Molecular evaluation of species delimitation and barcoding of *Daedaleopsis confragosa* specimens in Austria

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Abstract: Herbarium material of *Daedaleopsis confragosa*, *D. tricolor* and *D. nitida* collected in different regions of Austria, Hungary, Italy and France was molecularly analysed. Species boundaries were tested by sequencing the fungal barcoding region ITS rDNA. The results confirm that *Daedaleopsis confragosa* and *D. tricolor* cannot be separated on species level when using ITS data. The same conclusion has already been drawn for Czech specimens by KOUKOL & al. 2014 (Cech Mycol. 66: 107–119) with a multigene analysis.

Zusammenfassung: Herbarmaterial von *Daedaleopsis confragosa, D. tricolor* und *D. nitida* aus verschiedenen Regionen in Österreich, aus Ungarn, Italien und Frankreich wurde molekular analysiert. Die Artabgrenzung wurde durch Sequenzierung der pilzlichen Barcoderegion ITS rDNA getestet. Die Ergebnisse bestätigen, dass *Daedaleopsis confragosa* und *D. tricolor* unter Verwendung der ITS-Daten nicht auf Artniveau getrennt werden können. Diese Schlussfolgerung wurde bereits für tschechische Aufsammlungen anhand einer Multigen-Analyse durch KOUKOL & al. 2014 (Cech Mycol. 66: 107–119) vorgestellt.

The genus *Daedaleopsis* belongs to the family *Polyporaceae*. The species are lignicolous and found on numerous genera of hardwoods. They produce white rot and are widely distributed in Europe and Asia. In total the genus comprises approx. nine species, most of them being Asian taxa, only three of them occur in Europe. Two of them, *D. confragosa* (BOLTON) J. SCHRÖT. and *D. tricolor* (PERS.: FR.) BONDARTSEV & SINGER, are rather frequent and widespread in Central Europe. They were treated as separate species by some authors, e.g. BERNICCHIA (2005), RYVARDEN & MELO (2014), but others, e.g. JÜLICH (1984), consider the latter as a variety of *D. confragosa*, namely as *D. confragosa* var. *tricolor* (BULL.) BONDARTSEV & SINGER, due to the highly variable hymenophore and presence of intermediate forms. The apparent morphological variability and the absence of any differences in microscopical characters make it a challenge to define them clearly as separate species.

Nowadays molecular characteristics are a useful tool for solving disparities in biological classification, e.g. barcoding uses a short species-specific sequence from a standard part of the genome to identify organisms (http://www.ibol.org/about-us/what-

is-dna-barcoding/). The gene region that has been proposed and accepted for fungi as the primary standard barcode is the ITS region (BEGEROW & al. 2010, SCHOCH & al. 2012), although it has also been acknowledged that it does not always resolve species boundaries in some lineages.

Tab. 1. List of species sequenced in the present study with origin, herbarium and GenBank accession numbers.

Taxon	Origin	WU Herbarium no.	ITS GenBank Accession number
Daedaleopsis confragosa	Austria, Vienna, Hietzing, Lainzer Tiergarten, Dorotheerwald	8934	KR108000
D. confragosa	Austria, Lower Austria, Maissau, Grünhof-Fischteiche	27979	KR108001
D. confragosa	Austria, Lower Austria, Gänserndorf, Orth an der Donau	36548	KR108002
D. confragosa	Austria, Lower Austria, Schwarzau im Gebirge, Höllental	36547	KR108003
D. nitida	France, Ardeche, Bourg St. Andréol	16826	KR108007
D. nitida	Italy, Perugia, Spoleto, Monte Luco	29685	KR108008
D. nitida	Italy, Foggia, Foresta Umbra, Monte Nicola	31712	KR108009
D. tricolor	Austria, Lower Austria, Klausen-Leopoldsdorf, Mitterschöpfl	10311	KR108004
D. tricolor	Austria, Lower Austria, Maissau, Grünhof-Fischteiche	19193	KR108005
D. tricolor	Hungary, Nyíregyháza, Bátorliget, Fényi-erdö	26903	KR108006

The focus of the present paper was to barcode *D. confragosa* and to re-evaluate the species boundary between the two closely related taxa, *D. tricolor* and *D. confragosa,* by molecular analysis of nuclear ribosomal internal transcribed spacer (ITS) sequences from Austrian specimens, and to compare them with other Central European specimens and a species of the closely related genus *Hexagonia* s.l., represented by *Hexagonia nitida* DURIEU & MONT., which has already been included in the genus *Daedaleopsis*, as *D. nitida* (DURIEU & MONT.) ZMITR. & MALYSHEVA by ZMITROVICH & MALYSHEVA (2013).

Until recently, morphological features were used to distinguish the genera *Daedaleopsis* with poroid to lamellate hymenophore, and *Hexagonia*, with the typical hexagonal to angular poroid hymenium. One of the first studies (KO & JUNG 1999 a) dealing with phylogenetic relationships of the Trametoid group, already revealed the problem that these features may be unsuitable for generic distinction. In a phylogenetic analysis of trametoid fungi based on ITS and nLSU rDNA (ZMITROVICH & MALYSHEVA 2013) some species of *Hexagonia* and *Daedaleopsis* were placed in the same clade, whereas the generic type of *Hexagonia* was in a separate clade in the LSU analysis; however, without significant support. In WELTI & al. (2012) *Daedaleopsis* is nested within *Hexagonia* but the generic type, *H. hirta*, was not included in that analysis.

In the recent study by KOUKOL & al. (2014) the *D. confragosa–D. tricolor* species pair was analysed based on molecular data. They concluded that separation as distinct species cannot be maintained.

Material and methods

Material

Three specimens of *Daedaleopsis confragosa*, four of *D. tricolor* and three of *D. nitida* were obtained from the herbarium WU of the University of Vienna, collected in several regions around central Europe (Tab. 1).

The specimen of *D. confragosa* WU 36548 (see Tab. 1) shows intermediate features with a lamellate hymenophore near the pileus margin and poroid hymenophore on the oldest and central area, while the pileus surface is typical whitish to brownish and concentrically zoned.

DNA isolation, amplification and sequencing

DNA was extracted from hymenium of ten dried specimens, previously grinded under liquid nitrogen treatment. Extraction of DNA was done with the DNeasy Plant Mini Kit following the manufacturer's protocol.

PCR amplification of nuclear ribosomal internal transcribed spacer (ITS) region was performed with the primer pair ITS4 and ITS5 (WHITE & al. 1990) from 20 ng DNA in 10 µl PCR reagent. The region sequenced was approximately 600 bp long. Annealing temperatures and extension times were 50 °C for 20 sec and for amplification 72 °C and 50 sec, respectively. DNA was cycle-sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems, Warrington, UK) and the PCR primers. Sequencing was performed on an automated DNA sequencer (ABI 3730xl Genetic Analyzer, Applied Biosystems).

Tab. 2. ITS sequences from previous studies used for phylogenetic analyses in combination with those generated in the present research. Country of origin, Strain and GenBank accession numbers are given.

Species	Origin	Strain	GenBank Accession number
Daedaleopsis confragosa	Czech Republic	NK370	HG973497
D. confragosa	Czech Republic	NK3703	HG973500
D. confragosa	Latvia	M112	JF340288
D. confragosa	France	BRFM 1130	JX082372
D. confragosa	France	BRFM 1131	JX082373
D. confragosa	France	BRFM 1143	JX082375
D. confragosa	France	BRFM 1145	JX082376
D. confragosa	France	BRFM 947	FJ349623
D. confragosa	France	BRFM 948	GU731549
D. confragosa	USA	X-49	KC176338
D. confragosa	USA	X-78	KC176348
D. nitida	France	BRFM 1327	JN645082
D. septentrionalis	Finland	-	HG973499
D. tricolor	Czech Republic	NK374	HG973502
D. tricolor	Czech Republic	NK375	HG973495
D. tricolor	Czech Republic	NK376	HG973496
D. tricolor	France	BRFM 954	GU731548
D. tricolor	France	-	JN645096
Earliella scabrosa	Venezuela	-	JN165008
E. scabrosa	USA		JN165009
Polyporus arcularius	Germany	SBUG-M1244	AB070861
P. arcularius	Austria	TENN58370	AB070865

All the nucleotide sequences were deposited in GenBank under the accession numbers given in Tab. 1. Sequence data were edited and assembled in DNASTAR Lasergene SeqMan v. 7.1 (http://www. dnastar.com/).

Sequence alignment and phylogenetic analyses

Ten ITS sequences were newly generated and 22 additional ITS sequences were retrieved from GenBank (see Tab. 2). Two GenBank sequences of *Polyporus arcularius* were included as outgroup to root the trees, as it is phylogenetically closely related but distant enough not to belong to the ingroup. A total of 32 sequences was aligned and manually refined and minimized using the BioEdit v7.2.5 (HALL 1999) sequence alignment editor. Sequences were exported to nexus and phylip formats using Mesquite 3.04 (MADDISON & MADDISON 2015) for subsequent phylogenetic analyses.

The resulting sequence matrix was subjected to Maximum Likehood (ML) and Maximum Parsimony (MP) analyses. Maximum Likehood analysis was performed on the RAxML (STAMATAKIS 2006) as implemented in raxmlGUI 1.3 (SILVESTRO & MICHALAK 2012), using the ML + rapid bootstrap setting and the GTRI substitution model with 100 bootstrap replicates.

Maximum Parsimony bootstrap analysis was performed using PAUP 4.0a146 (SWOFFORD 2002). All molecular characters were unordered and given equal weight; analyses were performed with gaps treated as missing data; the COLLAPSE command was set to MinBrlen. 500 MP bootstrap replicates were performed, with 100 rounds of random sequence addition and subsequent TBR branch swapping (MULTREES option and steepest descent option not in effect) with each replicate limited to 1 million rearrangements.

Fig. 1. *Daedaleopsis tricolor*, basidioma with intermediate hymenophore, on *Fraxinus*, 2011-07-26 Wien: Hameau, phot. T. BARDORF.

Fig 2. Phylogram showing the best ML tree (-ln = -1458.344) revealed by RAXML from an analysis of the nuclear ITS rDNA matrix of *Daedaleopsis*, *Daedaleopsis (Hexagonia) nitida* and *Earliella scabrosa*. *Polyporus arcularius* was used to root the trees. Numbers above branches indicate Maximum Likelihood (MLBS) and Maximum Parsimony bootstrap (MLBS) support.

Results and discussion

Morphology and ecology

Daedaleopsis confragosa and *D. tricolor* have overlapping habitats, growing on several substrates, such as *Prunus avium*, *Fagus sylvatica, Alnus glutinosa, Quercus* sp. and many other deciduous tree species. *Daedaleopsis confragosa* is described as a rather variable species with mostly pale brownish zoned pileus surface and fluctuating shape and size of the pores. In contrast, dark red brown pileus surface and a lamellate hymenophore are found in *D. tricolor*. In addition, often intermediate forms exhibiting poroid and lamellate hymenophore in one and the same basidioma occur (Fig. 1). Thus macroscopic features show only minor morphological differences; microscopically both *Daedaleopsis* taxa have a trimitic hyphal system and white hyaline, smooth, cylindrical to ellipsoid spores. The spore size is variable and almost identical; *D. confragosa* has the dimension of 8–11 × 2–3 µm and *D. tricolor* 7–11 × 2–3 µm (see also KOUKOL & al. 2014), apparently also no new evidence to distinguish the taxa.

Molecular phylogeny

The quantity of DNA isolated and of ITS PCR products was often low, due to the age of herbarium specimens processed, but still usable for sequencing. ITS barcoding sequences were produced for ten specimens. The length of the alignment for the altogether 32 sequences was 580 sites with 100 parsimony informative characters. The ML tree computed by RAxML is presented in Fig. 2 with ML and MP bootstrap values given on the branches.

ML and MP bootstrap analyses group *Daedaleopsis confragosa* and the specimens morphologically identified as *D. tricolor* in one clade with collapsed branches, suggesting that they are genetically identical and should be treated only as varieties of a single species as was also suggested by KOUKOL & al. (2014).

In the phylogenetic analyses, *Earliella scabrosa* is sister species to the highly supported (MLBS: 100% and MPBS: 100%) *Daedaleopsis* clade. Within the latter, two subclades can be recognized at genus level, the *Daedaleopsis* clade (MLBS: 93%, MPBS: 95%) with *D. septentrionalis* being sister species to *D. confragosa* (MLBS: 66%, MPBS: 71%), and the highly supported (MLBS: 100% and MPBS: 100%) *Daedaleopsis (Hexagonia) nitida* clade.

Previous SSU phylogenetic studies on *Trametes consors* grouped *Hexagonia* and *Daedaleopsis* in the same clade together with *Pycnoporus, Coriolopsis aspera* and a *Microporus* species (KO & JUNG 1999 b). According to ZMITROVICH & MALYSHEVA (2013) *Hexagonia nitida* is not congeneric with *H. hirta*, the generic type. In consequence, they recombined *H. nitida* in *Daedaleopsis*, as. *D. nitida.* Finally, the genus *Hexagonia* ss. str. comprises the generic type and some other extra-European taxa.

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