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ORIGINAL ARTICLE

Ecological, morphological and phylogenetic survey of *Fomes fomentarius* and *F. inzengae* (*Agaricomycetes, Polyporaceae*) co-occurring in the same geographic area in Central Europe

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Abstract
The phenomenon of cryptic species is widespread among various fungal lineages. *Fomes inzengae* (Ces. & De Not.) Cooke has been recently recognized as a South European kin of wood-decay basidiomycete *F. fomentarius* (L.) Fr. due to the problematic morphological identification of both species, their taxonomic status has been disputed. The aim of this research is to examine the distribution, host preferences, morphological characters, and phylogenetic relationships between *F. fomentarius* and *F. inzengae* in the South Moravian region in Czechia (Central Europe), where both species occur sympatrically. The results revealed the ecological preferences of *Fomes* spp. along an altitudinal gradient, while *F. inzengae* is a lowland taxon, *F. fomentarius* dominates at higher altitudes in forests with abundant *Fagus sylvatica*. The main contact zone of the two taxa is located in the upper-colline vegetation belt (elevation ca. 400−550 m a.s.l.). The morphological analysis revealed that the basidiospore size, the width of skeletal hyphae in basidiomes, and the linear density of pores of both taxa are almost identical and can not be used for the identification of the two species. Multigene sequence analyses of ITS, LSU, *RPB1, RPB2,* and *TEF1* markers confirmed that *F. fomentarius* and *F. inzengae* are phylogenetically distinct species. The relationship of *F. inzengae* and *F. fomentarius* to *Globifomes graveolens* and *Hexagonia* spp. is discussed.

Keywords *Basidiomycota* · Divergence · Mycogeography · Polypores · Wood decay fungi

Introduction

Species identification and delimitation are challenging for morphologically similar and geographically overlapping fungal species which can be distinguished by DNA barcoding and phylogenetic analyses. Especially in the case of biotechnologically valuable species, the exact identification of species is necessary. *Fomes fomentarius* (L.) Fr. (*Polyporales, Polyporaceae*) belongs to a remarkable wood-decaying fungi because of its perennial, ungulate (hoof-shaped) basidiomes. The fungus was historically used as an important source of tinder, in handicrafts and European traditional medicine (Papp et al. 2017; Peintner et al. 2019). Also, the enzymatic activities of *Fomes fomentarius* s.l. are a subject of research (Větrovský et al. 2013).

Fomes (Fr.) Fr. is a small genus characterized by perennial basidiomes with a mycelial core consisting of distinct sclerids and large basidiospores. Although several hundred historic names of *Fomes* spp. have been published, only two species, *F. fomentarius* and *F. fasciatus* (Sw.) Cooke are currently widely accepted (Dai 2012; McCormick et al. 2013a, 2013b; Ryvarden and Melo 2014; Rivoire 2020).

Recent studies revealed a remarkable variability in DNA sequences of the ITS region between the ribosomal RNA gene (ITS) of *F. fomentarius* (Judova et al. 2012; Gáper et al. 2013) in Slovakia (Central Europe). The studies revealed two different genotypes with distinct host and habitat preferences. The variability between the genotypes can be quantified as 10 changes within 650 nucleotide positions of the ITS region or 97% sequence similarity between the respective

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genotypes (Judova et al. 2012). The two genotypes were later delimited as two different species *F. fomentarius* s.s. and *F. inzengae* (Ces. & De Not.) Cooke (Peintner et al. 2019). *F. inzengae* is a historical name—the respective basionym *Polyporus inzengae* Ces. & De Not. was published in 1861, and its combination in *Fomes* in 1885, but the species was for a long time treated as a synonym or a form of *F. fomentarius*. Badalyan et al. (2022) and Zhuykova and Mukhin (2022) discussed whether genetic divergence between *F. fomentarius* s.s. and *F. inzengae* within the ITS region (9–11 bp or 1.85% of nucleotide substitutions per site on average) is sufficient to treat both genetic lineages as separate species. DNA sequences of other gene regions have not been sufficiently applied for species delimitation in *F. fomentarius* s.l., but Pristas et al. (2013) confirmed that phylogenetic analyses based on DNA sequences of partial translation elongation factor 1-alpha gene (*TEF1*) and the large subunit (25S) of ribosomal RNA gene (LSU) clearly separated the two cryptic species, and topologies of phylograms based on different genetic markers were in agreement. The standard for species delimitation in fungi is the genealogical concordance method which uses multiple genetic loci to assess the limits of recombination among different genetic lineages by multigene phylogeny (Taylor et al. 2000). The topologies of phylograms based on either ITS, LSU, and *TEF1* sequences of *F. fomentarius* and *F. inzengae* seem to be in agreement (Judova et al. 2012; Pristas et al. 2013), so the two lineages could be treated as separate species. Another genetic marker useful for species delimitation in the *Fomes* sp. is the RNA polymerase II, the second largest subunit (McCormick et al. 2013a), but this has not been applied yet for the study of genetic variability in European *F. fomentarius* or *F. inzengae*.

Both, *Fomes fomentarius* and *F. inzengae* are characterized by perennial hoof-shaped (ungulate) basidiomes which can reach a width of up to 60 cm and a weight of several kilograms. The upper surface of the basidiome is smooth, zonate and sulcate, glabrous with a thick and hard crust, pale brown, reddish brown to gray, and cracked when old. The hymenophore is poroid, the pore surface is ochraceous to grey, and pores are small with thick, entire tomentose dissepiments. The context is brownish, tough-fibrous, thick, and homogenous, black with KOH. The mycelial core of varying size is developing at the upper part of the context next to the substrate composed of white hyphae and brown tissue. Hyphal system is trimitic, generative hyphae thin-walled, colorless, branched, with clamps, inconspicuous; skeletal hyphae thick-walled, aseptate, pale yellowish brown, binding hyphae yellowish brown, thick-walled, frequently branched, aseptate. The mycelial core is composed of a mixture of skeletal binding hyphae and irregularly shaped thick-walled sclerids. Cystidia are absent, and cystidioles are present. Basidia are cylindric, with four sterigmata, enlarged base, and a basal clamp. Basidiospores are cylindric, thin-walled, smooth, and negative in Melzer's reagent. Spore powder is whitish, apparent in spring during a short sporulation period.

F. fomentarius s.l. is growing on living and dead broad-leaved trees, rarely on conifers (Peintner et al. 2019) causing heart rot of the simultaneous white rot decay type (Schwarze 2007). The latent symptomless presence of the fungi has also been repeatedly confirmed in the functional sapwood of intact trees (Baum et al. 2003; Parfitt et al. 2010; own observation).

The information about the local distribution and host preferences of the two species is infrequent (Badalyan et al. 2022; Zhuykova and Mukhin 2022). Peintner et al. (2019) also proposed several microscopic characters (basidiospore size, linear density of pores, diameter of skeletal hyphae in pure culture and basidiomes) for the identification of *F. fomentarius* and *F. inzengae*, but the respective dimensions were not in agreement with our preliminary data.

The aims of this work are (A) to obtain the information about distribution, host, and habitat preferences of *Fomes fomentarius* and *F. inzengae* in the South Moravian Region (South-East part) of Czechia where the occurrence of both species could be expected, (B) the critical evaluation of morphological characters possibly applicable for morphological identification of the two *Fomes* spp. using the statistically relevant data, and (C) multigene phylogeny of the two species using standard markers: LSU, *TEF1*, the largest subunit of RNA polymerase II (*RPB1*), and the second largest subunit of RNA polymerase II (*RPB2*).

Material and methods

The basidiomes of *Fomes* spp. were collected during 2021–2022 in various woodland habitats (natural and managed forests, parks, and urban greenery). The research was focused on species distribution in diverse woodland habitats along the elevational gradient in the South Moravian Region of Czechia with several reference specimens from other regions of Czechia and Slovakia.

Morphological characters

The comparison of the linear density of pores (pores per cm on the hymenophore surface) was done under a stereomicroscope (Olympus SZX12). The hymenophore was photographed with an enclosed piece of millimeter paper, and the values were read from the photos in the Inscape v. 0.92.1 program. In total, 15 values per specimen were measured.

The spore prints were obtained after incubation of basidiomes on microscope slides for 1–3 days at room temperature. To avoid contamination by spores from the air, each basidiome was covered by a beaker or a bell jar.

The basidiospores were measured under the microscope, mounted in water or Melzer's reagent, 30 basidiospores per specimen were measured. For basidiospores, the factors *E* (the ratio of the spore length to its width for each spore) and *Q* (the mean of *E* values for each specimen) were determined. The skeletal hyphae were prepared from the context tissues, and the diameters of 30 different hyphae per specimen were measured. The microscopic measurements were made using the Olympus BX50 microscope (magnification 1000×), with a camera and the QuickPhoto Micro program.

The specimens with a sufficient number of spores were deposited in the herbarium of the Moravian museum in Brno (BRNM).

Morphometric charecteristics were analysed with R 4.1.2 (https://www.R-project.org/). For each parameter, the difference between the species (i.e. the effect of species on the respective parameter) was tested by means of comparing the full model with the corresponding null model (without the respective parameter) using the likelihood-ratio test. All traits except for *Q* were analysed by means of generalized linear mixed models (GLMM) implemented in the lme4 package (Bates et al. 2015) with individual specimen treated as random factor. *Q* was modelled by the Gamma generalized linear model (GLM) with the inverse link function (package stats; https://www.R-project.org/). Since *Q* is calculated from all specimen per species, it was not possible to account for the differences between specimen by including the random factor. The linear density of pores was modelled by the GLMM with the Poisson error structure. The presence of overdispersion was tested by a function created and recommended by Bolker, one of the authors of the lme4 package (https://bbolker.github.io/mixedmodels-misc/glmmFAQ.html#overdispersion). This is based on Pearson residuals that are less biased than deviance residuals for this type of computation (McCullagh and Nelder 2019). The skeletal hyphae width and spore length and width were analysed using the Gamma GLMM with the logarithmic link function. In the case of *E*, the Gamma GLMM with the inverse link function was used.

DNA sequencing

Cultures were isolated from the context tissue of the fresh basidiomes on Petri dishes with malt extract agar (Himedia, India) for 1–2 weeks at 21 °C. The small piece of mycelium of fresh culture was used as a template for PCR using Phire Plant Direct PCR Master Mix (Thermo Scientific). The amplifications of ITS, LSU, *TEF1, RPB1*, and *RPB2* were conducted according to standard protocols (Westphalen et al. 2022; Antonín et al. 2022).

Phylogeny

The ITS sequences were supplemented with those of *F. fomentarius, F. inzengae* and *Fomes fasciatus* published by Badalyan et al. (2022), Gáper et al. (2013), Judova et al. (2012), McCormick et al. (2013a), Náplavová et al. (2020), Peintner et al. (2019), and Zhuykova and Mukhin (2022). Moreover, the sequences of *Globifomes graveolens* (Schwein.) Murrill retrieved from the Genbank were added to the ITS dataset. Except the main ITS datasets including the large sampling of new previously published sequences, five regions were used, ITS, LSU, *RPB1, RPB2,* and *TEF1*, for the phylogenetic analysis with ten isolates available for each region, five per species. The phylogenetic trees were inferred for each of these regions individually as well as for their concatenation. Several closely related species pairs of *Polyporaceae* were added to all datasets of DNA sequences. Always, only specimens who have sequences of all five regions available were selected: *Funalia gallica* (Fr.) Bondartsev & Singer and *Funalia trogii* (Berk.) Bondartsev & Singer; *Hexagonia apiaria* (Pers.) Fr. and *Hexagonia glabra* Lév.; *Perenniporia tephropora* (Mont.) Ryvarden and *Perenniporia subtephropora* B.K. Cui & C.L. Zhao (Ji et al. 2023; Justo et al. 2017; Li et al. 2014; Zhao and Cui 2013). *Grifola frondosa* (Dicks.) Gray was selected as an outgroup of all datasets.

The main ITS dataset and multigene concatenated dataset were analysed by means of maximum likelihood (ML) and Bayesian inference (BI) algorithms, the single gene datasets were analyzed by BI only. The best-fitting partitioning schemes were found via PartitionFinder 2 (Lanfear et al. 2016) based on the corrected Akaike Information Criterion (AICc) with each codon being used as a separate data block to account for differences between the individual codon positions. For all the datasets, the analysis was done using both linked and unlinked branch lengths, with identical results obtained. The set of all partitioning schemes was evaluated (the option search = all;). The resulting partitioning schemes are listed in Supplementary Table 1.

The ML phylogenetic analysis was carried out with RAxML-NG 1.1.0 (Kozlov et al. 2019). The evolutionary model best describing the data was selected by PartitionFinder 2. All 84 models were used, including those with base frequencies estimated by ML (the parameter models = allx;). The MRE-based bootstopping test was applied to find out the necessary number of bootstrap replicates. The cutoff value was set to 0.01 (the option --bs-cutoff 0.01). Transfer Bootstrap Expectation (Lemoine et al. 2018) was used as a branch support measure. The presented phylogenetic trees are the best-scoring trees with the bootstrap support values mapped onto them.

The ML phylogenetic analysis was conducted within the BEAST 2 platform (Bouckaert et al. 2014). For all the datasets, the parameters were set up in the same way.

The uncorelated log-normal relaxed molecular clock was used (Drummond et al. 2006). The best-fit evolutionary models for individual partitions were determined through model averaging implemented in the bModelTest package (Bouckaert and Drummond 2017). All the analyses utilized the Metropolis-coupled MCMC (MC3) alrogithm implemented in the CoupledMCMC package (Müller and Bouckaert 2019).Three heated and one cold chains were used. The chain length was always set to 20,000,000 (60,000,000 for concatenated multigene dataset) and every 5000th generation was sampled. Target switch probability was set to 0.234 (Kone and Kofke 2005; Atchadé et al. 2011). The burn-in was set to 25%.

The posterior parameter estimates were summarized with Tracer 1.7.1 (Rambaut et al. 2018). To assess the accuracy of the posterior estimates, the trace plots were inspected for the presence of a "hairy caterpillar" pattern indicating that the chains have mixed properly and reached a stationary distribution and the ESS values were checked, with the values ≥ 200 considered as indicative of good mixing of the MCMC (standard approach). Parameter estimates were summarized with TreeAnnotator 2.6.0 (part of BEAST 2) and mapped onto the 50% majority-rule consensus tree created with SumTrees 4.4.0 (Sukumaran and Holder 2010). Edge lengths were calculated as mean lengths of the corresponding edges in the input array of trees.

Genetic differentiation

The extent of genetic differentiation between *Fomes fomentarius* and *F. inzengae* was expressed by the fixation index (FST) defined by Weir and Cockerham (1984) and formally proven by Michalakis and Excoffier (1996) using the Arlequin 3.5.2.2 software (Excoffier and Lischer 2010).

Maps of distribution

The spatial distribution maps of *Fomes* specimens (Figs. 1, 2 and 3) were constructed with Quantum GIS, v. 3.6.2, using a digital elevation model and a base map of Czechia in 1:100 000 scale available from the WMS server (https://geoportal.cuzk.cz), using the data of EuroGeographics© for the administrative boundaries. The borders of natural reserves were vectorized from a base map of Czechia in 1:25 000 scale, obtained from the WMS server.

Results

Distribution, host spectrum, and habitat preferences of *F. fomentarius* and *F. inzengae*

We analysed 48 specimens of *Fomes* spp. from Czechia and two specimens from Slovakia. The ITS sequencing

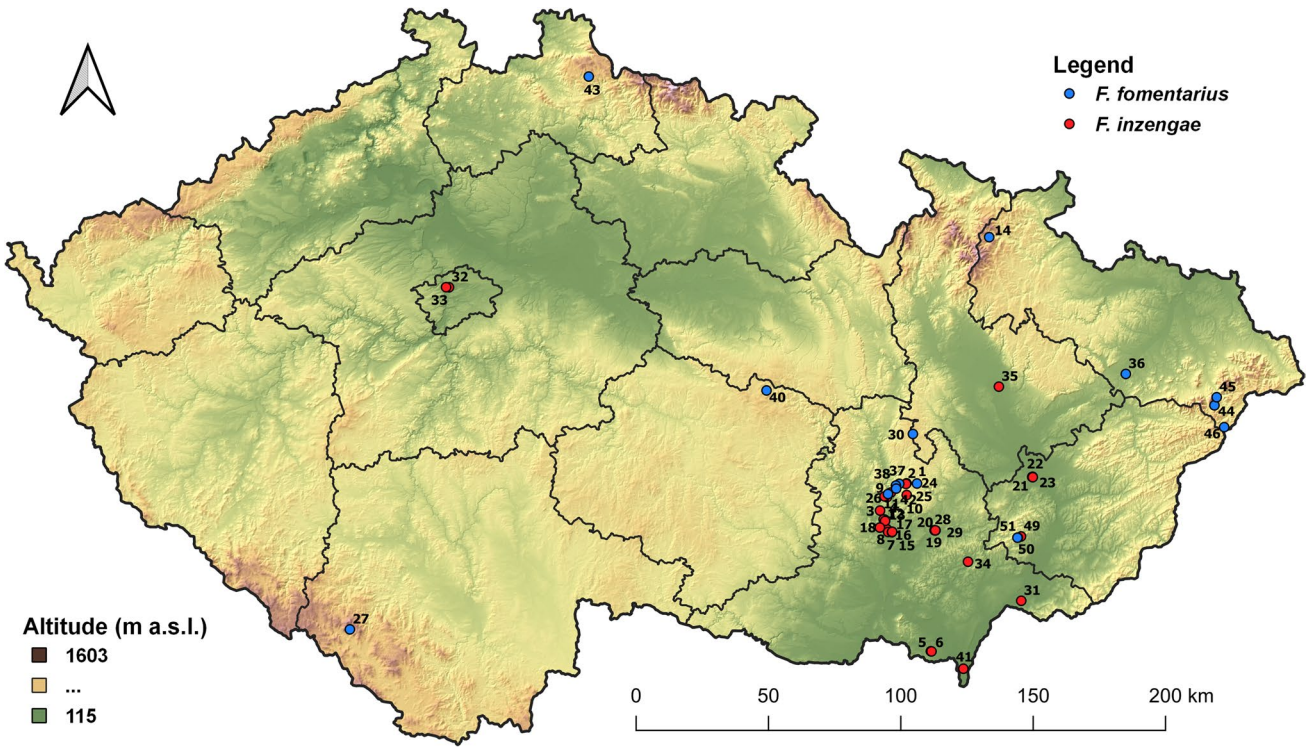

Fig. 1 Distribution maps of *Fomes* spp. specimens in Czechia

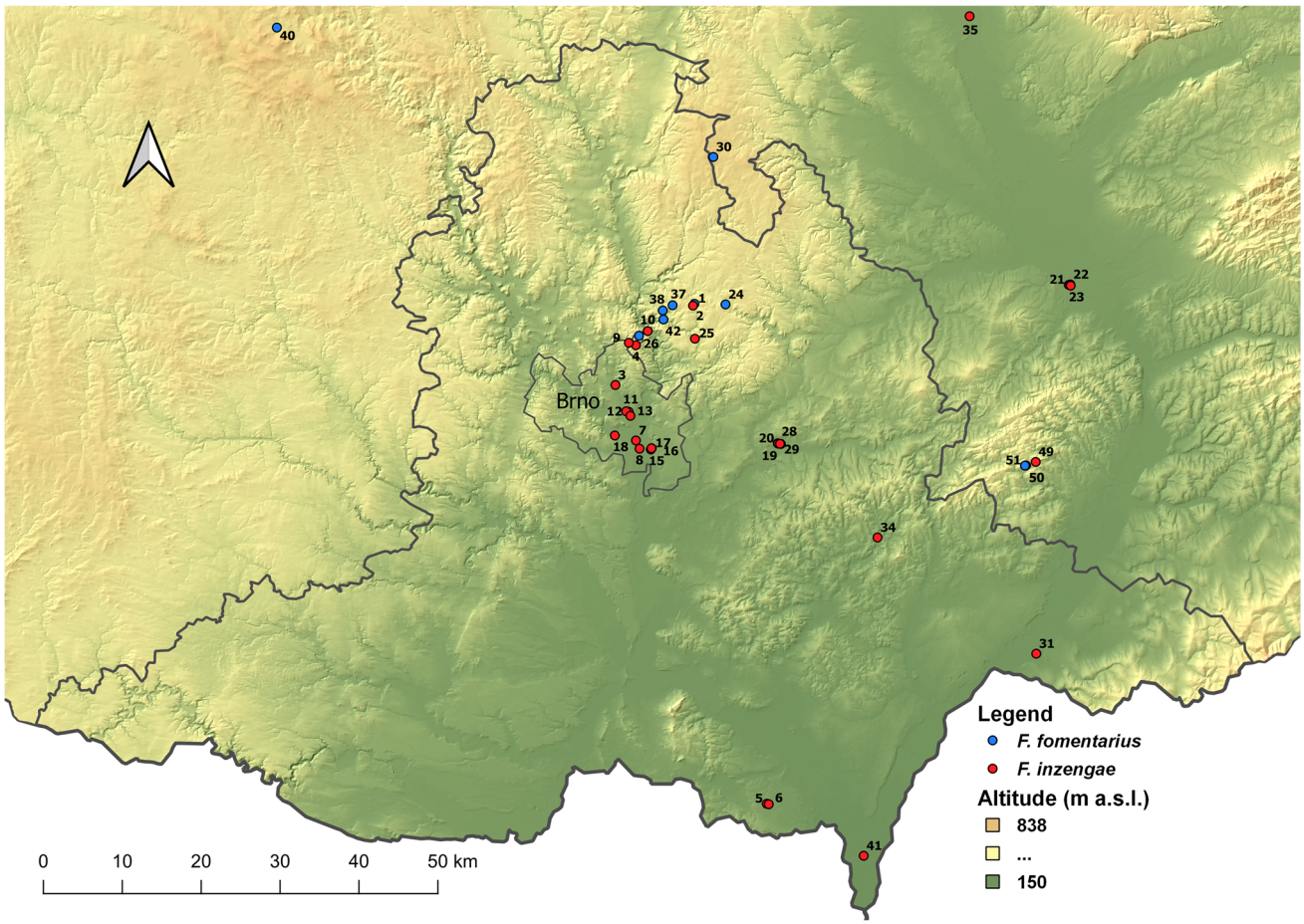

Fig. 2 Distribution maps of *Fomes* spp. specimens in the South Moravian region of Czechia

revealed 31 specimens of *F. inzengae* and 19 of *F. fomentarius* (Table 1, Figs. 1, 2, and 3). Results clearly delimited the ecological preferences of *F. fomentarius* and *F. inzengae* in the surveyed area. While *F. fomentarius* is distributed mainly at higher altitudes (400−970 m a.s.l.) with beech (*Fagus sylvatica*) and birch (*Betula* spp.) as dominant hosts, *F. inzengae* is a lowland species (154–490 m a.s.l.) with a wide spectrum of hosts (*Acer, Aesculus, Alnus, Betula, Fagus, Fraxinus, Quercus, Platanus, Populus, Salix, Sorbus,* and *Tilia*). The contact zone of the two *Fomes* spp. seems to take place in the upper-colline vegetation belt (elevation ca 400–550 m a.s.l.) within the Mesophyticum phytogeographical region comprising mainly various types of mesic beech or hornbeam (*Carpinus betulus*) forests (Chytrý et al. 2017). The center of distribution of *F. fomentarius* continues at higher altitudes in forests with predominant beech (submontane and montane belts).

In contrast, *F. inzengae* is distributed at altitudes up to ca. 500 m a.s.l. in the Thermophyticum phytogeographical region. Thermophyticum includes warm areas of lowland and colline belts characterized by the occurrence of basiphilous oak and oak-hornbeam forests or softwood (dominant *Salix* and *Populus*) and hardwood (frequent *Quercus*) floodplain forests in lowland basins. The town parks and other urban areas sampled in this study are also located in Thermophyticum. The beech forests, a common habitat of *F. fomentarius*, are almost absent in Thermophyticum.

While *F. fomentarius* was collected mostly at higher altitudes, here we noticed a few specimens of this species at lower altitudes (< 400 m a.s.l.). This can be explained by either topographic shading of deep valleys which contain patches of vegetation resembling to vegetation belts of higher altitudes (F38 and F42) or association to an exotic host in an arboretum (specimen F1; unfortunately the exact identification of the *Betula* sp. at species level could not be done because the specimen was sampled from dead wood). Another exception is the locality of the specimen F36; despite the low elevation (273 m a.s.l.), this area is located in upper-colline vegetation belt of Mesophyticum (Chytrý et al. 2017) and references therein. Most of the localities of *F. fomentarius* at lower altitudes are protected areas

Table 1 The list of *Fomes* spp. specimens included in this study

Code	ITS identification	Host	Locality	Coordinates	Herbarium
F01	*F. fomentarius*	*Betula* sp.	CZE, Křtiny, Arboretum of the Mendel University in Brno	49.3232678 N, 16.7417628 E	-
F02	*F. inzengae*	*Fagus sylvatica*	CZE, Křtiny, Arboretum of the Mendel University in Brno	49.3213444 N, 16.7390136 E	-
F03	*F. inzengae*	*Quercus petraea*	CZE, Brno, Královo Pole, Zamilovaný hájek grove	49.2390869 N, 16.5921158 E	BRNM 840273
F04	*F. inzengae*	*Fagus sylvatica*	CZE, Brno, Útěchov, beech forest	49.2822292 N, 16.6337158 E	BRNM 840274
F05	*F. inzengae*	*Quercus* sp.	CZE, Valtice, Rendezvous nature monument	48.7477653 N, 16.7885122 E	BRNM 840275
F06	*F. inzengae*	*Quercus* sp.	CZE, Valtice, Rendezvous nature monument	48.7470261 N, 16.7918550 E	BRNM 840277
F07	*F. inzengae*	*Salix alba*	CZE, Brno, Komárov, the Svratka river	49.1739728 N, 16.6190747 E	BRNM 840276
F08	*F. inzengae*	*Populus* × *canadensis*	CZE, Brno, Horní Heršpice, the Svratka river	49.1642322 N, 16.6240594 E	BRNM 840278
F09	*F. inzengae*	*Tilia sp.*	CZE, Brno, Útěchov	49.2857133 N, 16.6218222 E	BRNM 840279
F10	*F. inzengae*	*Fagus sylvatica*	CZE, Adamov, Hrádkovská pathway	49.2970886 N, 16.6564175 E	BRNM 840280
F11	*F. inzengae*	*Acer platanoides*	CZE, Brno, Lužánky park	49.2074319 N, 16.6109447 E	BRNM 840281
F12	*F. inzengae*	*Aesculus hippocastanum*	CZE, Brno, Lužánky park	49.2081119 N, 16.6066264 E	BRNM 840282
F13	*F. inzengae*	*Acer platanoides*	CZE, Brno, Náměstí 28. října park	49.2025708 N, 16.6133453 E	BRNM 840284
F14	*F. fomentarius*	*Fagus sylvatica*	CZE, Vrbno pod Pradědem, Skalní potok, nature reserve	50.1282694 N, 17.2943781 E	BRNM 840283
F15	*F. inzengae*	*Fraxinus excelsior*	CZE, Brno, Černovický hájek nature reserve	49.1622200 N, 16.6435569 E	BRNM 840285
F16	*F. inzengae*	*Alnus glutinosa*	CZE, Brno, Černovický hájek nature reserve	49.1635178 N, 16.6451983 E	BRNM 840286
F17	*F. inzengae*	*Populus* sp.	CZE, Brno, Černovický hájek nature reserve	49.1634722 N, 16.6448014 E	-
F18	*F. inzengae*	*Betula pendula*	CZE, Brno, Štýřice, Mahenova stráň hillside	49.1817794 N, 16.5833306 E	BRNM 840287
F19	*F. inzengae*	*Aesculus hippocastanum*	CZE, Slavkov u Brna, alley by the castle	49.1559386 N, 16.8642228 E	BRNM 840288
F20	*F. inzengae*	*Aesculus hippocastanum*	CZE, Slavkov u Brna, alley by the castle	49.1555853 N, 16.8647781 E	BRNM 840289
F21	*F. inzengae*	*Platanus* sp.	CZE, Kroměříž, Podzámecká zahrada (Chateau Garden)	49.3037914 N, 17.3947303 E	BRNM 840290
F22	*F. inzengae*	*Tilia cordata*	CZE, Kroměříž, Podzámecká zahrada (Chateau Garden)	49.3041200 N, 17.3975414 E	BRNM 840291
F23	*F. inzengae*	*Fraxinus excelsior*	CZE, Kroměříž, Podzámecká zahrada (Chateau Garden)	49.3027206 N, 17.3978900 E	BRNM 840292
F24	*F. fomentarius*	*Fagus sylvatica*	CZE, Křtiny, Bukovina, Rakovec nature reserve	49.3192822 N, 16.7956436 E	BRNM 840294
F25	*F. inzengae*	*Fagus sylvatica*	CZE, Křtiny, Březina, Březinka nature reserve	49.2835603 N, 16.7371178 E	BRNM 840293
F26	*F. fomentarius*	*Fagus sylvatica*	CZE, Brno, Útěchov, Coufavá nature reserve	49.2927275 N, 16.6406547 E	BRNM 840295
F27	*F. fomentarius*	*Fagus sylvatica*	CZE, Horní Vltavice, Boubínský prales, virgin forest	48.9637697 N, 13.8123331 E	BRNM 840296
F28	*F. inzengae*	*Fagus sylvatica*	CZE, Slavkov u Brna, castle park	49.1548669 N, 16.8693728 E	BRNM 840298
F29	*F. inzengae*	*Sorbus aucuparia*	CZE, Slavkov u Brna, castle park	49.1551722 N, 16.8679675 E	BRNM 840299
F30	*F. fomentarius*	*Fagus sylvatica*	CZE, Boskovice, Suchý, the Skály hill	49.4881533 N, 16.7978892 E	BRNM 840297
F31	*F. inzengae*	*Salix alba*	CZE, Strážnice, Petrov, the Baťa Canal	48.8890525 N, 17.2760239 E	BRNM 840300
F32	*F. inzengae*	*Acer platanoides*	CZE, Prague 6, Stromovka park	50.1051597 N, 14.4271636 E	BRNM 840301
F33	*F. inzengae*	*Platanus* × *hispanica*	CZE, Prague 6, Stromovka park	50.1060583 N, 14.4119692 E	BRNM 840302
F34	*F. inzengae*	*Populus nigra*	CZE, Ždánice, Dražůvky	49.0385103 N, 17.0215947 E	BRNM 840303

Table 1 (continued)

Code	ITS identification	Host	Locality	Coordinates	Herbarium
F35	*F. inzengae*	*Acer* sp.	CZE, Olomouc, Černovír, Černovírský les forest	49.6200969 N, 17.2682089 E	BRNM 840304
F36	*F. fomentarius*	*Betula* sp.	CZE, Bernartice nad Odrou, the Odra river	49.6184656 N, 17.9358994 E	BRNM 840305
F37	*F. fomentarius*	*Fagus sylvatica*	CZE, Adamov, Habrůvecká bučina nature reserve	49.3238572 N, 16.7035883 E	-
F38	*F. fomentarius*	*Fagus sylvatica*	CZE, Adamov, Habrůvecká bučina nature reserve	49.3189342 N, 16.6857678 E	BRNM 840306
F40	*F. fomentarius*	*Fagus sylvatica*	CZE, Svratka, Křižánky	49.6787314 N, 16.0551575 E	BRNM 840307
F41	*F. inzengae*	fallen hardwood	CZE, Lanžhot, Ranšpurk nature reserve	48.6783903 N, 16.9466828 E	BRNM 840308
F42	*F. fomentarius*	*Fagus sylvatica*	CZE, Adamov, Býčí skála nature reserve	49.3086353 N, 16.6855086 E	BRNM 840309
F43	*F. fomentarius*	*Fagus sylvatica*	CZE, Jizerské hory Mts., Josefův důl	50.7877778 N, 15.2427778 E	BRNM 840310
F44	*F. fomentarius*	*Fagus sylvatica*	CZE, Moravskoslezské Beskydy Mts., Čeladná, Podolánky	49.4790808 N, 18.3781449 E	-
F45	*F. fomentarius*	*Fagus sylvatica*	CZE, Moravskoslezské Beskydy, Ostravice, Malý Smrk Mt.	49.5056549 N, 18.3946141 E	BRNM 840311
F46	*F. fomentarius*	*Fagus sylvatica*	CZE, Moravskoslezské Beskydy Mts., Salajka nature reserve	49.4018592 N, 18.4158751 E	BRNM 840312
F47	*F. fomentarius*	*Fagus sylvatica*	Slovakia, Vihorlat hills, Morské oko nature reserve	48.9187000 N, 22.1915319 E	BRNM 840313
F48	*F. fomentarius*	*Fagus sylvatica*	Slovakia, Vihorlat hills, Sninský kameň, nature monument	48.9287314 N, 22.1887856 E	BRNM 840314
F49	*F. inzengae*	*Fagus sylvatica*	CZE, Chřiby hills, Buchlov, forest under the castle.	49.1065328 N, 17.3072206 E	BRNM 840317
F50	*F. fomentarius*	*Fagus sylvatica*	CZE, Chřiby hills, Holý kopec nature reserve	49.1036108 N, 17.2899364 E	BRNM 840315
F51	*F. fomentarius*	*Fagus sylvatica*	CZE, Chřiby hills, Holý kopec nature reserve	49.1035336 N, 17.2880697 E	BRNM 840316

characterized by natural forest vegetation with dominant beech trees and a certain amount of dead wood.

Morphometric charecteristics

The size of 967 basidiospores was measured (*F. fomentarius*: 270 spores/12 specimens; *F. inzengae*: 697 spores/26 specimens). Unfortunately, less than the planned 30 spores were measured at some specimens, due to their low abundance in hymenium. The basidiospore size of *F. fomentarius* (14.2)15.3–20.6(22.9) × (4.3)5.3–6.9(7.9) µm and *F. inzengae* (14.0)15.3–19.7(22.6) × (4.0)5.1–7.4(8.0) µm resulted almost identical and differences between their length, widths, and *Q* were non-significant (Table 2, Fig. S1). The diameters of skeletal hyphae (*F. fomentarius*: 155 values/5 specimens; *F. inzengae*: 151 values/5 specimens) were higher in basidiomes of *F. inzengae*, but the differences were not significant ($p = 0.085$). The detected dimensions were larger than those presented by Peintner et al. (2019), some measured hyphae of *F. inzengae* were >7 µm, especialy those of large basidiomes. The only significant difference between the two *Fomes* spp. was detected in the case of linear density of pores ($p = 0.009$), but the respective values overlap: *F. fomentarius*: (13) 15–25 (27) pores per cm; *F. inzengae:* (13) 16–27 (32) pores per cm. In total, 255 values/17 specimens for *F. fomentarius* and 450 values/30 specimens for *F. inzengae* were measured.

Phylogeny

All 50 specimens of *F. fomentarius* and *F. inzengae* were provided with ITS sequences. The 10 selected specimens (5 of *F. fomentarius* and 5 of *F. inzengae*) were subjected to multigene sequence analyses of ITS, LSU, *TEF1, RPB1*, and *RPB2* markers. The selected ITS sequences and all sequences of other markers are deposited in the GenBank database (Table 3), some of the ITS sequences were published by Cristini et al. (2023). The results of phylogenetic analyses of individual markers clearly delimited *F. fomentarius* and *F. inzengae* (Fig. S2). The resulting single-gene phylograms had identical topologies as the ITS phylogram of our dataset completed with previously published sequences (Fig. 4) and the phylogram of the multigene concatenated dataset (Fig. 5). Because incongruences among gene trees were not found and the two lineages are completely sorted, *F. fomentarius* and *F. inzengae*

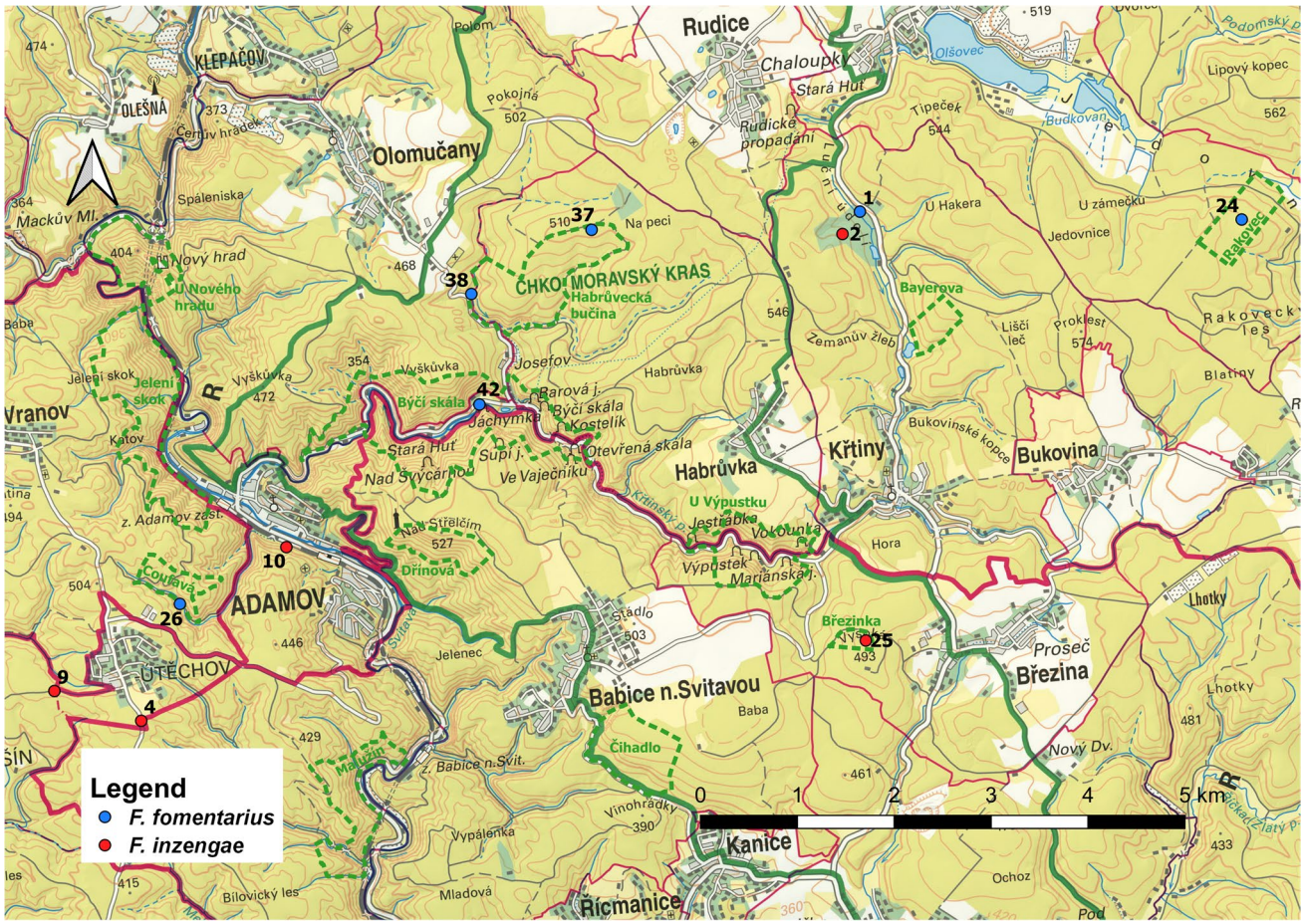

Fig. 3 Co-occurrence of *F. fomentarius* and *F. inzengae* in the northern part of the South Moravian region (vicinity of Adamov town), Czechia

are distinct, well delimited species. The specimens of *F. fomentarius* from North America (Fig. 4) can belong to a geographically separated species (*F. fomentarius* aff. USA). Similarly, two ITS sequences of *F. inzengae* from China and Korea differ from the others and may form a separate genetically distinct lineage close to *Globifomes graveolens* which resulted as a sister species of *F. inzengae*. *Hexagonia apiaria* resulted as closely related to *Fomes fasciatus* and *Fomes* sp. from Mexico and unrelated to *H. glabra*. The DNA sequence datasets of this study are available within the Supplementary Information files (SI1a-c).

Genetic differentiation

The fixation index (FST) calculated for the 50 ITS sequences (31 sequences of *F. inzengae* and 19 thats of *F. fomentarius*) resulted FST = 0.991, showing a very high genetic differentiation (Wright 1978).

Discussion

F. fomentarius and *F. inzengae* are undoubtedly genetically separated species. Even though the ranges of the two species overlap (the populations are sympatric), the DNA sequences support their complete genetic separation and a gene flow between them is improbable. Co-occurrence of *F. fomentarius* and *F. inzengae* at one locality is possible, but infrequent. Judova et al. (2012) revealed both species (referred to as genotypes A and B) at some localities in East Slovakia. We confirmed both *Fomes* species (specimens F01 and F02, Fig. 3) at only one locality, which is an arboretum, where the fungal diversity can be influenced by cultivation of non-native tree species.

Our results confirmed the geographical distribution pattern of *F. fomentarius* and *F. inzengae* in Czechia is in agreement with those of the previous studies (Peintner et al. 2019; Badalyan et al., 2022; Zhuykova and Mukhin 2022). The two species are ecologically segregated by altitudinal and latitudinal vegetation zonation. *F. inzengae* has a southern,

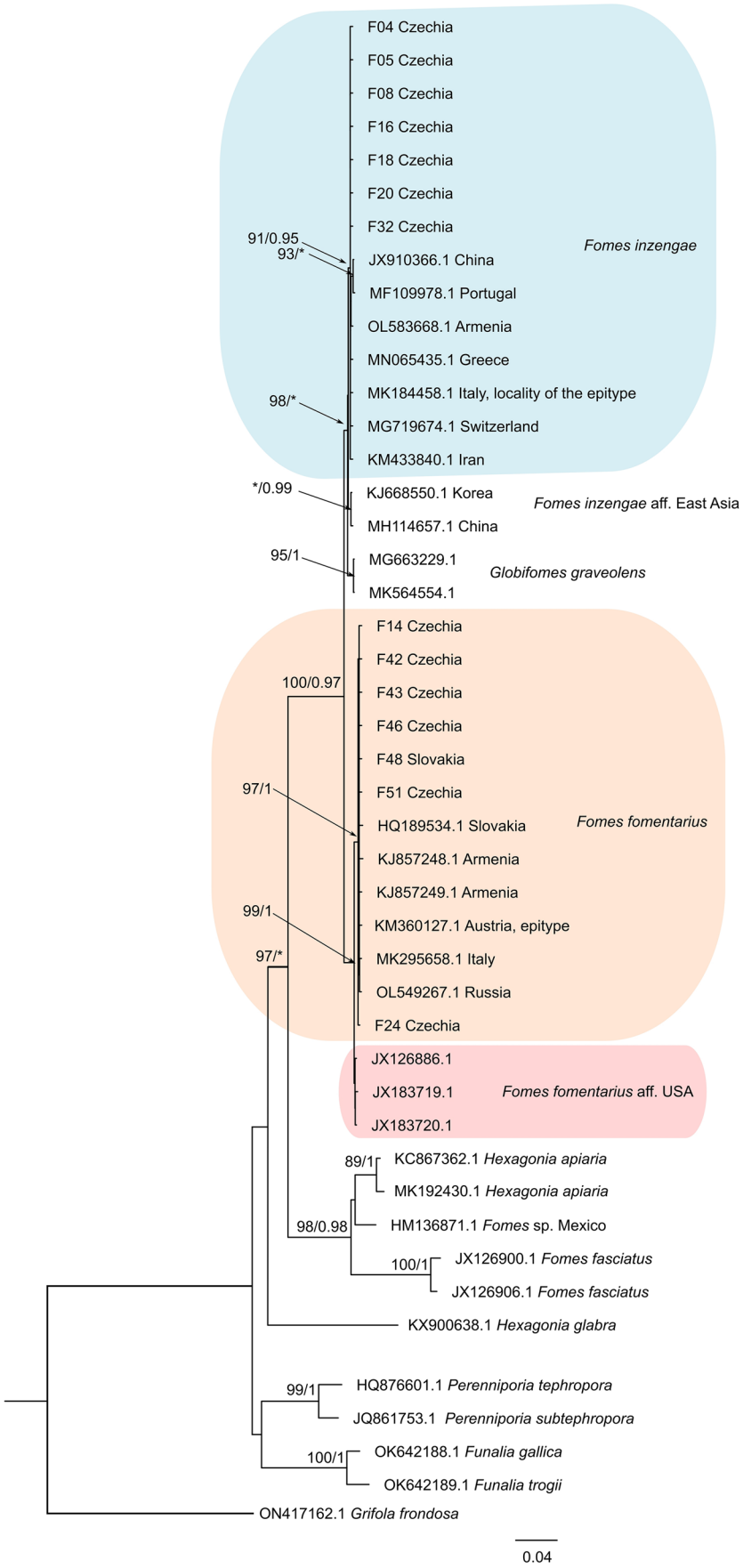

Fig. 4 The phylogenetic tree of *F. fomentarius* and *F. inzengae* specimens based on the Bayesian analysis of the ITS region. Numbers at branches indicate maximum likelihood bootstrap proportion and Bayesian posterior probability values. For legend to specimen codes, see "Table 1". The asterisks (*) mark low support (< 75 in maximum likelihood; < 0.9 in Bayesian analysis). The bar indicates the number of expected substitutions per position

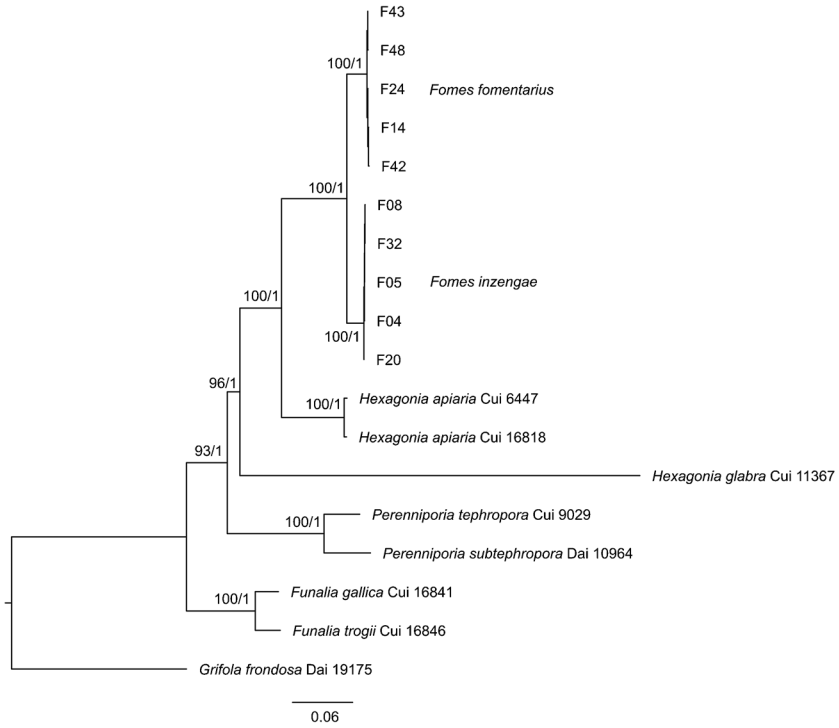

Fig. 5 The concatenated phylogenetic tree of *F. fomentarius* and *F. inzengae* specimens based on the maximum likelihood analysis of the combined ITS-LSU-*RPB1-RPB2-TEF1* dataset. Numbers at branches indicate maximum likelihood bootstrap proportion and Bayesian posterior probability values. For legend to specimen codes, see "Table 1". The bar indicates the number of expected substitutions per position

Table 2 Summary of morphometrical analysis of *F. fomentarius* and *F. inzengae* including the number of pores per cm, width of skeletal hyphae, and spore dimensions (spore length, width, and Q) including mean and standard deviation (SD). The outliers of 5th and 95th percentiles are given in parentheses. The ** indicates significant differences between the two species

	F. fomentarius			*F. inzengae*			
	Mean	SD	Range	Mean	SD	Range	*P*-value
Number of pores/cm	19.25	3.30	(13)15–25(27)	20.95	3.66	(13)16–27(32)	0.009**
Skeletal hyphae width [µm]	5.04	0.70	(3.6)3.9–6.3(6.9)	5.75	1.07	(3.8)4.2–7.8(8.4)	0.085
Spore length [µm]	17.87	1.58	(14.2)15.3–20.6(22.9)	17.35	1.40	(14.0)15.3–19.7(22.6)	0.959
Spore width [µm]	5.98	0.53	(4.3)5.3–6.9(7.9)	6.06	0.70	(4.0)5.1–7.4(8.0)	0.735
Q	2.93	0.25	2.6–3.3	2.88	0.26	2.4–3.2(3.4)	0.596

Mediterranean distribution (Peintner et al. 2019, Badalyan et al., 2022) extending its range to warmer areas in Central Europe, while *F. fomentarius* inhabits colder temperate woodlands. Apparently *F. fomentarius* is distributed mainly in mesic and (sub)montane beech forests in Central Europe. The range and distribution pattern of *F. fomentarius* and *F. inzengae* are similar to those of *Armillaria cepistipes* and *A. gallica* (Antonín et al. 2009) in the respective habitats. Náplavová et al. (2020) hypothesized the southern border of the occurrence of *F. fomentarius* s.s. (sublineage A2) in Europe being associated with the distribution of *Betula pendula* and the absence of *F. inzengae* on this host. Nevertheless, we confirmed *B. pendula* also as a host of *F. inzengae* (specimen F18).

F. fomentarius s.l. is reported not only from the Mediterranean and temperate biomes but also from subalpine birch forests in Fennoscandia (Ryvarden and Melo 2014). However, the identity of *Fomes* specimens in such a habitat is unclear. These may belong to *F. fomentarius* s.s. or a separate cryptic species. Nevertheless, the American lineage of *F. fomentarius* s.l. (named *F. fomentarius* aff. USA in this study—Fig. 4, or *F. fomentarius* II by McCormick et al. 2013a) is also confirmed from boreal forests in Alaska (McCormick et al. 2013b).

Especially when *Fomes* spp. can be dispersed with nursery stock due to its latent presence in the sapwood of healthy trees, its spread to an arboretum with a planted tree can not be excluded.

Table 3 DNA sequences generated in this study and deposited to the Genbank

Code	Taxon	Herbarium	ITS	LSU	*RPB1*	*RPB2*	*TEF1*
F01	*F. fomentarius*	-	OQ474923				
F14	*F. fomentarius*	BRNM 840283	OQ474924	OQ474924	OQ514040	OQ514020	OQ514030
F24	*F. fomentarius*	BRNM 840294	OQ474925	OQ474925	OQ514042	OQ514022	OQ514032
F36	*F. fomentarius*	BRNM 840305	OQ474926				
F38	*F. fomentarius*	BRNM 840306	OQ474927				
F40	*F. fomentarius*	BRNM 840307	OQ474928				
F42	*F. fomentarius*	BRNM 840309	OQ474929	OQ474929	OQ514044	OQ514024	OQ514034
F43	*F. fomentarius*	BRNM 840310	OQ474930	OQ474930	OQ514045	OQ514025	OQ514035
F46	*F. fomentarius*	BRNM 840312	OQ474931				
F48	*F. fomentarius*	BRNM 840314	OQ474932	OQ474932	OQ514046	OQ514026	OQ514036
F51	*F. fomentarius*	BRNM 840316	OQ474933				
F03	*F. inzengae*	BRNM 840273	OQ474913				
F04	*F. inzengae*	BRNM 840274	OQ474914	OQ474914	OQ514037	OQ514017	OQ514027
F05	*F. inzengae*	BRNM 840275	OQ474915	OQ474915	OQ514038	OQ514018	OQ514028
F08	*F. inzengae*	BRNM 840278	OQ474916	OQ474916	OQ514039	OQ514019	OQ514029
F10	*F. inzengae*	BRNM 840280	OQ474917				
F16	*F. inzengae*	BRNM 840286	OQ474918				
F18	*F. inzengae*	BRNM 840287	OQ474919				
F20	*F. inzengae*	BRNM 840289	OQ474920	OQ474920	OQ514041	OQ514021	OQ514031
F22	*F. inzengae*	BRNM 840291	OQ474921				
F32	*F. inzengae*	BRNM 840301	OQ474922	OQ474922	OQ514043	OQ514023	OQ514033

The most typical hosts of *F. fomentarius* s.s. are *Fagus* and *Betula*, less common *Acer, Alnus, Picea, and Populus* in Europe (Peintner et al. 2019); *Alnus, Betula, Prunus, Salix,* and *Sorbus* in the Ural regions in Russia and North Kazakhstan (Zhuykova and Mukhin 2022) and *Fagus* and *Quercus* in Armenia (Badalyan et al. 2022). Hosts of *F. inzengae* are *Quercus, Carpinus, Castanea, Cerasus, Platanus, Populus*, and *Abies* in Europe (Peintner et al. 2019), *Acer, Populus, Salix, Tilia* in the Ural regions in Russia and North Kazakhstan (Zhuykova and Mukhin 2022), and *Carpinus, Juglans, Fagus, Populus,* and *Salix* in Armenia (Badalyan et al. 2022). Most of these host genera of *F. inzengae* were also confirmed by our results. Obviously the host spectrum of *Fomes* spp. follows the local diversity of tree species including both native and non-native tree species (e.g. *Aesculus hippocastanum* and *Platanus*×*hispanica* are non-native hosts of *F. inzengae* revealed in this study), so the occurrence of *Fomes* spp. is not determined by specific host species, but by environmental conditions. Surprisingly, *F. inzengae* was also isolated from roots of *Festuca paniculata* in the alpine grassland in France (Mouhamadou et al. 2011), but its possibility to form basidiomes in such a habitat is questionable.

Identification of *F. fomentarius* and *F. inzengae* according to morphological characters of basidiomes is hardly possible. Because the morphological characters such as linear density of pores, spore size, and skeletal hyphae diameter overlap between *F. fomentarius* and *F. inzengae*, these characters can not be used for their reliable identification. Spore size of the *F. inzengae* detected by us do not correspond with those by Peintner et al. (2019), who published values of (9.0) 10–12 (12.5)×(2.8) 3.0–3.5 (3.8) µm, Q = (2.8) 3.0–3.6 (3.7), which are markedly smaller. Our results are closer to the values of *F. fomentarius* s.l. stated by different authors (Ryvarden and Melo 2014; Bernicchia 2005; Niemelä, 2005; Rivoire 2020).

Unfortunately, the only reliable method for identification of the two *Fomes* spp. is the DNA barcoding and ITS region is sufficiently informative to distinguish the species. Nevertheless, the ITS-RFLP analysis proposed by Judova et al. (2012) can help decrease the costs of DNA sequencing.

Our results also revealed that *Globifomes graveolens* seems to be closely related to *F. inzengae*. Although the more comprehensive phylogeny of this American species is needed, results indicate that even though the morphology of its basidiome composed of small petaloid pilei differs from *Fomes* spp., this apomorphic character is not in agreement with phylogenetic position of the species. Another phylogenetic problem that was revealed in *Hexagonia. H. apiaria* is likely to be closely related to *F. fasciatus* than to *H. glabra*. Therefore, a critical revision of the genus including the type species *H. crinigera* Fr. is desirable.

Conclusions

The results of our survey confirm that *Fomes inzengae* and *F. fomentarius* are well delimited species, according to Genealogical Concordance Phylogenetic Species Recognition, and gene flow between them is unlikely. In contrast, morphological characters proposed for the identification of the two species by Peintner et al. (2019) resulted as unreliable. Either basidiospore size, diameter of skeletal hyphae, or linear density of pores can not help to distinguish *Fomes inzengae* from *F. fomentarius*. Nevertheless, the trends of geographic distribution of the two species. in different phytogeographical regions is obvious, although coocurrence can not be excluded in some habitats. In conclusion, for correct identification of *F. inzengae* and *F. fomentarius*, DNA sequencing (ITS region is sufficiently informative) is necessary.

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Author contribution Michal Tomšovský and Tomáš Kudláček contributed to the study conception and design. Material preparation and data collection were performed by Michal Tomšovský and Sirapitcha Kaeochulsri. Data analyses were performed by Tomáš Kudláček and Sirapitcha Kaeochulsri. László Benedek Dálya was responsible for the construction of the maps of distribution. The first draft of the manuscript was written by Michal Tomšovský and all authors commented on the previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability DNA sequence data are freely available in the Genbank. The DNA sequence datasets generated during the study are available as Supplementary information files; the morphometric data are available from the corresponding author on request.

Declarations

Competing interests The authors declare no competing interests.

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