase A before use.

3. Add 130 **[195]** µl Buffer P3 **[and 400 µl Chloroform/IA Mix]**. Mix and incubate for 5 min on ice.
4. Recommended: Centrifuge the lysate for 5 min at 20,000 × g (14,000 rpm).
5. Pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuge for 2 min at 20,000 × g.
6. Transfer the flow-through into a new tube without disturbing the pellet if present. Add 1.5 volumes of Buffer AW1, and mix by pipetting.
7. Transfer 650 µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at ≥6000 × g (≥8000 rpm). Discard the flowthrough. Repeat this step with the remaining sample.
8. Place the spin column into a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 1 min at ≥6000 × g. Discard the flow-through.
9. **[Add 500 µl of Ethanol (100%) and centrifuge for 1 min at 6000 × g. Discard the flow-through].**

Note: Make sure that the membrane is colorless, otherwise repeat this step.

Remove the spin column from the collection tube carefully so that the column does not come into contact with the flow-through. Repeat this step with the remaining sample.

10.Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
11. Add 100 **[70]** µl Buffer AE for elution. Incubate for 5 **[10]** min at room temperature (15–25 °C). Centrifuge for 1 min at ≥6000 × g.

12. Repeat step 11 with **70 µl Buffer AE to have a total amount of 140 µl DNA extract.**

HS 2x CTAB DNA Extraction recipe – Quick Start

1. Disrupt 20-40 (max. 50) mg sample in 2 ml tube with 20 mg (2%) PVP-40.
2. Add 1 ml HS CTAB 2x
   1/10 vol. (10 µl) RNAse A (100mg/ml)
   1/10 vol (10 µl) Proteinase K (20 mg/ml)
   2 µl (0,2%) ß-mercaptoethanol
3. Incubate at least 1 hour in 65 °C.
4. Add 700 µl chloroform:isoamyl alcohol (SEVAG, CIA), then vortex it well. Then incubate on ice for 8 min.
5. Centrifuge for 15 min at 11000 × g.
6. Get supernatant (e.g. 800 µl) to a new 2 ml tube, and incubate it on ice for 5 min
7. Add 1/10 vol. (80 µl) sodium-acetate (3M, Ph 5.2)
8. Add 2/3 vol. (for e.g. 600 µl) ice-cold (-20°C) isopropanol.
9. Incubate at least overnight, maximum 1 week (for problematic samples) at -20 °C.
10. Centrifuge at 4°C for 15 min at maximum speed, then discard the supernatant.
11. Add 500 µl ethanol 80%. Detach pellet.
12. Centrifuge 5 min at maximum speed, then discard the supernatant.
13. Repeat step 11. and step 12.
14. Add 500 µl ethanol 100%. Detach pellet.
15. Centrifuge 5 min at maximum speed. Discard supernatant. Air-dry pellet.
16. Add 100-200 µl 1x (0.5× or 0.1×) TE or buffer AE (elution buffer) (45 °C). Mix.

Column Cleaning

1. Add 500 µl buffer PB (5× DNA volume). Mix, then load in column.
2. Centrifuge it 2 min at 13000 rpm. Discard flow-through.
3. Add 750 µl PE or NT3 (washing buffer)
4. Centrifuge it 2 min at 13000 rpm. Discard flow-through.
5. Centrifuge it 2 min at 13000 rpm and dry column. Then place it into a 1.5 ml collection tube.
6. Add 50 µl Milliq (65 °C). And incubate it for 20 min.
7. Centrifuge for 1 min at 13000 rpm. Keep flow-through.
8. Repeat Step 6 and 7. Extracted DNA volume should be 100 µl.

Plant Genomic DNA Purification Main Protocol

1. Prepare 10-25 mg dry tissue in a 2 ml microcentrifuge tube and add 5 glass beads.
2. Disrupt samples using a grinding mill (Tissuelyser II) at a speed of 30 cycles per second for 3 min (pre-cooled samples up to 6 min).
3. Spin down the samples for several seconds to collect all powder at the bottom of the tube.
4. Add 350 µl Lysis Buffer A and 40 µl PVP, then vortex it for 10-20 sec to mix thoroughly. Add 50 µl Lysis Buffer B and 20 µl RNase A separately, plus add 4 µl of Proteinase K. Vortex and incubate for 30 min at 65 °C. Invert or vortex the tube 4-5 times during incubation. (Do not mix Lysis Buffer B and RNase A before use)
5. Add 130 µl of Precipitation Solution and mix by inverting the tube 2-3 times. Add 100 µl Chloroform/IA Mix.
6. Incubate on ice for 10 min.
7. Centrifuge the lysate for 5 min at maximum speed (14000 rpm) at 4 °C.
8. Collect the supernatant (usually 450-550 µl) and transfer to a clean microcentrifuge tube. Add 400 µl of Plant gDNA Binding Solution and 400 µl of 96% ethanol and mix well with vortexing it).
9. Transfer half of the prepared mixture (600-700 µl) to the spin column. Centrifuge for 1 min at 6000 × g. Discard the flow-through. Repeat this step to the remaining sample.
10. Add 500 µl of Wash Buffer I to the column (ensure ethanol has been added to Wash Buffer I) Centrifuge for 1 min at 8000 × g. Discard the flow-through and place the column back into the collection tube.
11. Add 500 µl of Wash Buffer II to the column (ensure ethanol has been added to Wash Buffer II). Centrifuge for 3 min at maximum speed 20000 × g. Empty the collection tube.
12. Place the purification column back into the tube and re-spin the column for 1 min at maximum speed 20000 × g. Membrane drying step.
13. Discard the collection tube containing the flow-through solution and transfer the column to a sterile 1.5 ml microcentrifuge tube.
14. To elute genomic DNA add 50 µl of Elution Buffer to the center of the column membrane, incubate for 5 min at room temperature and centrifuge for 1 min at 8000 × g.
15. Repeat Step 14 to have a total of 100 µl DNA extract.

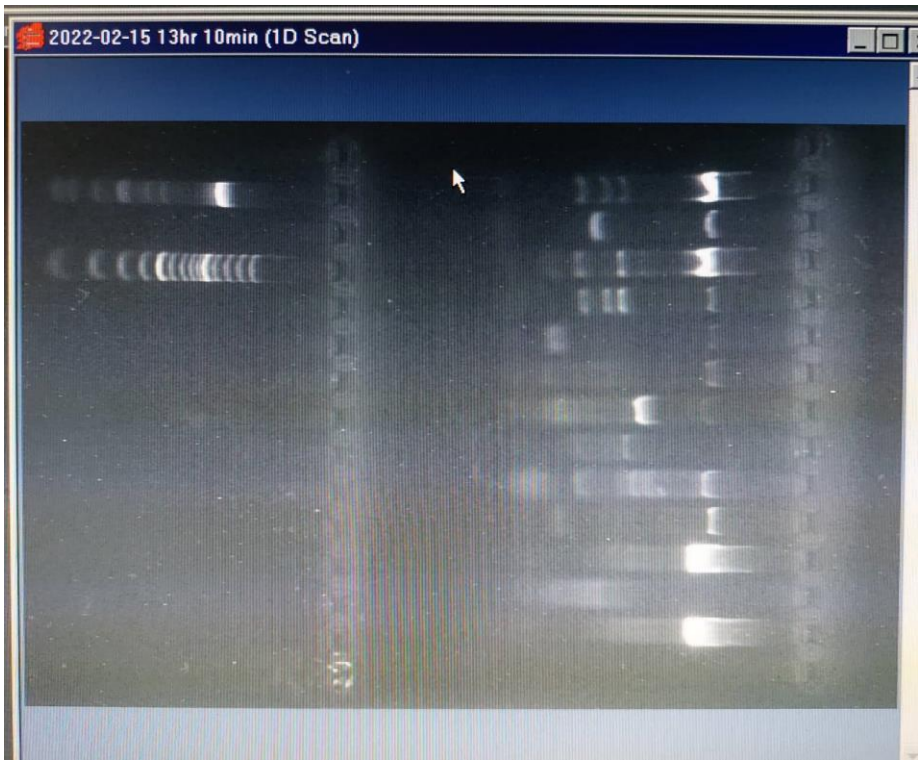

Fig. 21. Results after a successful PCR. The samples are from 26-57 (2022) starting from the upper right corner downwards, then the left side starting from above. The last pattern is a DNA ladder for comparison.

Fig. 22. Results after a second successful PCR where the primers LR5 and LR0R have been used. The samples are from 26-57 (2022) starting from the upper right corner downwards, then the left side starting from above. The last pattern is a DNA ladder for comparison.

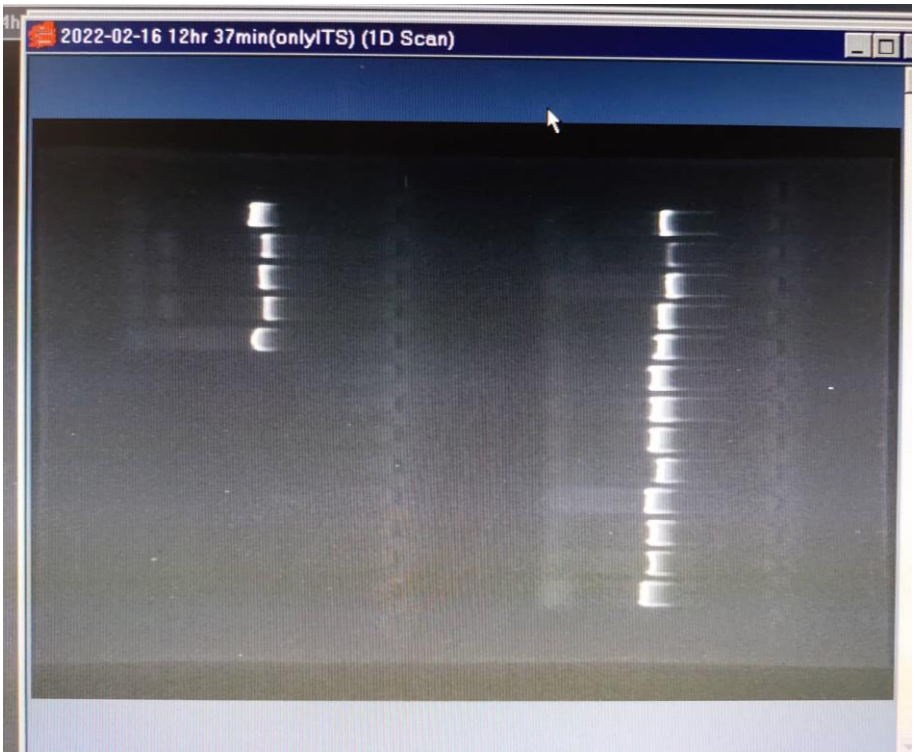

Fig. 23. Results after a second successful PCR where the primers ITS5 and ITS4 have been used. The samples are from 26-57 (2022) starting from the upper right corner downwards, then the left side starting from above.

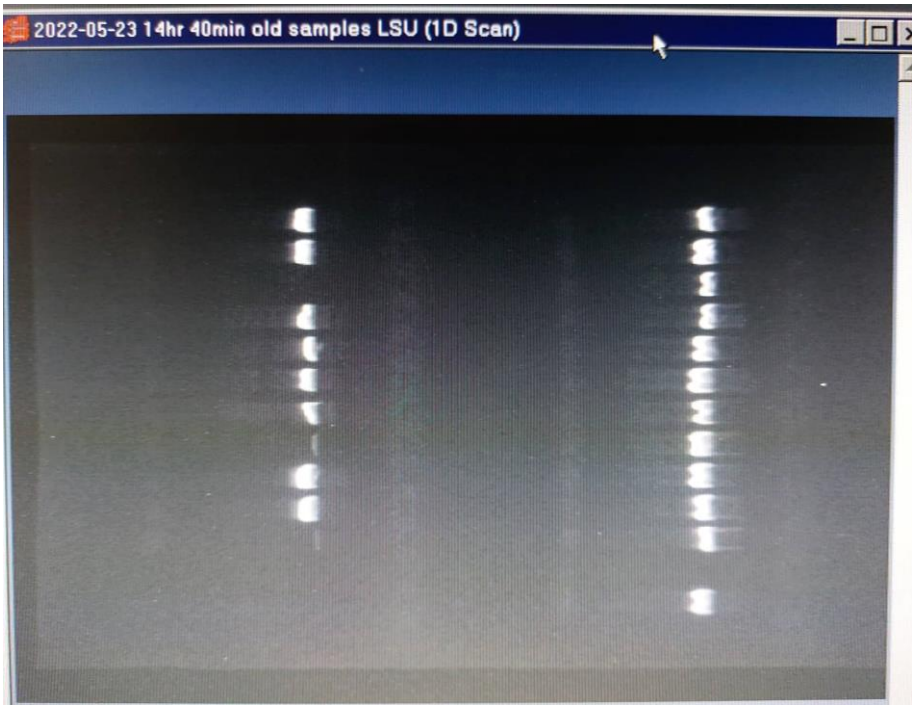

Fig. 24. Results after a successful PCR where the primers LR5 and LR0R have been used. The samples are from 1-25 (2020) starting from the upper right corner downwards, then the left side starting from above.