Mycol Progress
DOI 10.1007/s11557-013-0890-x

ORIGINAL ARTICLE

Morphological and molecular evidence for a new species of *Rhodotus* from tropical and subtropical Yunnan, China

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Received: 4 December 2012 / Revised: 16 January 2013 / Accepted: 18 January 2013

Abstract *Rhodotus* has been regarded as a monotypic genus, consisting of only one species, *R. palmatus*, for a long time. Morphological and phylogenetic studies were carried out on collections of *Rhodotus* from temperate, subtropical and tropical China. Our phylogenetic analysis of DNA sequences of three loci (the internal transcribed spacer, the large subunit nuclear ribosomal RNA, and the translation elongation factor-1 alpha) revealed that there are two phylogenetic species in the northern hemisphere, which is in concordance with morphological traits, supporting the division of *Rhodotus* into two distinct species. *Rhodotus asperior* is described as a new species that differs phenotypically from *R. palmatus* in its broadly ellipsoid to subglobose, more roughened basidiospores, longer cheilocystidia with slightly thickened wall, and its occurrence in tropical and subtropical environments. The discovery of this new taxon indicates *Rhodotus* has a wider distribution than previously thought.

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Keywords Geographic distribution · New taxon · Physalacriaceae · Species diversity · Taxonomy

Introduction

The genus *Rhodotus* was proposed by Maire (1926) to accommodate *Agaricus palmatus* Bull. The systematic position of *Rhodotus* remained unclear until molecular phylogenetic studies support it being a representative of the family Physalacriaceae (Moncalvo et al. 2002; Binder et al. 2006). This genus has since been regarded as monotypic with only the type species, *R. palmatus* (Bull.) Maire, originally described from Europe (Bulliard 1785; Maire 1926), and then also found in North America and Eastern Asia (Imai 1938; He 1992; Wang and Zhang 1992; Sundberg et al. 1997; Han et al. 2006), although variations were noted among different collections (Pouchet 1932; Imai 1938; Horak 1968; Krieglsteiner 1979; Kühner and Romagnesi 1984; Redhead 1989; Noordeloos 1995; Han et al. 2006; Ripkova 2003). *Rhodotus palmatus* is rare and considered endangered in Europe (ECCF 2005).

As part of our effort to study Physalacriaceae in China, we collected materials of *Rhodotus* from tropical and subtropical regions of Yunnan Province, Southwestern China. Compared to the descriptions of *R. palmatus*, these specimens showed significant divergences in morphological characters. To further understand the relationships among these *R. palmatus* specimens, we adopted both morphological and molecular approaches to examine these specimens collected from various locations.

The objectives of the present study are: (1) to compare the tropical and subtropical collections of *Rhodotus* with those from the temperate zones, and (2) to characterize

Published online: 09 February 2013

tropical and subtropical species of *Rhodotus* using morphological and molecular data.

Materials and methods

Specimens and morphological descriptions

Macro-morphological descriptions are based on the field notes and images of basidiomata. Color codes are from Kornerup and Wanscher (1981). Specimens were deposited in three herbaria: Herbarium of Cryptogams, Kunming Institute of Botany the Chinese Academy of Sciences (HKAS), Herbarium of Mycological Institute of Jilin Agricultural University (HMJAU), and Herbarium Mycological department, National Museum, Prague, Czech Republic (PRM). Herbarium codes used follow Thiers (continuously updated) with one exception: HKAS. Micro-morphological data were obtained from the dried specimens after sectioning and mounting in 5 % KOH solution. In the descriptions of basidiospores, the abbreviation [*n*/*m*/*p*] means *n* basidiospores measured from *m* basidiomata of *p* collections; *Q* is used to mean "length/width ratio" of a spore in side view; ***Q*** means average *Q* of all basidiospores ± sample standard deviation. The warts on the surface of the basidiospore were not included in the size. Basidiospores of dried specimens were examined with a Hitachi S-4800 scanning electron microscope (SEM) at 10.0 Kv.

DNA extraction, PCR and sequencing

The genomic DNA was extracted from herbarium vouchers, including one European and six Chinese collections of *Rhodotus* (Table 1). The process of extraction followed the CTAB methods described by Doyle and Doyle (1987) with minor modifications (e.g., prolongation of extraction time and higher temperature setting). For PCR reactions, the internal transcribed spacer (ITS) region was amplified using primer pair ITS1 and ITS4 (White et al. 1990), the large subunit nuclear ribosomal RNA (nrLSU) was amplified using LROR and LR5 (Vilgalys and Hester 1990), and the translation elongation factor-1 alpha (*tef1*-α) was amplified using combinations of EF1-595F, EF1-983F, and EF1-1567R (Rehner 2001; Rehner and Buckley 2005). PCR products were purified with a DNA purification kit (Bioteke) and subsequently sequenced on an ABI 3730 DNA analyzer using ABI BigDye 3.1 terminator cycle sequencing kit (Shanghai, China) with the same primers used for PCR amplifications. For PCR products that could not be sequenced directly, subcloning was carried out with DNA-A-Tailing Kit (Takara) prior to sequencing. Sequences generated in this study have been deposited in Genbank (Table 1).

For phylogenetic analyses, the sequences were initially compiled with SeqMan (DNASTAR Lasergene 9) and BioEdit (Hall 1999), then aligned using software Muscle (Edgar 2004), and finally manually adjusted where necessary. The alignments can be found in TreeBASE (Accession S13648).

Phylogenetic analyses

Based on our analysis of a larger dataset of Physalacriaceae, species of *Flammulina* were chosen as the outgroup in this study. Phylogenetic analysis was carried out in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using a Markov Chain Monte Carlo (MCMC) tree-sampling procedure.

Table 1 Sequences obtained in this study

Taxon	Voucher	Locality	GenBank accession		
			nrLSU	ITS	*tef1*–α
Rhodotus asperior	HKAS 56754 (holotype)	Yunnan, southwestern China	KC179745	KC179737	KC179730
R. asperior	HKAS 59387	Yunnan, southwestern China	KC179744	KC179736	KC179729
R. palmatus	HKAS 62858	Jilin, northeastern China	KC179746	KC179738	KC179731
R. palmatus	HMJAU 4302	Jilin, northeastern China	KC179748	KC179740	KC179733
R. palmatus [a]	HMJAU 5040	Jilin, northeastern China	KC179749	–	–
R. palmatus [b]	HMJAU 5040	Jilin, northeastern China	KC179750	KC179741	KC179734
R. palmatus [a]	HMJAU 6872	Jilin, northeastern China	KC179751	–	–
R. palmatus [b]	HMJAU 6872	Jilin, northeastern China	KC179752	KC179742	–
R. palmatus	PRM 889504	South Bohemia, Czech	KC179747	KC179739	KC179732
Flammulina rossica	HKAS 57924	Xizang, southwestern China	KC179743	KC179735	KC179728

[a,b] Indicate individuals of the same collection

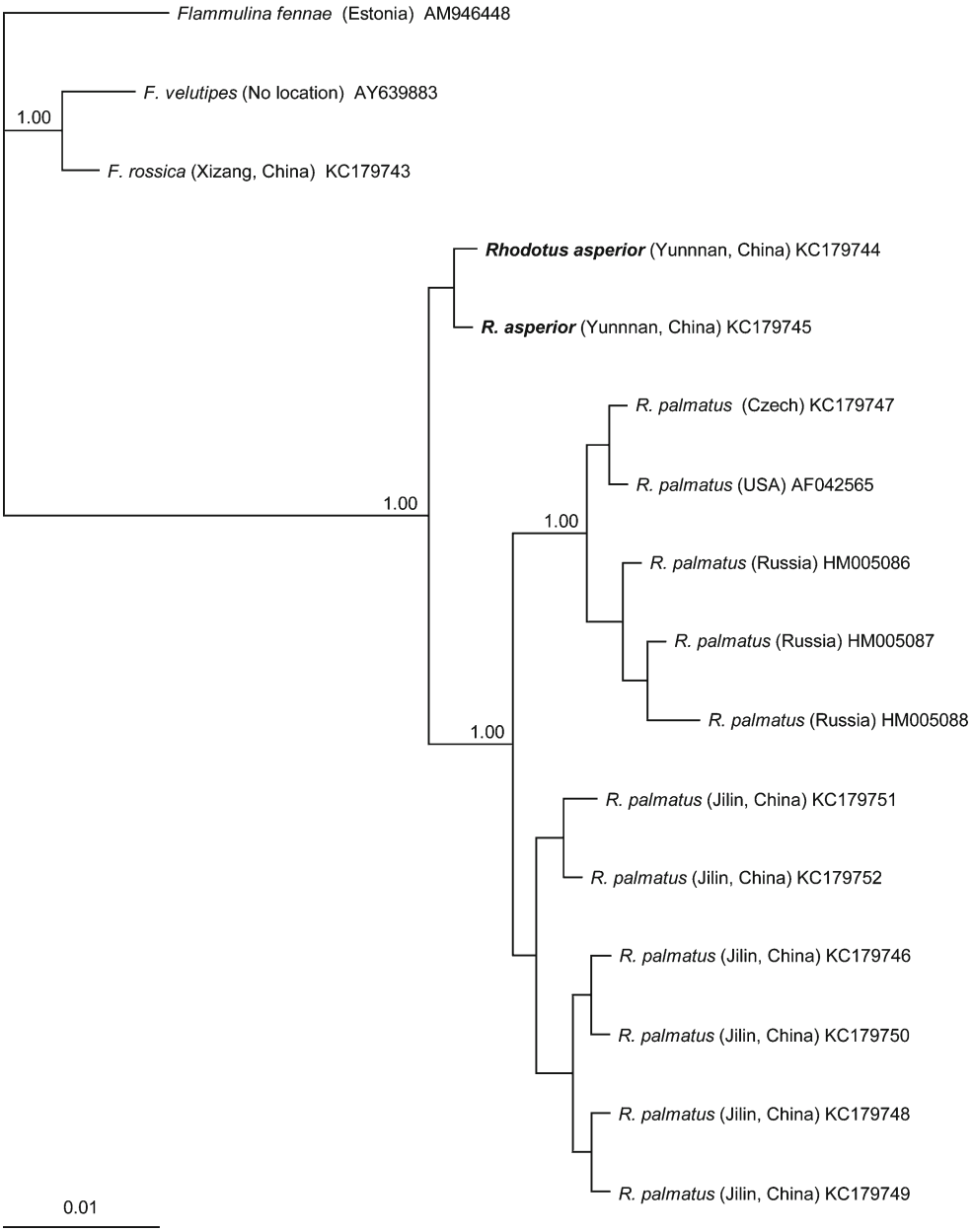

Fig. 1 Bayesian analysis based on nrLSU sequences showing the placement of the new species *R. asperior* within *Rhodotus*. Posterior probabilities values (> 0.90) are shown above the branches. The final dataset included a total of 16 sequences with a length of 856 characters (generations = 1,000,000; average standard deviation of split frequencies = 0.0035)

Nucleotide substitution models based on Akaike information criteria (AIC) data were obtained using MrModeltest 2.3. nrLSU region, ITS region, and the combined data used the same parameter with equal rates; *tef1*-α region was assigned with gamma rates.

All characters of datasets were treated as unordered and of equal weight. Posterior probabilities (PP) were determined twice by running cold and heated chains in parallel mode, samples saved every 1,000 generations. At the beginning of the run, the log likelihood values typically increase rapidly. This phase of the run is so called "burn-in" period, and the samples from this phase are typically discarded. By default, the value of burn-in is determined by 25 % of the total samples in Bayesian analysis. The trees in the burn-in period were excluded, and the 50 % majority-rule consensus tree of the remaining trees was calculated to determine Bayesian Posterior Probabilities (PP) of each clade. Runs were terminated once the average standard deviation of split frequencies went below 0.01.

The phylogenetic analyses were based on three fragments, nrLSU, ITS, and *tef1*-α. Two datasets were constructed and analyzed: the single-locus dataset of the nrLSU and the concatenated multi-locus dataset of nrLSU, ITS, and *tef1*-α. To test for phylogenetic conflict among the three fragments, the partition homogeneity (PH) or incongruence length difference (ILD) test was performed with 1,000 randomized replicates, using heuristic searches with simple addition of sequences in PAUP* 4.0b10 (Swofford

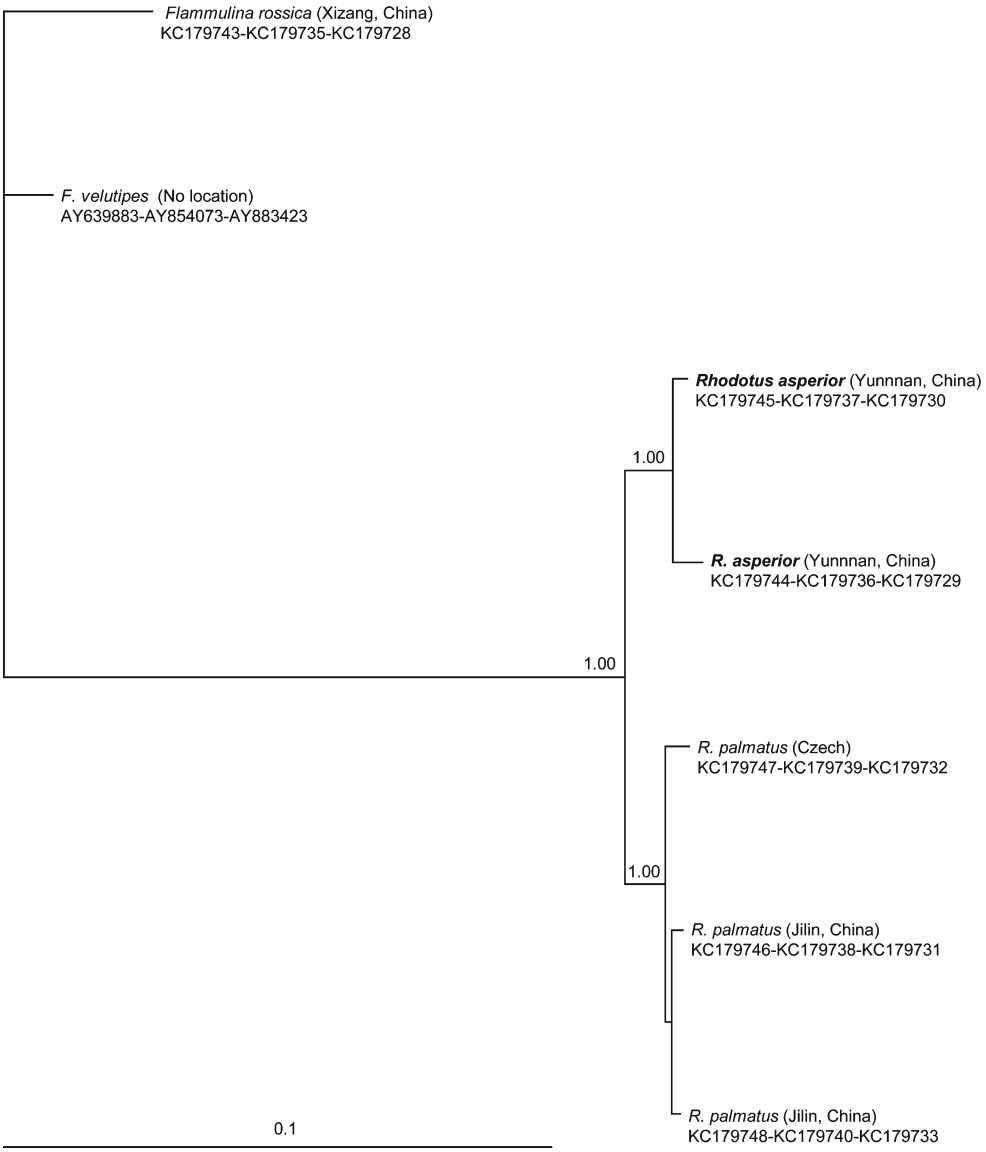

Fig. 2 Bayesian analysis based on a combined data set (nrLSU, ITS, and *tef1*-α) showing the placement of the new species *R. asperior* within *Rhodotus*. Posterior probabilities values (> 0.90) were shown above the branches. The final dataset included a total of seven samples with a length of 2,300 characters (nrLSU 850 characters, ITS 850 characters, *tef1*-α 600 characters; generations = 1,000,000; average standard deviation of split frequencies = 0.0015)

2004). The result of the partition homogeneity test showed that the phylogenetic signals present in the different gene fragments were not in conflict (P=1.00).

Results

Molecular phylogeny

In the phylogeny of nrLSU sequences, Bayesian analysis strongly supported the monophyly of *Rhodotus* with two clades: one corresponding to *R. asperior* (see below) from tropical and subtropical China yet without statistic support, and the other corresponding to *R. palmatus* with high statistic support. Within the *R. palmatus* clade, samples from Europe and North America formed one subclade with a high support value, while samples from northeastern China were clustered together yet without statistic support (Fig. 1). In the analysis of the combined multi-locus dataset of nrLSU, ITS, and *tef1*-α, both clades, viz. *R. asperior* lineage and *R. palmatus* lineage, received high statistic support (Fig. 2).

Taxonomy

1. *Rhodotus asperior* L.P. Tang, Zhu L. Yang & B. Tolgor, **sp. nov**.

(Figs. 3d–g, 4a–e, 6a)

MycoBank: MB 803212

Etymology: referring to the basidiospores which are more roughened than those of *R. palmatus*.

Holotypus: China, Yunnan Province, Yingjiang County, Tongbiguan, alt. 1,420 m, 16 July 2009, L.P. Tang 797 (HKAS 56754).

Fig. 3 Basidiomata of *Rhodotus* species. **a**–**c** *R. palmatus* (**a**, **b** from HKAS 62858; **c** from HMJAU 5040); **d**–**g** *R. asperior* (**d**, **e** from HKAS 56754, holotype; **f**, **g** from HKAS 59387, by courtesy of Dr. Y.C. Li)

Pileus 3–6 cm latus, convexus vel applanato-convexus, subviscidus vel viscidus, rufo-aurantiacus, aurantiacus, roseolus vel persicinus. *Lamellae* sinuatae vel adnexae, pallide incarnatae. *Stipes* 3–5×0.3–0.8 cm, subcylindricus vel sursum attenuatus, albidus vel griseolus. *Caro* pallide roseola vel pallide incarnata, non-discolorans. *Basidia* 35–50×8–11 μm, clavata, 4-sporigera. *Basidiosporae* 5–6.5×4.5–5.5 μm, lato-ellipsoideae vel subglobosae, echinulatae, non-dextrinoideae. *Cheilocystidia* lageniformia vel ventricosa, 40–68×5–10 μm. *Pleurocystidia* absentia. *Suprapellis hymeniformis. Fibulae praesentes.*

Basidiomata small to medium-sized. *Pileus* 3–6 cm in diam., hemispherical to convex when young, convex, broadly convex to applanate when mature; surface orange, reddish orange, reddish to peach-colored (6A2–6A5; 7A2–7A5; 8A2–8A5), subviscid to viscid when wet, sometimes reticulate-ridged or veined; margin incurved. *Lamellae* sinuate to adnexed, crowded to subdistant, reddish to pale meat-colored (6A2–6A4; 7A2–7A4; 8A2–8A4); edge even; lamellulae 2–3 tiers, not forked. *Stipe* more or less excentric, 3–5×0.3–0.8 cm, subcylindric or slightly attenuate upwards, dirty white to greyish; surface subviscid; base slightly enlarged. *Context* reddish, pink to whitish, unchanging in color when injured; taste unknown; smell indistinctive.

Basidiospores [60/3/3] 5–6.5(−7)×(4–)4.5–5.5(–6) μm, *Q*=(1.06–)1.08–1.3 (***Q***=1.18±0.74), broadly ellipsoid to subglobose, nearly hyaline, thin-walled, densely covered with obtuse warts which are 0.5–1.5 μm in height and 0.5–1 μm in width, without germ pore, non-amyloid, non-dextrinoid; apiculus up to 1.5 μm long and about 1 μm wide. *Basidia* 35–50×8–11 μm, clavate, hyaline, thin-walled, mostly 4-spored, occasionally 2- or 3-spored; sterigmata 4 μm long in length. *Cheilocystidia* abundant and crowded, lageniform to ventricose, 40–68×5–10 μm, hyaline, thin-walled to slightly thick-walled (wall ≤1 μm). *Pleurocystidia* absent. *Lamellar trama* slightly bilateral with a broad regular to subregular mediostratum composed of colorless, more or less gelatinized filamentous hyphae 3–12 μm in width. *Suprapellis* an ixohymeniderm 40–50 μm thick, composed of hyaline, occasionally yellowish brown to brownish vacuolar-pigmented, thin-walled, clavate, broadly clavate to sphaeropedunculate, sometimes fusiform elements (25–52×10–22 μm) occasionally with a short apical appendix up to 10 μm long, interspersed with abundant, colorless to brownish, filamentous hyphae 3–6 μm in width; *subpellis*

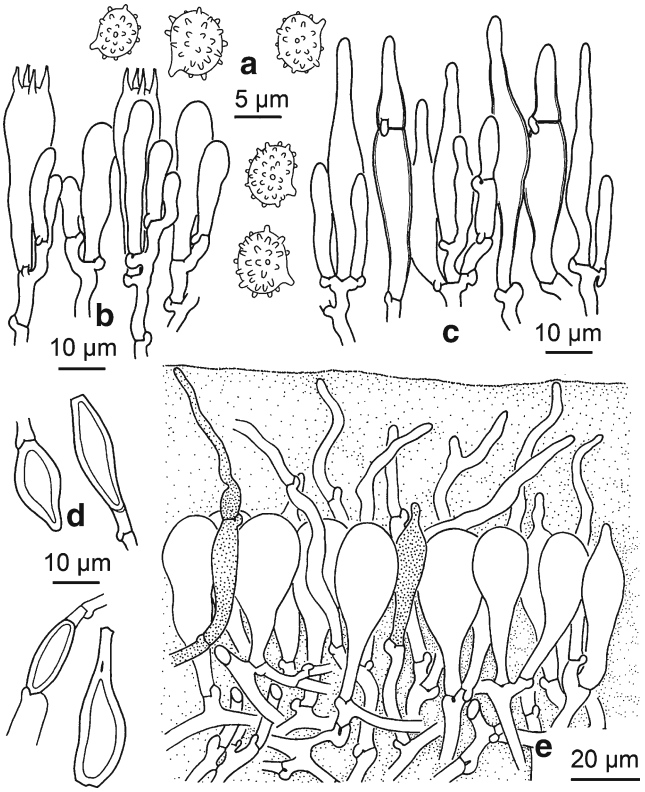

Fig. 4 Microscopic features of *Rhodotus asperior* (HKAS 56754, holotype). **a** Basidiospores; **b** hymenium and subhymenium with basidia at different stages of development; **c** cheilocystidia; **d** chlamydospores in pileal trama; **e** radial-vertical section of pileipellis

composed of colorless or almost colorless, barely-inflated filamentous hyphae 3–6 μm in width. *Chlamydospores* observed in the pileal and lamellar trama, ellipsoid to ventricose, occasionally nearly clavate, 10–25×6–10 μm, yellowish brown, thick-walled (wall ≤2.5 μm). *Clamp connections* abundant in every part of basidioma.

Habitat and known distribution: scattered or in clusters in small groups on dead trunk of broad-leaved trees of Fagaceae (*Castanopsis* and *Lithocarpus*); fruiting in July and August; so far known from two locations: Yingjiang, a tropical region, and Chuxiong, a subtropical region.

Specimens examined: China, Yunnan Province: Yingjiang County, Tongbiguan, alt. 1,420 m, in tropical evergreen broad-leaved forest dominated by *Lithocarpus* and *Castanopsis*, solitary or clusters on trunk, 16 July 2009, L.P. Tang 797 (HKAS 56754, holotype); same date and same location, Y.C. Li 1640 (HKAS 59387); Chuxiong, Jishan, alt. 2,300 m, in subtropical forest, clusters on trunk, substrate unknown, 25 August 1983, W.K. Zheng 8364 (HKAS 12068).

2. *Rhodotus palmatus* (Bull.) R. Maire, Bull. Soc. Mycol. Fr. 40: 308. 1926

(Figs. 3a–c, 5a–f, 6b–d)

Basidiomata small to medium-sized. *Pileus* 3–8 cm in diam., convex to broadly convex or nearly applanate when mature; surface orange, reddish to peach-colored, gelatinous or viscid when wet, decorated with obvious network of raised, round-edged, reticulate ridges; margin incurved, with apricot yellow to cream wrinkle. *Lamellae* sinuate to adnexed, crowded to subdistant, pale meat-colored, edge even; lamellulae 2–3 tiers, not forked. *Stipe* 3–6×0.3–1 cm, eccentric to nearly central, subcylindric or slightly attenuate upwards; nearly white to pale pink; base slightly enlarged. *Context* whitish to pink, unchanging.

Basidiospores [80/5/5] (5.5–)6–7×(5–)5.5–6.5(–7) μm, Q=1.00–1.16(–1.2) ($\boldsymbol{Q}$=1.06±0.06), globose to subglobose, nearly hyaline, thin-walled, covered with obtuse warts 0.5–1 μm in height and 0.5–0.8 μm in width, without germ pore, non-amyloid, non-dextrinoid; apiculus up to 1.5 μm long and about 1 μm wide. *Basidia* 25–52×6–9 (−11) μm, clavate, hyaline, thin-walled to slightly thick-walled (wall ≤1 μm), mostly 4-spored, occasionally 2- or 3-spored; sterigmata up to 4 μm in length. *Cheilocystidia* scarce to abundant, lageniform to ventricose to fusiform, 30–50(−58)×5–8(–10) μm, nearly hyaline, thin-walled. *Pleurocystidia* absent. *Lamellar trama* slightly bilateral with a broad regular to subregular mediostratum composed of colorless, more or less gelatinized filamentous hyphae 3–10 μm in width. *Suprapellis* an ixohymeniform layer 50–70(−100) μm thick, composed of thin to slightly thickened, clavate to sphaeropedunculate cells (30–50×8–17 μm) sometimes with an apical, sinuous apical appendix up to 40 μm long, interspersed with scattered to locally abundant, colorless to brownish, filamentous hyphae 2–5 μm in width; *subpellis* composed of colorless or almost colorless barely-inflated filamentous hyphae 3–7 μm in width. *Chlamydospores* not observed in the cited specimens. *Clamp connections* abundant in every part of basidioma.

Habitat and known distribution: clustered or occasionally solitary, on dead trunks of deciduous trees (*Acer*, *Aesculus*, *Populus*, or *Ulmus* etc.); fruiting period extends from summer to autumn in the northern temperate region (Bulliard 1785; Imai 1938; Horak 1968; Pegler and Young 1975; Krieglsteiner 1979; Kühner and Romagnesi 1984; He 1992; Wang and Zhang 1992; Noordeloos 1995; Sundberg et al. 1997; Han et al. 2006; Tolgor and Fan 2008).

Specimens examined: China, Jilin Province: Antu County, Erdaobaihe Town, on the ground in forest, 19 July 2001, B. Tolgor s. n. (HMJAU 6872); Changbai County, Shiwudaogou, on decayed wood, 28 June 2006, B. Tolgor s. n. (HKAS 62858); same location and data, B. Tolgor s. n. (HMJAU 4302); culture collection (wild basidioma from HMJAU 4302), 23 December 2006, Y.G. Fan s. n. (HMJAU 5040). Czech Republic: South Bohemia, Sumava Montains, Zatonska Hill, near Lenora Village, alt. 970 m, in natural forest, on trunk of *Ulmus*

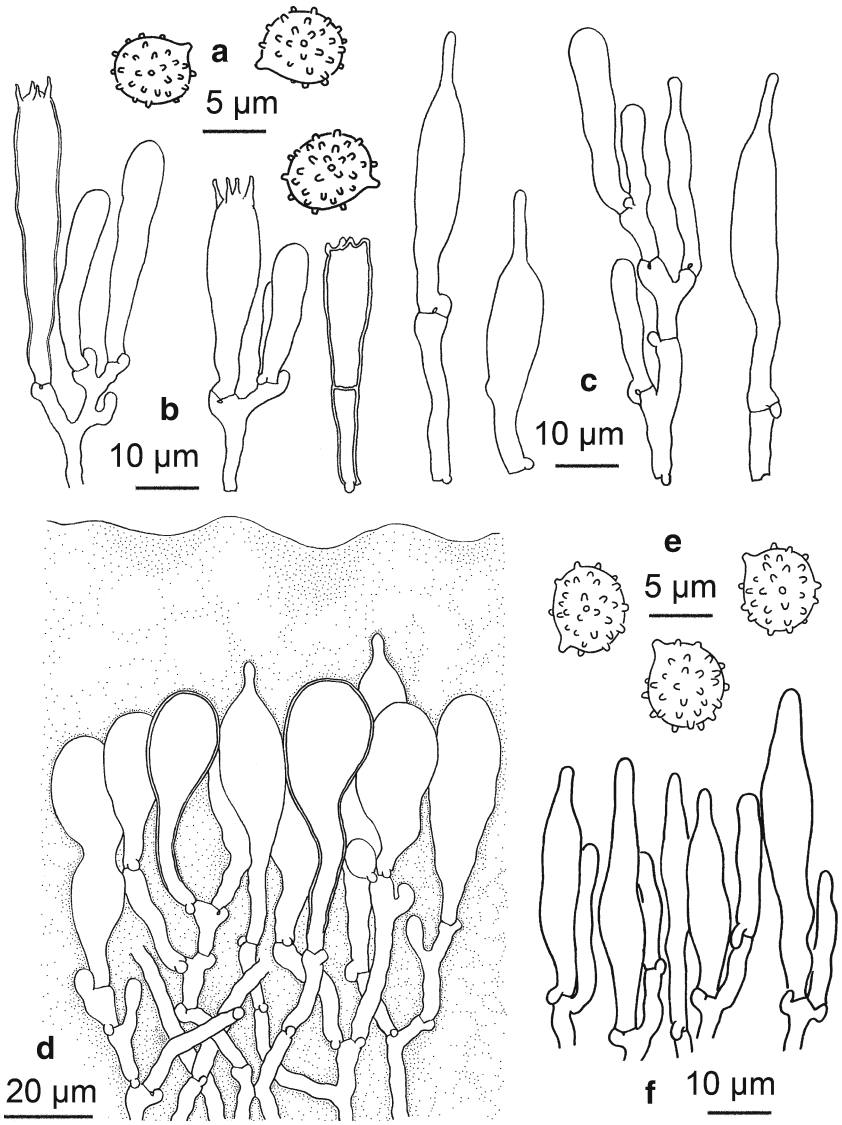

Fig. 5 Microscopic features of *Rhodotus palmatus*. **a**, **e** basidiospores; **b** basidia at different stages of development; **c**, **f** cheilocystidia; **d** radial section of pileipellis. (**a**–**d** from HKAS 62858; **e**, **f** from PRM 889504)

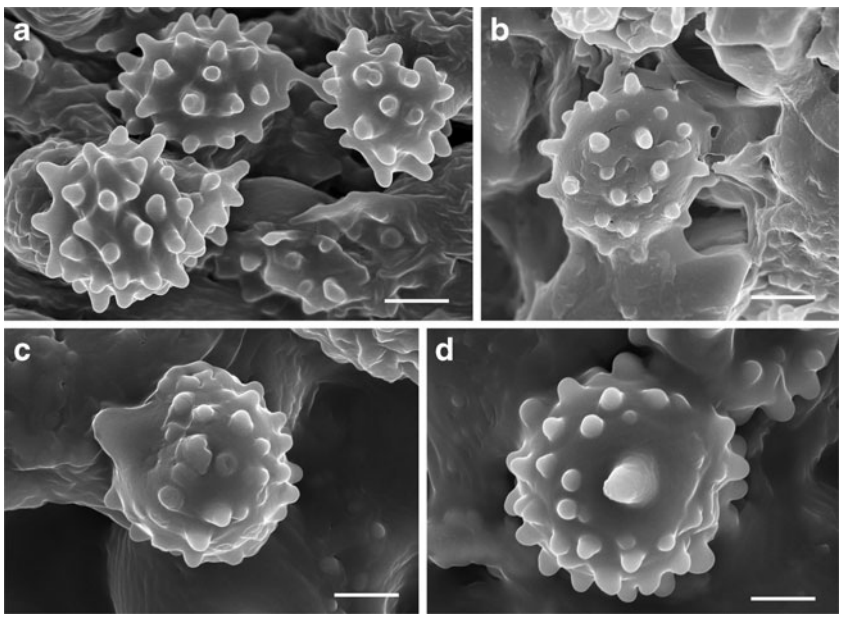

Fig. 6 Basidiospores of *Rhotodus* species from herbarium materials under SEM (S4800 10.0 Kv). **a** *R. asperior* (HKAS 56754, holotype); **b**–**d** *R. palmatus* (**b** from HKAS 62858; **c**, **d** from PRM 889504). Bars 2 μm

glabra, 14 October 1996, J. Holec 721/96 (PRM 889504).

Discussion

For species delimitation, we used multi-locus DNA data and phylogenetic species recognition based on genealogical concordance (Taylor et al. 2000). Our molecular data of three loci (nrLSU, ITS, and *tef1-α*) revealed that there are two phylogenetic species in the genus *Rhodotus*. Both phylogenetic species are in concordance with morphological traits, supporting the division of the samples of *Rhodotus* into two distinct species.

A few morphological traits are useful to delimit the species in *Rhodotus*. These include the form and size of the basidiospores, the length and width of the warts on the surface of the basidiospore, the size of the cystidia, and the growth substrate. The length and width of the warts on the surface of the basidiospore are also used for the delimitation of species in other agaric genera like *Laccaria* (Mueller 1992; Wang et al. 2004). *Rhodotus asperior* differs from *R. palmatus* in its broadly ellipsoid to subglobose basidiospores with longer and wider warts on the surface, longer cheilocystidia with a more or less thickened wall, and its occurrence on evergreen broad-leaved trees in subtropical and tropical regions. In contrast, *R. palmatus* has rounder basidiospores with smaller warts (warts ≤1 μm in height and <1 μm in width) on the surface of the basidospore, shorter and thinned cheilocystidia, and a preferred habitat on wood of deciduous trees (*Populus*, *Aesculus* or *Ulmus* etc.) in temperate or alpine regions of Europe, North America and Eastern Asia (Pegler and Young 1975; Krieglsteiner 1979; Kühner and Romagnesi 1984; Redhead 1989; Noordeloos 1995; Sundberg et al. 1997; Tolgor and Fan 2008).

Our molecular data indicated that there are variations among different populations of *R. palmatus*, although no obvious morphological characters could be found to distinguish them (Figs. 1, 2). Further sampling and future population genetic studies should provide new insights into the understanding of the evolution history and speciation of *Rhodotus* as a whole.

Acknowledgments The authors are very grateful to the following people: Dr. J. Borovička, Department of Nuclear Spectroscopy, Nuclear Physics Institute, the Academy of Sciences of the Czech Republic, for sending us a collection of *R. palmatus*; Dr. E. Horak, Innsbruck, Austria, for giving us good suggestion for the line-drawings; Dr. R.J. Vilgalys, Duke University, USA, for supplying accurate information about the GenBank sequence of the North American *R. palmatus*; Dr. Y.C. Li, Kunming Institute of Botany (KIB), Chinese Academy of Sciences (CAS), for providing valuable images of *R. asperior*; Dr. Z.X Ren and Mr. G. Wu, KIB, for helping us in using the SEM facility. The authors thank Dr. Hong Luo (DOE-Plant Research Laboratory, Michigan State University, USA) for polishing the English. This work was supported by the National Natural Science Foundation of China (No. 31170024), the Ministry of Science and Technology of China (2008FY110300), and the Knowledge Innovation Program of the CAS (No. KSCX2-EW-Z-9).

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