Mycoscience (2010) 51:291–299
DOI 10.1007/s10267-010-0039-6

FULL PAPER

Identification of Japanese *Astraeus*, based on morphological and phylogenetic analyses

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Received: 19 August 2009 / Accepted: 14 January 2010 / Published online: 12 March 2010

Abstract To clarify the diversity of *Astraeus* in Japan, 35 collections of *Astraeus* basidiomata from Japan and Thailand were examined for their morphological characteristics and the nucleotide sequences of the rDNA ITS region and compared with several worldwide *Astraeus* species. Japanese *Astraeus* specimens were separated into two groups based on basidiome size, shape of exoperidium, and ornamentation and size of basidiospores. The phylogenetic tree analyses supported the separation, and the morphological groups belonged to different clades. The Japanese *Astraeus* group 1, morphologically matched to *Astraeus hygromerticus* var. *koreanus*, showed the closest phylogenetic relationship with *Astraeus hygrometricus* from North American and Mediterranean regions, suggesting that the *Astraeus* group 1 can be indentified as *A. hygrometricus* var. *koreanus*. Another *Astraeus* group, group 2, morphologically matched to *A. hygrometricus* s.l., showed a distinct monophyletic clade that was separated from *A. hygrometricus* complexes, indicating an undescribed species.

Keywords Edible ectomycorrhizal fungi · Gasteromycetous mushrooms · ITS region · Astraeaceae · Basidiomata

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Introduction

Astraeus Morgan is a gasteromycetous genus with four described species and belongs to Astraeaceae, Boletales, Basidiomycota (Binder and Bresinsky 2002; Cannon and Kirk 2007; Kirk et al. 2008). *Astraeus* species are common ectomycorrhizal fungi found throughout the world. In Asia, *Astraeus* consists of three species with one variety: *A. hygrometricus* (Pers.) Morgan (Ito 1959; Imazeki and Hongo 1989), *A. hygrometricus* var. *koreanus* V.J. Staněk (Staněk 1958; Kreisel 1976; Imazeki and Hongo 1989), *A. odoratus* Phosri, Watling, M.P. Martín & Whalley (syn. *A. thailandicus* Petcharat; Phosri et al. 2004), and *A. asiaticus* Phosri, M.P. Martín & Watling (Phosri et al. 2007). The former species with the variety has been recorded from a wide range of locations in Asia, and the latter two from Southeast Asia. *Astraeus hygrometricus* is a cosmopolitan species. In North America and the Canary Islands, *A. pteridis* (Shear) Zeller is also known (Baseia and Galvão 2001; De Roman et al. 2005; Phosri et al. 2007). *Astraeus hygrometricus* and *A. pteridis* form ectomycorrhizal associations with several host tree species including *Pinus*, *Pseudotsuga*, *Alnus*, *Eucalyptus*, and *Castanea*, but *A. odoratus* and *A. asiaticus* have been reported only in dipterocarp forests (Trappe 1967; Malajczuk et al. 1982; Phosri et al. 2007). In vitro mycorrhizal synthesis in *Astraeus* has rarely been studied in *A. hygrometricus* and

A. pteridis and their host plant genera, e.g., *Pinus*, *Alnus*, *Picea*, and *Eucalyptus* (Molina 1979, 1981, 1982; Malajczuk et al. 1982; Molina and Trappe 1982; Danielson 1984). No information on in vitro mycorrhizal synthesis has been reported for *A. odoratus* and *A. asiaticus*.

The type species, *Astraeus hygrometricus*, was first described as *Geastrus hygrometricus* from France by Persoon in 1801 (Morgan 1889). As the species has been recorded over wide geographic regions, the descriptions vary considerably in terms of size, shape, and color of glebal mass, exterior nature of the peridium, and microscopic characteristics of the peridium and basidiospores (Arora 1986; Imazeki and Hongo 1989; Nouhra and De Toledo 1998; Baseia and Galvão 2001; De Roman et al. 2005; Phosri et al. 2007). Under such circumstances, it has been suggested, based on a molecular analysis, that *A. hygrometricus* sensu Persoon (the French type) may be different from *A. hygrometricus* sensu Morgan (the North American type) (Phosri et al. 2007). Furthermore, *A. asiaticus*, formerly identified as *A. hygrometricus* in Southeast Asia, was described recently as a new species based on its distinct morphological characteristics and independent phylogenetic position (Phosri et al. 2007). Because molecular analysis is useful for distinguishing a complex group of fungi at the species level (Bruns et al. 1998; Martin et al. 2002), taxonomic clarification of the *A. hygrometricus* specimens from other geographic regions is desired.

In Japan, *Astraeus* mushrooms are found commonly in wide areas, and they have hitherto been identified as *A. hygrometricus* and *A. hygrometricus* var. *koreanus* (Ito 1959; Imazeki and Hongo 1989). However, the difference between the two forms has not been well described, especially for microscopic characteristics. It is currently suggested that *A. hygrometricus* var. *koreanus*, described from North Korea, is different from typical *A. hygrometricus* because of its smaller size and pale basidomata color (Phosri et al. 2004). A reexamination of Japanese *Astraeus* using molecular techniques and morphological observations is thus timely. In the present study, we examined the population of *Astraeus* in Japan and compared it with several *Astraeus* species worldwide based on morphological characteristics and the nucleotide sequences of the internal transcribed spacer (ITS) region within rDNA.

Materials and methods

Sample collection and isolation

Fresh or dried *Astraeus* basidiomata were collected from various forest areas in Japan and dipterocarp forests in Thailand. The collection list is shown in Table 1. Collected basidiomata were freeze-dried for one night, oven dried at 60°C for one night, and kept in the laboratory. Several specimens were deposited in the herbarium of the National Museum of Nature and Science, Japan (TNS). Mycelial cultures were isolated from the inner tissue layer of fresh samples on modified Norkrans' "C" (MNC) agar plate medium (Yamada and Katsuya 1995). Grown mycelia were transferred to MNC slants, kept as stock cultures, and used for the following analyses.

Morphological characterization of basidiomata

All specimens were studied macroscopically and microscopically. In fresh basidiomata, macroscopic characteristics such as peridial thickness, glebal color, and exterior of the peridium were recorded and photographed. Several microscopic characteristics such as exoperidial tissues and basidiospores were observed in dried materials that were hand sectioned and mounted in 100% lactic acid. If necessary, 1% cotton blue in lactic acid was used. Basidiospores were observed under a differential interference contrast Nomarski microscope (Olympus BX51) with an oil immersion 100× objective lens, and at least 50 spores per sample were photographed. Spore images were processed by Adobe Photoshop (Adobe Systems, San Jose, CA, USA) and measured for diameter and spine length using the NIH image software, and their size was statistically calculated by computer. Spore ornamentation was further analyzed by scanning electron microscopy (SEM) following the method of Kaneko and Kakishima (2001). Dried *Astraeus* spores were dusted on double-sided adhesive tape on metal stubs, coated with platinum-palladium at 10 kV (Hitachi E1030, Tokyo, Japan), and observed by a scanning electron microscope (Hitachi S-4200).

ITS sequence analyses

DNA extraction and PCR amplification

Total DNA was extracted from a few milligrams of fresh or dried gleba of young basidiomata, basidiospores of mature basidiomata, or cultured mycelium on MNC agar following the method described by Gardes and Bruns (1993). Polymerase chain reaction (PCR) amplification of the ITS region within the rDNA was performed with 25 µl reaction mixture, in which 1× PCR buffer, 2.5 U Taq DNA polymerase (TaKaRa, Tokyo, Japan), 0.2 mM each deoxynucleotide triphosphate (dNTP), 0.5 µM each primer in pairs, and 1.5 mM $MgCl_2$ were included. The PCR was performed with the GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C for 3 min; 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 90 s; and a final step of 72°C

Table 1 List of *Astraeus* samples analyzed in this study

Sample ID	Origin of samples				Data obtained	Remarks[a]
	Country	Location	Putative host plants	Year		
ASWAN1 JP	Japan	Ichinomiya, Chiba	*Pinus thunbergii*	1987	M	
ASWAN2 JP	Japan	Sakae, Chiba	*P. thunbergii*	1988	M	
ASWAN3 JP	Japan	Chiba	ND	1993	M	
ASWAN4 JP	Japan	Noro, Chiba	*P. thunbergii*	1999	M	
ASWAN5 JP	Japan	Narita, Chiba	*P. thunbergii*	1999	M	
ASWAN6 JP	Japan	Ibaraki	*P. thunbergii*	2004	M	
ASWAN7 JP	Japan	Tokai, Ibaraki	*P. thunbergii*	2004	M, S	AB535105
ASWAN8 JP	Japan	Tokai, Ibaraki	*P. thunbergii*	2005	M	TNS-F-26390
ASWAN9 JP	Japan	Tokai, Ibaraki	*P. thunbergii*	2005	M, S	AB535106
ASWAN10 JP	Japan	Iwaki, Fukushima	*P. thunbergii*	2006	M, S	AB535107, TNS-F-26391
ASWAN11 JP	Japan	Iwaki, Fukushima	*P. thunbergii*	2006	M	
ASWAN12 JP	Thailand	Doi suthep, Chiangmai	ND	2004	M	
ASWAN13 TH	Thailand	Tak	ND	2005	M	
ASWAN14 TH	Thailand	Saiyoknoi, Kanchanaburi	ND	2005	M	
ASWAN15 TH	Thailand	Tak	ND	2005	M, S	AB535108
ASWAN17 JP	Japan	Sukagawa, Fukushima	ND (commercially harvested)	2007	M, S	AB507397, TNS-F-26392
ASWAN18 JP	Japan	Kohriyama, Fukushima	ND (commercially harvested)	2007	M, S	AB535109
ASWAN19 JP	Japan	Okaya, Nagano	*P. densiflora*	2007	M	AB535110
ASWAN20 JP	Japan	Machida, Tokyo	*Quercus* sp.	2007	M, S	
ASWAN21 JP	Japan	Hatoyama, Saitama	*Q. serrata*	2007	M, S	AB535111
ASWAN22 JP	Japan	Matsukawa, Nagano	*Quercus* sp.	2007	M	
ASWAN23 JP	Japan	Komagane, Nagano	*Picea abies*	2007	M	
ASWAN26 JP	Japan	Komagane, Nagano	*Tilia japonica*	2007	M	
ASWAN27 TH	Thailand	Bankog, Uttaradit,	Dipterocarpaceae	2007	M	TNS-F-26393
ASWAN28 JP	Japan	Kumakogen, Ehime	*Quercus* sp.	2007	M, S	AB535112
ASWAN29 TH	Thailand	Numpad, Uttaradit	Dipterocarpaceae	2007	M, S	AB507404
ASWAN33 TH	Thailand	Numpad, Uttaradit	Dipterocarpaceae	2007	M	TNS-F-26394
ASWAN34 TH	Thailand	Numpad, Uttaradit	Dipterocarpaceae	2007	M, S	AB535113
ASWAN35 TH	Thailand	Numpad, Uttaradit	Dipterocarpaceae	2007	M, S	AB507406
ASWAN37 JP	Japan	Minowa, Nagano	*P. densiflora*	2007	M	
ASWAN45 JP	Japan	Minowa, Nagano	*P. densiflora*	2007	M	
ASWAN56 JP	Japan	Matsumoto, Nagano	*Cedrus* sp.	2007	M, S	AB535114
ASWAN58 JP	Japan	Komagane, Nagano	*Quercus* sp., *P. densiflora*	2007	M	
ASWAN59 JP	Japan	Shinano, Nagano	*Q. serrata*, *Q. crispula*	2007	M	
ASWAN60 JP	Japan	Shinano, Nagano	*Q. serrata*, *Q. crispula*	2007	M	TNS-F-26395

ND not determined, *M* morphology, *S* sequence of rDNA internal transcribed spacer (ITS) region

[a] Numbers indicate the accession number of DNA sequence in GenBank, or specimen number in the herbarium of National Museum of Nature and Science, Japan (TNS)

for 10 min. The ITS1–5.8S–ITS2 region was amplified using the primer pair NS5/TW14 for the first PCR and NS7/ITS4B for the second PCR (Gardes and Bruns 1993). PCR products were electrophoresed on 1.5% agarose gel immersed in TBE buffer [90 mM Tris-borate, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] and visualized with ethidium bromide staining. All PCR products were digested with endonucleases (*Cfr*13, *Rsa*I, and *Hae*III), and their restriction fragment length polymorphism (RFLP) was analyzed (Yamada et al. 2001) to screen the samples for further sequence analysis.

DNA sequencing

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Sequencing was performed on both strands using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems).

Reactions were performed in 10 µl with 1 µl purified PCR product, 1.3 µM primers (ITS1, ITS2, ITS3, ITS4B, or NS7), 1 µl 5× sequencing buffer, and 1 µl Terminator Ready Reaction Mix. The reaction proceeded under the following conditions: 96°C for 1 min; and 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Cycle sequencing reaction products (10 µl) were purified with ethanol and then sequenced using an ABI Prism 3100 genetic analyzer (Applied Biosystems).

DNA sequence alignment and phylogenetic analyses

The DNA sequences determined and deposited in Genbank (DDBJ) are listed in Table 1. The DNA sequences were initially aligned and then manually refined, using MEGA, version 4 (Tamura et al. 2007). Phylogenetic analyses were constructed by maximum parsimony method and Bayesian method. Heuristic searches for the most parsimonious tree were performed using PAUP* 4.0 b10 (Swofford 2002). All sites were treated as unordered and unweighted, with gaps treated as missing data. Searches used 1 K stepwise additions, a simple option set, and tree bisection-reconnection (TBR) branch swapping. Tree scores, including tree length, consistency index (CI), retention index (RI), and reconstructed consistency index (RC), were also calculated. The confidence levels of the branching points were determined by 1 K BS replicates. The trees generated were rooted to *Boletus edulis* Bull. as the outgroup taxon. Bayesian method was conducted on the same aligned dataset. First, PAUP and MrModeltest v 2.2 (Nylander et al. 2004) were used to determine the best nucleotide substitution model via the Akaike information criterion. Phylogenetic analyses were performed with MrBayes v 3.1 (Ronquist and Heuelsenbeck 2003) applying the best model. The MCMC method of four chains started from a random tree and lasted 1 M generations. Trees were saved each 100 generations, resulting in 10 K saved trees. The analysis was set to use the GTR + I + G model. All trees sampled before stationary were discarded using a 20% safety margin [burn-in of 2000 trees (200K generation)]. A 50% majority-rule consensus cladogram was computed from the remaining trees. The proportions of this tree corresponded to Bayesian posterior probabilities (BPP).

Results

Twenty-six samples of *Astraeus* basidiomata from Japan and 9 samples from Thailand were examined. Based on the macroscopic and microscopic characteristics, Japanese samples were divided into two groups (Table 2). Thailand collections were identified as *A. odoratus* or *A. asiaticus* (Phosri et al. 2007), both of which were different from Japanese *Astraeus* based on split ray shape, size of the mature basidiocarp, and shape and length of the basidiospore spine. The morphological and ecological characteristics of the two Japanese *Astraeus* groups are described as follows:

Japanese *Astraeus* group 1 Fig. 1a,c–e

Basidiomata subglobose or depressed globose when young, hypogeous to somewhat epigeous, sessile, 13.6–18.8 mm in diameter, becoming superficial, outer peridial wall splitting to star shaped when mature (Fig. 1a), firm, smooth to slightly rough, encrusted with soil debris; basal mycelium consisting of darkish to blackish rhizomorphs. Exoperidium pale brownish, thick, hard, and composed of several layers, 0.5–1 mm thick when mature, splitting into 15–22 acute rays, hygroscopic; inner layer white at first, smooth, becoming yellow to brown, thick, developing extensively longitudinal cracks (Fig. 1a). Endoperidium sessile, depressed globose, 9.4–14.8 mm in diameter, whitish when young, becoming yellow to pale brownish when mature, soft, thin, opening at an apical through a rupturing mount of poorly delimited peristome. Gleba whitish when young, becoming brownish and then dark brownish to black at maturity, columella absent. Paracapillitium long, hyaline, thick walled but not cyanophilic, lumen continuous, sometimes branched, interwoven,

Table 2 Comparison of characteristics of Japanese *Astraeus* and *Astraeus hygrometricus* from North America and Mediterranean regions

Morphological characteristics and sample ID	Japanese *Astraeus* observed in this study		*A. hygrometricus* in North America and the Mediterranean[a]
	Group 1	Group 2	
Number of split acute rays	15–22	5–13	6–15
Basidiome size (diameter, mm)	13.6–18.8	19.5-31.9	20.0–25.0
Basidiospore size (µm)	7.59–12.88	5.15–9.59	5.18–13.89
Basidiospore ornamentation	Short, moderately narrow, dense	Short, slender, moderately dense	Short, narrow, dense
Sample ID (ASWAN-X JP)	7, 8, 9, 10	1, 2, 3, 4, 5, 6, 11, 17, 18, 19, 20, 21, 22, 23, 26, 28, 37, 45, 56, 58, 59, 60	

[a] Based on the descriptions of Arora (1986), Nouhra and De Toledo (1998), and Phosri et al. (2004, 2007)

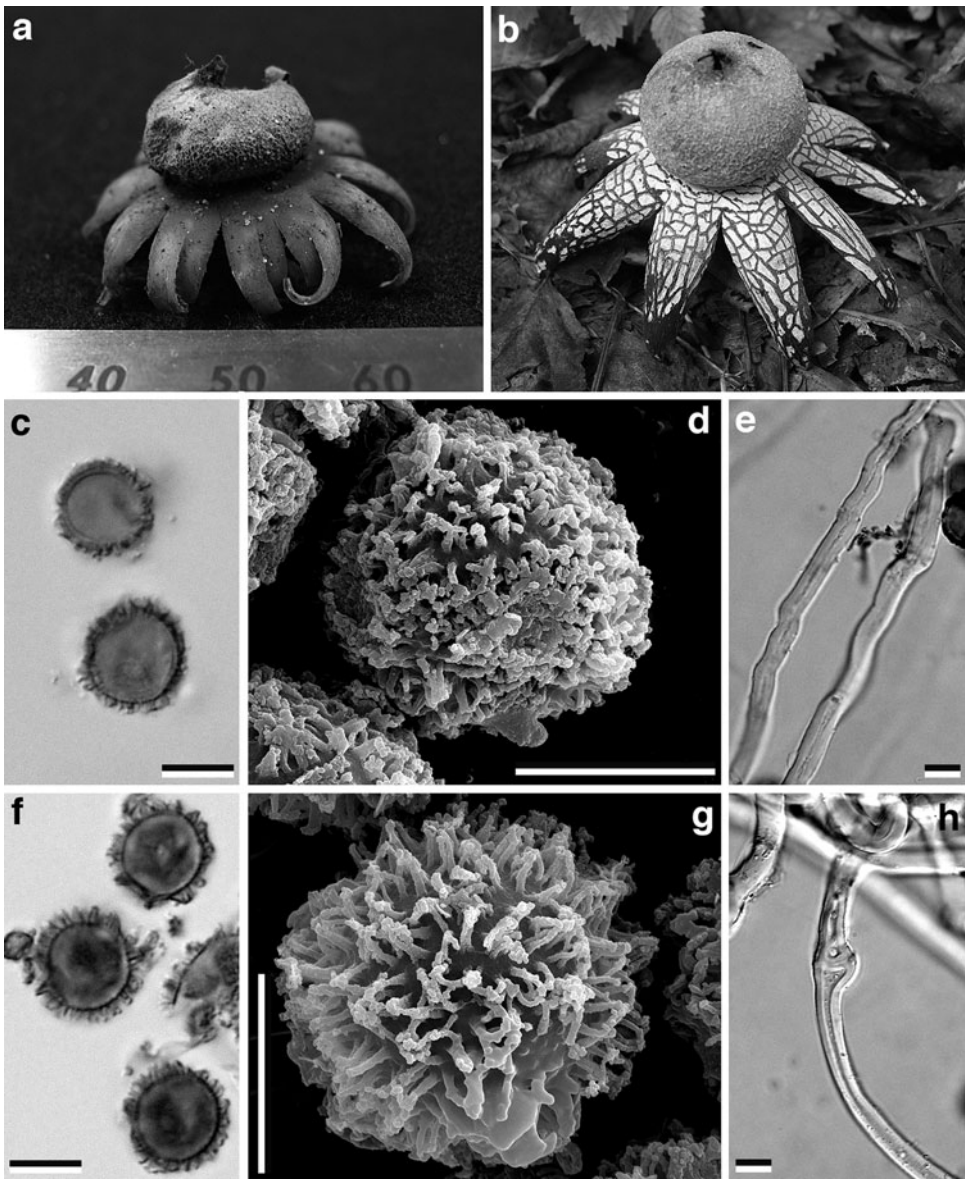

Fig. 1 Morphology of two Japanese *Astraeus* groups. Japanese *Astraeus* group 1 (ASWAN8 JP) (**a**, **c**, **d**, **e**) and group 2 (ASWAN 60 JP) (**b**, **f**, **g**, **h**). **a**, **b** Mature basidiomata. **c**, **f** Basidiospores under a light microscope. **d**, **g** Basidiospores under a scanning electron microscope (SEM). **e**, **h** Paracapillitium under a light microscope. *Bars* 5 μm

3.9–5.9 μm in diameter, septum infrequently present, some of which have a clamp-connection-like structure (see Fig. 1e). Basidiospores globose, cell wall thickened, spiny, 6.3–11.3 μm in diameter, pale brown, moderately densely ornamented with short, rounded, fibrils, moderated narrow, translucently reddish to darkish brown, 0.8–1.6 μm long (see Fig. 1c–d).

Habitat Growing in dry to humid areas and associated with pine trees.

Distribution In the central part of Honshu Island, the main island of Japan, including Ibaraki and Fukushima.

Specimens examined Iwaki-shi, Fukushima Pref., Japan, October 28, 2006, coll. T. Kasuya, TNS-F-26391.

Japanese *Astraeus* group 2 Fig. 1b,f–h

Basidiomata globose to subglobose when young, hypogeous to somewhat epigeous, sessile, 19.5–31.9 mm in diameter, becoming superficial, outer peridial wall splitting to star shaped when mature (Fig. 1b), firm, smooth to slightly rough, covered with thin dark brownish to blackish mycelial layer, basal mycelium consisting of darkish to blackish rhizomorphs, strong odor when fresh. Exoperidium pale to dark brownish, thick, hard, composed of several layers, 0.5–1 mm thick, when mature splitting into 5–13 acute rays, hygroscopic; inner layer white at first, smooth, becoming pale gray, brown to dark brown, thick, developing extensive longitudinal cracks with trapezoid or rhombus pattern when mature (Fig. 1b). Endoperidium sessile, subglobose to somewhat depressed globose,

14.5–26.4 mm in diameter, whitish when young, becoming grayish or yellowish to pale brownish at maturity, soft, thin, opening at an apical when mature through a rupturing mount of poorly delimited peristome. Gleba whitish when young, becoming purple to pale brownish and then dark brownish at maturity, columella absent. Paracapillitium long, hyaline, thick walled but not cyanophilic, lumen continuous, sometimes branched, interwoven, 3.6–6.6 μm in diameter, septum infrequently present, some of which have a clamp-connection-like structure (Fig. 1h). Basidiospores globose, cell wall moderately thickened, spiny, 4.6–9.8 μm in diameter, pale reddish to darkish brown, densely ornamented with short, rounded, fibrils, slender, translucently reddish to darkish brown, 0.6–1.7 μm long (Fig. 1f,g).

Habitat In dry to humid areas associated with *Pinus*, *Picea*, *Cedrus*, *Quercus*, and *Tilia*; fruiting in rainy summer season (June to September).

Distribution Commonly distributed throughout Japan.

Specimens examined Sukagawa-shi, Fukushima Pref., Japan, June 17, 2007, coll. M. Hiroi, TNS-F-26392; Shinano-machi, Nagano Pref., Japan, October 26, 2007, coll. W. Fangfuk, TNS-F-26395.

Based on macroscopic character, mature basidiomata of the Japanese *Astraeus* groups 1 and 2 were different in the number of split acute rays and basidiome size. The difference in basidiospore size was distinct under a light microscope, and a difference in basidiospore ornamentation was observed under SEM. Other characteristics of the groups were quite similar.

Phylogenetic analyses of the ITS sequences

All 35 specimens were amplified in the ITS region, and their RFLP analysis showed that each of the four morphologically separate groups had a single RFLP pattern. Therefore, selected specimens in each group were analyzed for ITS sequencing, i.e., three from the *Astraeus* group 1, six from the *Astraeus* group 2, three from *A. odoratus*, and one from *A. asiaticus*. The 13 ITS sequences from the selected Japanese and Thai specimens in this study were analyzed phylogenetically with 17 sequences of *Astraeus* species and *Boletus edulis* (outgroup) obtained from the GenBank database. The aligned dataset of these 30 sequences consisted of 863 characters, of which 503 characters were constant, 132 variable characters were parsimony uninformative, and 228 characters were parsimony informative. Heuristic searches of unweighted characters in PAUP resulted in 10,000 trees, with the following score: tree length = 593 steps, CI = 0.794, RI = 0.893, RC = 0.709, and homoplasy index (HI) = 0.206. One of the maximum-parsimony (MP) trees, shown as Fig. 2, formed two distinct monophyletic Japanese *Astraeus* clades with strong bootstrap (BS) support, i.e., 99% in *Astraeus* group 2 and 60% in *Astraeus* group 1. The MP tree topology supported four lineages and eight clades. Lineage A included *Astraeus* group 1 (clade I), *A. hygrometricus* North American group (clade II), *A. hygrometricus* Mediterranean group (clade III), and *A. hygrometricus* French group (clade IV) with strong (60–100%) BS support. In lineage B, *A. asiaticus* was placed in clade V (BS = 100%), which was closely related with *A. pteridis*, clade VI (BS = 100%). The *Astraeus* group 2 was located in clade VII, lineage C (BS = 99%), whereas *A. odoratus* was located in clade VIII, lineage D. The tree topology obtained from the Bayesian method was different from that of the MP tree (Fig. 3). Although all eight clades of the MP tree were supported in the Bayesian tree as the same number of clades, lineage A in the MP tree was divided into two independent lineages. In the Bayesian analysis, the branching pattern between clades was generally different from that in the MP tree. In the Bayesian tree, *Astraeus* group 1 consisted a lineage with *A. hygrometricus* North American and Mediterranean groups. Another Japanese *Astraeus* group consisted of a lineage with the *A. odoratus* and *A. hygrometricus* French group.

Discussion

Japanese *Astraeus* specimens were separated into two groups, based on their morphological characteristics, both of which matched the descriptions of *A. hygrometricus* var. *koreanus* and *A. hygrometricus* s.l. Although the phylogenetic analysis supported the morphological separation, it suggested that *Astraeus* group 1 is *A. hygrometricus* var. *koreanus* and *Astraeus* group 2 is an undescribed *Astraeus* species (see Figs. 2, 3). These two Japanese *Astraeus* species were readily distinguished from *A. odoratus* based on their acute-shaped rays of the exoperidium when splitting and short to moderate spines of basidiospore ornamentation (Petcharat 2004; Phosri et al. 2004, 2007). *Astraeus hygrometricus* var. *koreanus* is different from *A. asiaticus* and *A. hygrometricus* complex (clades II, III, VI in Fig. 2) based on a pale basidiocarp color, a greater number of exoperidial rays, and a smaller scale size of the basidiomata (Arora 1986; Nouhra and De Toledo 1998; Petcharat 2004; Phosri et al. 2004, 2007). However, the only morphological differences between the Japanese undescribed *Astraeus* species and *A. asiaticus* were the shape of the spine and the size of the basidiospores (see Fig. 1; Petcharat 2004; Phosri et al. 2004, 2007). Under SEM, the undescribed Japanese *Astraeus* species showed distinct characteristics for the spines of the basidiospores, which were slender when compared with the other species.

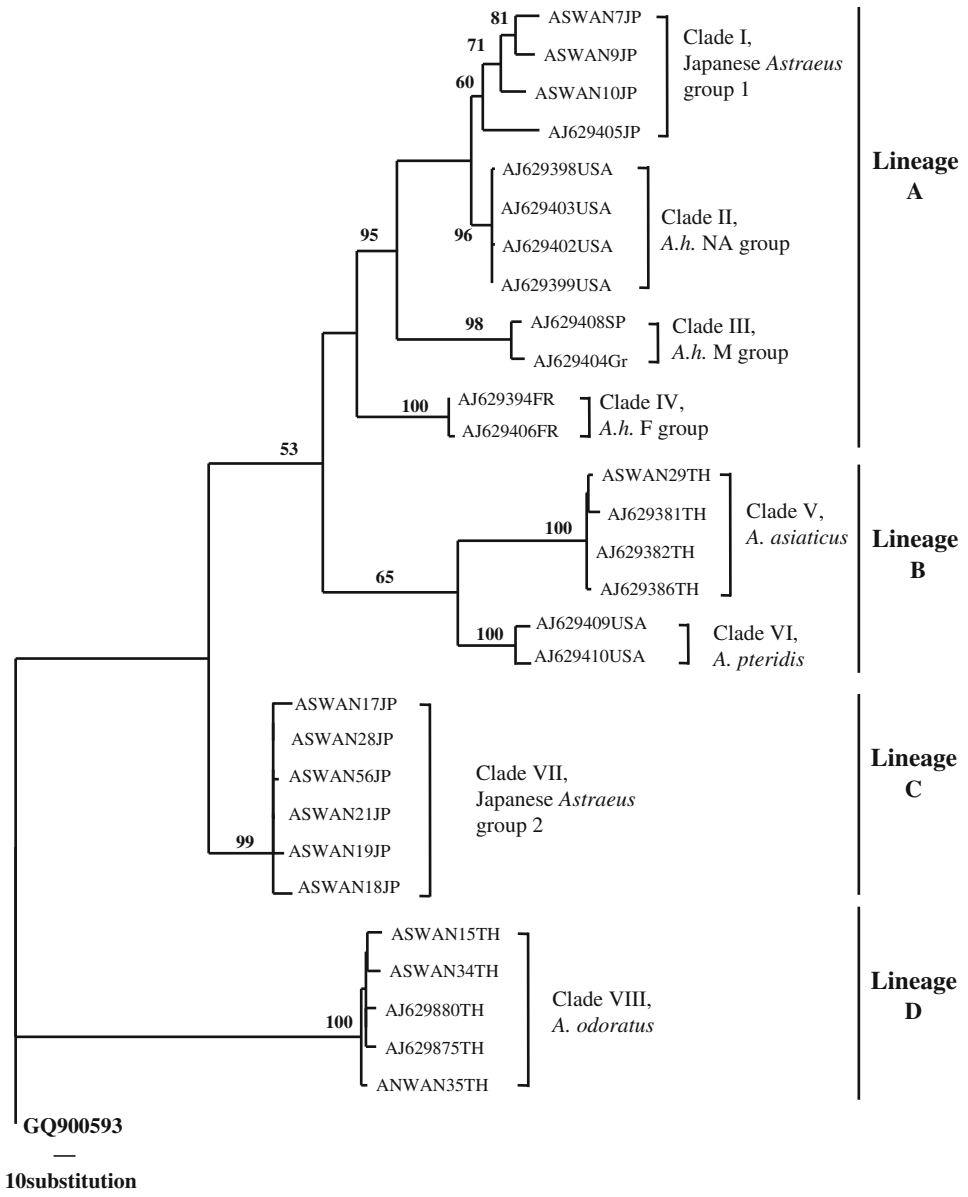

Fig. 2 Maximum-parsimony (MP) phylogenetic tree of *Astraeus* internal transcribed spacer (ITS) sequences. Bootstrap-supported values (>50%) are shown *on the respective branches*. In the tree, sequences obtained in this study are indicated as specimen number, whereas sequences obtained from GenBank are indicated by accession number. Each sequence ID is appended the country name in the last: JP, Japan; SP, Spain; GR, Greece; FR, France; TH, Thailand. Please see Table 1 for the accession numbers obtained in this study. *A. h.*, *Astraeus hygrometricus*; NA, North American; M, Mediterranean; F, French

Phosri et al. (2007) showed five *Astraeus* phylogroups based on ITS sequence analyses and suggested the same number of species. Our phylogenetic data also showed similar grouping pattern as clades (see Figs. 2, 3). However, we could distinguish the North American *A. hygrometricus* group defined by Phosri et al. (2007) as three distinct clades in lineage A, i.e., North American, Mediterranean, and Japanese clades, based on the improved phylogenetic analyses with sufficient specimen numbers. Phosri et al. (2007) analyzed only a single Japanese *Astraeus* specimen ASTRAE_94 (described as AJ629405JP in this study) and grouped it together with other specimens from North America and Mediterranean regions. *Astraeus hygrometricus* var. *koreanus* from Japan can be separated from *A. hygrometricus* in North America and the Mediterranean regions based on the small size of the basidiomata and a greater number of exoperidial rays (Coker and Couch 1928; Arora 1986; Nouhra and De Toledo 1998). Phosri et al. (2007) indicated that the Japanese *Astraeus* (ASTRAE_94) basidiospores were morphologically different from North American and Mediterranean collections, i.e., smaller spore size, varying between 6.8 and 9 μm in diameter, and longer, occasionally coalescent, densely arranged spines, 0.8–1.6 μm in length. The ASTRAE_94 sequence (named AJ629405JP in this study) was located in clade I of *A. hygrometricus* var. *koreanus*. However, the morphology of the three specimens in clade I, i.e., ASWAN 7 JP, ASWAN 9 JP, and ASWAN 10 JP, were not necessarily matched with that of ASTRAE_94, but matched the North American and Mediterranean specimens (Phosri et al.

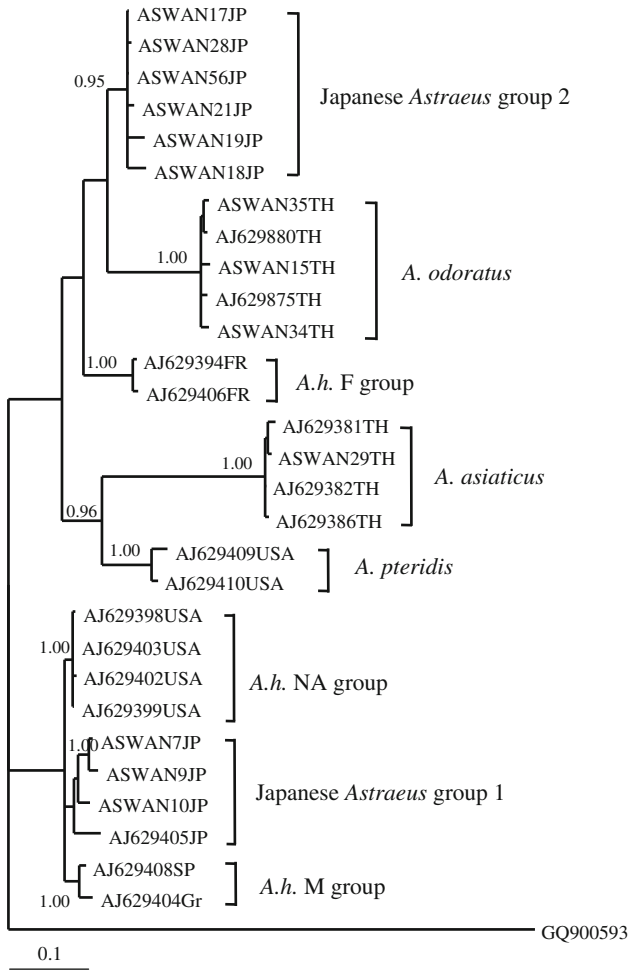

Fig. 3 Majority-rule consensus tree of *Astraeus* ITS sequences obtained from the Bayesian analysis. Bayesian posterior probabilities (BPPs) are indicated *above branches*. Values equal to and greater than 0.95 were considered significant. *A. h.*, *Astraeus hygrometricus*; NA, North American; M, Mediterranean; F, French

2007). Therefore, the *A. hygrometricus* var. *koreanus* specimens could be regarded as the same species as *A. hygrometricus* sensu Morgan, or a new species tentatively named as *A. koreanus* (V.J. Staněk) Kreisel. Because we did not analyze *A. hygrometricus* var. *koreanus* specimens from the type locality, i.e., North Korea (Staněk 1958), further study is required to clarify this issue.

The undescribed Japanese *Astraeus* species, formerly identified as *A. hygrometricus* (Ito 1959; Imazeki and Hongo 1989), was located in a distant clade from *A. hygrometricus* s.l. clades irrespective of the phylogenetic analysis applied (see Figs. 2, 3), suggesting the importance of reevaluating the taxonomy of the cosmopolitan *A. hygrometricus*. As discussed by Phosri et al. (2007), *A. hygrometricus* sensu Persoon and *A. hygrometricus* sensu Morgan may be different species, and our Bayesian tree (Fig. 3) strongly supported the idea. If this is the case, the name of *A. hygrometricus* should be revised. Furthermore, although only one *Astraeus* species, *A. hygrometricus*, has been described from Europe (Phillips 1981), European *A. hygrometricus* specimens could be divided into two distinct phylogroups in this study, i.e., Mediterranean and French groups. In this study, we could not clarify the evolutional divergence pattern of *Astraeus* spp. because of the different branching patterns of the phylogenetic trees (Figs. 2, 3), which was the same as the results obtained by Phosri et al. (2007). Multigene analyses may be required for the clarification, as has been suggested in various fungal taxa.

Based on phylogenetic trees (see Figs. 2, 3), four different clades, *A. odoratus*, *A. asiaticus*, and *A. hygrometricus* var. *koreanus*, and one undescribed Japanese *Astraeus* species, were found from Asia, whereas two *Astraeus* clades, i.e., *A. hygrometricus* North American group and *A. pteridis*, were found from North America, and another two clades, i.e., the *A. hygrometricus* Mediterranean and French groups, were found from Europe. The higher phylogroup richness of *Astraeus* in Asia suggests the possibility of a geographic origin in Asia. Further geoecological studies of *Astraeus* are necessary in the surrounding regions of Asia to clarify this hypothesis.

In conclusion, two *Astraeus* species were recognized from Japan, i.e., *A. hygrometricus* var. *koreanus* and an undescribed *Astraeus* species, the latter of which requires further taxonomic investigation and treatment.

Acknowledgments We thank Susumu Takamatsu, Graduate School of Bioresources, Mie University, Japan, for teaching and discussing phylogenetic analyses. We also acknowledge Hiromi Sasaki, Naoki Endo, Masaru Hiroi, Taiga Kasuya, Numyen Kamonkunanon, Siwat Kamonkunanon, and Pakron Ket-In for their assistance with mushroom collections. This study was supported, in part, by a Grant-in-Aid for Scientific Research (no. 19380085) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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