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Research article

The phylogenetic analysis of Armenian collections of medicinal tinder polypore *Fomes fomentarius* (Agaricomycetes, Polyporaceae)

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Abstract
The medicinal tinder polypore *Fomes fomentarius* is widely distributed in all floristic regions of Armenia on different woody substrates. The phylogenetic analysis of Armenian collections using ITS barcoding revealed that it is taxonomically complex species represented by sublineages A2 and B2 (in ratio 1:1) of European phylogenetic lineages A and B, which correspond to two cryptic sympatric species *F. fomentarius sensu stricto* and *F. inzengae*, respectively. These species are phylogenetically almost equidistant from Fomes fasciatus by the level of nucleotide divergence (6.75% and 7.17%, respectively). Nucleotide divergence between these two species is 1.85% which does not exceed the average level of intraspecific ITS variability in basidiomycetes fungi (3.33%). It is suggesting that *F. fomentarius s.s.* and *F. inzengae* are possibly not taxonomically separate species, but sympatric cryptic subspecies of *F. fomentarius sensu lato*. Both taxa significantly differ by their ecology and distribution: *F. fomentarius s.s.* is mainly found on *Betula* spp. trees and widespread in temperate forests, while *F. inzengae* has been recorded on *Carpinus* sp., *Fagus* sp., *Populus* sp. and other deciduous trees in subtropical latitudes. In Armenia, *F. fomentarius s.s.* was found on *Fagus* sp. and *Quercus* sp., while *F. inzengae* - on *Carpinus* sp., *Juglans* sp., *Fagus* sp., *Populus* sp., and *Salix* sp. trees. Although the species rank of Mediterranean subtropical species *F. inzengae* remains disputable it has been originally described for the mycobiota of Armenia.

Keywords
Armenia, cryptic, *Fomes inzengae*, *Fomes fomentarius* sensu stricto, ITS barcoding, polypore, sympatric

Introduction

The tinder polypore *Fomes fomentarius* (L.) Fr. (Polyporaceae, Agaricomycetes) is a very common bracket fungus largely distributed and growing nearly all habitat zones in the forests of Eurasia and North America (Gilbertson and Ryvarden, 1986; Ryvarden and Gilbertson, 1993; Mukhin and Votintseva, 2002). As a parasite, *Fomes fomentarius sensu lato* infects a very large number of deciduous, rarely coniferous trees and continues to habitat them as a white-rot saprotrophic fungus (Větrovský et al., 2011; Pristaš et al., 2013). In Eurasia, this fungus is predominantly reported on the remnants of *Betula*

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sp. and *Fagus* sp. trees (Bondartsev, 1953; Jülich, 1984; Ryvarden and Gilbertson, 1993; Mukhin, 1993; Bondartseva, 1998), while in North America on *Betula* sp. and *Alnus* sp. trees (Gilbertson and Ryvarden, 1986; Farr et al., 1989; McCormick et al., 2013; Gáper and Gáperová, 2014). Wide ecological distribution and high occurrence of *F. fomentarius s.l.* makes it one of the main destructors of deciduous tree debris and, accordingly, CO_2 emitters in forest ecosystems (Mukhin et al., 2021).

Tinder polypore is also regarded as a producer of several pharmacologically active compounds, such as phenolics, flavonoids, polysaccharides, triterpenoids and ketones with antibacterial, antifungal, antiviral, anticancer, immunomodulatory and antioxidant effects (Peintner et al., 1998; Grienke et al., 2014; Badalyan and Shahbazyan, 2015; Badalyan and Gharibyan, 2016; Gáper et al., 2016; Mukhin et al., 2018; Badalyan et al., 2019a). However, there is insufficient fundamental knowledge on species structure and genetic variability, pathways of biosynthesis of their bioactive molecules and genes behind for biotechnological cultivation of medicinal mushrooms, including *F. fomentarius s.l.*, to develop biotech products and mycopharmaceuticals (Gáper et al., 2016; Kües and Badalyan, 2017).

It has been described that *F. fomentarius s.l.* is genetically inhomogeneous species and consists of several phylogenetic lineages (A, B) and sublineages (A1, A2, B1, B2) (Júdová et al., 2012; McCormick et al., 2013; Pristaš et al., 2013; Gáper et al., 2016; Mukhin et al., 2018; Náplavová et al., 2020). Moreover, phylogenetic lineages A and B were suggested as two sympatric cryptic species (Júdová et al., 2012; Pristaš et al., 2013). The area of distribution of sublineage B2 is limited in the European subcontinent (England, Italy, Latvia, Slovakia, and Slovenia) with an Eastern border in the Urals (Mukhin et al., 2018), whereas sublineage A2 has a wider distribution and is found not only in Europe (Gáper et al., 2016; Náplavová et al., 2020), but also in the Urals (border between the European and Asian subcontinents) and Southern Siberia (Altai, Western Sayan and Baikal region) (Mukhin et al., 2018). In Europe, *F. fomentarius s.l.* is considered a complex species, consisting of two cryptic species: *F. fomentarius sensu stricto* and *F. inzengae* (Ces. & De Not.) Cooke (Peintner et al., 2019). Phylogenetic heterogeneity of this species has been also described in China (Gáper et al., 2016; Mukhin et al., 2018; Peintner et al., 2019).

The Armenian highlands is mountainous region of Western Asia, one of biogeographically interesting and important regions of speciation. *F. fomentarius s.l.* is one of the most widespread polypore described in all floristic regions of Armenia (Takhtajyan, 1954) on *Carpinus* sp., *Fagus* sp., *Quercus* sp., and *Populus* sp. (Melik-Khachatryan and Martirosyan, 1971), *Fraxinus* sp., *Junglans regia* L., *Salix alba* L., *Ulmus* sp. trees and other broad leaves substrates (Badalyan and Gharibyan, 2008; 2016; 2017). The current phylogenetic analysis of *F. fomentarius s.l.* in the territory of Armenia has been carried out originally. This paper discusses the species structure and intraspecific genetic variability of Armenian collections of medicinal tinder polypore fungus.

Materials and Methods

Fungal material

This work was performed on 11 dikaryotic strains of *F. fomentarius s.l.* from the fungal culture collection of the Laboratory of Fungal Biology and Biotechnology of the Yerevan State University (FCC-YSU, Yerevan, Armenia) (Badalyan and Gharibyan, 2017). The 11 original strains were isolated from *F. fomentarius s.l.* basidiocarps collected from living and dead deciduous trees (*Carpinus* sp., *Fagus* sp., *Juglans regia*, *Populus* sp., *Quercus* sp., *Salix alba*) in 6 localities of Aparan, Ijevan, and Yerevan floristic regions of Armenia during 2002-2016 (Table 1). The morphological identification and species diagnosis of basidiocarps were performed using traditional taxonomic keys and microscopy (Ryvarden and Gilbertson, 1993) (Fig. 1).

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For molecular studies the cultures were grown on 1.5% malt-extract agar in Petri dishes during 10 days at 25 °C. The mycelial samples with agar were dried at room temperature (22 ± 2 °C) for further usage in DNA isolation and phylogenetic analysis. The published sequences KJ857249 and KJ857248 of strains Ff/1 and Ffa/2 were taken from the GenBank (Badalyan et al., 2015).

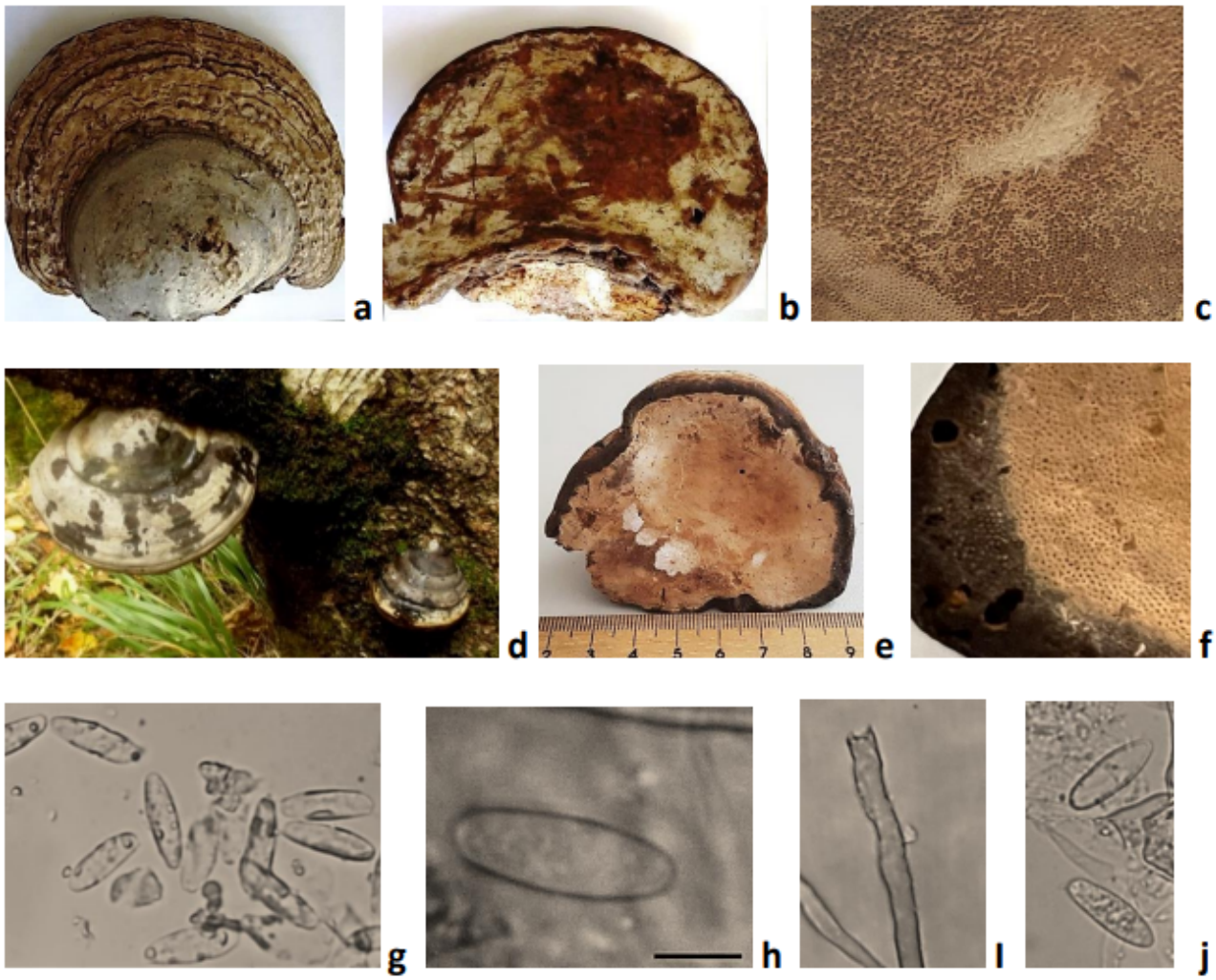

Fig. 1 - *Fomes inzengae:* (a, b) basidiocarps (21×14 cm) in Ff/24 and (c) hymenium in Ff/15, (g) basidiospores in Ff/15 (× 600) and (h) in Ff/26 isolates (Size bar correspond 10 µm). *Fomes fomentarius s.s.*: (d, e) basidiocarps, (f) hymenium, (i) basidium and (j) basidiospores in Ff/18 isolates (× 600).

DNA isolation and PCR amplification

The extraction of DNA and PCR amplification with visualization were performed in the Laboratory of Molecular Research of Plants and Fungi of the Institute of Natural Sciences and Mathematics of the Ural Federal University (Yekaterinburg, Russia). DNA isolation was performed using a DNeasy Plant Mini Kit (QIAGEN) without RNase treatment dissolving DNA in 100 µL Tris-EDTA buffer.

Two primers, ITS1F and ITS4b (Gardes and Bruns, 1993), were used to amplify ITS regions of nuclear ribosomal DNA by PCR which was carried out in the C1000 Touch Amplifier (Bio-Rad Laboratories) in 25 µL reaction mixture containing deionized water, 1× buffer solution with 2.5 µM $MgCl_2$, 0.2 µM dNTP, 0.1 µM of each primer, 0.08 u $µl^{-1}$ Taq DNA polymerase (Evrogen Joint Stock Company) and 5 µl of DNA solution. The PCR program included a pre-denaturation at 95 °C for 5

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min, 35 cycles of a denaturation at 95 °C for 30 sec, an annealing at 55 °C for 45 sec, an elongation at 72 °C for 45 sec, and a final elongation at 72 °C for 10 min. The reactions were monitored by electrophoresis in a 1.2% agarose Tris-borate-EDTA gel stained with ethidium bromide. The length of fragments was monitored by a 100 bp DNA ladder (Invitrogen). The gel was visualized with a Gel Doc XR+ Gel Documentation System (Bio-Rad Laboratories).

The enzymatic purification or purification by PCR products electrophoresis and sequencing in both directions were performed at Sintol LLC (Moscow, Russia). The primary data processing was carried out using Sequencing Analysis Software v. 5.3.1 (Applied Biosystems): Finch TV v. 1.4.0 (Geoprisa, Inc.) and MEGA v. 7.0.18 (Kumar et al., 2016). Identification of sequences was performed with a search for structurally similar sequences in the GenBank database using the Blast algorithm (blast.ncbi.nlm.nih.gov). The obtained sequences were submitted to the GenBank database and accession numbers were received (Table 1).

Table 1 - List of studied dikaryotic collections of *Fomes fomentarius s.l.*

Catalogue number	Isolate	Location/Date of collection	Host tree	GenBank code	Sublineage	Genetically identified species
5201	Ff/1	Yerevan, 2002; YR	*Quercus* sp.	KJ857249	A2	*F. fomentarius s.s.*
5204	Ffa/2	Berdavan, 2011; IJ	*Fagus* sp.	KJ857248	A2	*F. fomentarius s.s.*
5206	Ff/8	Berdavan, 2014; IJ	*J. regia*	OL583665	B2	*F. inzengae*
5207	Ff/11	Berdavan, 2014; IJ	*Fagus* sp.	OL583666	A2	*F. fomentarius s.s.*
5208	Ff/12	Berdavan, 2014; IJ	*Fagus* sp.	OL583667	A2	*F. fomentarius s.s.*
5215	Ff/15	Solak, 2015; AP	*S. alba* stump	OL583668	B2	*F. inzengae*
5216	Ff/16	Solak, 2015; AP	*S. alba* stump	OL583669	B2	*F. inzengae*
5218	Ff/18	Marmarik, 2015; AP	Deciduous tree	OL583670	A2	*F. fomentarius s.s.*
5221	Ff/24	Baghanis, 2016; IJ	*Fagus* sp.	OL583671	B2	*F. inzengae*
5222	Ff/25	Yerevan, 2016; YR	*Populus* sp.	OL583672	B2	*F. inzengae*
5223	Ff/26	Dilijan, 2016; IJ	*Carpinus* sp.	OL583673	B2	*F. inzengae*

(AP) – Aparan, (IJ) – Ijevan, (YR) - Yerevan floristic regions of Armenia (Takhtajyan, 1954)

Phylogenetic and statistical analysis

Two groups of sequences were selected from the GenBank database (www.ncbi.nlm.nih.gov/genbank/) for phylogenetic analysis. The first, reference for *F. fomentarius s.s.* and *F. inzengae* according to Peintner et al. (2019). From the sequences belonging to the *F. fomentarius s.s.* and *F. inzengae*,

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epitypes and isolates close to them were selected. The clade "*Fomes* sp. Asia" and "*F. fomentarius* II" were fully included in the final array. The analysis included a total of 23 ITS sequences. The second group included sequences reference for sublineages A2 and B2. They were generated from 20 ITS1-5.8S-ITS2 sequences according to Gáper et al. (2016) and Mukhin et al. (2018) and deposited in the GenBank. The sequences from Armenia were added to reference groups and aligned using the Muscle Algorithm with manual verification of obtained results. The *Fomes fasciatus* (Sw.) Cooke sequence was selected as an outgroup.

Phylogenetic trees were constructed using the maximum likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985). All positions with site coverage less than 90% were excluded. The trees are drawn to scale with branch lengths in the same units as the evolutionary distances used to derive the phylogenetic tree. The evolutionary analysis was carried out in MEGA7 (Kumar et al., 2016). The database for calculating DNA polymorphism included 11 sequences obtained in this study and 5 *F. fasciatus* sequences (Peintner et al., 2019). The alignment was performed by the Muscle Algorithm with manual verification of results. The number of single nucleotide substitutions, deletions and insertions in pairwise sequence comparisons of both individual lineages and between them was manually calculated. The nucleotide diversity ($\boldsymbol{\pi}$) and divergence (average number of nucleotide substitutions per site between species, D_{xy}) were calculated using the DnaSP v. 6.12 (Rozas et al., 2017).

Results and Discussion

The phylogenetic analysis of Armenian collections showed that 5 out of 11 isolates are included in the cluster with reference sequences for A2, and 6 in the cluster with the reference sequences for sublineages B2 (Fig. 2). Thus, the phylogenetic sublineages A2 and B2 of *F. fomentarius s.l.* are distributed in 1:1 ratio in all studied floristic regions in Armenia.

The Armenian isolates that cluster with sublineage A2 reference sequences are combined at the same time into one group with reference sequences for *F. fomentarius s.s.*, and isolates cluster with sublineage B2 reference sequences are included in the same group with the reference ones for *F. inzengae* (Figs 2–3). Thus, in Armenia both phylogenetic sublineages are represented, described for the tinder fungus in Europe and considered by Peintner et al. (2019) as two cryptic sympatric species: *F. fomentarius s.s.* and *F. inzengae* (Ces. & De Not.) Cooke. They are the very close cryptic species regarding to basidiocarp morphology. However, basidiocarps of *F. inzengae* differ from *F. fomentarius s.s.* by smaller hymenial pores (0.31 mm *vs* 0.36 mm in *F. fomentarius s.s.*), therefore by their large number (31-34 pores / cm *vs* 27-30 pores / cm in *F. fomentarius s.s.*). *Fomes inzengae* also differs by smaller size of basidiospores (9-12.5 × 3-4 µm *vs* 13.5-18 × 4.5-6.5 µm in *F. fomentarius s.s.*) and thicker skeletal hyphae (3.2-6.9 µm) compared to *F. fomentarius s.s*. (3.0-6.4 µm).

F. inzengae has been observed in Great Britain, Italy, Slovakia, Slovenia, Switzerland, France, Iran, and China. However, it is widely distributed in the forests of subtropical latitudes of the Mediterranean region (Peintner et al., 2019). The genus *Fomes* is represented only by *F. inzengae* species in the Iberian Peninsula (Garrido-Benavent et al., 2020). Moreover, 35 out of 36 DNA isolates obtained from *F. fomentarius s.l.* basidiocarps collected in Greece, Italy, Spain, and Portugal belong to the phylogenetic lineage B (*F. inzengae*), and only one from Northern Greece – to sublineage A2 (*F. fomentarius s.s.*) (Náplavová et al., 2020).

Previous studies have shown that all *Fomes* strains grow well at temperatures 25-30 °C and do not show any significant differences, particularly at 25 °C. However, *F. inzengae* (sublineage B2) have more than 30 °C optimal growth temperature range and grew significantly faster at higher temperature, whereas mycelia of *F. fomentarius s.s.* (sublineage A2) a fungus of northern, temperate

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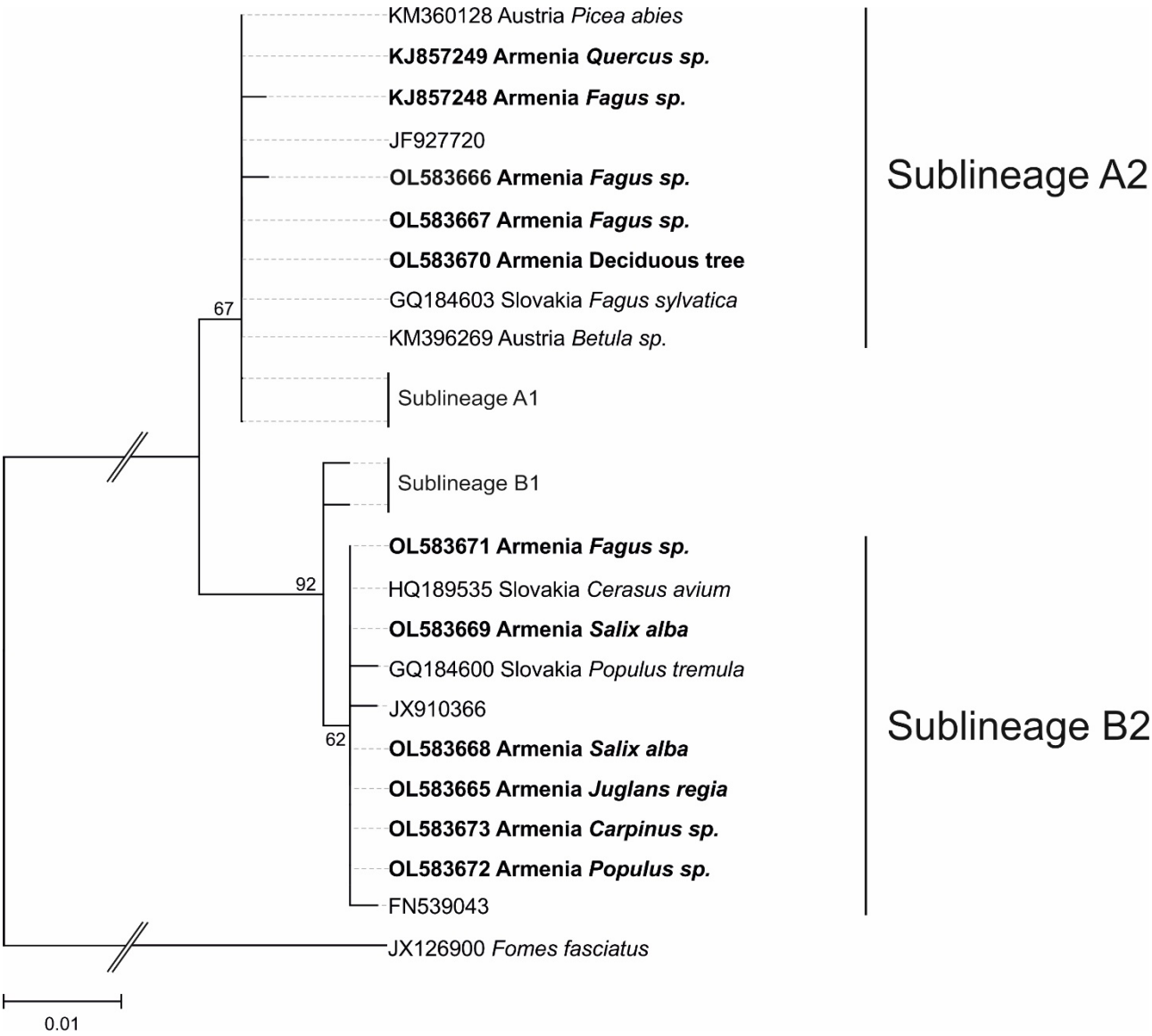

Fig. 2 - Phylogenetic tree of *Fomes fomentarius s.l.* isolates from Armenia and reference for phylogenetic sublineages A2 and B2 based on 521 positions of ITS sequences. The bootstrap values are greater than 50% next to the nodes.

latitudes, grow faster at lower temperatures (10 and 20 °C) (Peintner et al., 2019). The southern distribution of *F. inzengae* and its adaptation to warm and dry climate conditions are also evidenced by substrate spectrum (*Carpinus* sp., *Castanea* sp., *Quercus* sp., *Platanus* sp., *Populus* sp., and rarely *Cerasium* sp. and *Abies* sp. trees) and form of basidiocarps with increased water absorption ability from the air (Peintner et al., 2019). A distinctive feature of substrate spectrum of *F. fomentarius s.s.* is the presence of *Betula* sp. tree which is absent in the substrate spectrum of *F. inzengae* (Mukhin et al., 2018). In Europe, the southern boundary of *F. fomentarius s.s.* (sublineage A2) distribution is possibly determined by the corresponding distribution boundary of *Betula pendula* Roth (Náplavová et al., 2020). The characteristics of substrate spectra and preferences provided by *F. fomentarius s.s.* and *F. inzengae* are the possibility of sympatric existence where their ranges overlap. In most locations of Central Europe, phylogenetic sublineages A2 (*F. fomentarius s.s.)* and B2 (*F. inzengae*) occur simultaneously on substrates different to sympatric species (Júdová et al., 2012; Peintner et al., 2019).

According to our data, these two taxa are similarly distributed in Aparan, Ijevan and Yerevan floristic regions of Armenia. The studied collections, identified as *F. inzengae*, have been isolated

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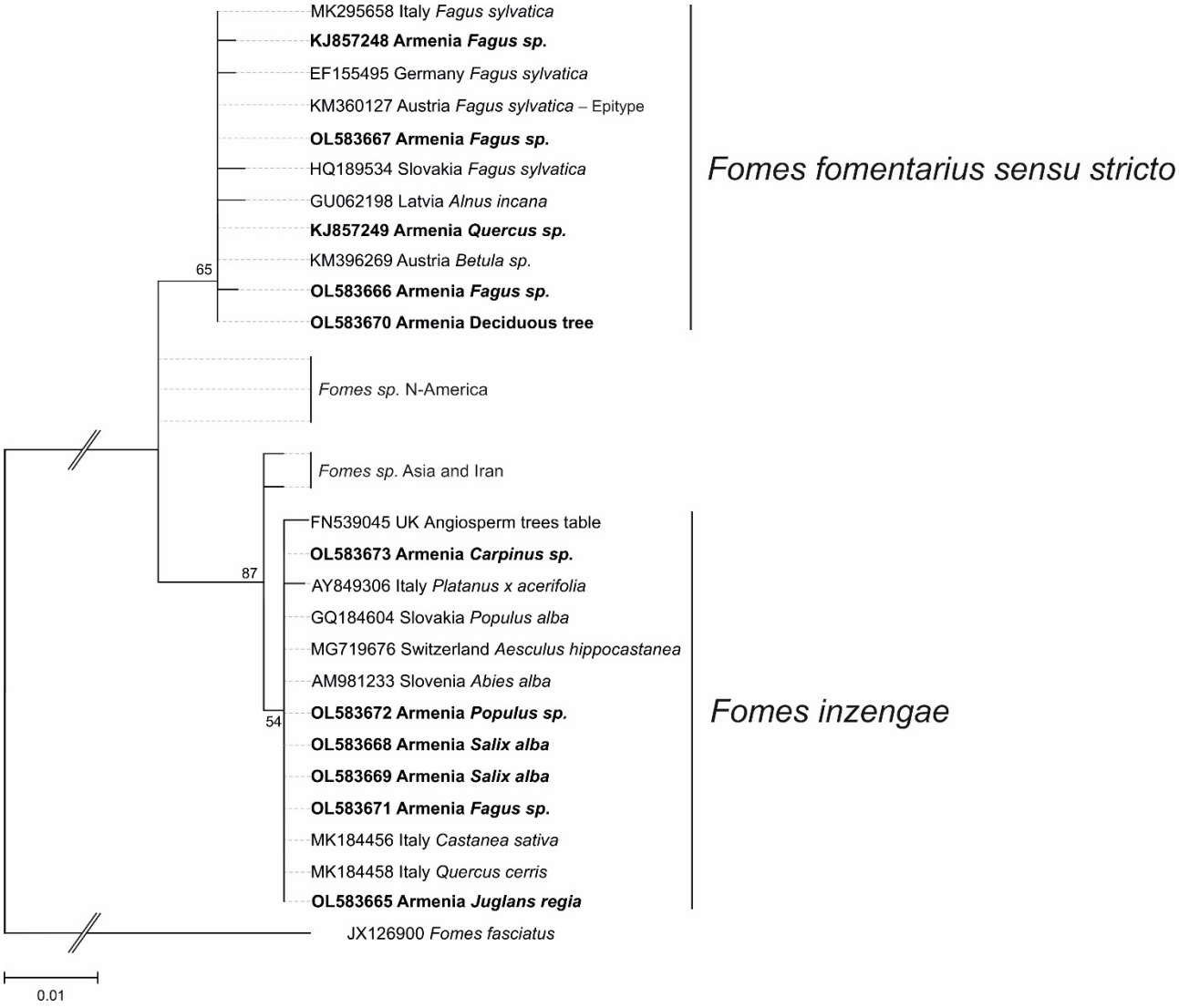

Fig. 3 - Phylogenetic tree of *Fomes fomentarius s.l.* isolates from Armenia and reference for *F. fomentarius s.s.* and *F. inzengae* based on 516 positions of ITS sequences. The bootstrap values are above 50% next to the nodes.

from basidiocarps growing on *Carpinus* sp., *Juglans* sp., *Fagus* sp., *Populus* sp., and *Salix* sp. trees, while *F. fomentarius s.s.* on *Fagus* sp. – one of the host trees in Europe (Peintner et al., 2019) and *Quercus* sp. (Table 1).

The sympatric and cryptic characters of *F. fomentarius s.s.* and *F. inzengae* are beyond doubt, but their species rank, in our opinion, is disputable. The phylogenetic divergences between Armenian isolates of *F. fomentarius s.s.* and *F. inzengae* species and *F. fasciatus* species constitute 35-37 bp (6.75%) and 36-38 bp (7.17%), respectively. The genetic divergence between *F. fomentarius s.s.* and *F. inzengae* is significantly smaller (9-11 bp or 1.85% of nucleotide substitutions per site on average) which is significantly higher compared to individual differences in the nucleotide diversity of *F. fomentarius s.s.* and *F. inzengae* from Armenia (0-0.158%) but does not exceed the average level of intraspecific ITS variability for basidiomycetes fungi (3.33%) (Nilsson et al., 2008).

Similar data were reported by Dresch et al. (2015) and Peintner et al. (2019). The authors of this study have revealed genetic differences between *F. fasciatus*, *F. fomentarius s.s.* and *F. inzengae* in Europe is 41-62 bp, between *F. fomentarius s.s.* and *F. inzengae* is 9-18 bp, and between individual strains of *F. inzengae* and *F. fomentarius s.s.* are 0-1 bp and 0-3 bp, respectively. In our opinion, these

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data show the absence of phylogenetic differences between Armenian isolates of *F. fomentarius s.s.* and *F. inzengae* at the species level and, may therefore, represent cryptic subspecies of one taxonomic species *F. fomentarius s.l.*

The cultural studies of dikaryotic Armenian collections support data of current phylogenetic analysis (Badalyan et al., 2019b). The results showed that *F. fomentarius s.s.* strains grow in up to 35 °C (only on inoculum) with an optimum growth temperature 25 °C (3.0–4.4 mm d^{-1} and 2.1–4.2 mm d^{-1} on MEA - Malt Extract Agar - and PDA - Potato Dextrose Agar -, respectively), meanwhile *F. inzengae* strains tolerate temperature 35 ºC and above (up to 40 °C, only on inoculum) with optimum 30 °C and higher growth rate (4.8-6.0 mm d^{-1} and 4.0-5.3 mm d^{-1}, on MEA and PDA, respectively) (Badalyan et al., 2019b). This corresponds to similar data reported by Peintner et al. (2019), according to which the highest growth rate of *F. inzengae* isolates was 5.50 mm d^{-1} and of *F. fomentarius s.s.* – 4.25 mm d^{-1} on 3% MEA at 30 °C. Peintner et al. (2019) have also shown that all *Fomes* sp. strains grow well at temperatures 25-30 °C and do not show any significant differences, particularly at 25 °C. However, *F. inzengae* (sublineage B2) have more than 30 °C optimal growth temperature range and grew significantly faster at higher temperature, whereas mycelia of *F. fomentarius s.s.* (sublineage A2) a fungus of northern, temperate latitudes, grow faster at lower temperatures (10 and 20 °C). The morphological differences between colonies of *F. inzengae* and *F. fomentarius s.s.* have also been revealed. Asexual chlamydospores were described in cultures of both species, while oidia – mainly in *F. fomentarius s.s.* strains (Badalyan et al., 2019a). Further morphological observation of Armenian *Fomes* sp. collections is warranted.

Conclusion

The tinder polypore *F. fomentarius* (L.) Fr. in the territory of Armenia was described as a taxonomically complex species represented by two cryptic sympatric phylogenetic sublineages (A2 and B2) corresponding to two species: *F. fomentarius s.s.* and *F. inzengae*. *F. inzengae* is a Mediterranean, subtropical species which was originally described for the mycobiota of Armenia although its species rank is remaining disputable. Both taxa *F. inzengae* and *F. fomentarius s.s.* could be possibly represented as cryptic sympatric subspecies evolved from *F. fomentarius s.l.*

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