

Wawelia amyloasca, sp. nov., – the only species of *Wawelia* (Xylariaceae) discovered in the field

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The new species *Wawelia amyloasca* is described from the dung of Siberian roe deer in the Russian Far East. This is the first published record of *Wawelia* for more than 20 years, the only record outside Europe, and the only discovery made in the field, even though the genus was described in 1908. The new taxon is characterized by the longest stromata in the genus reaching 120 mm, ascospores with polar pad-like appendages, and asci with a well-noticeable amyloid apical apparatus, which is unique within the genus. *Wawelia* is accommodated within the family Xylariaceae together with other coprophilous genera, which was proved by the phylogenetic analysis using four markers, ITS, LSU, BenA, and RPB2, the latter two being obtained for the first time for the genus. A complete bibliography is given and a comparative table including all known species of *Wawelia* is presented.

Key words: apical apparatus, Ascomycota, coprophilous fungi, molecular phylogeny, rare species. – 1 new species.

In 1908, B. Namysłowski cultivated coprophilous fungi on rabbit dung using moist chamber cultures in the Botanic Garden of the Jagiellonian University (Kraków, Poland). He was supplied with this substrate by one of the garden workers, who lived on the Wawel hill, where the Royal Castle was located, and raised rabbits there. Namysłowski found a curious fungus from a previously unknown genus and described it as *Wawelia regia* Namysl. (Namysłowski 1908). The scientist included the genus in the subfamily “Waweliaceae” in the family “Hypocreales”. Later F. Vincens studied the type material of *W. regia* and supposed that this genus was related to Xylariaceae rather than Hypocreaceae, which was based on the presence of an ascospore germ slit, a feature not mentioned by Namysłowski (Vincens 1918). Since then, almost 50 years passed without any records of *Wawelia* in the world. In 1956, *W. regia* was rediscovered by B. Gumińska, while she was incubating rabbit droppings in the same botanical garden in Kraków (Gumińska 1957). Her material was sent to G. Doguet and E. Müller, who examined it (Müller 1959; Doguet 1960, 1961a, b, 1963) and concluded that the genus *Wawelia* belonged to the family Melanosporaceae because of the features of asci: they were thin-walled and

lacked an iodine-positive apical apparatus, which is present in typical xylariaceous species (Müller 1959). Doguet followed the perithecia development of *W. regia* during the experiment and pointed out that this species should be either classified as a member of the family Melanosporaceae or maintained in the initially suggested family of its own, Waweliaceae (Doguet 1961b).

For a long time, the genus *Wawelia* remained monotypic. W. Wojewoda speculated that the incredible rarity of this fungus could be explained by the fact that it originated from tropical or subtropical regions and spores or whole fragments of mycelium were accidentally brought to the Kraków Botanic Garden with the introduction of exotic plants (Wojewoda 1983). However, the same year, a new species, *W. octospora* Minter & J. Webster, was described from England, but it was characterized by 8-spored instead of 4-spored asci as in the case of *W. regia* (Minter & Webster 1983). Subsequently, three more species of *Wawelia* were introduced: *W. effusa* N. Lundq. from Sweden and Hungary (Lundqvist 1992), *W. argentea* J. Webster, and *W. microspora* J. Webster, both from England (Webster et al. 1999). As such, the genus *Wawelia* currently comprises five species, all described from Europe.

Although the genus *Wawelia* is quite small and there are not many studies focusing on it, some of them still appear little known and are rarely or never cited. Therefore, we provide full bibliography considering this taxon (Tab. 1).

Despite a number of mycological studies and specific interest of some researchers in coprophilous fungi in Europe, *Wawelia* has never been observed in the field before (Gumińska 2000). All the known specimens (less than 15–20 in the world) were obtained from the substrate incubated in moist chambers. Moreover, only *W. regia* has been recorded by someone else besides its discoverer (Gumińska 1957).

In July 2020, we were lucky enough to encounter extremely long filiform stromata of a mature but unknown coprophilous ascomycete during a myxomycete inventory in Kedrovaya Pad Nature Reserve in the south-west of Primorsky Krai (Russia) (Fig. 1). Further research proved that this specimen represents an undescribed species of the genus *Wawelia*, because it possesses several unique features, distinguishing it from other taxa.

Materials and methods

Field and morphological study

Stromata were collected in the field and then fully dried out at room temperature. Microscopic morphological features were studied using temporary slides prepared with water using a Micromed 3, Var. 3 LED M microscope (Shenzhen, China). The amyloid reaction of apical apparatus was revealed via Lugol's iodine and Melzer's solution. Ascospore appendages and ascus walls were observed in Indian ink. All measurements were performed in water. Measurements are given as follows: (minimum) mean-standard deviation – mean + standard deviation (maximum). Some images were obtained using a CamScan S2 scanning electron microscope (Cambridge Instruments Co Ltd, Cambridge, UK). Specimens were preliminary sputter coated with gold-palladium alloy. Tissue types of stromata and perithecia were interpreted following Hengstmengel (2020).

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from 1 g of dry stroma using the cetyltrimethyl ammonium bromide (CTAB) method. Samples were grounded in liquid nitrogen and incubated in eppendorfs for 60 min at 65 °C with 700 µl extraction buffer (2 % CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl

pH 8). After 5 min cooling, 500 µl chloroform were added. Samples were vortexed and then centrifuged at 13000 g for 10 min. This aqueous phase was transferred to a new 1.5 ml tube and 400 µl cold isopropanol + 70 µl 5M CH₃COOK were added. Samples were vortexed and then centrifuged at 13000 g for 10 min. Supernatant was removed and the DNA was washed with 70 % ethanol, then centrifuged at 13000 g for 5 min. The step was repeated twice. The DNA pellet was dried thoroughly and dissolved in TE buffer. The DNA samples were stored at -20 °C until use.

PCR was performed with the use of ScreenMix supermix (Evrogen JSC, Moscow, Russia) in T100 Thermal Cycler (Bio-Rad, CA, USA). Primer pairs used were as follows: ITS1/ITS4 for ITS (White et al. 1990), LROR/LR5 for LSU (Vilgalys & Hester 1990), Bt2a/Bt2b for benA β-tubulin locus (Glass & Donaldson 1995), and fRPB2-5F/fRPB2-7R for RPB2 (Liu et al. 1999).

Amplification products were cut out from the gel and purified on the silica spin-columns using a Cleanup Standard kit (Evrogen JSC, Moscow, Russia). Sequencing reactions were performed by Evrogen JSC (Moscow, Russia) following the BigDye terminator protocol on the Applied Biosystems 3730xl automatic sequencer (Applied Biosystems, Inc, CA, USA) with both forward and reverse primers. Newly generated sequences were deposited in the NCBI (GenBank) nucleotide database.

Phylogenetic analyses

Two datasets were analyzed, ITS+LSU and BenA+ITS+RPB2. Sequences were aligned using MUSCLE implemented in UNIPRO Ugene v46.0 (Okonechnikov et al. 2012) and then optimized with TrimAl v1.3 (Capella-Gutierrez et al. 2009) with automated 1 option using webserver version implemented in Phylemon 2.0. (Sánchez e al. 2011) in the first case and aligned via MAFFT version 7 (Katoh et al. 2019) using the auto strategy and then manually optimized using MEGA X (Kumar et al. 2018) in the second case. The phylogenetic sampling was based on taxonomic studies of Xylariaceae (Daranagama et al. 2015, 2018), coprophilous Xylariaceae (Becker et al. 2020), and polyphyletic genera of Xylariaceae (Konta et al. 2020). *Graphostroma platystomum* CBS 270.87 and *Biscogniauxia nummularia* MUCL 51395 (Graphostromataceae) were chosen as the outgroup in both cases.

The ITS+LSU dataset comprised 36 sequences concatenated in SequenceMatrix (Vaidya et al. 2011) with a total length of 1854 characters includ-

Tab. 1. Bibliography of the genus *Wawelia* Namysl.

Literary source	Language	Content of the article regarding <i>Wawelia</i>	Figures and tables
Namysłowski (1908)	French	Description of the genus <i>Wawelia</i> and the type species, <i>W. regia</i> ; commentary on cultivation, development, and taxonomic position of the new genus and species	Five hand drawings of stromata, perithecia, asci, paraphyses, and anamorphic stage; one black-and-white photograph of <i>W. regia</i> type specimen
Vincens (1918)	French	Short commentary on the ascospore morphology of <i>W. regia</i> and its taxonomic position within the family Xylariaceae	One hand drawing of ascospores of the <i>W. regia</i> type specimen
Gumińska (1957)	English	Report of the second record of <i>W. regia</i> on <i>terra typica</i> (Krakow, Poland). Morphological description of the new specimen	Six black-and-white photographs of the second <i>W. regia</i> specimen: stromata (3), asci and paraphyses (2), and ascospores (1)
Müller (1959)	German	Description of <i>W. regia</i> morphology (Gumińska's material), commentary on the genus taxonomic position	Four hand drawings of the second <i>W. regia</i> specimen: stromata, perithecium (general view and section), and ascus with spores
Doguet (1960)	French	Detailed description of different ways of treating and cultivating <i>W. regia</i> (Gumińska's material and fresh cultures isolated from it) and their influence on ascospore germination and development of fertile stromata with perithecia	Three tables
Doguet (1961a)	French	Detailed cytological report on <i>W. regia</i> asci development (Gumińska's material and fresh cultures isolated from it) and nuclei distribution in ascospores	Forty-two hand drawings of different asci development stages and normal and abnormal ways of nuclei distribution in ascospores
Doguet (1961b)	French	Detailed description of <i>W. regia</i> morphology (Gumińska's material and fresh cultures isolated from it) and perithecium development. Commentary on taxonomic position of the genus based on the observations provided	Twenty hand drawings: ascogenous hyphae, paraphyses, asci, ascospores, anamorph (6), and perithecium development stages (14); 14 tables
Doguet (1963)	French	Detailed description of the ascospore treatment required for their activation (dormancy breaking)	Seven graphs and 13 tables
Gumińska (1963)	Polish	Short review of four out of five articles of Müller and Doguet considering <i>W. regia</i>	–
Rogers (1981)	English	Short commentary on the issue that <i>Wawelia</i> should not be considered synonymous to <i>Sarcoxydon</i> (based on the study of <i>W. regia</i> material in Royal Botanic Gardens, Kew)	–
Wojewoda (1983)	Polish	Brief information on the history of <i>W. regia</i> discovery, different opinions on its taxonomic position, and assumption about the native range of this fungus	–
Minter & Webster (1983)	English	Description of the second <i>Wawelia</i> species (<i>W. octospora</i>). Commentary on its taxonomic position, a way of spore release, and preferred conditions for the development of fertile stromata	Five hand drawings of stromata, perithecia, asci, paraphyses, and anamorph of <i>W. octospora</i>
Gumińska (1987)	Polish	Review of the Minter & Webster (1983) article with a <i>W. octospora</i> description and additional commentary	Two schematic hand drawings, comparing morphology of stromata, perithecia, and asci of <i>W. regia</i> and <i>W. octospora</i>

Literary source	Language	Content of the article regarding <i>Wawelia</i>	Figures and tables
Lundqvist (1992)	English	Description of the third <i>Wawelia</i> species (<i>W. effusa</i>). Commentary on the perithecia development and taxonomic position of the genus	Eight hand-drawn illustrations (Fig. 1 A-H) of stroma, perithecium (general view and section), asci, and ascospores of <i>W. effusa</i> ; five black-and-white photos of stromata, perithecia, and the outer layer of <i>W. effusa</i>
Webster et al. (1999)	English	Description of the fourth and fifth <i>Wawelia</i> species (<i>W. argentea</i> and <i>W. microspora</i>). Identification key of species known at that time. Commentary on preferred conditions for the development of fertile stromata	Eleven black-and-white photographs of <i>W. argentea</i> (6) and <i>W. microspora</i> (5)
Gumińska (2000)	Polish	Overview of the history of the genus research	Five schematic hand drawings, comparing stromata and asci morphology of different <i>Wawelia</i> species
Wojewoda & Karasiński (2010)	English	Short review of the history of <i>W. regia</i> discovery	–
Daranagama et al. (2018)	English	Description of the genus morphology and <i>W. octospora</i> holotype. NB: there are a few inaccuracies about <i>Wawelia</i>	Nine colored photographs of <i>W. octospora</i> herbarium specimen and morphological features; three black-and-white reprinted illustrations of <i>W. octospora</i> from Minter & Webster (1983)

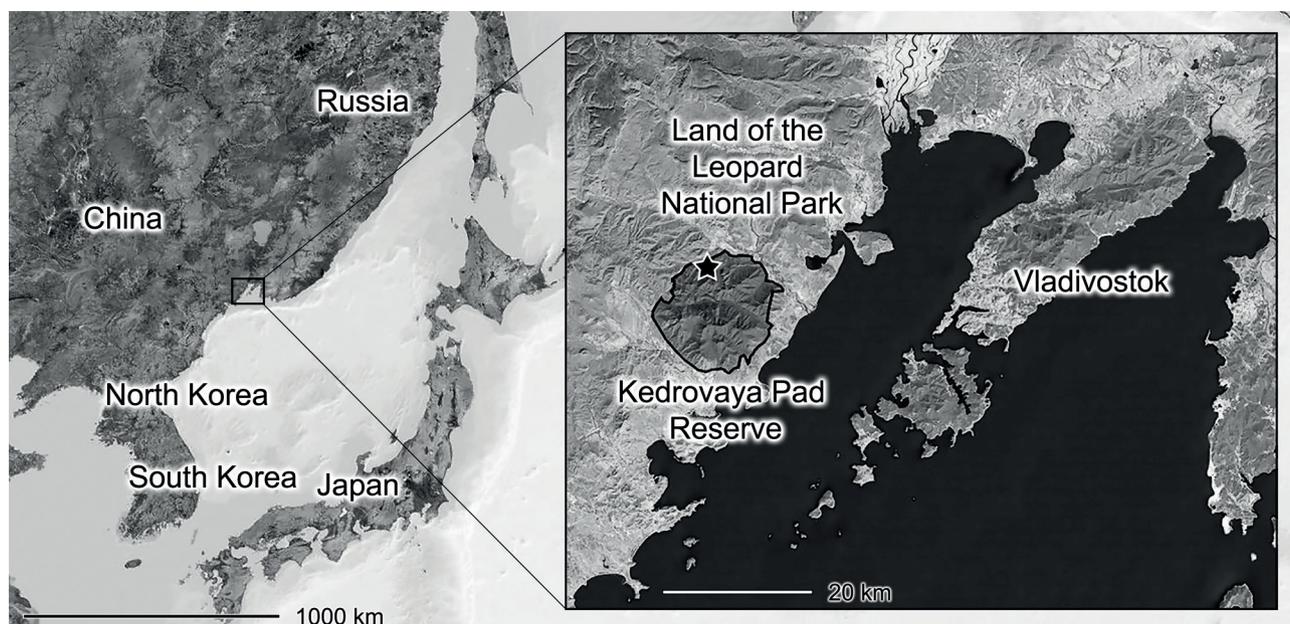


Fig. 1. Type locality of *Wawelia amyloasca*.

ing gaps (ITS, 1–486, GTR+G model; LSU, 487–1770, GTR+G) (Tab. 2). The best-fit models were estimated based on the Akaike Information Criterion (AIC) using FindModel web server (<http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>). Maximum likelihood (ML) analysis of the ITS+LSU dataset with two partitions (Chernomor et al. 2016)

was run in IQ-TREE v1.6.12 (Nguyen et al. 2015); ultrafast bootstrap was performed with 100000 samples (Hoang et al. 2018). Bayesian inference (BI) analysis was performed with MrBayes v. 3.2.6 (Ronquist et al. 2012); two sets of four chains were run for 8 million generations, trees were sampled every 100th generation. The convergence of MCMC chains

Tab. 2. Specimens and GenBank accession numbers of DNA sequences used in this study.

Species	Strain / voucher	GenBank accession numbers				References
		ITS	LSU	RPB2	BenA	
<i>Arthroxyllaria elegans</i>	CBS 537.79	AF432179	–	–	–	Seifert et al. (2002)
<i>Biscogniauxia nummularia</i>	MUCL 51395	KY610382	KY610427	KY624236	KX271241	Wendt et al. (2018)
<i>Brunneiperidium gracilentum</i>	MFLUCC 14-0011	KP297400	–	KP340528	KP406611	Daranagama et al. (2015)
<i>B. involucreatum</i>	MFLUCC 14-0009	KP297399	–	KP340527	KP406610	Daranagama et al. (2015)
<i>Daldinia concentrica</i>	CBS 113277	AY616683	KY610434	–	–	ITS: Triebel et al. (2005), LSU: Wendt et al. (2018)
<i>D. dennisii</i>	CBS 114741	JX658477	KY610435	–	–	ITS: Stadler et al. (2014), LSU: Wendt et al. (2018)
<i>D. loculatoides</i>	CBS 113279	MH862918	–	KX271246	KY624247	ITS: Vu et al. (2019), RPB, BenA: Wendt et al. (2018)
<i>D. macaronesica</i>	CBS 113040	KY610398	–	KY624294	KX271266	Wendt et al. (2018)
<i>Dematophora bunodes</i>	CBS 123597	MN984619	MN984625	–	–	Wittstein et al. (2020)
<i>D. buxi</i>	JDR 99	GU300070	–	GQ844780	GQ470228	Hsieh et al. (2010)
<i>D.necatrix</i>	CBS 349.36	AY909001	–	KY624275	KY624310	Wendt et al. (2018)
<i>Graphostroma platystoma</i>	CBS 270.87	JX658535	DQ836906	KY624296	HG934108	ITS: Stadler et al. (2014), LSU: Zhang et al. (2006), RPB2: Koukol et al. (2015), BenA: Wendt et al. (2018)
<i>Hypocopra anomala</i>	TTI-000339	–	–	MT901033	MT901030	Becker et al. (2020)
<i>H. rostrata</i>	TTI-000009	MT896134	MT903246	MT901034	MT901031	Becker et al. (2020)
<i>Hypoxyylon fragiforme</i>	MUCL 51264	KC477229	KM186295	KM186296	KX271282	ITS: Stadler et al. (2013), LSU, RPB2: Daranagama et al. (2015), BenA: Wendt et al. (2018)
<i>H. monticulosum</i>	MUCL 54604	KY610404	KY610487	KY624305	KX271273	Wendt et al. (2018)
<i>Kretzschmaria deusta</i>	CBS 163.93	KC477237	–	KY624227	KX271251	Stadler et al. (2013)
<i>K. deusta</i>	CBS 288.30	MH855142	MH866592	–	–	Vu et al. (2019)
<i>K. guyanensis</i>	HAST 89062903	GU300079	–	GQ478214	GQ844792	Hsieh et al. (2010)
<i>Nemania beaumontii</i>	HAST 405	GU292819	–	GQ844772	GQ470222	Hsieh et al. (2010)
<i>N. serpens</i>	CBS 679.86	KU683765	–	KU684284	KU684188	U'Ren et al. (2016)
<i>N. serpens</i>	AT-114	DQ631942	DQ840075	–	–	Tang et al. (2007)
<i>Podosordaria mexicana</i>	WSP 176	GU324762	–	GQ853039	GQ844840	Hsieh et al. (2010)
<i>P. muli</i>	WSP 167	GU324761	–	GQ853038	GQ844839	Hsieh et al. (2010)
<i>Poronia erici</i>	DSM 107106	MN954396	MN954397	MN956523	MN956524	Peric & Wendt (2017)
<i>P. pileiformis</i>	WSP 88113001	GU324760	–	GQ853037	GQ502720	Hsieh et al. (2010)
<i>P. punctata</i>	CBS 656.78	KT281904	KY610496	KY624278	KX271281	Wendt et al. (2018)
<i>Rhopalostroma angolense</i>	CBS 126414	KY610420	KY610459	–	–	Wendt et al. (2018)

Species	Strain / voucher	GenBank accession numbers				References
		ITS	LSU	RPB2	BenA	
<i>Rh. indicum</i>	CBS 113035	MH862909	MH874483	–	–	Vu et al. (2019)
<i>Rosellinia aquila</i>	MUCL 51703	KY610392	–	KY624285	KX271253	Wendt et al. (2018)
<i>R. australiensis</i>	CBS 142160	KY979742	KY979797	–	–	Crous et al. (2017)
<i>R. corticium</i>	MUCL 51693	KY610393	–	KY624229	KX271254	Wendt et al. (2018)
<i>R. necatrix</i>	CBS 349.36	AY909001	KF719204	–	–	Peláez et al. (2008)
<i>Sarcoxyylon compunc- tum</i>	CBS 359.61	KT281903	KY610462	KY624230	KX271255	Wendt et al. (2018)
<i>Stromatoneurospora phoenix</i>	BCC 82040	MT703666	MT735133	MT742605	MT700438	Becker et al. (2020)
<i>Thamnomycetes dendroideus</i>	CBS 123578	FN428831	KY610467	KY624232	KY624313	ITS: Stadler et al. (2010), LSU, RPB2 and BenA: Wendt et al. (2018)
<i>Wawelia amyloasca</i>	LE F-334908	OP687954	OP687953	OP700449	OP700450	Present study
<i>W. regia</i>	CBS 110.10	MH854595	MH866123	–	–	Vu et al. (2019)
<i>Xylaria adscendens</i>	JDR 865	GU322432	–	GQ844818	GQ487709	Hsieh et al. (2010)
<i>X. arbuscula</i>	CBS 126415	MH864101	KY610463	KY624287	KX271257	ITS: Vu et al. (2019), LSU, RPB2 and BenA: Wendt et al. (2018)
<i>X. bambusicola</i>	JDR 162	GU300088	–	GQ844801	GQ478223	Hsieh et al. (2010)
<i>X. bambusicola</i>	BCC22739	MT710944	MT735135	–	–	Becker et al. (2020)
<i>X. brunneovinosa</i>	isolate ZE	OL656078	OL704809	–	–	Direct submission
<i>X. carpophila</i>	isolate Z191	MZ621171	MZ703409	–	–	Direct submission
<i>X. crozonensis</i>	HAST 398	GU324748	–	GQ8448361	GQ502697	Hsieh et al. (2010)
<i>Xylaria cubensis</i>	BCC20646	MT703672	MT735142	–	–	Becker et al. (2020)
<i>X. curta</i>	HAST 494	GU322444	–	GQ844831	GQ495937	Hsieh et al. (2010)
<i>X. discolor</i>	Y.M.J 1280	JQ087405	–	JQ087411	JQ087414	Ju et al. (2012)
<i>X. grammica</i>	HAST 479	GU300097	–	GQ844813	GQ487704	Hsieh et al. (2010)
<i>X. grammica</i>	BCC20655	MT703670	MT735138	–	–	Becker et al. (2020)
<i>X. hypoxylon</i>	CBS 122620	KY610407	KY610495	KY624231	KX271279	Wendt et al. (2018)
<i>X. ianthinovelutina</i>	HAST 553	GU322441	–	GQ844828	GQ495934	Hsieh et al. (2010)
<i>X. ianthinovelutina</i>	isolate 246	MZ620706	MZ703179	–	–	Direct submission
<i>X. multiplex</i>	HAST 580	GU300098	–	GQ844814	GQ487705	Hsieh et al. (2010)
<i>X. nigripes</i>	isolate ZK	OL656080	OL704811	–	–	Direct submission
<i>X. polymorpha</i>	MUCL 49884	KY610408	KY610464	KY624288	KX271280	Wendt et al. (2018)
<i>X. telfairii</i>	HAST 421	GU324737	–	GQ8448350	GQ502686	Hsieh et al. (2010)
<i>X. telfairii</i>	BCC23019	MT703674	MT735139	–	–	Becker et al. (2020)

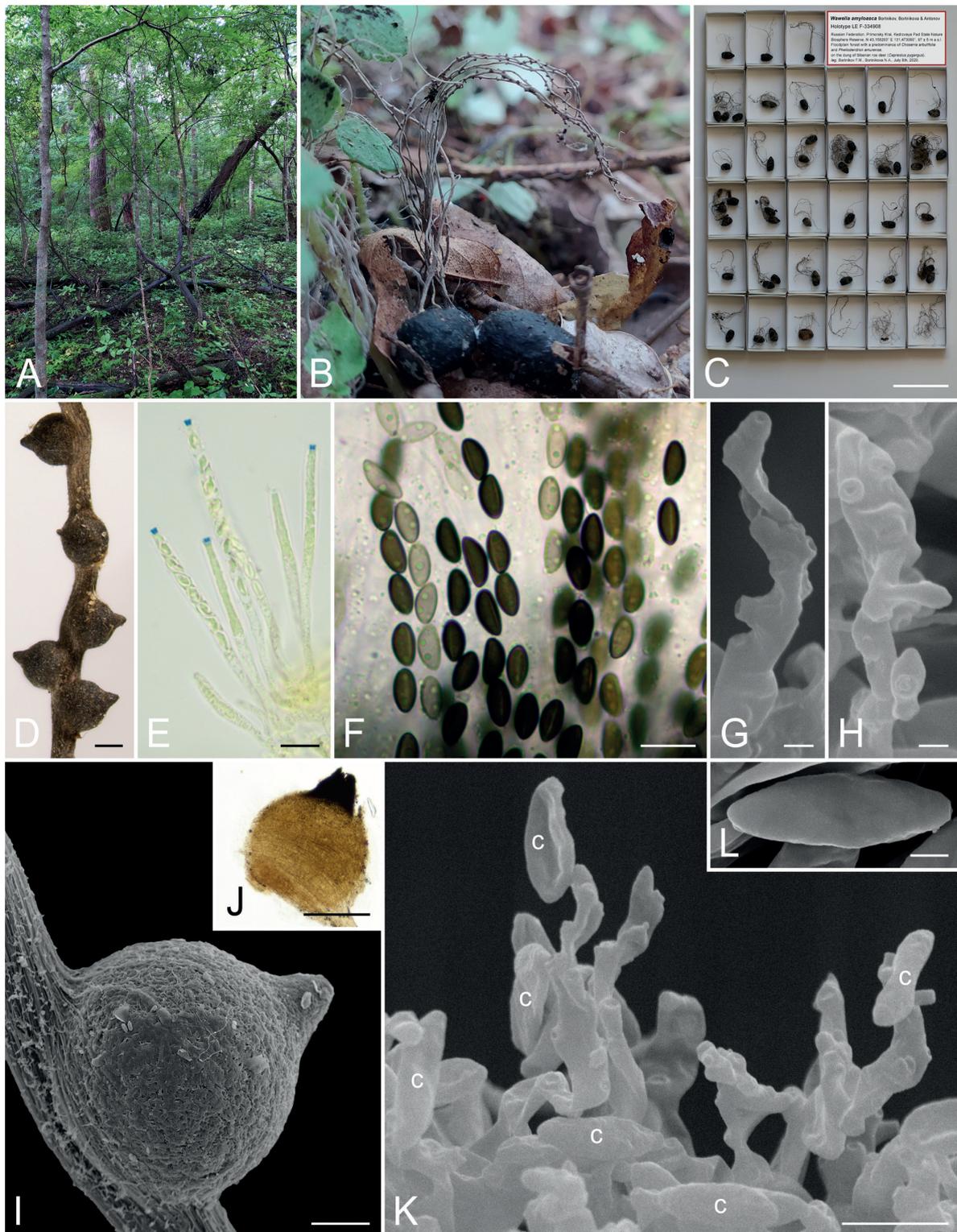


Fig. 2. *Wawelia amyloasca* (holotype): **A.** Type locality of *Wawelia amyloasca*. **B.** Stromata in the field. **C.** Herbarium specimen (holotype). **D.** Part of stroma. **E.** Immature asci with distinctive J+ apical apparatuses. **F.** Immature and mature ascospores. **G, H.** Conidiogenous cells fragments (SEM). **I.** Perithecium (SEM). **J.** Perithecium viewed with transmitted light; strongly melanized neck is well-noticeable. **K, L.** Anamorphic structures on the surface of stroma: conidiophores with conidia (c) and conidial secession scars. Scale bars: C = 50 mm; D = 200 µm; E, F = 10 µm; G, H = 1 µm; I = 100 µm; J = 200 µm; K = 5 µm; L = 1 µm.

was estimated using Tracer 1.7.2 (Rambaut et al. 2018). The result with the minimum ESS (effective sample size) above 5400 and the PSRF (potential scale reduction factor) equal to 1 was accepted.

The BenA+ITS+RPB2 dataset included 40 concatenated sequences with a total length of 1963 characters with gaps: BenA (1–419, K80+I+G model), ITS (420–1157, SYM+G model), RPB2 (1158–1963, GTR+I+G model). The most suitable models were selected based on the Bayesian Information Criterion (BIC), calculated in jModelTest v2.1.10 (Darriba et al. 2012). ML analysis with three partitions was run in IQ-TREE v2.1.2 (Minh et al. 2020); bootstrapping was performed using standard bootstrap with 200 bootstrap replicates. BI was performed in MrBayes v. 3.2.6; two sets of four chains were run for 2 million generations, trees were sampled every 100th generation. All chains were converged to <0.005 average standard deviation of split frequencies, and a burn-in of 25 % was used.

Bayesian posterior probabilities (BPP) were exported to the ML-tree. Branch support values are

shown only for clades with ML bootstrap higher than 60 % and BPP above 0.6. Clades with supports higher than 90 % ML and 0.98 BPP simultaneously are marked with thicker lines. Trees were visualized in FigTree v1.4.4 and then prepared and edited using Inkscape 1.2.

Results

Taxonomy

Wawelia amyloasca Bortnikov, Bortnikova & Antonov, **sp. nov.** – Figs. 2–4.

Mycobank: MB 841454

E t y m o l o g y. – The name refers to the amyloid apical apparatus, which is a unique feature within the genus *Wawelia*.

H o l o t y p u s. – Russian Federation, Primorsky Krai, Kedrovaya Pad Nature Reserve, N 43.158293° E 131.473090°, 97 ± 5 m a.s.l. (Fig. 1), floodplain forest with a predominance of *Chosenia arbutifolia* (Pall.) A.K. Skvortsov and *Phellodendron amurense* Rupr., on dung of the Siberian roe deer (*Capreolus pygargus* Pallas, 1771), 8 Jul. 2020, *leg.* F.M. Bortnikov & N.A. Bortnikova, (LE F-334908). GenBank ITS: OP687954; LSU: OP687953; BenA: OP700450; RPB2: OP700449.

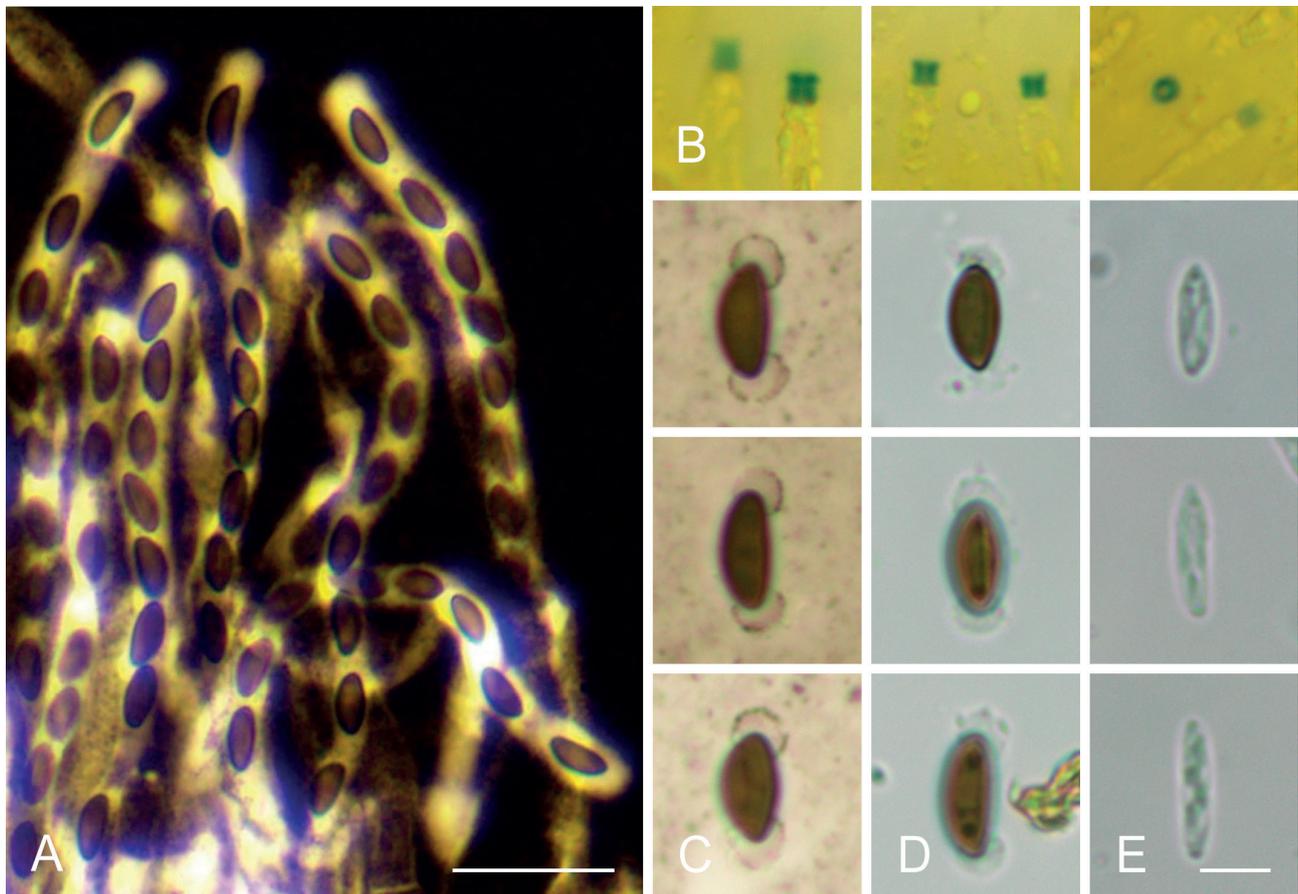


Fig. 3. *Wawelia amyloasca* (holotype): **A.** Asci in Indian ink. **B.** Amyloid apical apparatuses in Melzer's solution. **C, D.** Ascospores with non-cellular appendages in Indian ink (**C**) and water (**D**). **E.** Conidia. Scale bars: A = 20 µm, B–E = 5 µm.

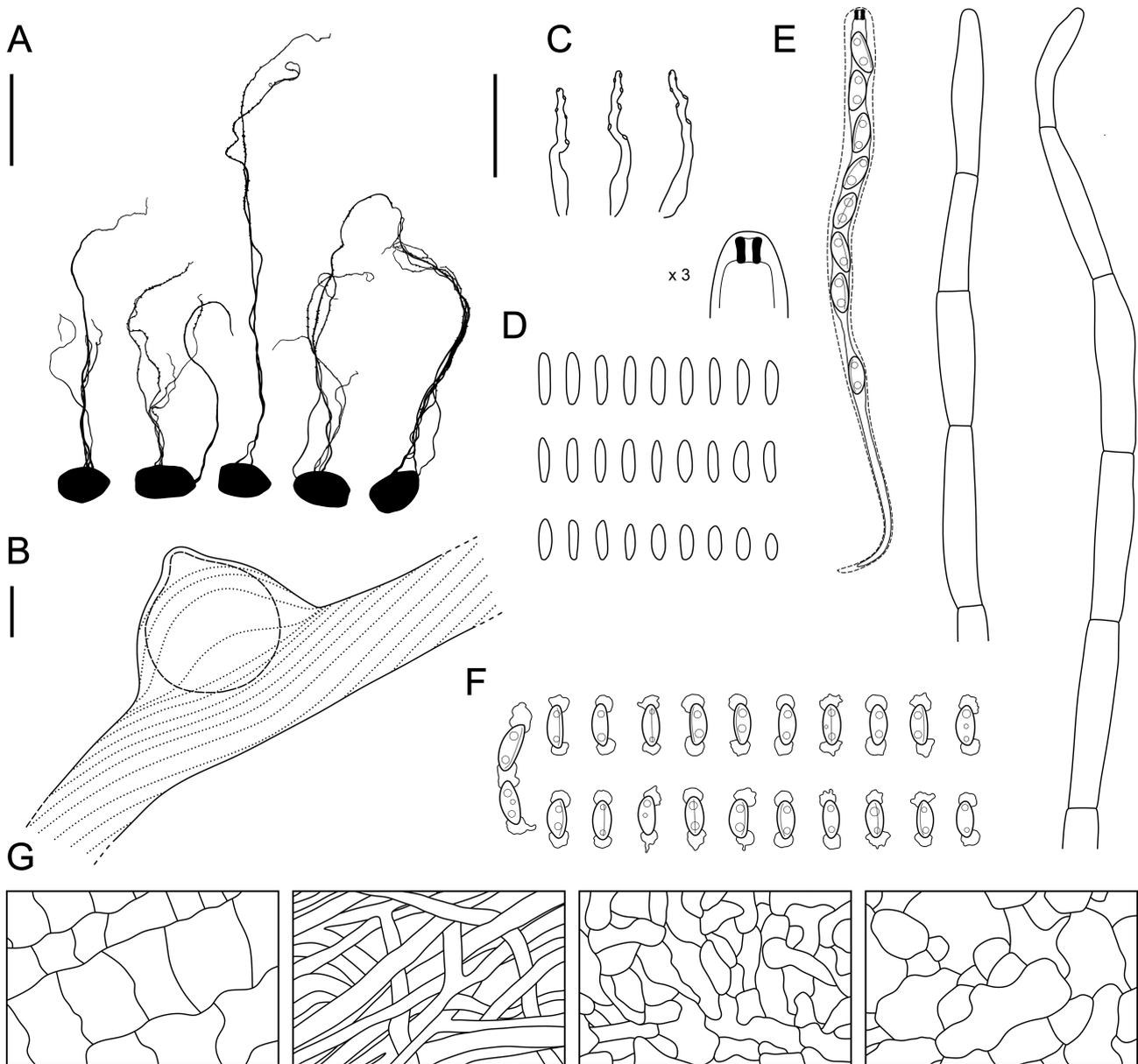


Fig. 4. *Wawelia amyloasca* (holotype): **A.** Stromata on Siberian roe deer droppings. **B.** Schematic illustration of an immature perithecium, developing under a stromal layer. **C.** Conidiogenous cells. **D.** Conidia. **E.** Ascus and paraphyses, with a more detailed illustration of the apical apparatus (dashed lines indicate ascus walls which are almost invisible in water, but well-contrasted in Indian ink). **F.** Ascospores. **G.** Structure of the perithecial wall from the outer layer to the inner one. Scale bars: A = 20 mm, B = 100 μ m, C–G = 20 μ m.

Diagnosis. – *Wawelia amyloasca* differs from other species of *Wawelia* by its stromata up to 120 mm long, asci with a distinctive amyloid apical apparatus, and ascospores with polar pad-like appendages.

Description. – Stromata filiform, flexuous, unbranched, (30)40–120 mm (on average 50–70 mm) long, 0.1–0.3 mm wide, tapering toward the apex, cylindrical or sometimes slightly flattened,

flexible when fresh; initially whitish grey, due to conidiogenesis on the surface, blackish brown at maturity; with yellow hairs in the lower part. Ectostroma melanized, rather soft, never carbonaceous or brittle, made up of 2–3 layers of *textura porrecta* composed of closely packed cells about 3.4–4.4 μ m wide and 25–90 μ m long; entostroma hyaline, composed of somewhat sinuous and loosely assembled

cells similar to the cells of ectostroma. – **P e r i t h e c i a** evenly distributed or sometimes aggregated in the upper 3/4 of the stroma, individual or rarely in twos or threes under the common outer layer, stromal, with fully exposed contours; dark greyish brown to black, globose, slightly asymmetrical, (295)310–420 (460) μm long and (230)290–380(435) μm wide, with opaque black, eminent, conical ostioles, glabrous, yellowish brown in transmitted light; tissue surrounding perithecia composed of four layers, with the outer two being stromal and the inner two being perithecial. The outermost layer composed of large, melanized, thick-walled *textura prismatica* cells, 10–20 (up to 30) μm wide, which are presumably the overgrown cells of ectostroma – *textura porrecta* (Fig. 4b). The following layer made up of loose cells, mainly lined along the stroma, though often twisted and curved (*textura intricata*), that previously composed the peripheral layer of entostroma. The third layer consists of small, very densely packed cells of *textura epidermoidea*. The innermost layer lining the lumen of perithecium composed of large, flat cells of *textura epidermoidea*, which are bigger and have a more isodiametrical form than those of the layer above. – **A s c i** cylindrical, unitunicate, containing eight uniseriately arranged ascospores, thin-walled, 100–150 \times 5.0–6.1 μm in total length, with the ascospore-bearing part 70–80 μm long and the stipe up to 60(70) μm long; apical apparatus bluing in Lugol's and Melzer's solutions with or without KOH pre-treatment, tubular to urn-shaped, 1.6–1.9 \times 1.6–2.0 μm . – **A s c o s p o r e s** unicellular, broadly ellipsoid in front view, inequilateral in side view, smooth, (6.9)7.6–8.5(9.8) μm long, (3.1)3.5–3.9(4.2) μm wide, 8.0 \times 3.7 μm on average (n = 150), dark olive-brown to dark greyish brown, mostly biguttulate, with conspicuous bright longitudinal germ slit along the entire length on the ventral side, bearing non-cellular, pad-like or irregular appendages 3.5–4 μm long at both ends. – **P a r a p h y s e s** thin-walled, septate, 1.5–2 times longer than asci, composed of sac-like cells 4.7–6.7(7.7) μm wide, slightly tapering toward the tips. – **A n a m o r p h** *Geniculosporium*-type, detaching from the surface of stroma, varying in length, 20–40 μm (rarely to 150 μm), straight or flexuous, sometimes zigzag-like, almost hyaline, with slightly darker conidial secession scars and thicker walls near detachment point (better viewed by SEM). – **C o n i d i a** hyaline, oblong ellipsoid, (5.1)7.0–9.2(10.6) \times (1.7)2.1–2.7(3.1) μm , 8.1 \times 2.4 μm on average (n = 100), with rounded or slightly narrowed tips, sometimes with prominent, darker, truncated base and secession scar.

Phylogeny

Phylogenetic analyses resulted in two phylogenetic trees with three congruent major clades, corresponding to the families Xylariaceae, Hypoxyalaceae, and Graphostromataceae (genera of this family were used as the outgroup). All major clades are characterized by maximum support ML bootstrap values and BPP. Division of the Xylariaceae s.s. clade into two subclades is maximally supported in the ITS+BenA+RPB2 phylogeny. However, another situation is observed on the ITS+LSU tree: two subclades are also present, but poorly supported. We suppose this can be explained by the unexpected clustering of *Kretzschmaria deusta* (Hoffm.) P.M.D. Martin with the “coprophilous” clade. *Wawelia amyloasca* clusters with *Hypocopra* spp., *Podosordaria* spp., *Poronia* spp., *Stromatoneurospora phoenix* (Kunze ex Fr.) S.C. Jong & E.E. Davis and *Sarcoxyylon compunctum* (Jungh.) Cooke with 69 % ML, 1.00 BPP (Fig. 5) and with the same taxa plus *Arthroxyllaria elegans* Seifert & W. Gams with 98 % ML and 1.00 BPP (Fig. 6). *Hypocopra* (Fr.) J. Kickx f., *Podosordaria* Ellis & Holw., *Poronia* Willd., and *Arthroxyllaria elegans* are coprophilous fungi and *Stromatoneurospora phoenix* is a pyrophilous one. However, the latter has recently been proven to be closely related to coprophilous xylariales fungi by a combination of morphological, chemotaxonomic, and molecular data (Becker et al. 2020). *Sarcoxyylon compunctum* is a rather rare tropical species, lacking detailed studies on its morphology, ecology, and phylogeny. Nevertheless, despite typically inhabiting wood, *Sarcoxyylon* Cooke clusters together with coprophilous and pyrophilous genera of Xylariaceae according to the published data and the present study (Becker et al. 2020, Konta et al. 2020, Voglmayr & Beenken 2020, Xie et al. 2020, Pi et al. 2021).

Wawelia amyloasca forms a separate clade with the maximum support values (100 % ML, 1.00 BPP) together with *W. regia*, the type species of the genus and the only one currently available for intrageneric comparison. There are two partial sequences present in GenBank, ITS and LSU. Phylogeny obtained using ITS+BenA+RPB2 might be confusing since ITS is the only marker present for both species of *Wawelia* and branch lengths are practically equal (Fig. 5). However, the p-distance between ITS sequences of *W. amyloasca* and *W. regia* is 5 %, and zooming-in Fig. 5 allows to see their difference. Also, the ITS+LSU tree (Fig. 6) clearly shows dissimilarity of branch lengths. These sequences were

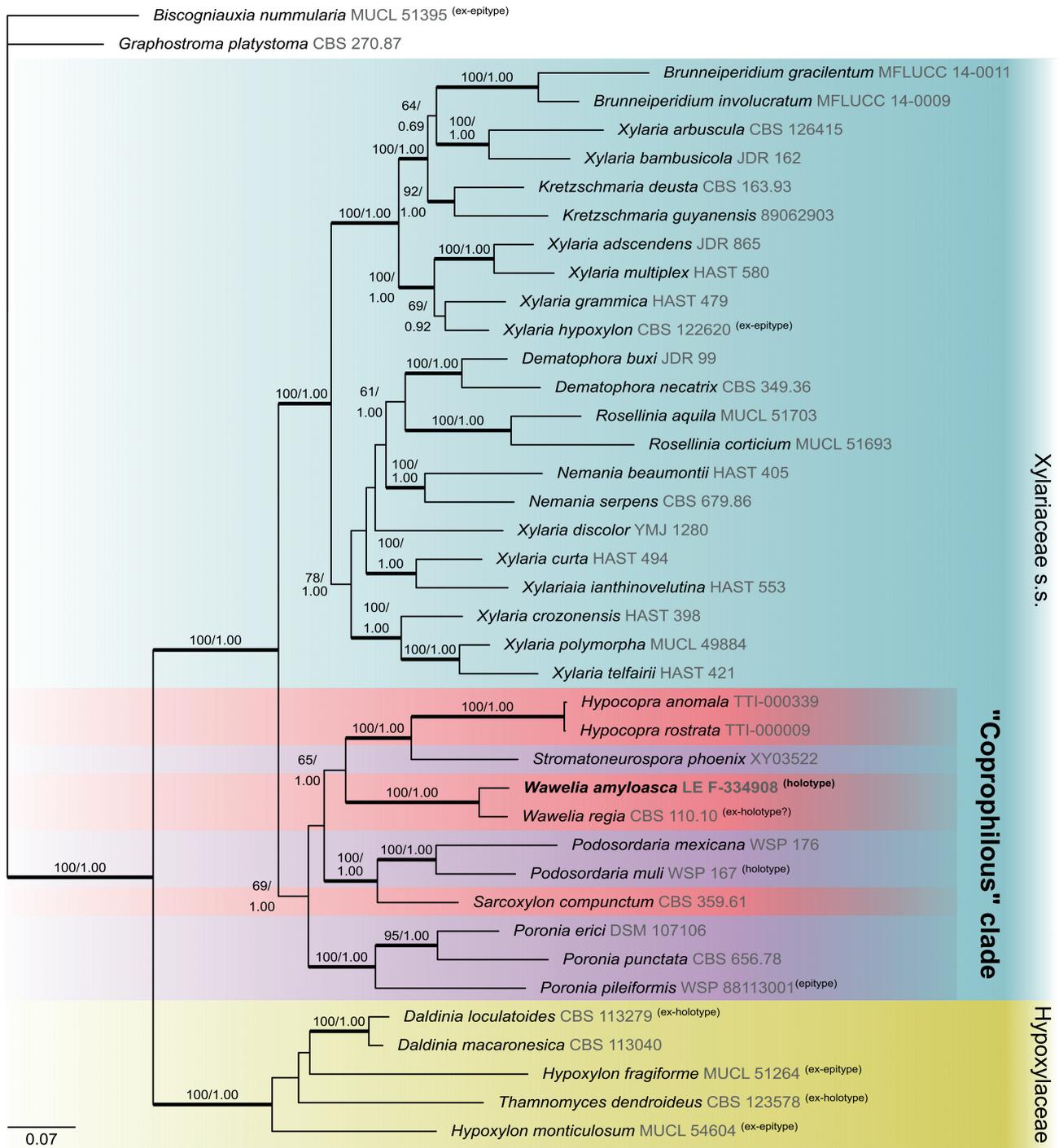


Fig. 5. Three-locus (ITS+BenA+RPB2) phylogenetic tree was constructed by BI analysis. Support values are given only for branches with maximum likelihood bootstrap > 60 % and Bayesian posterior probabilities > 0.60. Statistically significant branches (ML > 90 % and BPP > 0.98) are marked with thicker lines, the bar indicates the number of substitutions per site. New taxon is highlighted in bold.

probably obtained from the holotype since it was cultured by B. Namysłowski himself (as indicated on the CBS website) and can be considered an ex-holotype.

A sister taxon to the *Wawelia*-clade is *Arthroxy-laria elegans*, which was the second result of the NCBI BLAST search of *W. amyloasca* ITS. Only one marker (ITS) is available for this monotypic genus.

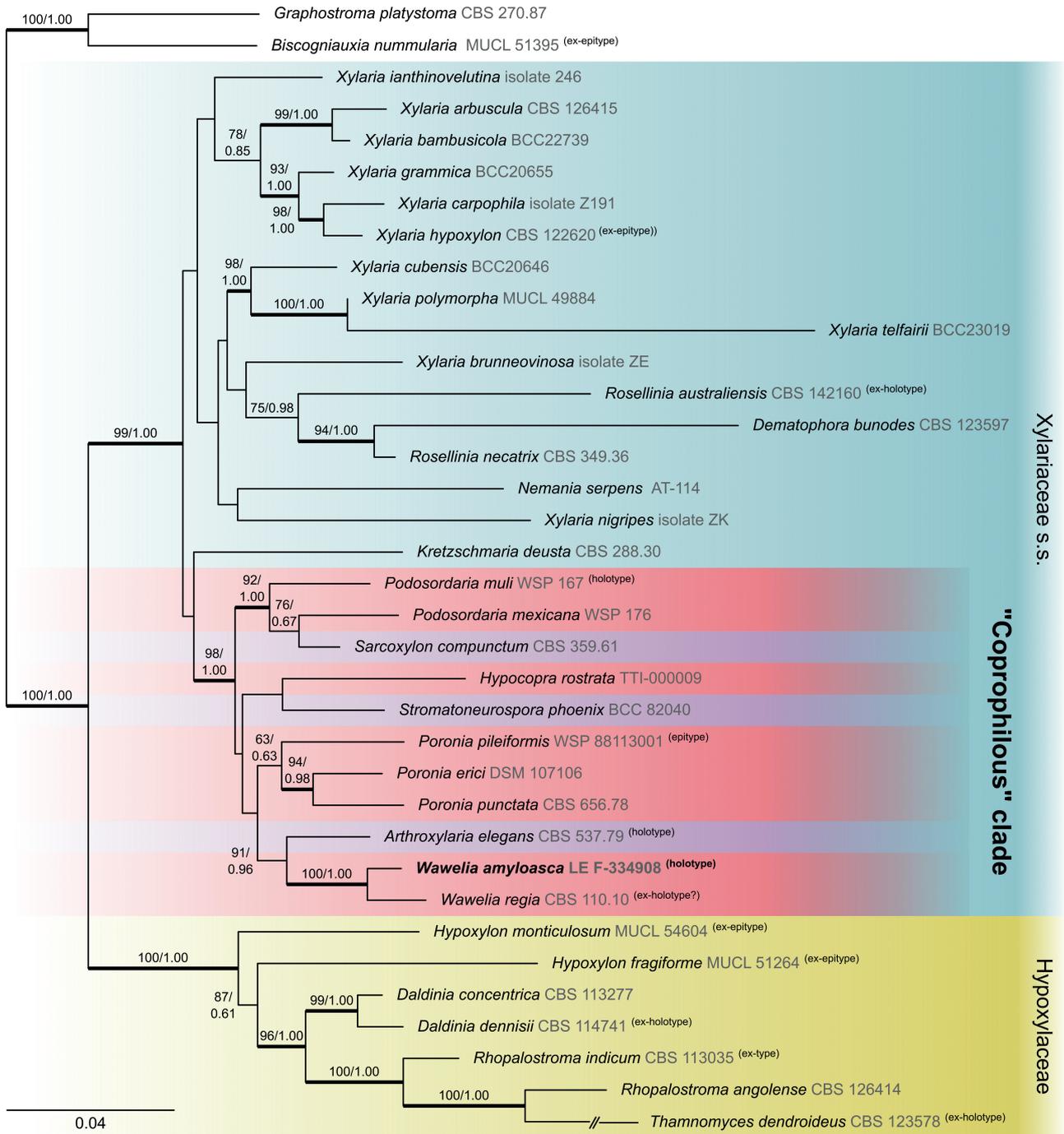


Fig. 6. Two-locus (ITS+LSU) phylogenetic tree was constructed by ML analysis. Support values are given only for branches with maximum likelihood bootstrap > 60 % and Bayesian posterior probabilities > 0.60. Statistically significant branches (ML > 90 % and BPP > 0.98) are marked with thicker lines, the bar indicates the number of substitutions per site. New taxon is highlighted in bold.

ITS+LSU phylogeny shows rather high support (91 % ML, 0.96 BPP) for the bigger clade of *Wawelias* and *A. elegans*.

It is worth mentioning that obtaining partial sequences of *benA* and *RPB2* for *W. amyloasca* was

not an easy task: all efforts to sequence the former locus with reverse Bt2b primer and the latter – with forward RPB2-5f primer failed, so only one-way reads of these loci were used.

Discussion

Wawelia amyloasca displays typical characteristics for the genus *Wawelia*, such as a) filiform, flexuous stromata (similar to those of *W. argentea*, *W. microspora*, and *W. octospora*), forming on the surface of herbivorous animal droppings; b) perithecia developing under the outer layer of stroma and later erupting through it, thus making the stromal layer a part of the perithecial wall (typical for all the species); c) cylindrical, thin-walled asci and dark smooth ascospores with a longitudinal germ slit; d) non-palisadic anamorph of *Geniculosporium*-type, developing on the surface of stroma.

Besides *Wawelia* spp., there are other species with filiform stromata in Xylariaceae s.l., such as *Xylaria filiformis* (Alb. & Schwein.) Fr., *X. hippotrichoides* (Sowerby) Sacc., *Thamnomycetes* spp. However, they all noticeably differ from *Wawelia amyloasca* morphologically. *Xylaria filiformis* is characterized by filiform stromata up to 72 mm long, but ascospores are much larger ($13\text{--}16 \times 5\text{--}7 \mu\text{m}$ vs. $8.0 \times 3.7 \mu\text{m}$ of *W. amyloasca*) and conidiophores form a compact palisadic layer along the stromata (Rogers & Samuels 1986, Hashemi et al. 2014). Stromata of *Xylaria hippotrichoides* are also filiform, although usually branched. Ascospores of this species possess similar appendages, which are much bigger – $14\text{--}20 \mu\text{m}$ long (Sowerby 1799, Dennis 1981, Minter & Webster 1983). All species of the genus *Thamnomycetes* Ehrenb. have been recorded only from Africa and the Neotropics and are characterized by brittle, strongly melanized stromata that are often branched and contain KOH-extractable pigments. The anamorph of *Thamnomycetes* is *Nodulisporium*-type, while *Wawelia* has a *Geniculosporium*-type anamorph. Molecular data show that *Thamnomycetes* is placed within the family Hypoxylaceae, which has recently been segregated from Xylariaceae (Stadler et al. 2010, Daranagama et al. 2018). Despite all of the aforementioned similarities, these species inhabit plant debris and never animal dung.

A number of genera and particular species belonging to Xylariaceae s.l. are coprophilous, but only some of them are attributed to *Xylaria* (San Martin et al. 1998a). Nevertheless, even then it was questioned, whether they belong to the genus *Xylaria*, and assumptions about their position within the coprophilous genus *Podosordaria* were made (San Martin et al. 1998b). *Xylaria pileiformis* (Berk.) Curr. is currently regarded as *Poronia pileiformis* (Berk.) Fr. (Ju & Rogers 2001, Hsieh et al. 2010); besides, its stroma has a long stalk and a hemispheri-

cal fertile apex, which makes this species different from all *Wawelia* members (San Martin et al. 1998a, Deepna Latha & Manimohan 2012). *Xylaria equina* San Martin & Guervara can be easily distinguished from *W. amyloasca* by massive stromata, smaller ascospores surrounded by a hyaline mucilaginous sheath, and a different shape of apical apparatus (San Martin et al. 1998a). *Xylaria coprophila* Wehm. differs by non-filiform stromata of smaller size (up to 1.5 mm long) and larger ascospores ($9\text{--}10.5 \times 3.5\text{--}4.5 \mu\text{m}$ vs. $7.6\text{--}8.5 \times 3.5\text{--}3.9 \mu\text{m}$ of *W. amyloasca*) (Wehmeyer 1942). *Xylaria guepini* (Fr.) Ces. has slightly smaller ascospores ($5\text{--}7.5 \times 2.7\text{--}4.4 \mu\text{m}$ vs. $7.6\text{--}8.5 \times 3.5\text{--}3.9 \mu\text{m}$ of *W. amyloasca*). This species was previously regarded along with coprophilous Xylarias (San Martin et al. 1998a), but even then, its preference for soil rich in organic matter was accentuated. Recently, *X. guepini* has been thoroughly studied, and its ecology has been finally settled (Hsieh et al. 2022). Taking all the above-stated morphological and ecological features into account, placing the new species in the coprophilous genus *Wawelia* is justified.

Wawelia amyloasca is characterized by several unique features, distinguishing it from the species of the genus described before (Tab. 3). Firstly, this species was found on dung of the Siberian roe deer (*Capreolus pygargus*), while all other species of *Wawelia* were described from leporoid dung, although it has to be mentioned that *W. effusa* has also been recorded from the European roe deer droppings (*C. capreolus* Linnaeus, 1758) besides rabbit ones (Lundqvist 1992). Secondly, stromata of *W. amyloasca* reach up to 120 mm in length, while stromata of other species do not exceed 30 mm. However, the fact that stromata of the new species have developed under natural conditions and not in a limited space of moist chamber has to be considered. It might be assumed that the comparison of two specimens collected in the field might show less difference in the length of stromata. Thirdly, perithecia of *W. amyloasca* do not bear any hairs on the surface, while the other species are characterized by more or less hairy perithecia and tend to have even longer hairs around ostioles. In particular, *W. octospora* is characterized by such long hairs that they might tangle in a ball up to $1000 \mu\text{m}$ in diameter (as in *Chaetomium* Kunze). Fourthly, ascospores of *W. amyloasca* bear appendages, which are sometimes poorly-visible due to their weak refraction (Figs. 3C–D, 4F). Pad-like appendages are common for other coprophilous members of Xylariaceae, for instance, *Poronia australiensis* (Læssøe, C.A. Pearce & K.D. Hyde) J.D. Rogers, Y.M. Ju &

Tab. 3. Morphological comparison of *Wawelia* spp.

	<i>W. amyloasca</i>	<i>W. microspora</i>	<i>W. octospora</i>	<i>W. argentea</i>	<i>W. regia</i>			<i>W. effusa</i>	
Literary source	Present study	Webster et al. (1999)	Minter & Webster (1983)	Webster et al. (1999)	Namyslowski (1908)	Gumińska (1957)	Müller (1959)	Doguet (1961b)	Lundqvist (1992)
Shape of stroma	Filiform, straight or flexuous, usually unbranched, sometimes slightly flattened	Filiform, unbranched near the base, cylindrical, then curved and branched; flattened or with longitudinal ridges	Filiform, flexuous, unbranched or rarely branched	Filiform, branched or unbranched, wavy and curved, cylindrical or slightly flattened	Half-cylindrical, half-conical, sometimes more or less flattened, sometimes furcate	Conical, 1-2 times branched, sometimes quite flattened	Varies greatly. Besides individual bulbous structures, there are branched and quite delicate ones	Shape and size vary. Sometimes simple, conical; sometimes branched in every dimension; sometimes spherical; sometimes flattened	Prostrate on the substrate
Length of stroma, mm	(30-) 40-120	20-30	2-25	< 30	5-12	< 10	< 5	5-10	
Width of stroma, mm	0.1-0.3	0.1-0.3	0.1-0.3	0.1-0.5	1-2 mm	NA	NA	NA	
Size of perithecia, µm	310-420 × 290-380	250 × 230-360	350-500	< 400 × 400	240 (on average) (up to 300)	NA	300-400	250-400	650-960 × 615-720
Asci	8-spored	8-spored	8-spored	4-spored	4-spored				8-spored
Size of asci, µm	100-150 × 5.0-6.1	75-88 × 5	70-90 × 6-8	87-93 × 10-12 (holotype), 50-67 × 10-11 (another specimen)	60-80 × 8	52.9-71.3 × 8 (on average)	55-65 × 7-8	55-80 × 7-8	110-140 × 13-15
Paraphyses	1.5-2 longer than asci (on average), 4.7-6.7 (-7.7) µm wide	Longer than asci, up to 8 µm wide	Slightly longer than asci	Much longer than asci, up to 12 µm wide	120 × 6 µm	105.8-142.6 × 6 µm	NA	100-120 × <10 µm	Longer than asci, 5-12 µm wide
Ascospores, µm	7.6-8.5 × 3.5-3.9	7.5-8 × 3-4	9-12 × 6-8	15-18 × 9-12 (holotype), 12-15 × 9.5-12.5 (another specimen)	8 × 6 (on average)	9.2-11.5 × 6	9-11 × 5.5-7	8-11 (on average 9.3)	15-19 × 9-10
Conidia, µm	7.0-9.2 × 2.1-2.7	3-4 × 2	8-12 × 2-4	3-4 × 2-2.5	4-6 × 2	NA	NA	5-6 × 3	NA
Country of record and dung type	Russia: Siberian roe deer (<i>Capreolus pygargus</i>)	England: rabbit (<i>Oryctolagus</i>), hare (<i>Lepus</i>)	England: European rabbit (<i>Oryctolagus cuniculus</i>)	England: rabbit (<i>Oryctolagus</i>)	Poland: European rabbit (<i>Oryctolagus cuniculus</i>)	Poland: European rabbit (<i>Oryctolagus cuniculus</i>)			Sweden: hare (<i>Lepus</i>), Hungary: roe deer (<i>Capreolus capreolus</i>)
Conditions of record	In the field	After incubation for several weeks under limited water access conditions at about 20-25 °C	Under low humidity conditions (90 and 95 % RH) and at room temperature (around 20 °C)	After incubation for several weeks under limited water access conditions at about 20-25 °C	Not stated explicitly; presumably similar to those of Gumińska	In closed glass dishes, under high humidity conditions at room temperature (around 20 °C)	Gumińska's material		After incubation in moist chambers, on almost dried-out substrate

F. San Martin and *Podosordaria elephantis* J.D. Rogers & Y.M. Ju (Hyde et al. 1996, Deepna Latha & Manimohan 2012), although they have never been recorded for any *Wawelia* species. Fifthly, the combination of measurements of ascospores and conidia is unique within the genus.

Finally, the most important and truly distinctive feature of *Wawelia amyloasca* is the presence of an amyloid apical apparatus. Doguet stated that the apical apparatus of *W. regia* had neither a ring nor a nasse; moreover, it was reduced to a circular thickening of the ascus inner layer which formed a rather well-developed apical dome, bluing slightly in Cotton Blue (Doguet 1961b). All other species also do not have a noticeably amyloid reaction, and that was one of the main arguments that E. Müller and G. Doguet presented to exclude *Wawelia* from the family Xylariaceae (Müller 1959, Doguet 1961b). The presence of the J+ apical apparatus in *W. amyloasca* clearly suggests positioning of the genus *Wawelia* in Xylariaceae.

Furthermore, biology of *W. amyloasca* is of great interest. Four out of five species of *Wawelia* (except for *W. regia*) are apparently xerophilous, since substrates for incubation were collected on the windy seashore cliffs or sand dunes with scarce vegetation, and fertile stromata were cultivated under conditions of low humidity or on almost dry substrate (Tab. 3). On the contrary, *W. amyloasca* does not seem to be xerophilous. Since the specimen was found in the field, we cannot be certain under which conditions stromata and perithecia were formed. Nevertheless, the specimen was encountered not on the dry south slopes, but in a floodplain forest less than 50 m from a stream (Fig. 2A), where humidity is generally higher. In addition, according to the weather station 4.3 km from the type locality, there were 210 mm of rainfall during two months before the record (58.3 mm between May 8 and June 7 and 151.6 mm between June 8 and July 7). All these facts allow us to consider that *W. amyloasca* is not a xerophilous species as *W. argentea*, *W. effusa*, *W. microspora*, and *W. octospora*.

The reasons for the apparent extreme rarity of *Wawelia* species are still unknown due to the lack of data on their distribution and physiology. It is known that ascospores of *Wawelia* can be dormant and high temperature is required to trigger their activation. Germination also depends on other factors, such as substrate composition and the presence of metabolites from other organisms (Doguet 1960). It can be suggested that under natural conditions ascospores, activated after passing through the animal digesting system, do not remain in this

state for a long time, and if the environment is not suitable for the formation of stromata, fungus may die before reproduction. We believe that new *Wawelia* discoveries might shed light on true reasons of its extreme rarity in the world.

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