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A new host record of *Allocryptovalsa castaneae* (Diatrypaceae) in *Aquilaria* from China

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Abstract

In a microfungi survey conducted in Yunnan Province, China, decaying branches of *Aquilaria* sp. (Thymeleaceae) with fungal fruiting bodies were collected. Based on morphology combined with DNA sequence data showed that our new collection belongs to *Allocryptovalsa* in Diatrypaceae, Xylariales, Sordariomycetes. Maximum likelihood and Bayesian analyses (combining ITS and TUB genes) were conducted to elucidate the phylogenetic placement of our new collection, which was confirmed as a new host record of *Allocryptovalsa castanae*. This study provides a comprehensive description, photo plate, morphological comparison, and an updated phylogenetic tree.

Key words – Diatrypaceous fungi, Diatrypales, Saprophytic fungi, Thymeleaceae, Yunnan Province

Introduction

Sordariomycetes O.E. Erikss. & Winka is one of the largest classes of Ascomycota Caval.-Sm., which comprises a highly diverse range of fungi mainly characterized by non-lichenized, flask-shaped fruiting bodies or less frequently cleistothecial ascomata and unitunicate asci (Zhang et al. 2006, Maharachchikumbura et al. 2016, Hyde et al. 2020). In Sordariomycetes, there are seven subclasses *viz.* Diaporthomycetidae Senan., Maharachch. & K.D. Hyde, Hypocreomycetidae O.E. Erikss. & Winka, Lulworthiomycetidae Dayar., E.B.G. Jones & K.D. Hyde, Pisorisporiomycetidae

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Bundhun, Maharachch. & K.D. Hyde, Savoryellomycetidae Hongsanan, K.D. Hyde & Maharachch., Sordariomycetidae O.E. Erikss & Winka, and Xylariomycetidae O.E. Erikss & Winka (Hyde et al. 2020).

Xylariales Nannf. is a large order in the subclass Xylariomycetidae O.E. Erikss. & Winka, which was accepted by Maharachchikumbura et al. (2016). According to Wijayawardene et al. (2022), currently, 20 families are listed in this order.

Diatrypaceae Nitschke was introduced by Nitschke (1870) with *Diatrype* Fr. as the type genus that belongs to Xylariales (Maharachchikumbura et al. 2016). Currently, 26 genera and more than 1,500 species are accepted in this family (Li et al. 2023). This family can mostly be found in terrestrial habitats and can also be found in marine habitats (Hyde & Rappaz 1993, Chalkley et al. 2010, Abdel-Wahab et al. 2014, Jones et al. 2015, Dayarathne et al. 2016, Li et al. 2016, Du et al. 2022). Members of this family are common worldwide, typically occurring as saprobes, pathogens, or endophytes (Acero et al. 2004, Trouillas & Gubler 2004, 2010, Trouillas et al. 2010, 2011, Grassi et al. 2014, Paolinelli-Alfonso et al. 2015, Du et al. 2022, Li et al. 2023). Diatrypaceae has allantosporous taxa, which possess allantoid ascospores (Li et al. 2023). The sexual morph is characterized by having perithecial ascomata, ostiolate with short to long necks, and allantoid ascospores (Trouillas et al. 2010, Mehrabi et al. 2015, Dayarathne et al. 2016, De Almeida et al. 2016, Li et al. 2016). Senanayake et al. (2015) summarized that the asexual morph of this family had acervular and astromatic conidiomata, branched conidiophores, and filiform, allantoid or rarely straight conidia with flattened base and blunt apex. However, in most cases, it is difficult to differentiate diatrypaceous species based on asexual morph (Glawe and Rogers 1986, De Almeida et al. 2016). In addition, because most of the characteristics overlap among the genus, Diatrypaceae species identification based only on morphological characteristics is difficult (Glawe & Rogers 1984, Rappaz 1987, Mehrabi et al. 2016). Thus, morphology together with the DNA sequence data of ITS and TUB are used for the identification of Diatrypaceae species (Acero et al. 2004, Trouillas & Gubler 2010, Trouillas et al. 2011, Dayarathne et al. 2016, De Almeida et al. 2016, Shang et al. 2017).

Allocryptovalsa Senwanna, Phookamsak & K.D. Hyde was introduced by Senwanna et al. (2017) with the type species *A. polyspora* Senwanna, Phookamsak & K.D. Hyde collected on a dead twig of *Hevea brasiliensis* (Willd. ex A. Juss.) Muell. Arg. from Thailand. Eleven species have been recorded in Index Fungorum (2024). Most species in *Allocryptovalsa* are saprobes on dead wood, dead petiole, or lignified canes on the ground (Senanayake et al. 2023), reported in *Acer palmatum* Thunb., *Aquilaria sinensis* (Lour.) Spreng., *Castanea mollissima* Blume, *Elaeis guineensis* Jacq., *Ficus carica* L., *Hevea brasiliensis*, *Sambuscus nigra* L., and *Vitis vinifera* L. from America, Australia, China, India and Thailand (Chethana et al. 2023). The general features of this genus are immersed stromata, ostiolar with periphyses, unbranched, septate paraphyses, polysporous asci, and oblong to allantoid ascospores, while, the asexual morph was reported with hyaline, elongate-allantoid conidia (Senwanna et al. 2017, Zhu et al. 2021).

In this paper, our fungal collection is introduced as a new host record for *Allocryptovalsa castaneae* from Yunnan, China. We provide a full description, a photo plate of micromorphological characteristics, and phylogenetic analysis.

Materials & Methods

Sample collection, morphological observation, and isolation

The decaying branches of *Aquilaria* sp. with fungal fruiting bodies were collected from Nujiang, Yunnan Province, China. The specimens were packed in plastic sealed bags with collection details such as collector, collection date, host, and location (Rathnayaka et al. 2024), and taken to the mycological laboratory at Qujing Normal University (Tian et al. 2024). The fruiting bodies on the host surface were observed by OPTEC SZ650 dissecting stereomicroscope. Freehand sections of fruiting bodies were done by razor blade, and thin pieces of fungal structures (ascomata, ostiole, peridium, asci, and ascospores) were mounted onto slides in water and observed under an OLYMPUS compound microscope (Tokyo, Japan) and were photographed by the OLYMPUS DP74 (Tokyo, Japan) digital camera fitted to the compound microscope. The photo plate was processed using Adobe Photoshop CS3 Extended version 10.0 software (Adobe Systems, USA), and the size of micro-structures were measured by the Tarosoft ® Image Frame Work (v.0.9.0.7) program.

Single spore isolation was carried out according to the method described by Senanayake et al. (2020). The germinated ascospores were transferred into a fresh potato dextrose agar (PDA) (OXOID, UK) and incubated at 28 °C for two weeks. The cultural characteristics, including color, shape, and size were recorded. The newly obtained cultures were stored in two tubes of 1 mL sterile water, two tubes of 1 mL PDA, stored at room temperature (20–25 °C) in the dark, and two tubes of 1 mL 10% glycerol stored at –80 °C. The specimen was deposited at the Herbarium of Guizhou Medical University, Guiyang, China (GMB-W), and the living culture was deposited at Guizhou Medical University Culture Collection (GMBCC).

DNA extraction, PCR amplification and sequencing

The sterile scalpel was used to scrape the fresh mycelia cultured in PDA at 28 ℃ for two weeks, and the mycelia were stored in 1.5 mL sterile Eppendorf tubes. DNA extraction was performed using the Biospin Fungus Genomic DNA Extraction Kit-BSC14M1 (BioFlux®, P.R. China), following the manufacturer's guidelines. The reference DNA was stored at −20 °C for long-term storage. Primer pairs ITS4/ITS5 (White et al., 1990) and T1/Bt2b (Glass and Donaldson 1995, O'Donnell and Cigelnik 1997) were used to amplify Internal transcribed spacer (ITS) and beta-tubulin (TUB) sequences, respectively. The ITS and TUB loci amplifications were performed by polymerase chain reaction (PCR) according to the conditions of Table 1. The total volume of the polymerase chain reaction (PCR) was 25 µL, including 12.5 µL of 2 × Power Taq PCR MasterMix (mixture of Easy Taq TM DNA Polymerase, dNTPs, and optimized buffer, Beijing Bomaide Biotechnology Co., Ltd., Haidian District, Beijing, China), 8.5 µL of double-distilled water (ddH_2O), 1 µL of each forward primers and reverse primers, and 2 µL of DNA template. Purification and sequencing of PCR

products were carried out at Sangon Biotech Shanghai (Co., Ltd.) in Shanghai, P.R. China. The newly generated nucleotide sequences data were submitted to GenBank (https://www.ncbi.nlm.nih.gov) and accession numbers were obtained.

Table 1. Details of genes/loci with PCR primers and PCR conditions.

Genes/loci	PCR primers (forward/ reverse)	PCR conditions	References
ITS	ITS5/ITS4	Initiation step of 95 °C: 3 min; 95 °C: 30 s, 55 °C: 50 s, 72 °C: 30 s (35 cycles); final elongation step of 72 °C: 10 min and final hold at 4 °C	White et al. (1990)
TUB	T1/Bt2b	Initiation step of 95 °C: 3 min; 94 °C: 1 min, 55 °C: 50 s, 72 °C: 1 min (35 cycles); final elongation step of 72 °C: 10 min and final hold at 4 °C	Glass & Donaldson (1995), O'Donnell & Cigelnik (1997)

Phylogenetic analyses

The representative species used in phylogenetic analyses were selected based on previous publications (Trouillas et al. 2011, Konta et al. 2020, Zhu et al. 2021, Chethana et al. 2023). Forward and reverse raw sequences were assembled by the Genious program (9.1.8) (Kearse et al. 2012) (https://www.geneious.com/), and initial identifications were performed by using a standard BLASTn search in GenBank. Sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/), and their accession numbers were listed in Table 2. The single gene sequence matrix was aligned by MAFFT v. 7 (Katoh & Standley 2013) (https://mafft.cbrc.jp/alignment/server). The ambiguous regions were removed by trimAl.v1.2rev59 (Capella-Gutiérrez et al. 2009), and manually improved in BioEdit v. 7.0 whenever necessary, and then combined by using BioEdit v. 7.0. Multiple aligned sequences were manually combined by BioEdit v. 7.0. (Hall 2004). Finally, the combined sequences were converted in ALTER (http://www.sing-group.org/ALTER/) from FASTA to PHYLIP or NEXUS format (Glez-Peña et al. 2010). Phylogenetic analyses were conducted on the CIPRES Science Gateway platform (https://www.phylo.org/portal2/ home. action), using the tool RAxML-HPC v.8 on XSEDE (8.2.12) for maximum likelihood (ML) and MrBayes on XSEDE (3.2.7a) for Bayesian inference (BI). Maximum likelihood analysis was done using the GTR + I + G model with 1,000 bootstrap repetitions. Bayesian analyses were performed by six simultaneous Markov chains that were run for 5,000,000 generations, sampling every 100^{th} generation, ending the run automatically when the standard deviation of split frequencies dropped below 0.01 with a burn-in fraction of 0.25. Phylograms were visualized with the FigTree v1.4.0 program (Rambaut 2014) and reorganized in Microsoft PowerPoint (2021).

Table 2. Names, strains, GenBank accession numbers, host/substrates, and origins of the taxa used in this study.

Species	**Strains**	**GenBank accession numbers**		**Hosts/Substrates**	**Origins**	**References**
		ITS	**TUB**			
Allocryptovalsa aquilariae	KUNCC 22-10819^{T}	OP454035	OP572197	*Aquilaria sinensis*	China	Chethana et al. 2023
Allocryptovalsa aquilariae	KUNCC 22-12389	OP456373	OP572198	*Aquilaria sinensis*	China	Chethana et al. 2023
Allocryptovalsa aceris	HKAS 121126^{T}	MZ727001	OK043823	*Acer palmatum*	China	Senanayake et al. 2023
Allocryptovalsa castanea	CFCC 52427	MW632944	NA	*Juglans regia*	China	Zhu et al. 2021
Allocryptovalsa castanea	CFCC 52428^{T}	MW632945	NA	*Castanea mollissima*	China	Zhu et al. 2021
Allocryptovalsa castanea	CFCC 52429	MW632946	NA	*Castanea mollissima*	China	Zhu et al. 2021
Allocryptovalsa castanea	**GMBCC1072**	**PQ137081**	**PQ001358**	***Aquilaria* sp.**	**China**	**This study**
Allocryptovalsa castanea	**GMBCC1072**	**PQ137081**	**PQ001358**	***Aquilaria* sp.**	**China**	**This study**
Allocryptovalsa castanea	**GMBCC1072**	**PQ137081**	**PQ001358**	***Aquilaria* sp.**	**China**	**This study**
Allocryptovalsa castaneicola	CFCC 52432^{T}	MW632947	NA	*Castanea mollissima*	China	Zhu et al. 2021
Allocryptovalsa cryptovalsoidea	HVFIG02^{T}	HQ692573	HQ692524	*Ficus carica*	Australia	Trouillas et al. 2011
Allocryptovalsa cryptovalsoidea	HVFIG05	HQ692574	HQ692525	*Ficus carica*	Australia	Trouillas et al. 2011
Allocryptovalsa elaeidis	MFLUCC 15-0707^{T}	MN308410	MN340296	*Elaeis guineensis*	Thailand	Konta et al. 2020
Allocryptovalsa polyspora	MFLUCC 17-0364^{T}	MF959500	MG334556	*Hevea brasiliensis*	Thailand	Senwanna et al. 2017
Allocryptovalsa rabenhorstii	WA07CO	HQ692620	HQ692522	*Vitis vinifera*	Australia	Trouillas et al. 2011
Allocryptovalsa rabenhorstii	WA08CB	HQ692619	HQ692523	*Vitis vinifera*	Australia	Trouillas et al. 2011
Allocryptovalsa sichuanensis	HKAS 107017	MW240633	MW775592	NA	NA	Samarakoon et al. 2022
Allocryptovalsa truncata	NFCCI-4520^{T}	MK990279	NA	NA	India	Hyde et al. 2020
Allocryptovalsa xishuangbanica	KUMCC 21-0829	ON041131	ON081501	NA	NA	Maharachchikumbura et al. 2022
Allocryptovalsa xishuangbanica	KUMCC 21-0830^{T}	ON041128	ON081498	NA	NA	Maharachchikumbura et al. 2022

Cryptovalsa ampelina	A001	GQ293901	GQ293972	NA	Australia	NA
Cryptovalsa ampelina	DRO101	GQ293902	GQ293982	NA	USA	NA
Eutypella australiensis	CNP03^{T}	HM581945	HQ692479	*Acacia longifolia* subsp. *sophore*	Australia	Trouillas et al. 2010
Eutypella cearensis	HUEFS 131070	KM396639	NA	Unidentified plant	Brazil	Almeida et al. 2016
Eutypella cerviculata	CBS 221.87	AJ302468	NA	*Alnus glutinosa*	Switzerland	NA
Eutypella citricola	CFCC 52433	MW632948	MW656396	*Morus alba*	China	NA
Eutypella leprosa	STEU 8190	MF359638	MF359673	NA	South Africa	NA
Eutypella microtheca	CBS 128337	MH864886	NA	*Citrus paradisi*	Australia	Trouillas et al. 2011
Eutypella microtheca	UCRDC67	KF620386	KF620421	NA	NA	Trouillas et al. 2011
Eutypella persica	IRAN 2540C^{T}	KX828144	NA	*Alnus* sp.	Iran	Mehrabi et al. 2019
Eutypella quercina	IRAN 2543C^{T}	KX828139	NA	*Quercus* sp.	Iran	Mehrabi et al. 2019
Eutypella semicircularis	MP4669	JQ517314	NA	*Alnus acuminata*	Panama	Chacón et al. 2013
Eutypella vitis	UCD2291AR	HQ288224	HQ288303	*Vitis vinifera*	USA	NA

Note: Newly generated sequences are in bold; type species are denoted with the superscript "^{T}". NA = Not available.

Results

Phylogenetic analyses

Phylogram generated from maximum likelihood analysis based on combined ITS and TUB sequence data. Thirty-one taxa were included in the combined analyses, which comprised 1650 characters (ITS = 557 bp, TUB = 1093 bp) after alignment. The best-scoring RAxML tree with a final likelihood value of -5660.865529 is presented. The matrix had 442 distinct alignment patterns, with 51.47% of undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.230548, C = 0.266056, G = 0.233167, T = 0.270229; substitution rates: AC = 0.918975, AG = 3.893505, AT = 1.877094, CG = 0.847580, CT = 5.357519, GT = 1.000000; gamma distribution shape parameter α = 1.760228.

NCBI BLASTn searches of our collection showed 99.80% similarity to *Allocryptovalsa castaneae* (MW632945.1) in the ITS sequence, and 99.72% similarity to *A. sichuanensis* (MW775592.1) in the TUB sequence. The final RAxML is similar to the phylogenetic trees in a recently published article (Chethana et al. 2023) (Fig. 1) inferred from ITS and TUB sequences also demonstrated that our new strain was nested together with strains of *A. castaneae* (CFCC 52429, CFCC 52428 and CFCC 52427). Therefore, the new isolate is identified as a new strain of *A. castaneae*. Strains of *A. castaneae* (GMBCC1072) were sister to *A. sichuanensis* (HKAS 107017) with 96% ML and 1.00 BYPP statistical support.

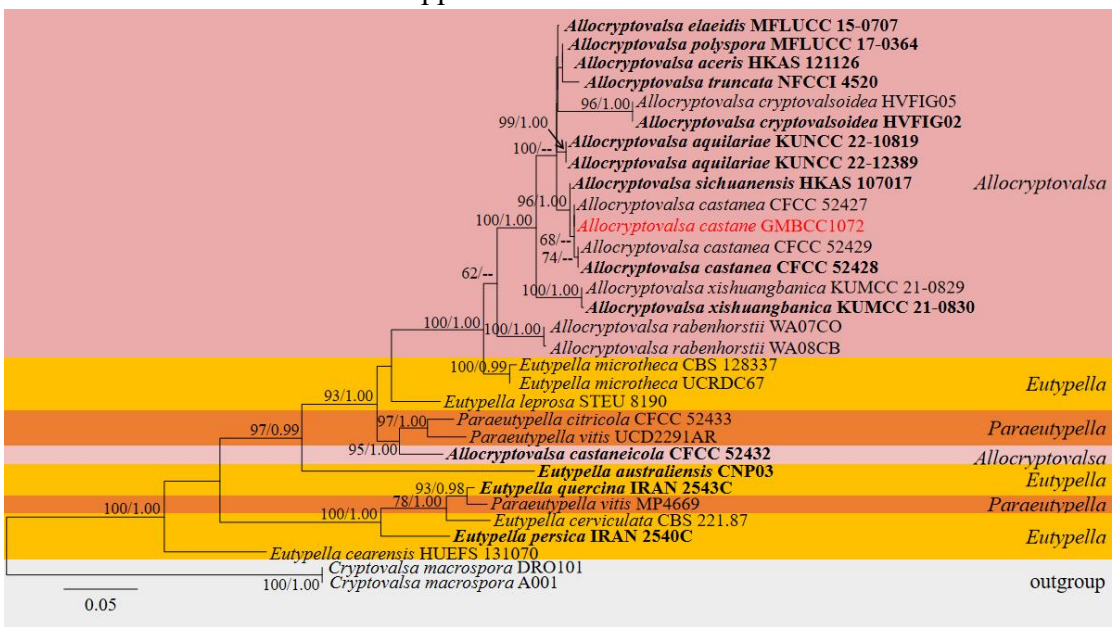

Figure 1. Maximum likelihood phylogenetic tree generated from the combined ITS and TUB loci sequence data. Maximum likelihood bootstrap values equal to or greater than 60% and BYPP equal to or greater than 0.90 were given above each node of the phylogenetic tree. New strains are marked red, while ex-type strains are in bold.

Taxonomy

Diatrypaceae Nitschke (1869)

Allocryptovalsa Senwanna, Phookamsak & K.D. Hyde (2017)

Allocryptovalsa castaneae N. Jiang & X.L. Fan (2021), **Fig. 2**

Index Fungorum number: IF837777; *Facesoffungi number*: FoF 14960

Saprobic on dead branches of *Aquilaria* sp. **Sexual morph:** *Ascostromata* solitary to gregarious, immersed in the bark, erumpent through the surface of the bark, with 3–8 perithecia arranged irregularly. *Ascomata* (excluding neck) 355–390 µm high × 400–440 µm diam. ($\bar{x}$ = 368 × 413 µm, n = 5), gregarious, immersed, subglobose, brown to dark brown, not wrapped in white entostroma. *Ostiolar canal* 160–195 µm high × 125–150 µm diam. ($\bar{x}$ = 171 × 135 µm, n = 5), central, not protrude or protrude a little outside from the substrate, near cylindrical, the upper part is wider than the lower part, straight, dark brown to black, with periphyses. *Peridium* 25–50 µm wide, composed of two type layers, outer layer is comprising several layers of thick-walled, dark brown to black cells of *textura angularis* to *textura prismatica*, which are not fully fused with the host tissue, inner layer comprising 3–5 layers of thin-walled, hyaline cells of *textura angularis* to *textura prismatica*. *Hamathecium* 2–4 µm wide, hyaline, with granulate, filamentous paraphyses, branched, septate, slightly constricted at the septa, tapering towards the apex. *Asci* 105–160 × 15–25 µm ($\bar{x}$ = 140 × 20 µm, n = 30), spore-bearing part length (35–)60–95(–110) µm ($\bar{x}$ = 79 µm, n = 30), polysporous, clavate to elongate obovoid, thin-walled, with 10–50 µm pedicels, apically rounded, narrowing towards lower region. *Ascospores* 15–20 × 4–6 µm ($\bar{x}$ = 18 × 5 µm, n = 30), crowded, oblong to allantoid, hyaline when immature, turning pale yellowish at maturity, aseptate, thin-walled slightly curved, smooth-walled, granules, without appendages and mucilaginous sheath. **Asexual morph:** Undetermined.

Culture characteristics – Ascospores germinated on PDA within 24 h at 28 ℃ and germ tubes were produced from both ends. Colonies on PDA reached 6 cm diam. after two weeks at 28 ℃. Colonies obverse: flat, circular, flossy, filiform margin, while white to cream, smooth in reverse.

Material examined – CHINA, Yunnan Province, Nujiang, Lushui City, agarwood plantation, on dead branches of *Aquilaria* sp. (Thymelaeaceae), 21 April 2023, T.Y. Du, NJZ21, (GMB-W1072, new host record), living culture, GMBCC1072.

Known hosts – *Aquilaria* sp. (This study), *Castanea mollissima*, and *Juglans regia* (Zhu et al. 2021).

Known distribution – China (Zhu et al. 2021, this study).

Notes – According to the phylogenetic analyses, our fungal collection (GMBCC1072) clustered with *Allocryptovalsa castanea* strains (CFCC 52427, CFCC 52428 and CFCC 52429) (Fig. 1). Our fungal collection shares similar morphology to *A. castanea* (BJFU CF2020518, holotype) in asci and ascospores, but a bit different by our new collection has lighter ascospores in color than *A. castanea* (BJFU CF2020518, holotype) (Zhu et al. 2021). This study is the first to find *A. castanea* species in the plant genus *Aquilaria*; therefore, our fungal collection is introduced here as a new host record of *A. castanea* based on morphological and phylogenetic analyses.

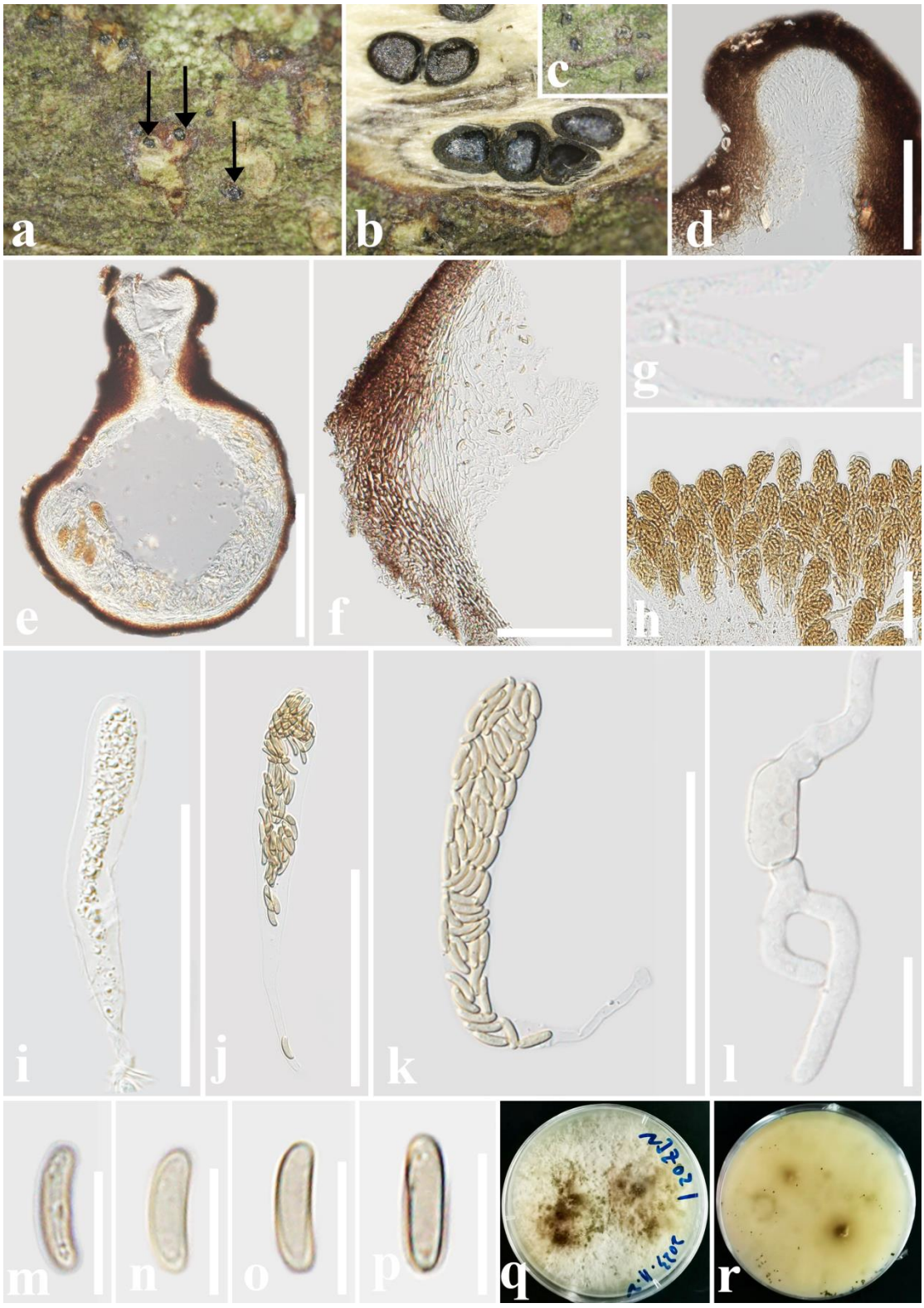

Figure 2. ***Allocryptovalsa castaneae*** (GMB-W1072). a–c Appearance of ascomata on the host (the arrows indicate the ascomata). d Ostiole. e The longitudinal section of an ascoma. f Peridium. g Paraphyses. h–k Asci. l A germinated ascospore. m–p Ascospores. q, r Culture characteristic on PDA after two weeks. Scale bars: d, f, j, k = 100 µm, e = 200 µm, i, h = 50 µm, l = 20 µm, m–p, g = 10 µm.

Discussion

Phylogenetic analyses showed that our new isolate clustered with *Allocryptovalsa castaneae* strains (CFCC 52427, CFCC 52428, and CFCC 52429) (Fig. 1). Moreover, nucleotide pairwise comparison between the ex-type strain of *A. castaneae* (CFCC 52428) and our new isolate (GMBCC1072) was identical in TUB and only one gap difference in ITS. Therefore, the new isolate is identified as *A. castaneae*, and this is the first record of *A. castaneae* from *Aquilaria* species. In contrast, *A. castaneae* has been reported from *Castanea mollissima* and *Juglans regia* in China as saprobic species previously (Zhu et al. 2021).

The generic status of the genera in Diatrypaceae has been unstable; many Diatrypaceae species were transferred from one genus to another (Konta et al. 2020). Moreover, the current molecular phylogenetic study of Diatrypaceae only uses ITS and TUB gene sequences, which do not distinguish this family well. Hence, we believe that other primers or more suitable primers are needed for Diatrypaceae to support phylogenetic study in the future. Furthermore, most species in Diatrypaceae lack DNA sequences in the NCBI database. Hence, new and known species materials should be collected, and their DNA sequences should be obtained to support their placement.

Aquilaria is a plant of the Thymeleaceae that can produce expensive resin - agarwood. There is relatively little research on saprophytic fungi of *Aquilaria* (Du et al. 2024). At present, there are only 11 species in the genus *Allocryptovalsa*, and two species *viz. A. aquilariae* T.Y. Du & Tibpromma (Chethana et al. 2023) and *A. rabenhorstii* (Nitschke) C. Senwanna, Phookamsak & K.D. Hyde have been discovered on *Aquilaria* (Hyde et al. 2024). The new record introduced in this study is the third species associated with *Aquilaria*.

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