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Contributed Paper

Inonotus andersonii and *I. krawtzewii*: Another Case of Molecular Sequencing-Based Diagnosis of Morphologically Similar Species

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ABSTRACT

Inonotus andersonii, originally described from America, but thought to be distributed worldwide, and *I. krawtzewii*, only known from the type locality – Russian Far East, are similar in morphology and were considered as conspecific. To test this view and to check their distribution, we sampled collections of so-called *I. andersonii* from Asia, America and Europe, and compared them to the type specimens of *I. krawtzewii*. After a combination of phylogenetic analysis based on ITS and *tef1a* sequences, and morphological examination, we found that *I. andersonii* and *I. krawtzewii* are different species, and Asian and European collections previously identified as *I. andersonii* represent *I. krawtzewii* and not *I. andersonii*. *Inonotus andersonii* differs from *I. krawtzewii* by both ITS and *tef1a* sequences, and by the presence of hyphoid setae. *Inonotus krawtzewii* is redescribed according to the lectotype.

Keywords: Hymenochaetaceae, ITS, polypore, taxonomy, *tef1a*

1. INTRODUCTION

Inonotus P. Karst., belonging to the Hymenochaetaceae, is one of the most important polypore genera that, in a wide sense, comprising more than 100 species [1], many of which are important medicinal fungi [2] and forest pathogens [3]. Some species in this genus, however, are not well known, such as *Inonotus krawtzewii* (Pilát) Pilát, which was originally described as *Xanthochrous krawtzewii* Pilát based on two collections (Pl. 8 & 96) from Russian Far East [4]. Later, Kotlaba and Pouzar [5] designated Pl. 96 (= PRM 607951) as lectotype and Pl. 8 (= PRM 628384) as paralectotype. However, Černý [6], Lowe [7] as well as Kotlaba and Pouzar [5] considered this species conspecific with *Inonotus andersonii* (Ellis & Everh.) Černý that was originally described from eastern USA [8] and was thought to be widely distributed, including western USA, Europe and Asia [1].

When studying fresh collections of American *Inonotus andersonii*, we noticed several subtle morphological and ecological differences compared to specimens collected in Europe and Asia and previously also identified as *I. andersonii*. Re-examination of the type specimens of *Inonotus krawtzewii* from the Russian Far East confirmed the differences between collections from America and Eurasia.

It has been repeatedly reported that morphologically similar specimens from different regions might represent different species [9-17]. With the aid of molecular phylogeny, numerous cryptic species, including those of *Inonotus* [18-20], have been revealed [21-23].

In the present paper, we sampled the type specimens of *I. krawtzewii* and specimens previously identified as *I. andersonii* from USA, Europe and Asia to explore whether or not these represent the same species from morphological and phylogenetic perspectives.

2. MATERIALS AND METHODS

2.1 Morphological Study

The studied specimens are deposited in the private herbarium of J. Vlasák (JV), the Prague Museum Herbarium, Czech Republic (PRM), the herbarium of Institute of Applied Ecology, Chinese Academy of Sciences, China (IFP), and the Botanical Museum, Finnish Museum of Natural History, Finland (H). They were microscopically examined from slide preparations stained with Cotton Blue, Melzer's reagent and 5% potassium hydroxide (KOH) using a Nikon E80i microscope at magnification up to × 1000 and phase contrast illumination following [24]. In presenting the size range of basidiospores, 5% of the measurements were excluded from each end of the range and are given in parentheses. In the text the following abbreviations are used: L = mean basidiospore length (arithmetical average of all basidiospores), W = mean basidiospore width (arithmetical average of all basidiospores), Q = L/W ratio, and n (a/b) = the number of basidiospores (a) measured from given number of specimens (b). Drawings were made with the aid of a drawing tube. Special color terms follow [25].

2.2 Molecular Phylogeny

DNA was isolated and sequenced as described by [26]. Amplification was performed with ITS5 and ITS4 primers [27] for ITS, and EF983F and EF2218R primers [28] for *tef1a* in 25 µl reaction mixture using 55°C annealing temperature. With ITS primers, 30 cycles were used and 0.5 µl of crude amplified DNA was used directly for sequencing. With *tef1a* primes, 37 cycles were used and the amplified DNA was purified from agarose gel using Machery-Nagel NucleoSpin kit before subsequent sequencing. Sequencing was performed in the Genomics laboratory of Biology Centre, Academy of Sciences of the Czech Republic, České Budějovice, on ABI 3730xl DNA analyzer, using BigDye Terminator 3.1 kit.

Besides the newly generated sequences, other related ITS and *tef1a* sequences used for phylogenetic analyses were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/; Table 1). The ITS dataset was firstly used to explore the relationship between *Inonotus krawtzewii* and *I. andersonii*. *Inonotus nidus-pici* Pilát, occupying a separate position from all other sampled species [29], was selected as outgroup. Then, the combined ITS and *tef1a* dataset was used to further confirm the phylogenetic distance between the two species. *Inonotus*

obliquus (Ach. ex Pers.) Pilát was assigned as outgroup according to the topology from our ITS phylogeny. The two datasets were aligned using MAFFT 6.935 [30-31] with Q-INS-i options. After a few adjustments, the alignments are used for subsequent phylogenetic analyses.

The combinability of ITS and *tef1a* sequences in the alignment from the combined ITS and *tef1a* dataset was evaluated by incongruence length difference (ILD) test [32] implemented in PAUP* 4.0b10 [33] with heuristic search and 1,000 homogeneity replicates. The ILD test gave a P value far greater than 0.01, indicating the combination of ITS and *tef1a* sequences was suitable for phylogenetic analysis.

raxmlGUI 1.2 [34-35] with GTR + G model and auto FC option [36] in bootstrap (BS) replicates was utilized to construct maximum likelihood (ML) tree. Maximum parsimony (MP) analysis was performed by PAUP* 4.0b10 with 1,000 BS replicates as follows: heuristic search, starting tree obtained via stepwise addition, tree-bisection-reconnection branch swapping, steepest descent option not in effect and 'MULTREES' option in effect. All nucleotide characters were equally weighted and gaps were treated as missing. The topology from ML analysis along with BS values above 50% from both ML and MP analyses is presented.

Table 1. Information of sequences used in the phylogenetic analysis.

Species	Voucher No.	Locality	GenBank Accession No.	
			ITS	*tef1α*
Inonotus andersonii (Ellis & Everh.) Černý	JV 1209/22-J	Arizona, USA	KF446592	KF446608
I. andersonii	JV 1209/57-J	Arizona, USA	KF446593	KF446609
I. andersonii	JV 1209/66	Arizona, USA	KF446594	
I. andersonii	L(61)11-14-C	Ohio, USA	AM269781	
Inonotus cuticularis (Bull.) P. Karst.	JV 1109/89-J	Texas, USA	KF446595	KF446610
I. cuticularis	QFB-888	unknown	AF237730	
Inonotus hispidus (Bull.) P. Karst.	JV 0407/31	Morocco	KF446596	KF446611
I. hispidus	CBS 386.61	UK	AY558602	
Inonotus krawtzewii (Pilát) Pilát	JV 8709/27	Slovakia	KF446597	KF446612
I. krawtzewii	JV 8709/35	Slovakia	KF446598	KF446613
I. krawtzewii	JV 0511/3	Czech Republic	KF446599	KF446614
I. krawtzewii	PRM 607951 (= Pl. 96, lectotype)	Russian Far East	KF446600	
I. krawtzewii	PRM 628384 (= Pl. 8, paralectotype)	Russian Far East	KF446601	
I. krawtzewii	PRM 807112	Inner Mongolia, China	KF446602	
I. krawtzewii	Wang 550 (IFP)	Shaanxi, China	KF446603	
I. krawtzewii	Yuan 6568 (IFP)	Gansu, China	KF446604	
Inonotus obliquus (Ach. ex Pers.) Pilát	JV 0408/36	Czech Republic	KF446605	KF446615
Inonotus ulmicola Corfixen outgroup	H 6012614	Finland	KF446606	
Inonotus nidus-pici Pilát	JV 0107/6	Czech Republic	KF446607	

3. RESULTS

3.1 Phylogenetic Analysis

A total of 16 ITS and eight *tef1a* sequences were newly generated for this study and deposited in GenBank (Table 1). The ITS dataset, comprising 19 sequences, resulted in an alignment with 848 characters, of which 602 are constant, 85 parsimony-uninformative and 161 parsimony-informative. The ML search stopped after 250 BS replicates. A total of 540 equally most-parsimonious trees of 338 steps (CI = 0.867, RI = 0.869) were recovered by MP analysis. The tree from ITS dataset (Figure 1) supported four collections of *Inonotus andersonii* from America as a separate clade from the type specimens of *Inonotus krawtzewii*, which formed a clade with the collections of so-called *I. andersonii* from Asia and Europe.

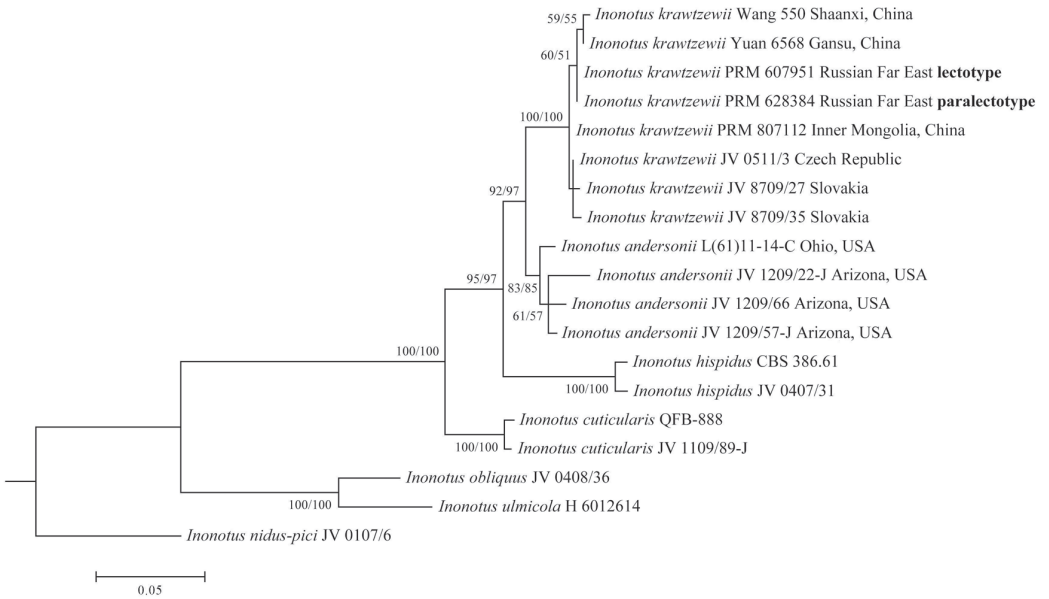

Figure 1. Phylogenetic relationship of *Inonotus andersonii*, *I. krawtzewii* and related species inferred from ITS dataset.

The combined ITS and *tef1a* dataset includes eight collections. Among 1670 aligning characters of this dataset, 1349 are constant, 244 parsimony-uninformative and 77 parsimony-informative. The BS search for the ML analysis stopped after 250 replicates. MP analysis resulted in three equally most-parsimonious trees of 370 steps (CI = 0.941, RI = 0.794). The resulting tree from combined ITS and *tef1a* dataset (Figure 2) supported the European collections previously identified as *Inonotus andersonii* formed a clade that was separated from the clade including two American collections of *Inonotus andersonii*.

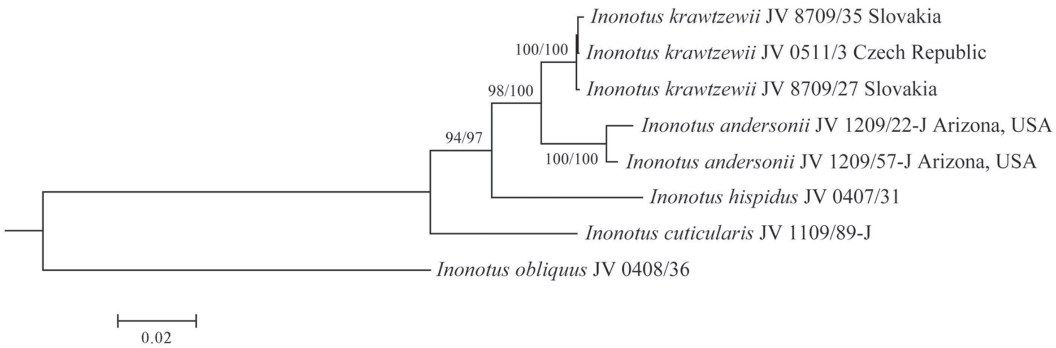

Figure 2. Phylogenetic relationship of *Inonotus andersonii*, *I. krawtzewii* and related species inferred from combined ITS and *tef1a* dataset.

Taxonomy

Inonotus krawtzewii (Pilát) Pilát, Annls mycol. 38(1): 81 (1940) (Figure 3)

Basionym: *Xanthochrous krawtzewii* Pilát, Bull. trimest. Soc. mycol. Fr. 48(1): 31 (1932)

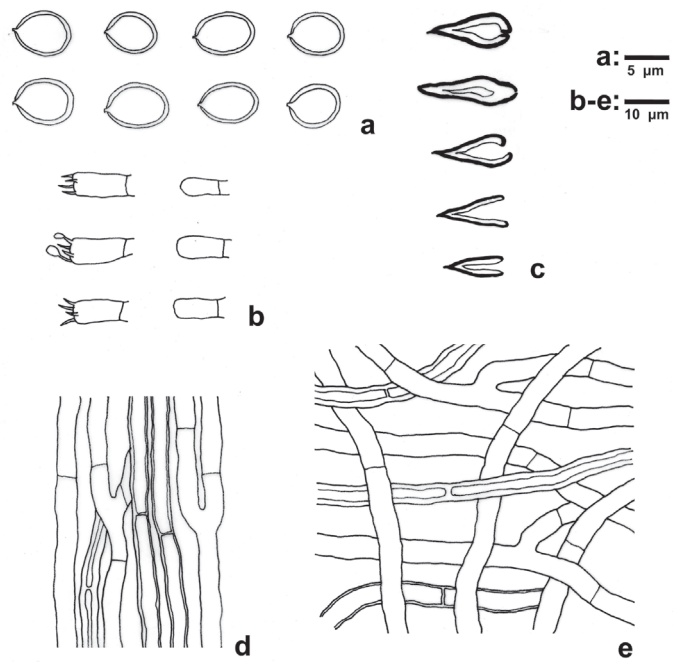

Figure 3. Microscopic structures of *Inonotus krawtzewii* (drawn from lectotype).

Fruitbody – Basidiocarps annual, resupinate, inseparable, corky and without odor or taste when dry. Pore surface brown; pores angular, 1-2 per mm; dissepiments thin, entire to strongly lacerate. Subiculum dark brown, corky, up to 4 mm thick. Tubes golden brown, paler than pore surface, fragile to brittle, up to 10 mm long.

Hyphal structure – Hyphal system

monomitic; generative hyphae simple septate; tissues darkening but otherwise unchanged in KOH.

Subiculum – Generative hyphae mostly pale yellowish, thin- to slightly thick-walled, frequently simple septate, rarely branched, flexuous, interwoven, 4-6 μm in diam.; some hyphae dark brown, distinctly thick-walled, frequently simple septate, unbranched, 3-5 μm in diam.

Tubes – Tramal hyphae mostly pale yellowish, thin- to slightly thick-walled, a few hyphae brown, thick-walled with a narrow lumen, frequently simple septate and branched, straight, parallel along the tubes, 3-5 μm in diam. Hymenial setae abundant, subulate to ventricose with a sharp or round apex, dark brown, thick-walled, 13-28 × 5-10 μm; basidia clavate, hyaline, thin-walled, with four sterigmata and a simple septum at the base, 10-15 × 4-8 μm; basidioles similar to basidia in shape, but slightly smaller.

Spores – Basidiospores ellipsoid, yellowish, thick-walled, smooth, neither amyloid nor dextrinoid, weakly cyanophilous to cyanophilous, (6-)6.1-7(-7.2) × (4.9-)5-5.8(-5.9) μm, L = 6.56 μm, W = 5.33 μm, Q = 1.23 (30/1).

Specimen examined – RUSSIA. Distr. Amur, *Quercus mongolica*, 1 October 1928, PRM 607951 (= Pl. 96, lectotype).

4. DISCUSSION

In our phylogenetic analysis from ITS (Figure 1), the Asian and European collections, previously identified as *Inonotus andersonii*, formed a clade with the type specimens of *I. krawtzewii*. This clade was separated from a clade containing four collections of *I. andersonii* from America, the type locality. The phylogeny from combined ITS and *tef1a* datasets (Figure 2) further confirmed the so-called *I. andersonii* from Europe and America as two clades. *Inonotus krawtzewii* represented an independent lineage from *I. andersonii*, and the Asian and European collections could be *I. krawtzewii*, but not *I. andersonii*.

We further checked the morphological differences between these two clades. Both *Inonotus andersonii* and *I. krawtzewii* have abundant hymenial setae and a few brown, thick-walled hyphae in trama. However, *Inonotus andersonii* differs from *I. krawtzewii* in the presence of hyphoid setae, which occasionally exist and penetrate from either tube bottom or trama, like those in *Phellinopsis* [23]. This kind of hyphoid setae is never found in *I. krawtzewii*. Dai [24] also mentioned this difference between *I. andersonii* from America and the so-called Chinese *I. andersonii*, but he did not consider this difference to be enough to delimit taxonomical rank at species level. After our phylogenetic analyses, the presence or absence of hyphoid setae is evidenced as a stable character in distinguishing *I. andersonii* and *I. krawtzewii*. This phenomenon is similar to the *Elmerina* and *Protomerulius* study [37], which proved the presence or absence of gloeocystidia, a polymorphic feature previously considered, as reliable morphological evidence in delimiting the two genera with the aid of phylogenetic analyses.

Resupinate basidiocarps of both *Inonotus andersonii* and *I. krawtzewii* develop under a layer of living sapwood, and split off outer wood and bark as the fungus matures, killing, at the same time, this part of the tree. In our experience, *I. krawtzewii* kills by maturation mostly the whole tree, but *I. andersonii* usually just a part of it, producing bark-less lesions up

to 1 m long and covered with tubes. Surprisingly, the pileate species *I. hispidus* (Bull.) P. Karst. is their closest relative, but *I. obliquus* and *I. ulmicola* Corfixen with similar ecology on birch and elm, respectively, are more distant, judging by ITS-based phylogeny (Figure 1). This might be a nice example of convergent evolution.

Other specimens studied – *Inonotus andersonii*. USA. Arizona, St. Rita Mt., Madera Canyon, *Quercus arizonica*, 2 September 2012, JV 1209/22-J; USA. Arizona, Chiricahua Mt., Turkey Creek, *Quercus arizonica*, 5 September 2012, JV 1209/57-J; USA. Arizona, Chiricahua Mt., Portal Area, *Quercus arizonica*, 6 September 2012, JV 1209/66. – *Inonotus hispidus*. MOROCCO. High Atlas Mt., Asni Village, *Juglans* sp., 21 July 2004, JV 0407/31. – *Inonotus krawtzewii*. CHINA. Inner Mongolia, September 1917, PRM 807112; CHINA. Shaanxi, Zhouzhi County, Louguantai National Forest Park, fallen trunk of angiosperm, 19 September 2005, Wang 550 (IFP); CHINA. Gansu, Pingliang, Kongdongshan, fallen trunk of angiosperm, 12 September 2012, Yuan 6568 (IFP); CZECH REPUBLIC. Valtice-Randezvous, *Quercus cerris*, 18 November 2005, JV 0511/3; RUSSIA. Distr. Amur, *Quercus mongolica*, 1 October 1928, PRM 628384 (= Pl. 8, paralectotype). SLOVAKIA. Drieňovo, Cabrad Castle, *Quercus cerris*, 22 September 1987, JV 8709/27; SLOVAKIA. Zvolen, Certova Skala, *Quercus cerris*, 25 September 1987, JV 8709/35. – *Inonotus obliquus*. CZECH REPUBLIC. Hluboka, Libochovka NPR, *Fagus sylvatica*, August 2004, JV 0408/36. – *Inonotus ulmicola*. FINLAND. Helsinki, Kalio, *Ulmus* sp., 24 September 2009, H 6012614.

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