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Article *in* Mycological Progress · August 2009

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ORIGINAL ARTICLE

Morphological and molecular characterization of the *Armillaria cepistipes – A. gallica* complex in the Czech Republic and Slovakia

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Received: 12 June 2008 / Revised: 7 April 2009 / Accepted: 17 April 2009

Abstract *Armillaria cepistipes* and *A. gallica* (Basidiomycota, *Physalacriaceae*) are morphologically similar species, and they are often nearly indistinguishable using DNA-based methods targeting the ITS region of ribosomal DNA. The aim of this study was to examine morphological and ecological features of *A. cepistipes* and *A. gallica*, and to test other DNA-based methods to distinguish the two species. Our results revealed discriminative macro- and micromorphological features between these two species, especially the presence of a distinct central pileus ocella, the shape of the annulus, the character of the velar stipe remnants and the length of the terminal cells of the pileus scales. Ecologically, *A. gallica* generally prefers warmer areas in lowlands (oak and alluvial forests), while *A. cepistipes* is more common in hilly and lower montane beech forests in Central Europe. Nevertheless, despite differences in ecological preferences, certain locations between 300 and 500 m a.s.l. are known to sympatrically support both species. The sequences of the translation elongation factor 1-alpha showed high interspecific variability, and this gene is a more appropriate candidate for distinguishing *A. gallica* from *A. cepistipes*.

Keywords *Armillaria* · *Armillaria cepistipes* · *Armillaria gallica* · PCR-RFLP · Translation elongation factor 1-alpha

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Introduction

Five European annulate *Armillaria* species were distinguished 30 years ago based on intersterility tests (Korhonen 1978; Korhonen and Hintikka 1980). Since then, thorough anatomical and morphological studies (including Antonín 1986, 1990; Marxmüller 1992; Termorshuizen and Arnolds 1997; Volk and Burdsall 1995) have further differentiated the taxonomy of the group. The most detailed morphological studies were performed by Marxmüller (1980, 1982, 1986, 1987) and by Romagnesi and Marxmüller (1983). The primary purpose of these studies was to describe characteristics capable of distinguishing between the species. A survey of macroscopic characters of all European annulate species was published by Nierhaus-Wunderwald (1994). A summary of the taxonomic and morphological studies was published by Pegler (2000), and it included a key to the European species. Romagnesi and Marxmüller (1983) and Termorshuizen and Arnolds (1987) published

Published online: 21 May 2009

earlier identification keys. A survey of Italian *Armillaria* species including colour photographs was published by Cherubini (2003).

All current data suggest that the most difficult *Armillaria* group to distinguish is the *A. cepistipes – A. gallica* assemblage. *Armillaria cepistipes* and *A. gallica* are macroscopically nearly indistinguishable species that occur in the Northern hemisphere in similar biomes of temperate forest ecosystems. *Armillaria cepistipes* occurs from approximately 250 m a.s.l. to the mountain spruce zone at an altitude of approximately 1,200 m in Central Europe. The primary centres of its distribution are beech stands at altitudes of 500–900 m. *Armillaria gallica* is distributed predominantly in lowlands, and it spreads to higher elevations along rivers and streams. The distribution of both species overlaps at altitudes of 300–500 m (Jankovský 2003). Indeed, Prospero et al. (2003) reported sympatric occurrence of both species at elevations as high as 1,400 m in the Swiss Alps.

Based on data from France, England and Italy, Guillaumin et al. (1993) characterised 142 species from 30 families as hosts to *Armillaria* species. *A. gallica* has been observed on 40 host species in 14 different families, 10 of which were coniferous species. Of these *A. gallica* hosts, *A. cepistipes* is found only on *Tilia platyphyllos*. Jankovský (2003) recorded *A. cepistipes* on two conifers and four broadleaved hosts and *A. gallica* on 13 conifers and 37 broadleaved hosts from the area of South Moravia (Czech Republic). *Armillaria gallica* is reported to be an opportunistic pathogen, invading trees weakened by defoliation or drought (Marçais and Bréda 2006). *Armilaria cepistipes* is also a weakly pathogenic species (Guillaumin et al. 1993), and both species are less likely to kill trees than the more virulent fungus *A. ostoyae* (Prospero et al. 2004).

PCR-RFLP methods have been developed for rapid and cost-effective DNA-based identification of *Armillaria* samples (Harrington and Wingfield 1995; Lochman et al. 2004a). This new method uses an *Armillaria*-specific primer pair targeting the ITS region of nuclear ribosomal DNA, and the nested-PCR assay is suitable for low-quality DNA obtained from rhizomorphs and older herbarium specimens. However, some *Armillaria* specimens tested with this PCR-RFLP method have yielded ambiguous restriction patterns. Since multiple copies of nuclear ribosomal DNA (rDNA) are present in fungal genomes, some heterozygous specimens were detected. If a copy of the gene includes a base transition, the change may cause the elimination of a particular restriction site (Lochman et al. 2004a). These individuals showing an intermediate restriction pattern should be studied in detail to determine if they are an interspecific hybrid or a different species.

To reveal evolutionary relationships between closely related basidiomycete species, more genetic sequence must be characterised. The partial sequence of gene coding translation elongation factor 1- alpha (tefa), which includes two partial exons and one complete exon, as well as two introns, may be a promising tool in distinguishing closely related fungal species (Kauserud et al. 2007). The sequences of this single-copy protein-coding gene region have been analysed in order to study relationships between different *Armillaria* species (Maphosa et al. 2006), but this study focused mainly on southern hemisphere species, and it did not address European specimens of *A. cepistipes* or *A. gallica*.

The aim of the current study was to examine morphological features of *A. cepistipes* and *A. gallica*, to screen samples for their PCR-RFLP patterns, and to obtain DNA sequences of their tefa region. With this analysis, we hoped to further determine the phylogeny of European *Armillaria* species.

Material and methods

Morphological study

The descriptions of macroscopic and microscopic characters are entirely based on the first author's own observations. A total of 51 specimens were observed (see Table 1). Microscopic studies were generally made from dried material using an Olympus BX light microscope. Microscopic preparations were mounted using Congo-red, 10 % KOH and Melzer's reagent.

The following abbreviations are used: Q = the mean value of the length-width quotient in all collections studied, L = number of entire lamellae, and l = number of lamellulae between each pair of entire lamellae. All herbarium specimens are preserved in the herbarium of the Moravian Museum, Dept. of Botany, Brno, Czech Republic (BRNM). Colour abbreviations follow Kornerup and Wanscher (1983). Identification of all observed specimens was confirmed by PCR-RFLP.

Molecular study

PCR-RFLP and rDNA sequencing

The identification of each examined specimen of the *A. cepistipes – A. gallica* group was checked by PCR-RFLP. The DNA was isolated either from dried herbarium specimens or from fresh cultures grown on Petri dishes with Malt-extract agar (3% Malt-extract, 0.5% peptone, 1.5% agar; Himedia, Mumbai, India) using the PowerSoil DNA kit (MoBio, Carlsbad, USA) according to the manufacturer's instructions. The obtained cultures are deposited in the Laboratory of Forest Protection, Mendel University, Brno (MUAF; for strain numbers, see Table 1).

PCR-RFLP identification of the samples was performed using the *Armillaria*-specific primer pair AR1/AR2

Table 1 List of *Armillaria* specimens studied

Species	Country	Locality	Substrate	BRNM[a] Herbarium no.	MUAF[b] Culture no.
borealis	Czech Republic	Českomoravská vrchovina (Czech-Moravian Highlands), Ransko	*Picea abies*	699842	501
cepistipes	Slovakia	Nízké Tatry (Low Tatra Mts.), Liptovská Teplička	*Alnus* sp.	706816	612
cepistipes	Slovakia	Nízké Tatry (Low Tatra Mts.), Lupčianská dolina	*Picea abies*	699840	530
cepistipes	Czech Republic	Vsetínské vrchy (Vsetín Hills), Kutaný	*Fagus sylvatica*	706814	516
cepistipes	Czech Republic	Brno, Soběšice ponds	*Acer* sp. or *Alnus* sp.	706818	613
cepistipes	Czech Republic	Českomoravská vrchovina (Czech-Moravian Highlands), Ransko	*Fraxinus excelsior*	706819	503
cepistipes	Czech Republic	Železné hory (Iron Mts.), Velký Polom	*Picea abies*	706829	586
cepistipes	Czech Republic	Svitavy, Janov, Psí kuchyně	*Picea abies*	705382	–
cepistipes	Slovakia	Nízké Tatry (Low Tatra Mts.), Dúbravská dolina valley	*Picea abies, Salix caprea*	695701	526
cepistipes	Slovakia	Nízké Tatry (Low Tatra Mts.), Lupčianská dolina	*Picea abies*	695717	–
cepistipes	Czech Republic	Bílé Karpaty (White Carpathian Mts.), Velká Javořina	*Faxinus excelsior, Fagus sylvatica*	706831	–
cepistipes	Czech Republic	Křtiny, Arboretum of Mendel University	On soil, from buried wood (*Fagus sylvatica, Picea abies*)	706834	651
cepistipes	Czech Republic	Lomnice near Tišnov, Nový Svět	*Alnus glutinosa*	706836	592
cepistipes	Czech Republic	Hostýnské vrchy (Hostýn Hills), Čerňava	*Fagus sylvatica*	706837	507
cepistipes	Czech Republic	Hostýnské vrchy (Hostýn Hills), Čerňava	*Fagus sylvatica*	706838	506
cepistipes	Czech Republic	Vsetínské vrchy (Vsetín Hills), Kutaný	*Fagus sylvatica*	706839	513
cepistipes	Czech Republic	Vsetínské vrchy (Vsetín Hills), Kutaný	*Fagus sylvatica*	706840	517
cepistipes	Czech Republic	Vsetínské vrchy (Vsetín Hills), Kutaný	*Fagus sylvatica*	706841	515
cepistipes	Czech Republic	Jedovnice, Rakovec	*Fagus sylvatica*	706877	–
cepistipes	Czech Republic	Vsetínské vrchy (Vsetín Hills), Kutaný	*Fagus sylvatica*	706842	514
cepistipes	Czech Republic	Hrubý Jeseník Mts., Praděd	*Picea abies*	706843	–
cepistipes	Czech Republic	Brno, Soběšice ponds	*Carpinus betulus*	706849	615
cepistipes	Czech Republic	Železné hory (Iron Mts.), Velký Polom	*Picea abies*	706865	585
cepistipes	Czech Republic	Hostýnské vrchy (Hostýn Hills), Čerňava	*Fagus sylvatica*	706866	505
cepistipes	Czech Republic	Vsetínské vrchy (Vsetín Hills), Kutaný	*Fagus sylvatica*	706867	509
cepistipes	Czech Republic	Vsetínské vrchy (Vsetín Hills), Kutaný	*Fagus sylvatica*	706869	–
cepistipes	Czech Republic	Jeseníky Mts., Šerák	*Picea abies*	706872	523
ectypa	Austria	Upper Austria, Obertrumer See	On soil, among *Sphagnum*	704974	–
gallica	Czech Republic	Hostýnské vrchy (Hostýn Hills), Čerňava	*Fagus sylvatica*	705380	–
gallica	Czech Republic	Třeboň, Stará řeka	*Quercus robur*	705069	–

Table 1 (continued)

Species	Country	Locality	Substrate	BRNM[a] Herbarium no.	MUAF[b] Culture no.
gallica	Austria	Upper Austria, Kemanting	*Picea abies*	705070	–
gallica	Czech Republic	Brno, Soběšice ponds	Broadleaved tree	706817	614
gallica	Czech Republic	Brno, Soběšice ponds	Broadleaved tree	706825	619
gallica	Czech Republic	Nové Mlýny, Křivé jezero	*Quercus robur*	706882	–
gallica	Czech Republic	Brno, Soběšice ponds	*Quercus petraea, Carpinus betulus, Alnus* sp.	706826	617
gallica	Czech Republic	Lanžhot, Ranšpurk	*Quercus robur*	706827	554
gallica	Czech Republic	Valtice, Rendezvous	*Quercus cerris*	706828	550
gallica	Czech Republic	Jedovnice Rakovec	*Fagus sylvatica*	706832	634
gallica	Czech Republic	Lednice, castle park	*Quercus* sp.	706835	575
gallica	Czech Republic	Brno, Soběšice ponds	broadleaved tree	706847	616
gallica	Czech Republic	Brno, Soběšice ponds	*Alnus glutinosa*	706848	618
gallica	Czech Republic	Valtice, Rendezvous	*Quercus cerris*	706857	551
gallica	Czech Republic	Valtice, Rendezvous	*Quercus cerris*	706856	552
gallica	Czech Republic	Lanžhot, Ranšpurk	*Quercus robur*	706858	555
gallica	Czech Republic	Brno, Řečkovice	*Carpinus betulus*	706879	621
gallica	Czech Republic	Šerkovice, Besének valley	Stump of a broadleaved tree	706880	622
gallica	Czech Republic	Jedovnice, Rakovec	On soil	706833	630
gallica	Czech Republic	Brno, Soběšice ponds	Wood of a broadleaved tree	706881	623
gallica	Czech Republic	Czech-Switzerland National Park, Vosí vrch	*Betula pendula*	706883	–
mellea	Czech Republic	Brno, Soběšice ponds	*Alnus glutinosa*	706813	591
ostoyae	Czech Republic	Českomoravská vrchovina (Czech-Moravian Highlands), Ransko	*Picea abies*	695627	502
ostoyae	Czech Republic	Beskydy Mts., Klíny	*Fagus sylvatica*	706815	537

[a] *BRNM* Herbarium of the Moravian Museum, Dept. of Botany, Brno, Czech Republic

[b] *MUAF* Culture collection of the Department of Forest Protection and Wildlife Management, Mendel University in Brno

(Lochman et al. 2004a) using the DynaZyme™ DNA-polymerase with appropriate buffer (Finnzymes, Espoo, Finland). The DNA was amplified through PCR, using the Mastercycler® ep thermocycler (Eppendorf, Hamburg, Germany). The PCR products were cleaved by *Hinf*I restriction endonuclease (recognition site G/ANTC; Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. DNA fragment sizes were estimated on 3 % agarose (Serva, Heidelberg, Germany) gel using Phi X174 DNA/HinfI Marker (Fermentas) as DNA molecular size marker.

To confirm the identification, the ITS and IGS-1 regions of rDNA of selected specimens (see Table 2) of various Central European *Armillaria* species were sequenced using the primer pairs ITS1F/ITS4 and LR12R/O1 (for details, see Kim et al. 2006). The results were compared to previously published sequences (Chillali et al. 1998; Keča et al. 2006; Lochman et al. 2004b) and deposited in the NCBI Nucleotide Sequence Database (see Table 2 for the respective GenBank accession numbers).

Table 2 Table of newly sequenced specimens of *Armillaria* species

Species	BRNM Herbarium no.	MUAF Culture Collection Strain no.	tefa sequence	ITS sequence	IGS-1 sequence
A. borealis	699842	501	EU251402	EU257713	EU257708
A. cepistipes	706814	516	EU251395	EU257715	EU257709
A. cepistipes (h)	695717	–	EU251396	EU257716	EU257710
A. cepistipes (h)	706816	612	EU251397	–	–
A. cepistipes	706818	613	EU251393	–	–
A. cepistipes	706829	586	EU251392	EU257719	–
A. cepistipes (h)	706834	651	EU251398	–	–
A. cepistipes	706836	592	EU251394	–	–
A. gallica	706817	614	EU251391	–	–
A. gallica	706832	634	EU251388	–	–
A. gallica	706833	630	EU251389	–	–
A. gallica	706835	575	EU251390	EU257718	EU636240
A. ectypa	704974	–	EU251403	EU257720	EU257712
A. mellea	706813	591	EU251399	–	–
A. ostoyae	695627	502	EU251401	EU257714	–
A. ostoyae	706815	537	EU251400	EU257717	EU257711

(h) indicates specimens with an intermediate RFLP profile between *A. cepistipes* and *A. gallica*, the putative hybrids. The DNA sequences are deposited in the EMBL database under the accession numbers given. For geographic origin and substrate, see Table 1

Sequencing the tefa region

To study the affinity of *A. cepistipes* and *A. gallica*, we obtained the sequences of the tefa gene region of both species and the putative interspecific hybrid. To elucidate the infrageneric phylogenetic position of the species, representatives of other European *Armillaria* species (*A. borealis, A. ectypa, A. mellea*, and *A. ostoyae*) were analysed.

The primer pair EF595F/EF1160R (Kauserud and Schumacher 2001) was used to amplify the tefa gene region. If amplification was initially unsuccessful, a nested PCR was performed using the primer pair EF1-526F/EF1-1567R (O'Donnell et al. 1998) in the first reaction and the primers EF595F/EF1160R in the second one. The PCRs were performed in 25-µl total reaction volume containing 50 ng of DNA, 20 pmol of each primer, 0.2 mM each dNTP, and 1U of DynaZyme™ DNA-polymerase. PCR amplifications were performed under the following cycling conditions: 94°C / 3 min., 50°C / 30 s, 72°C / 1 min. (1×), 94°C / 30 s, 50°C / 30 s, 72°C / 30 s (33×) and 94°C / 30 s, 50°C / 30 s, 72°C / 5 min (1×). The PCR products were purified with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) prior to sequencing.

The sequences were analysed with an ABI PRISM 3100 Avant DNA sequencer (Applied Biosystems, Foster City, USA) at the Department of Animal Morphology, Physiology and Genetics, Faculty of Agriculture, Mendel University, Brno, using the ABI PRISM BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems). All samples were sequenced with both forward and reverse primers used in the PCR. The sequences were deposited in the NCBI Nucleotide Sequence Database, and GenBank accession numbers are given in Table 2. Additional tefa sequences published by Maphosa et al. (2006) were included in the study (the isolate numbers, country origin and GenBank accession numbers are given in parentheses): *Armillaria borealis* (CMW 3172, Finland, DQ435623), *Armillaria borealis* (CMW 3182, Germany, DQ435624), *A. cepistipes* (CMW 6909, USA, DQ435630), *A. gallica* (CMW 6901, USA, DQ435628), *A. gallica* (CMW 3717, USA, DQ435629), *A. gemina* (CMW 6888, USA, DQ435626), *A. nabsnona* (CMW 6905, USA, DQ435631), *A. ostoyae* (CMW 3162, USA, DQ435625) and *A. socialis* (as *A. tabescens*; CMW 3158, USA, DQ435641).

All sequences were edited manually using BioEdit version 4.7.1. (Hall 1999). Phylogenetic trees were constructed using two approaches: Bayesian analysis and maximum parsimony. Bayesian analysis was performed using the MrBayes 3.0 software (Ronquist and Huelsenbeck 2003). In MrBayes, base frequencies, rates for six different types of substitutions, the number of invariant sites, and the shape parameter of the gamma correction for the rate heterogeneity with four discrete categories were allowed to vary; 2×10^6 generations of the Markov Chain Monte Carlo were run with four simultaneous chains at a heating temperature of 0.2. The chains were sampled every 100th generation. When the likelihood scores of trees sampled approached similar values, they were considered to have converged. After this convergence point, the trees were

included in the consensus tree computation. The value of the cold chain was plotted against generation number, so the number of discarded trees is not random. Maximum parsimony (MP) was performed with PAUP 4.0 (Swofford 1999). MP was performed using 1,000 repeated heuristic tree searches wherein the starting tree was constructed by addition of random taxa and swapped using the TBR algorithm. Support for the MP topology was estimated using 1,000 bootstrap-replicates.

Results

Morphological observations

Armillaria cepistipes Velen., Čes. Houby 2: 283. 1920 (as A. cepaestipes)

Figure 1 and 2

Pileus 20–100 mm, (broadly) conical with obtuse centre, then convex-conical, plano-conical to applanate, with a less distinct, broad, obtuse umbo; margin involute, then straight, inflexed, to uplifted and undulate when old; yellow-ochraceous (4A3), ochraceous (4–5A3), ochraceous brown (6B–C4), ochre-brown (7D–E6–7), red-brown (7–8E5), usually paler towards margin. **Pileus scales** cover the entire pileus when young, very close at centre, then becoming more distant towards margin, (almost) absent at margin, pyramidal-fibrillose at centre, ± erect to adpressed towards margin, remaining only at centre when older (central ocella), brown (6C–D5–6, 7E–F7), paler towards margin; some forms with (bluish) grey-brown (5C3–6D4) pileus colours with dark grey (black)-brown (6E–F3–4) scales. **Lamellae** moderately close, emarginate and decurrent with a vein nearly up to annulus, whitish to cream coloured, then brownish to reddish (fleshy) brown (6C5, 6–7D5–6), edge concolorous, sometimes red-brown when old. **Annulus** distinctly radially fibrillose, sometimes thin membranaceous-fibrillose, irregularly radial cracking, white to whitish with scattered yellowish to yellow (2–3A4–5) remnants of velum universale, sometimes with brown marginal hairs. **Stipe** up to 100×9–11 (20) mm, cylindrical, clavate to bulbose (up to 30 mm) at base, whitish to flesh-tinged above annulus, up to grey-brown (4–5B4, 5B3–4, 6E–F4) below it. Stipe velar remnants covering the whole surface when young, tomentose-fibrillose, then breaking up into scattered small fibrillose flocci, yellow, sometimes also grey-brown. **Context** with a fungal smell and unpleasant astringent taste.

Basidiospores 7.0–9.0 (10)×4.5–6.5 (7.0) µm (mean= 8.3×5.3 µm), Q=1.7–(1.56)–1.8, mostly broadly ellipsoid to subglobose (cepistipes-type, according to Marxmüller 1982, see "Notes" below), mixed with less frequent to rather common ellipsoid ones, both thin- and thick-walled. **Basidia** 29–45×8.5–11 µm, 4-spored, rather narrowly clavate, clamped; crassobasidia present. **Cheilocystidia** 14–35×6.0–11 µm, regular to often irregular, clavate, subcylindrical, mostly single, sometimes in fascicles, 2-celled or in the form of chains of cells in lower part, thin- to slightly thick-walled, clamped. **Pileipellis** a cutis of radially arranged, cylindrical, ± thin-walled, clampless hyphae with irregularly cylindrical to coralloid terminal cells. **Pileus scales** consisting of 25–125 (140) × 7.5–30 µm, ellipsoid, subfusoid to cylindrical, ± slightly thick-walled, hyaline to brownish cells, 0–1(2) septate; terminal cells 15–90 (125) × 5.0–25 µm, subulate, conical, ellipsoid to cylindrical. **Stipe velar hyphae** similar to those of pileus scales. **Caulocystidia** 15–60×10–20 (30) µm, clavate, vesiculose, subulate, thin- to slightly thick-walled, single or in chains.

Ecology and pathology In the Czech Republic and Slovakia, the species is mainly distributed in beech stands at altitudes of 500–900 m a.s.l. Habitats range from oak–beech stands (250 m a.s.l) to mountain spruce stands at 1,200 m a.s.l. The fungus grows apparently in soil, on buried wood remnants or on stumps and stems of broadleaved and coniferous trees. It mostly occurs as a saprophyte in litter, decomposing buried wood such as roots, stumps and coarse woody debris. In weakened trees (see below for a list of hosts), it also behaves as a weak nectrotrophic parasite, infecting roots and penetrating the basal part of stems, causing heart rot to live trees. *Armillaria cepistipes* can also cause mortality of trees in secondary spruce stands similar to *A. ostoyae*, creating ecological conditions unsuitable for spruce planting. Rhizomorphs with a typical oval cross-section are common in the soil under loose bark forming nets. Carpophores grow individually or in small clusters from the soil or on fallen stems, occasionally directly from rhizomorphs. The species occurs sympatrically with *A. gallica* and infrequently with *A. mellea* at lower elevations in oak–beech or pure beech stands. *Armillaria cepistipes* co-occurs with *A. ostoyae* and *A. borealis* in some stands in the highlands and mountains (higher than 500 m a.s.l.). Except for *A. borealis*, it is the dominant *Armillaria* species at higher altitudes.

Hosts of *A. cepistipes*. Conifers: *Abies alba, Picea abies, Pinus sylvestris.* **Angiosperms:** *Alnus glutinosa, A. incana, Betula* spp., *Fraxinus excelsior, Fagus sylvatica, Quercus* spp., *Carpinus betulus, Salix* spp., *Sorbus aucuparia*.

Fruitation: September to early November (until first frost).

Armillaria gallica Marxm. & Romagn., Bull. Soc. Mycol. Fr. 103: 154. 1987

Figure 3

Synonyms: *Armillaria mellea* var. *bulbosa* Barla, Bull. Soc. Mycol. Fr. 3: 143. 1887; *Armillariella bulbosa* (Barla) Romagn., Bull. Soc. Mycol. Fr. 89: 199. 1973; *Armillaria*

Fig. 1 *Armillaria cepistipes*. Czech Republic, Janov near Litomyšl, Psí kuchyně, 16 September 2006, V. Antonín 06.92. Photo: V. Antonín

Fig. 3 *Armillaria gallica*. Czech Republic, Hostýn Hills, Čerňava, 2 September 2006, V. Antonín 06.57. Photo: V. Antonín

bulbosa (Barla) Kile and Watling, Trans. Br. mycol. Soc. 81(1): 135. 1983. – *Armillaria lutea* Gillet, Les Hymenomycetes: 83. 1874 ss. e.g. Pegler (2000), Termorshuizen and Arnolds 1987 (Mycotaxon 30: 108), Termorshuizen (1995). – ? *Armillaria inflata* Velen., Čes. Houby 2: 283. 1920 (see Antonín 1990).

Pileus 25–100 mm, broadly conical with broad obtuse umbo and involute margin when young, then applanate with low obtuse umbo and inflexed margin, light or dark brown to pinkish brown, pallescent up to whitish towards margin when young, then yellow-ochraceous (± 5A3) to brown (6-7D-E4-6). **Pileus scales** long fibrillose (up to 1(–2) mm), close and almost pyramidal at centre, scattered, single, adpressed to erect towards margin, dark brown (6–7E–F6–8), grey-brown at centre, concolorous with pileus surface, yellowish to whitish towards margin; soon glabrescent towards margin, centre ± (scatteredly) squamose. **Lamellae** moderately close, emarginate and decurrent with a vein, whitish, then pale fleshy brown, with concolorous edge. **Annulus** fibrillose to thin fibrillose-membranaceous, breaking up star-like, white, often with brown marginal scales, often with yellowish or brownish velar remnants below. **Stipe** up to 120×12 mm, usually cylindrical with often distinctly clavate to (sub)bulbous base (up to 25 mm), white to pale fleshy above annulus, fleshy brown (7C3–4), brown (7D4, 7E-F4), sometimes with greyish tinge at base. Yellow stipe velar remnants common and scattered, fibrillose or fibrillose-tomentose (3A–B5). **Context** with indistinct or unpleasant camembert smell and unpleasant astringent taste.

Fig. 2 *Armillaria cepistipes* (grey form). Czech Republic, White Carpathian Mts., Velká Javořina, 10 October 2007, V. Antonín 07.373. Photo: V. Antonín

Basidiospores 7.5–11 (12) × 5.0–6.5 µm (mean=8.9×5.5 µm), Q = (1.5) 1.6–(1.61)–1.8, often ellipsoid-amygdaloid (gallica-type, according to Marxmüller 1982, see notes below), mixed with less frequent (broadly) ellipsoid ones, both thin- and thick-walled. **Basidia** 32–45×7.5–9.0 µm, 4-spored, (narrowly) clavate, clamped; crassobasidia present. **Cheilocystidia** 15–25×5.0–12 µm, usually regular, less frequently irregular, clavate, subcylindrical, (sub)fusoid, mostly single, sometimes in fascicles, 2 (3)-celled or in chains of cells in lower part, thin- to slightly thick-walled, clamped. **Pileipellis** a cutis of radially arranged, ± cylindrical, thin- to slightly thick-walled, clampless hyphae with cylindrical, usually irregular to coralloid terminal cells. **Pileus scales** consisting of 10–125 (175) × 5.0–25 µm, ellipsoid, subfusoid to cylindrical, slightly thick-walled, hyaline to brownish cells, 0–2-septate; terminal cells: 15–130×5.0–25 µm, subulate, ellipsoid to cylindrical. **Stipe velar hyphae** similar to those of the pileus scales. **Caulocystidia** 20–55×11–23 µm, (broadly) clavate, vesiculose, fusoid-clavate, thin- to slightly thick-walled.

Ecology and pathology In the Czech Republic and Slovakia, the species grows at lower altitudes in floodplain forests, wet alder stands, thermophilic oak and hornbeam stands. It also grows in parks and secondary plantations, on roots, stumps and stems of broadleaved trees and less frequently on coniferous hosts. *Armillaria gallica* mostly

occurs as a saprophyte in litter, decomposing buried wood, roots, stumps and coarse woody debris lying on the ground. It can occasionally behave as a nectrotrophic parasite in weakened trees, where the fungus attacks roots and penetrates the basal part of stem and causing a heart rot. *Armillaria gallica* occurs sympatrically with *A. mellea* in thermophilic oak stands or with *A. socialis* in floodplain forests. The species could co-occur with *A. cepistipes, A. ostoyae*, and, occasionally, with *A. borealis* in the higher elevation regions of its distribution.

Hosts of *A. gallica*. Conifers: *Abies* spp., *Juniperus* spp., *Larix decidua, Picea* spp., *Pinus* spp., *Pseudotsuga menziesii, Taxus baccata, Thuja plicata*. **Angiosperms:** *Acer* spp., *Aesculus hippocastanum, Alnus glutinosa, Armeniaca vulgaris, Betula* spp., *Carpinus betulus, Cerasus avium, Cornus mas, Corylus* spp., *Fagus sylvatica, Fraxinus* spp., *Gleditschia triacanthos, Malus domestica, Padus serotina, Populus* spp., *Prunus domestica, Quercus* spp., *Robinia pseudacacia, Rosa* spp., *Salix* spp., *Sambucus* spp., *Sorbus* spp., *Syringa vulgaris, Tilia* spp., *Ulmus* spp.

Fruiting: September to October. Although *A. gallica* produces fruiting bodies early, its fruiting period is preceded by *A. borealis* and followed by *A. ostoyae* and *A. cepistipes* at localities of sympatric occurrence.

Notes

According to the descriptions above, it is clear that *A. cepistipes and A. gallica* are very similar. *A. cepistipes* differs from *A. gallica* by (1) scales, which disappear very soon except for the centre (± distinct central ocella developed), (2) mostly irregularly breaking annulus (often star-like breaking in *A. gallica*), (3) stipe surface generally covered only with sparse fibrillose velar remnants (rich fibrillose or fibrillose-tomentose velar remnants in *A. gallica*), and (4) basidiospores of the "cepistipes-type", 7.0–9.0 (10) × 4.5–6.5 (7.0) µm, mostly broadly ellipsoid to subglobose, mixed with less frequent ellipsoid ones (of the "gallica-type", 7.5–11 (12) × 5.0–6.5 µm, often ellipsoid-amygdaloid, mixed with less frequent (broadly) ellipsoid ones in *A.gallica*). All differences between the taxa are summarised in Table 3. Several times, a colour form of *A. cepistipes* with distinctly (blue-) grey (grey-brown) pileus and scales was found. It seems to be associated with broadleaved trees, found only on *Fagus* and *Fraxinus* in relatively wet environments at higher elevations. *A. gallica* has never been found to correlate to this type of form.

According to the original description by Velenovský, along with his unpublished drawings, *A. inflata* Velen. 1920 may represent an older name for this particular fungus. However, his specimens were not preserved and, therefore, it is better to consider this antiquated name a nomen dubium (Antonín 1990).

Several collections (BRNM herbarium nos. 706834, 706877, 695717, 706816; see details in Table 1) contain intermediate PCR-RFLP profiles between *A. cepistipes* and *A. gallica* (Fig. 4). These were characterised by a grey-brown to pale ochraceous brown, entirely **squamulose pileus with tomentose centre** covered with adpressed, at margin adpressed to erect, grey-brown to (pale) brown scales, later slowly glabrescent towards margin, a thin fibrillose-membranaceous annulus, white above, grey below, rarely with yellow velar remnants at margin, a cylindrical stipe with distinctly clavate to bulbous base (**like *A. cepistipes***) and **white to whitish** basal mycelium. Their basidiospores are 6.5–10×4.0–6.5 µm large (average 8.5×5.4 µm, Q=1.3–(1.8)–1.9), both broadly ellipsoid to subglobose (cepistipes-type according to Marxmüller 1982), and ellipsoid to subamygdaloid (gallica-type according to Marxmüller 1982), cheilocystidia 20–50×7.0–15 µm, **regular to irregular**, pileus scales consisting of 15–80 (120) × 7.5–40 µm large, ellipsoid, subfusoid to cylindrical cells, with conical, ellipsoid to cylindrical, 12–70 (120) × 7.5–20 µm large terminal cells. These morphs have been found on spruce and alder hosts. These specimens were preferentially selected for DNA sequencing of the tefa region.

Molecular study

PCR-RFLP and sequencing of rDNA

Altogether, 47 specimens of the *Armillaria cepistipes – A. gallica* group were studied for specific RFLP patterns. The majority of specimens belonged to one of the earlier published RFLP profiles (Lochman et al. 2004a). Twenty-two of the specimens belonged to *A. cepistipes*, 21 to *A. gallica*, and 4 specimens possessed the intermediate pattern between those of *A. gallica* and *A. cepistipes*. The restriction pattern of *A. gallica* contains five fragments characterised by lengths of 294, 227, 69, 63 and 43 bp. The *A. cepistipes* restriction pattern contained four fragments of lengths 294, 227, 132 and 43 bp. Intermediate profiles were occasionally detected with lengths 294, 227, 132, 69, 63 and 43 bp. This polymorphism was caused by a single transition (C-T) in a gene copy. All the ITS and IGS-1 sequences of selected samples (except the *A. ectypa*; see Table 1) showed 99–100% identity with previously published sequences (according to Chillali et al. 1998; Keča et al. 2006; Lochman et al. 2004b). The comparison of nucleotide polymorphism is shown in Table 4.

Sequencing the tefa region

Four specimens of *Armillaria cepistipes*, four of *A. gallica* and three with intermediate restriction patterns were chosen for the tefa gene region sequencing. The tefa alignment

Table 3 Summary of distinguishing characters between *A. cepistipes* and *A. gallica*

	A. cepistipes	***A. gallica***
Pileus scales	Fibrillose to tomentose at centre, often in erect groups, except for centre (± well-developed distinct central ocella), very soon glabrescent	Long fibrillose, almost pyramidal at centre, soon glabrescent towards margin, centre ± (scatteredly) squamose
Pileus	Entirely bluish grey-brown form present	Entirely bluish grey-brown form never present
Annulus	Breaking up mostly irregularly	Breaking up often star-like
Stipe	Always with distinctly clavate to bulbous base (± more distinctly than *A. gallica*); velar remnants sparse, fibrillose	Often with distinctly clavate to (sub)bulbous base; velar remnants distinct, fibrillose or fibrillose-tomentose
Basidiospores	7.0–9.0 (10) × 4.5–6.5 (7.0) µm, mean=8.3×5.3 µm, mostly broadly ellipsoid to subglobose (cepistipes-type), mixed with less frequent ellipsoid ones	7.5–11 (12) × 5.0–6.5 µm, mean=8.9×5.5 µm, often ellipsoid-amygdaloid (gallica-type), mixed with less frequent (broadly) ellipsoid ones
Cheilocystidia	14–35×6.0–11 µm, regular to often irregular	15–25×5.0–12 µm, usually regular, less frequently irregular
Terminal cells of pileus scales	15–90 (125) × 5.0–25 µm	15–130×5.0–25 µm
Hosts	*Quercus*, *Carpinus*, less frequently other broadleaved trees	*Fagus, Picea*, less frequently *Abies, Fraxinus*

contained 498 characters, including 370 conserved, 127 variable and 74 parsimony-informative sites.

The tefa sequences of *A. cepistipes* and *A. gallica* showed significant differences, and all sequences of the collections with intermediate PCR-RFLP profiles are grouped together with those of *A. cepistipes* in a strongly supported clade (sequence comparison in Table 4). Genetic distance between the species was calculated to be 0.0526–0.0571 by the Kimura two-parameter model. The distances were significantly lower within *A. gallica* sequences (0.0000–0.0061) and within *A. cepistipes* (0.0000–0.0020 including specimens with intermediate PCR-RFLP profiles). According to these results, all sequenced specimens of the putative hybrids belong to the species *A. cepistipes*.

Both phylogenetic methods produce consistent results, and the topology of the different trees differs only in the internal branching of *A. mellea* (Fig. 5). In terms of MP analysis, the most parsimonious trees resulted in a 50 % majority-rule MP consensus tree. The equally parsimonious trees were found through MP searches with the following scores: score of best trees = 206, tree length = 206, consistency index (CI) = 0.7282, retention index (RI) = 0.7956, re-scaled consistency index (RC) = 0.5793, and homoplasy index (HI) = 0.2718. The Bayesian consensus tree was calculated from trees saved from the likelihood score convergence point (10,000 generations), and the first 100 trees were discarded as burn-in.

Fig. 4 *Armillaria cepistipes* (putative hybrid). Slovakia, Low Tatra Mts., Lupčianská dolina, 13 September 2005, V. Antonín 05.158. Photo: V. Antonín

The resulting phylograms include the well-supported main clade of subgenus *Armillaria*, while subgenus *Desarmillaria* Herink (*A. ectypa* and *A. socialis*) forms the outgroup. The following three subclades were identified inside the subgenus *Armillaria*.

1. Subclade formed by two sister branches, the first one of newly sequenced *A. cepistipes* specimens and the second one of the *A. ostoyae* – *A. borealis* group, including sequences of extra-European *A. gemina* and *A. ostoyae* specimens.
2. Subclade of putative European *A. borealis* sequences (published by Maphosa et al. 2006). Compared with our newly sequenced specimen of *A. borealis*, it appears that both sequences probably belong to different species.
3. Subclade of *A. gallica* specimens grouped with extra-European specimens of *A. cepistipes, A. gallica* and *A. nabsnona*. The exact position of *A. mellea* is only weakly supported in both analyses.

The phylogenetic positions of most species resemble those based on earlier published ITS or IGS-1 data (Anderson and Stasovski 1992; Chillali et al. 1998; Keča et al. 2006). The only exception is *A. cepistipes*, which we

Table 4 Comparison of nucleotide polymorphism of selected aligned *A. cepistipes* and *A. gallica* tefa, ITS and IGS sequences

Specimen	tefa position no.							
	1–7	46	69	87	93	99	139	143–145	150–152
A. cepistipes EU251392	GGAACTG	C	C	G	T	T	T	ACT	TCC
A. cepistipes EU251395	GGAACTG	C	Y	G	T	T	T	ACT	TCC
A. cepistipes (h) EU251396	GGAACTG	C	C	G	T	T	T	ACT	TCC
A. gallica EU251391	GGAACTG	A	C	A	C	C	C	GCC	-GT
A. gallica EU251390	GGAACTG	C	C	A	C	C	C	GCC	-GT

Specimen	tefa position no.								
	157–160	170	262	271	310–312	323–324	340	349–351	447
A. cepistipes EU251392	GCAT	G	G	C	CCC	CC	C	CTT	C
A. cepistipes EU251395	GCAT	G	G	C	CCC	CC	C	CTT	C
A. cepistipes (h) EU251396	GCAT	G	G	C	CCC	CC	C	CTT	C
A. gallica EU251391	CCAA	T	T	A	TGC	T-	T	TCY	T
A. gallica EU251390	CCAA	T	T	A	TGC	T-	T	TCY	T

Specimen	ITS position no.								
	1–7	120	138	432	544	644	670	688	726
A. cepistipes EU257719	GAAACTT	T	C	T	G	C	T	A	T
A. cepistipes EU257715	GAAACTT	T	C	T	A	C	T	A	T
A. cepistipes (h) EU257716	GAAACTT	C	C	C	A	C	C	A	T
A. gallica EU257718	GAAACTT	C	T	C	A	T	C	R	C

Specimen	IGS position no.								
	1–7	101	343	439	447	553	638	682	801
A. cepistipes EU257709	AACGATG	T	A	G	A	A	C	A	T
A. cepistipes (h) EU257710	AACGATG	C	R	T	G	A	T	A	C
A. gallica EU636240	AACGATG	C	G	G	G	G	T	G	C

Approximately 90% of the polymorphic positions of each gene region are shown. For detailed data on the sequenced specimens, see Tables 1 and 2

found to be more proximal to the *A. ostoyae – A. borealis* clade than to *A. gallica*.

Discussion

According to anatomic and morphological characters, there are observable differences between *A. cepistipes* and *A. gallica* (for a summary, see Table 3). However, as previously reported by other authors (Marxmüller 1980, 1982, 1986, 1987; Romagnesi and Marxmüller 1983), the morphological similarity between the two species is very high. In many cases, the two species are indistinguishable in the field when using classical taxonomic methods, particularly in the case of older carpophores. Other distinguishing characters used by Pegler (2000) and Termorshuizen and Arnolds (1987)—colour of scales, carpophores, and their character (slender and/or robust)—do not include the entire variability of those features. Marxmüller (1982) also distinguished typical forms of basidiospores for each species. However, these typically formed basidiospores are mixed with unspecific ellipsoid ones.

According to the tefa sequences, *A. cepistipes* and *A. gallica* are clearly distinguishable, while collections with intermediate PCR-RFLP profiles apparently belong to *A. cepistipes* species. The topology of the tefa-derived phylogram does not support close taxonomic relationships of the two species; *A. cepistipes* is rather proximal to the *A. ostoyae – A. borealis* group.

The morphological similarities between *A. gallica* and *A. cepistipes* may be explained by the similar selection pressures on the species and/or by a common evolutionary history. The genus *Armillaria* likely originates in the southern hemisphere (Coetzee et al. 2003; Maphosa et al. 2006), and the morphological similarity of most annulate *Armillaria* species can be explained by their short evolutionary history in the northern hemisphere. This hypothesis

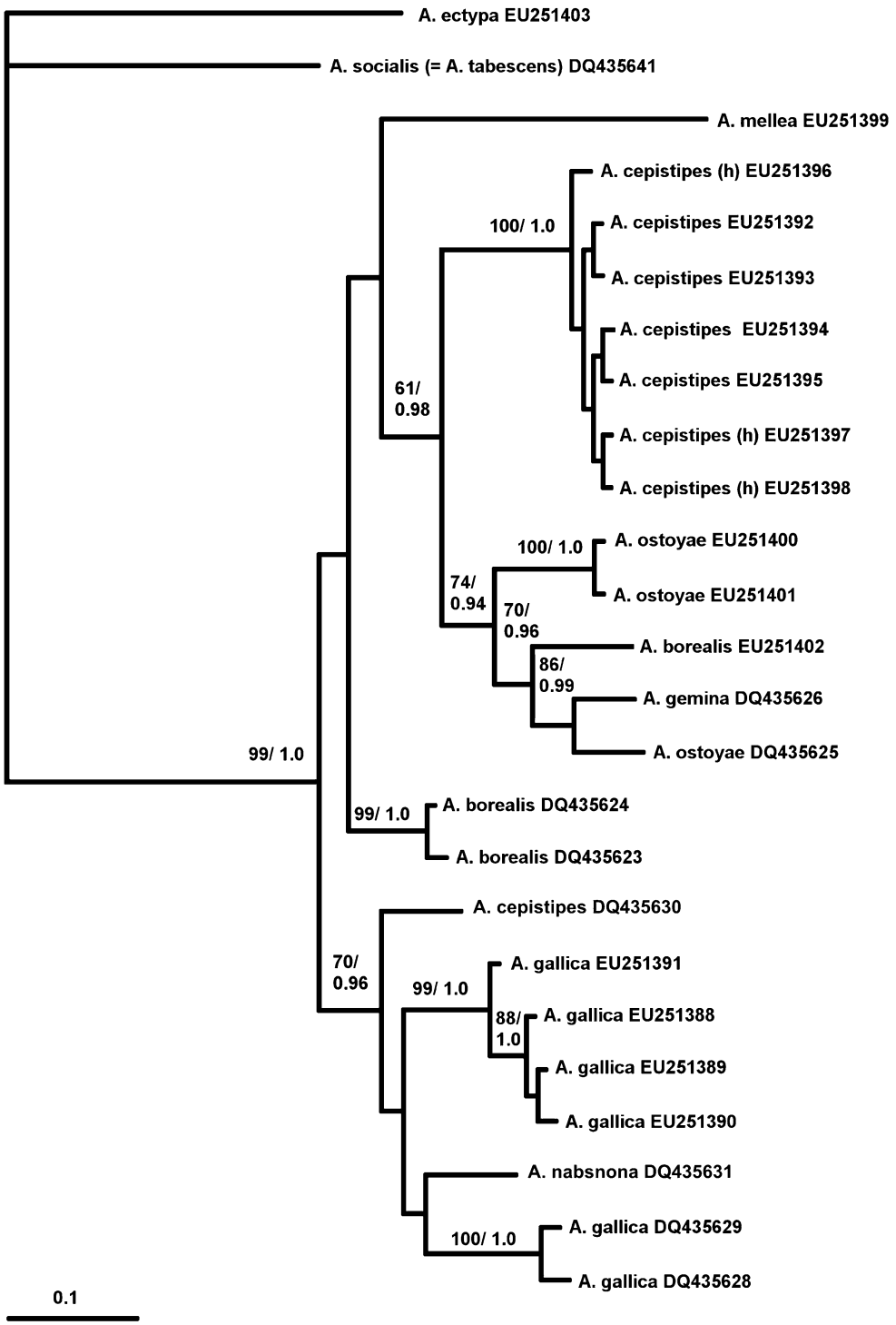

Fig. 5 Phylogenetic tree of the genus *Armillaria* based on sequences of the tefa region. Newly obtained sequences were completed with those published by Maphosa et al. (2006). These sequences refer to isolates from USA, only these of *A. borealis* have origin in Germany and Finland, respectively. All available information on these isolates see in "Material and Methods". The tree was constructed using the Bayesian method (for details, see text). The bootstrap values of the maximum parsimony analysis are shown above the nodes if they are higher than 50%; the posterior probability values of the Bayesian analyses are shown if they are higher then 0.5. *Scale bar* represents one change per 10 positions

is supported by minimal differences between their ITS regions (see Table 4). The IGS1 region is suitable for distinguishing *Armillaria ostoyae* and *A. borealis* in Europe (Anderson and Stasovski 1992; Chillali et al. 1998; Keča et al. 2006), but the position of some specimens of *A. cepistipes* and *A. gallica* was not previously resolved. Generally, polymorphism among ITS sequences of northern hemisphere *Armillaria* species of subgenus *Armillaria* is relatively low, compared to rather high ITS polymorphism within *A. socialis* (Antonín et al. 2006). Nonetheless, PCR-RFLP methods targeting the ITS region proposed by Lochman et al. (2004a) are well suited to rapid identification of at least Central European *Armillaria* specimens. The advantage of this method compared to a similar one proposed by Harrington and Wingfield (1995) is that it utilises *Armillaria*-specific primers and thus avoids non-specific DNA amplification.

The interspecific hybridisation between *A. cepistipes* and *A. gallica* is very unlikely according to the tefa data that we present. Kauserud et al. (2007) revealed interspecific hybrids among cryptic species of *Coniophora puteana* group, characterised by numerous heterozygous positions in three DNA regions, including tefa and ITS. In contrast, our tefa sequences of putative hybrids contain very few heterozygous positions—the only one heterozygous position was found in sequence no. EU251397—so the specimens with intermediate RFLP pattern should be treated as genuine *A. cepistipes* samples. In general, tefa sequences appear to be a useful tool for differentiating *Armillaria* species.

The current study confirmed considerable genetic differences between European and American representatives of *A. cepistipes, A. gallica* and *A. ostoyae*. Although Harrington and Wingfield (1995) found remarkable genetic differences between European and American specimens of *A. cepistipes* and *A. gallica*, a complex study comparing *Armillaria* species of both continents has not yet been worked out.

Acknowledgements These studies were supported by the Czech Science Foundation (project no. 526/05/0086) and by the Ministry of Education, Youth and Sports, project no. MSM 6215648902.

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