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The first record of *Hebeloma vinosophyllum* (*Strophariaceae*) in Southeast Asia

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Abstract – Basidiomata of a *Hebeloma* sp. were collected from two urea-plots in a pine (*Pinus kesiya*) forest, Da Lat City, Lam Dong Province, Vietnam. Based on morphology, molecular phylogeny, and mating compatibility, the specimens were identified as *Hebeloma vinosophyllum*. This is the first record of *H. vinosophyllum* in Southeast Asia and of *H. vinosophyllum* occurring in *P. kesiya* forests, one of two dominant pines in Southeast Asia.

Key words – *Porphyrospora*, cortina, cheilocystidia, ammonia fungi

Introduction

Hebeloma vinosophyllum was originally described by Hongo (1965) from Japanese specimens. This species was reported to resemble *H. sarcophyllum* (Peck) Sacc. in having a light brown to purplish red spore print but to differ in the shape of the hymenial cystidia (Hongo 1965, Singer 1986, Vesterholt 2005). In 1975, Sagara reported *H. vinosophyllum* as a member of the "ammonia fungi

... a chemo-ecological group of fungi that develop reproductive structures exclusively or relatively luxuriantly on soil after a sudden addition of ammonia, of other nitrogenous materials that react as bases by themselves or on decomposition, or of alkalis" (Sagara 1975). Basidiomata of *H. vinosophyllum* were recorded not only from the forest floor sites augmented with urea but also from soils naturally disturbed by decomposition of dead animal bodies (Sagara 1976, 1992, Takayama & Sagara 1981, Fukiharu et al. 2000a,b).

Hebeloma vinosophyllum has been recorded from different vegetation in various geographical regions of Japan. The fungus has been collected from *Castanopsis*, *Quercus*, and *Pinus* forests (Table 1) in middle and western Honshu (Hongo 1965, Sagara 1975, Suzuki 1987, Yamanaka 1995, Fukiharu et al. 1995, Fukiharu et al. 2000a,b, Kasuya 2002, Imamura & Yumoto 2004), and Shikoku and Kyushu (Sagara 1975). However, this fungus has not been recorded for urea applications in *Pinus* and *Quercus* forests in Hokkaido (Sagara 1975) or eastern Honshu (Sagara 1975, Fukiharu & Horigome 1996), or in *Castanopsis* and *Quercus* forests on Ryukyu Island (Fukiharu & Hongo 1995).

Outside Japan, *H. vinosophyllum* has been recorded from a fagaceous-coniferous forest (*Quercus*, *Castanopsis*, *Castanea*, *Cunninghamia*, *Pinus*, etc.) near SanMing City, Fujian Province, China (Hongo et al. 1996; no voucher specimen deposited – Dr. Noriko Kinjo, pers. comm.) and possibly in South Korea (Lee 2011; species name listed without details). These collection records suggest that *H. vinosophyllum* has an East Asian distribution (Fukiharu & Horigome 1996).

During a survey of ammonia fungi in southern Vietnam, specimens belonging to *Hebeloma* sect. *Porphyrospora* Konrad & Maubl. ex Vesterh. (Vesterholt 2005) were collected. We identified these Vietnamese specimens based on morphology, molecular phylogeny, and compatibility between the strains from Vietnam and stock cultures of *H. vinosophyllum* from Japan.

Materials & methods

Collection

Urea-treated plots were established in a *Pinus kesiya* Royle ex Gordon forest (more than 30 years old), ca. 1500 m altitude, at Xuan Tho, Da Lat City, Lam Dong Province, Vietnam. Commercial granulated urea fertilizer was applied to 2 plots (1 × 1 m) on the forest floor in the amount of 600 g/m^2. About two months after urea application, basidiomata of *Hebeloma* sp. were observed, collected, and subcultured onto agar medium. Cultures were maintained on MY agar medium [malt extract 10 g/L (Difco, Detroit, USA), yeast extract 2 g/L (Difco, Detroit, USA) and agar 15 g/L (Nacalai Tesque, Japan)] at 20°C in darkness. Basidiomata were dried at 60°C for 24 h and deposited in the herbarium of the Natural History Museum and Institute, Chiba, Japan (CBM). Cultures were deposited in Faculty of Biology, University of Science, Ho Chi Minh City, Vietnam (HCMUS).

Other specimens and cultures of *H. vinosophyllum* in Japan, *H. aminophilum* in Australia, and *H. porphyrosporum* in Italy were deposited in the Natural History Museum and Institute, Chiba, Japan (CBM); Forest Microbiology Division, Forestry and Forest Products Research Institute, Ibaraki, Japan (CHU); and Biological Resource Center, National Institute of Technology and Evaluation, Japan (NBRC).

Observation

Morphological observations were recorded from both dry and fresh field specimens. Color notation follows Kornerup & Wanscher (1981).

The microscopic characteristics were observed using differential interference contrast (DIC) on Labophot-2 (Nikon, Tokyo, Japan) or Olympus B51 (Tokyo, Japan) microscopes after hand-sectioning and mounting in 10% ammonium hydroxide aqueous solution. The basidiospores were also observed by a scanning electron microscope (SEM) (Hitachi S-800; Tokyo, Japan) at 15.0 kV after rehydrating in 25% aqueous ammonia, fixing in 2.5% osmic acid, and coating with platinum-palladium in an ion sputter-coater (E-1030; Hitachi, Tokyo, Japan). Abbreviations used: Q = mean length/width ratio measured from "n" number of spores; m = mean spore length and width.

Phylogenetic analysis

Total nuclear DNA (nDNA) was extracted from disintegrated tissue of dried mycelia and basidiomata using 200 µl 0.5 mm glass beads (Yasui Kikai, Tokyo, Japan) and 500 µl TES buffer [50 mM Tris-HCl, pH 7.5, 20 mM EDTA, 1% sodium dodecyl sulfate (SDS)] by vigorous shaking (FastPrep System; mp-Biomedicals, Solon, OH, USA) at 6.5 m/s for 45 s. Soluble fractions were recovered by centrifugation. DNA was purified using TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)–saturated phenol/chloroform/isoamyl alcohol (25:24:1, Nippon Gene, Tokyo, Japan) extraction followed by an isopropyl alcohol precipitation. After desiccation of the DNA pellet, DNA was dissolved in a 30 µl TE buffer. For some samples, genomic DNA was further purified using NucleoSpin Extract II (Macherey-Nagel, Duren, Germany), following the manufacturer's recommendations.

The primer pairs ITS1 and ITS4 or ITS5 and ITS4 (White et al. 1990) were used to amplify the ITS regions of ribosomal DNA (rDNA). The primers LR0R and LR5 (Vilgalys and Hester 1990) were used to amplify 28S rDNA (large subunit, LSU). Polymerase chains reactions (PCR) were carried out using KOD FX (Toyobo, Tokyo, Japan) following the manufacturer's instructions. PCR products were purified using NucleoSpin Extract II, and DNA fragments were directly sequenced using the BigDye Terminator ver3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the protocol provided. Reactions were cleaned up using the Centri Sep (Princeton Separations, Adelphia, NJ, USA), before analyzing by capillary electrophoresis on a 3130x DNA Analyzer (Applied Biosystems). Sequences were assembled and edited using ATSQ software (Genetyx, Tokyo, Japan) and deposited in GenBank/EMBL/DDJB (Table 1).

Two data sets (LSU, ITS) were established that included sequences from Vietnamese specimens, several sequences of Japanese *H. vinosophyllum,* and sequences downloaded from GenBank (Table 2). The data sets were aligned using Clustal X ver. 1.81 (Jeannmougin et al. 1998), and the resulting alignments were manually refined. For phylogenetic analyses, each data set (LSU = 892 bp, ITS = 625 bp) was analyzed using

Table 1. Collection details of *Hebeloma* specimens and cultures in the phylogenetic analysis.

Taxon	Voucher no./Isolate no.	Origin	GenBank acc. no.	
			ITS	LSU
H. vinosophyllum	CBM:FB32636*/CBM-BC69	Japan	AB1742172	AB1742341
	CBM:FB12306*/CBM-BC307	Japan	AB1742173	AB1742342
	CBM:FB12325*/CBM-BC314	Japan	AB1742174	AB1742343
	CBM:FB12381*/CBM-BC315	Japan	AB1742175	AB1742344
	CBM:FB14335*/CBM-BC337	Japan	AB1742176	AB1742345
	CBM:FB14285*/CBM-BC366	Japan	AB1742177	AB1742346
	CBM:FB14216*/CBM-BC376	Japan	AB1742178	AB1742347
	CBM:FB14520*/CBM-BC384	Japan	AB1742179	AB1742348
	CBM:FB14502*/CBM-BC407	Japan	AB1742180	AB1742349
	CBM:FB15552*/CBM-BC437-3	Japan	AB1742181	AB1742350
	CBM:FB15553*/CBM-BC438-2	Japan	AB1742182	AB1742351
	CBM:FB15556*/CBM-BC440-2	Japan	AB1742183	AB1742352
	CBM:FB24700/CBM-BC533**	Japan	AB1742184	AB1742353
	—/NBRC107913	Japan	AB1742185	AB1742354
	—/CHU4001*	Japan	AB1742186	AB1742355
	CBM:FB39191*/HCMUS-C1	VietNam	AB1742187	AB1742356
	CBM:FB39192*/—	VietNam	AB1742188	AB1742357
	CBM:FB39267*/HCMUS-C2	VietNam	AB1742189	AB1742358
H. aminophilum	—/CHU5001*	Australia	AB1742190	AB1742359
	—/CHU5002***	Australia	AB1742191	AB1742360
	CBM:FB35472*/—	Australia	AB1742192	AB1742361
H. porphyrosporum	CBM:FB24804/—	Italy	AB1742193	AB1742362

Basidiomata appeared * in urea plot, ** around decaying unidentified animal, or *** around decaying snake.

Tree-Puzzle 5.2 (Schmidt et al. 2002). The maximum likelihood (ML) tree (Felsenstein 1981) of each data set was inferred based on quartet puzzling algorithm (Strimmer & Haeseler 1996) with the options of 1000 puzzling steps, model of substitution HKY (Hasegawa et al. 1985).

Table 2. Published sequences in the phylogenetic analysis

Taxon	GenBank acc. no.	
	ITS	LSU
Hebeloma fastibile	AF325643	AY033139
H. sarcophyllum	AF124715	——
H. vinosophyllum (NBRC32945)	[03294501]*	——
H. vinosophyllum (Boyle et al. 2006)	AY320398**	——
Alnicola lactariolens	AY818352	AY818353
Anamika angustilamellata	AY575919	AY575919
An. indica	AF407163	AF407164

* Retrieved from DNA resource of Biological Resource Center, National Institute of Technology and Evaluation, Japan.

** Used only for alignment with sequence from Vietnamese *H. vinosophyllum*.

Mating test

The dikaryotic stock culture of Vietnamese *Hebeloma* sp. (HCMUS-C2) was cultured on Ohta's medium (Ohta 1990) with 15 g agar, at 20°C, 12 h/12 h light/dark cycle. Light was irradiated at ca. 140 μmol $m^{-2}s^{-1}$. Basidiomata appeared in vitro after ca. 3 weeks, when a small piece of sterile filter paper was placed under the hymenophores for collecting basidiospores. Monokaryotic strains were obtained by germinating these in vitro spores.

Mating tests between monokaryotic tester strains of Vietnamese *Hebeloma* sp. and dikaryotic stock cultures of Japanese *H. vinosophyllum* (isolates CBM-BC69 and CHU4001) were compared. Mating types were determined by pair-mating with each other. The mating tests were conducted by placing a pair of mycelial discs (a monokaryotic tester strain vs. a dikaryotic culture) on opposite sides of 90 mm MY agar plates, incubated at 25°C in darkness, and replicated 3 times. After two weeks, mycelium from the monokaryotic colony was removed from the edge farthest from the dikaryotic colony and examined microscopically; a compatible crossing was indicated by the presence of clamp connections.

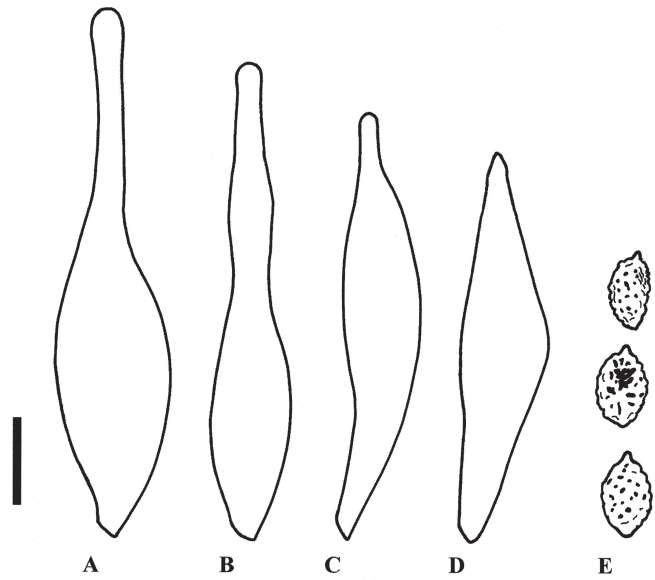

Plate 1. *Hebeloma vinosophyllum*. A–D: Hymenial cystidia. E: Basidiospores. Scale bar = 10 μm.

Taxonomy

Hebeloma vinosophyllum Hongo, J. Jap. Bot. 40: 314. 1965 Plates 1–2

[Description based on Vietnamese specimens] Pileus 20–70 mm broad, at first hemispherical, then becoming plano-convex to plane; cream to light

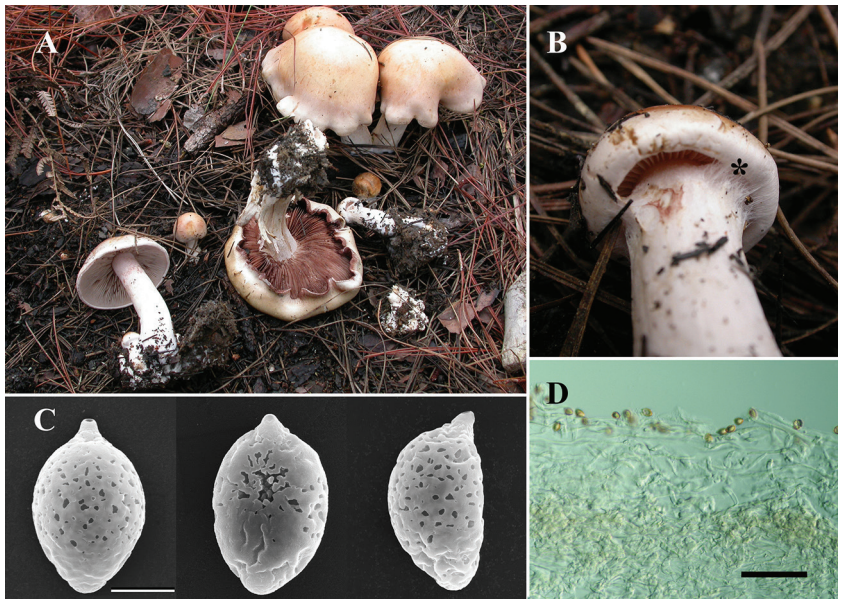

Plate 2. *Hebeloma vinosophyllum* from the urea plot in Viet Nam. A: Basidiomata in nature. B: Cortina (partial veil; *) of a young basidioma. C: Scanning electron micrographs of basidiospores. D: Ixotrichodermium structure of pileipellis. Scale bars: C = 3 µm; D = 30 µm.

vinaceous (7–9A2–3), usually darker at the top; surface glabrescent, smooth, viscid to glutinous in wet condition; margin at first inrolled and entire, then plane or eroded-undulating, sometimes still incurved at maturity. Universal veil absent, partial veil fibrillose, white, dry, at first cortinoid (following Vesterholt 2005), leaving a few fibrils on the stipe, disappearing with age. Lamellae crowded, 2–4 mm width, 5–30 mm length, adnexed; pinkish brown (8–9C3–5) to light vinaceous (8–9A3–5) and darker at maturity. Stipe up to 105 x 15 mm, thickened downward; surface somewhat smooth to longitudinal striate, floccose-pruinose at the apex; fleshy-fibrous, stuffed at first, then hollow; cream (7A2–3) to light clay (5D5–6). Context pale white (7–8A2–3); smell musty; taste weakly bitter. Spore print vinaceous to brownish red (9–10C3–4).

Basidiospores m = 9.7 ± 0.6 × 6.1 ± 0.4 µm (8.4–11.3 × 5.3–7.2 µm, n = 50, Q = 1.59), amygdaliform to citriform. Basidia 30–40 × 8–9 µm (without sterigmata), clavate, four-spored; sterigmata 3–5 µm long. Pleurocystidia 48–60 × 8–12 µm, numerous, ventricose-rostrate to lageniform, narrowly utriform or mucronate. Cheilocystidia similar to pleurocystidia but less numerous. Pileipellis an ixotrichodermium, composed of gelatinized cylindrical cells 50–60 µm in length; hypodermium cellular composed of globose cells 7–9 µm in width.

Habitat – In Vietnam, scattered, gregarious, or subcespitose on urea-plots in *Pinus kesiya* forest, ca. 1500 m, 2 months after urea application. Elsewhere in warm temperate East Asia scattered, gregarious, or subcespitose on soil under *Castanopsis*, *Quercus*, and *Pinus*, usually 6-12 months after urea application or following decomposition of dead animal bodies.

Distribution (Plate 3): Japan (middle to western Honshu, Shikoku, and Kyushu); China (SanMing, Fujian Province); Vietnam (DaLat City, LamDong Province); possibly South Korea (confirmation needed).

Specimens examined: **VIETNAM**. **Lam Dong**: Da Lat City, Xuan Truong - Xuan Tho (UTM, 49P, 023053, 1319015), 24 Jun 2009, B.N Truong (CBM FB-39191; culture HCMUS-C1); 25 Jun 2009, B.N Truong (CBM FB-39192); 14 Jun 2009, B.N Truong (CBM FB-39267; culture HCMUS-C2). **JAPAN**. **Shiga**: Otsu City, Ishizune, 17 Jul 1961, T Hongo (TNS-F-39101, holotype)

Plate 3. Biogeographic distribution of *Hebeloma vinosophyllum* in Japan (Honshu: Saitama, Tochigi, Tokyo, Chiba, Shiga, Shizuoka, Kyoto, Tottori; Shikoku: Kochi; Kyushu: Oita, Miyazaki), South Korea, China (Fujian Province), and Vietnam (Da Lat). Δ = no deposition of voucher specimen, O = collection site of voucher specimen.

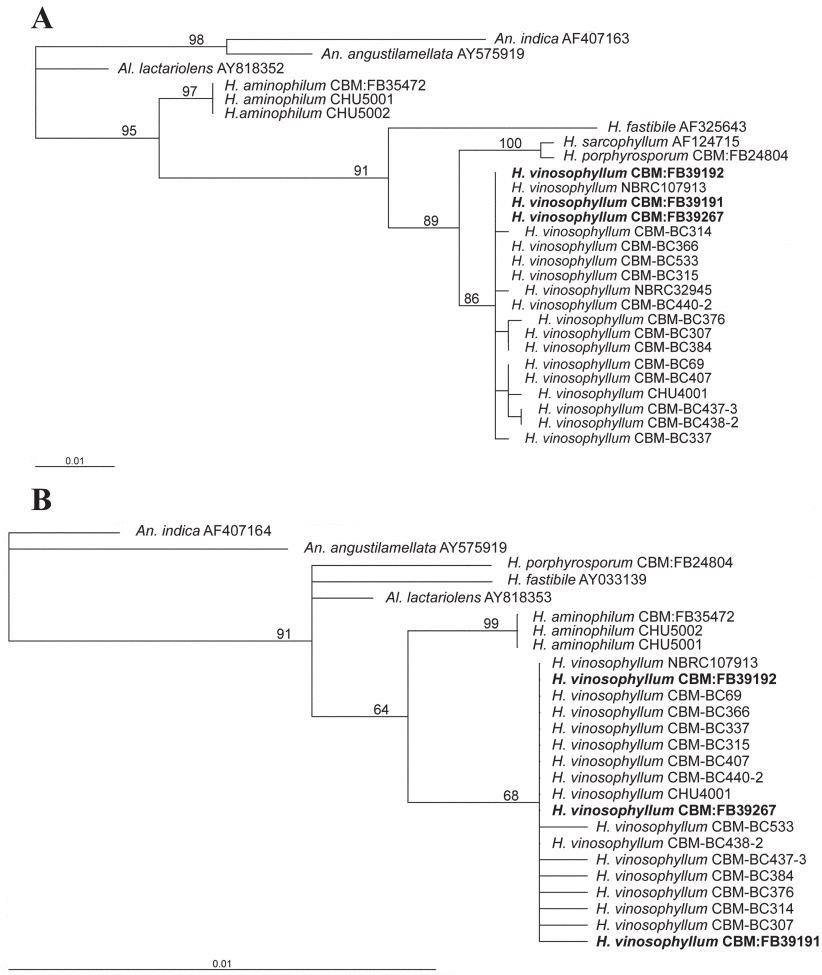

Plate 4. The maximum likelihood phylogenetic tree based on ITS data set (A) and LSU data set (B) of *Hebeloma vinosophyllum* and allied species. The numbers on each branch represent bootstrap percentage support values. The scale bar shows the ratio substitutions/site. Trees are rooted by outgroup *Anamika indica* and *An. angustilamellata*. Vietnamese specimens are in bold. Abbreviation: *Al.* = *Alnicola*.

Phylogenetic analysis

Dikaryotic cultures and dried specimens of Vietnamese *H. vinosophyllum* have identical ITS and LSU sequences. Sequence alignments of dikaryotic *H. vinosophyllum* cultures from Japan and dried *H. vinosophyllum* specimens

from Vietnam showed 99.55% (887/891 bp) similarity of the LSU and 98.52% (600/609 bp) similarity of the ITS regions. The phylogenetic tree (Plate 4) grouped the dikaryotic cultures and the Vietnamese specimens in the same clade as those from Japan (supporting values = 68% in LSU and 86% in ITS).

We could not analyze the holotype specimen (TNS-F-39101) of *H. vinosophyllum* molecularly due to its long storage with para-formaldehyde. The lower ITS similarity of 95.4% (587/615 bp) for GenBank AY320398 (Boyle et al. 2006, labeled as *H. vinosophyllum*) suggests that that material is misidentified.

Mating test

The di-mon mating tests between monokaryotic strains from Vietnam and dikaryotic cultures of Japanese *H. vinosophyllum* showed clamp connections (Table 3), indicating that all Vietnamese monokaryotic strains were compatible with Japanese *H. vinosophyllum* cultures.

Table 3. Dikaryon-monokaryon mating tests between *Hebeloma vinosophyllum* (Japan) and *Hebeloma* sp. (Vietnam)*

		Monokaryotic strains of Vietnamese *Hebeloma* sp. HCMUS-C2							
		A_1B_1		A_2B_2		A_1B_2		A_2B_1	
		C2-03	C2-36	C2-02	C2-37	C2-09	C2-38	C2-01	C2-35
Dikaryotic stock cultures of Japanese *H. vinosophyllum*	CBM-BC69	+	+	+	+	+	+	+	+
	CHU4001	+	+	+	+	+	+	+	+

*+ = clamp connections formed

Discussion

Except for forming more abundant cheilocystidia, all Vietnamese specimens were similar to the holotype specimen of *H. vinosophyllum* (TNS-F-39101) based on morphological analysis. The di-mon mating tests indicate that Vietnamese *Hebeloma* sp. belongs to the same biological species as *H. vinosophyllum*. The phylogenetic analyses (Plate 4) also indicate that all Vietnamese and Japanese specimens belong to the same species.

This is the first record of *H. vinosophyllum* in Southeast Asia (Plate 3) and of *H. vinosophyllum* associating with *P. kesiya,* one of two dominant pines in Southeast Asia (Zonneveld et al. 2009).

As a putative ectomycorrhizal fungus (Fukiharu 1991, Sagara 1995, Imamura & Yumoto 2008), *H. vinosophyllum* could habituate from hosts of

Northeast Asian fagaceous and/or pinaceous species to those of Southeast Asian *Pinus* species, or vice versa. Further studies of applications of urea to forests up to the northern limit of *P. kesiya* in central Asia (Zonneveld et al. 2009) and to fagaceous (possibly *Quercus* and *Castanopsis*) and pinaceous forests in Ryukyu islands, Taiwan, southern China, and Southeast Asia will help resolve the geographic range, host tree species, and potentially the origin of *H. vinosophyllum*.

Despite the presence of a cobweb-like partial veil (cortina), *H. vinosophyllum* could belong to *Porphyrospora* (Vesterholt 2005) based on its brownish red spore deposit and amygdaliform-citriform spores.

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