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Phylogenetic Relationships of Korean *Sparassis latifolia* Based on Morphological and ITS rDNA Characteristics

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Recent studies based on morphological characteristics and molecular analyses have revealed that the characteristics of *Sparassis crispa* from Asia are not concordant with those of collections from Europe and North America. Consequently, the Asian isolate was redefined as *Sparassis latifolia*. This study is the first report of *Sparassis latifolia* collected in Korea. The taxonomic relationships and replacement of *Sparassis* species were inferred from a comparison of the morphological characteristics and by molecular sequence analysis of the internal transcribed spacer (ITS) rDNA regions. In particular, this study focused on the phylogenetic relationships inferred from the biogeographical distribution of isolates within the genus *Sparassis*.

Keywords: *Sparassis latifolia*, *Sparassis crispa*, *Sparassis radicata*, geographical distribution, phylogenetic relationship

Introduction

The *Sparassis* Fr. species belonging to the family *Sparassidaceae* Herter is distributed throughout the northern temperate forests. The generic and infrageneric delimitations of *Sparassis* are based mainly on colour of the basidiomata, shape of the flabella, size of the basidiospore and the host plant. On the boundaries of the genus *Sparassis*, their morphological circumscription agreed with the phylogenetic relationships (Desjardin *et al*., 2004; Wang *et al*., 2004). These reports mentioned, however, that the Asian isolate of *S*. cf. *crispa* is morphologically different from *S. crispa* of Europe. The taxonomic division of *Sparassis* has been refined since by Dai *et al*. (2006). They re-evaluated the Asian *Sparassis* isolate as a new species, *S. latifolia*, based on macromorphological, microscopic, mating type, and phylogenetic analyses. Their molecular data suggested a new hypothesis for the correlation between the morphological characteristics and the geographical distribution within the genus *Sparassis*. They proposed that *Sparassis* be classified into three groups, *S. crispa* from Europe and eastern North America, *S. radicata* from western North America, and *S. latifolia* from Asia, according to phylogenetic relationships and placement (Dai *et al*., 2006). The holotype of *S. latifolia* collected from the Jilin region of China was found to be distributed broadly in eastern Asia (Dai *et al*., 2006). Korea, which is situated in eastern Asia and located close to northeastern China, had only the species *Sparassis crispa* among species of the genus *Sparassis*. The authors' collections of *S. latifolia* from Korea described in this report were new compared with collections from China.

The objectives of this study were; (1) to represent the first published records of *S. latifolia* in the Republic of Korea; and (2) to discuss the re-identification of Korean *Sparassis* isolates.

Materials and Methods

Morphological studies

Morphological descriptions of all specimens are based on fresh basidiocarps. Microscopic characteristics described pertain to dried material mounted in H_2O, 3% KOH, and Melzer's reagent, using bright field (Leica DM 2500). For basidiospores, the factors L (the arithmetic mean of the spore lengths), W (the arithmetic mean of the spore width), E (quotient of the mean spore length and the mean spore width), and Q (mean of Evalues) were used to describe the morphological characteristics. Specimens are preserved at the Korea Forest Research Institute (KRFI), the Republic of Korea.

Molecular techniques

Genomic DNAs were obtained from the mycelia of their cultures. The DNA was extracted with cetyl-trimethylammonium bromide (CTAB) buffer and purified with a phenol: chloroform: isoamyl alcohol (in ratio of 25: 24: 1) mixture using the modified method of Lee and Taylor (1990).

Polymerase chain reaction (PCR) was performed by the method of White *et al*. (1990). Primers specific to the internal transcribed spacer (ITS) regions of rDNA, named ITS1 and ITS4, were used for the selective amplification. The PCR was conducted in total 50 μl reaction volumes containing 1.2 μl of template DNA mixture, 5 μl of 10× buffer (0.5 M KCl, 0.1 M Tris-HCl; pH 8.0, 0.1% Triton X-100, 15 mM $MgCl_2$), 1 μl of 2.5 mM dNTPs, 0.4 μl of 100 pM primer ITS1, 0.4 μl of 100 pM primer ITS4, and 0.4 μl of Taq polymerase (5 unit/μl). Temperature cycling was performed with denaturation for 30 sec at 94°C, annealing for 30 sec

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Table 1. List of *Sparassis* species used in this study. Species names in bold designated sequenced newly for this study.

Species	Isolate Number	Specimen Number	Locality	GenBank accession no.
				ITS
Sparassis brevipes	GER22	RB8/78	GERMANY	AY218439
Sparassis brevipes	GER24	ILKKA-96-1044	GERMANY	AY218441
Sparassis crispa	AME27	TENN45811	USA/WA	AY218444
Sparassis crispa	AME28	TENN44575	USA/GA	AY218445
Sparassis crispa	AME9	ZW-Clarku003	USA/MA	AY218430
Sparassis crispa	**CZ1**	**BRMN 686439**	**CZECH REPUBLIC**	**JQ586250**
Sparassis crispa	ENG10	BMS2857	ENGLAND	AY218431
Sparassis crispa	FIN3	YCDA12637	FINLAND	AY218425
Sparassis crispa	FIN4	SAVOLAINEN	FINLAND	AY218426
Sparassis crispa	FRA5	ILKKA94-1587	FRANCE	AY218427
Sparassis crispa	GER23	RB9/6/87	GERMANY	AY218440
Sparassis crispa	GER25	DORISLABER	GERMANY	AY218442
Sparassis crispa	**KFRI 0639**	-	**USA**	**JX566462**
Sparassis crispa	**KFRI 0642**	-	**NETHERLANDS**	**JX566465**
Sparassis cystidiosa	THAI	DEDesjardin7410	THAILAND	AY256891
Sparassis latifolia	CHN1	YCDAI2145	CHINA	AY218423
Sparassis latifolia	CHN17	HMAS60590	CHINA	AY218435
Sparassis latifolia	CHN19	HKAS15728	CHINA	AY218436
Sparassis latifolia	CHN2	YCDAI2470	CHINA	AY218424
Sparassis latifolia	CHN20	HKAS32363	CHINA	AY218437
Sparassis latifolia	CHN21	HKAS17477	CHINA	AY218438
Sparassis latifolia	**KFRI 0123**	-	**JAPAN**	**JX566460**
Sparassis latifolia	**KFRI 0245**	-	**KOREA**	**JX566461**
Sparassis latifolia	**KFRI 0640**	-	**JAPAN**	**JX566463**
Sparassis latifolia	**KFRI 0641**	-	**KOREA**	**JX566464**
Sparassis latifolia	**KFRI 0643**	-	**KOREA**	**JX566466**
Sparassis latifolia	**KFRI 0644**	-	**KOREA**	**JX566467**
Sparassis latifolia	**KFRI 0645**	-	**KOREA**	**JX566468**
Sparassis latifolia	**KFRI 0691**	-	**KOREA**	**JX566470**
Sparassis latifolia	**KFRI 0700**	**KFRI 0700**	**KOREA**	**JQ586251**
Sparassis latifolia	**KFRI 0720**	-	**KOREA**	**JX566472**
Sparassis latifolia	**KFRI 0721**	-	**KOREA**	**JX566473**
Sparassis latifolia	**KFRI 0722**	-	**KOREA**	**JX566474**
Sparassis latifolia	**KFRI 0723**	**KFRI 0723**	**KOREA**	**JQ586252**
Sparassis latifolia	**KFRI 0724**	-	**KOREA**	**JX566475**
Sparassis latifolia	**KFRI 0746**	-	**KOREA**	**JX566476**
Sparassis latifolia	**KFRI 0748**	-	**KOREA**	**JX566477**
Sparassis latifolia	**KFRI 0749**	-	**KOREA**	**JX566478**
Sparassis latifolia	**KFRI 0819**	-	**KOREA**	**JX566479**
Sparassis latifolia	**KFRI 0921**	-	**KOREA**	**JX566480**
Sparassis latifolia	**KFRI 0922**	-	**KOREA**	**JX566481**
Sparassis latifolia	**KFRI 0923**	**KFRI 0923**	**KOREA**	**JQ586253**
Sparassis latifolia	**KFRI 0926**	-	**KOREA**	**JX566482**
Sparassis latifolia	**KFRI 1074**	-	**KOREA**	**JX566483**
Sparassis latifolia	**KFRI 1075**	-	**KOREA**	**JX566484**
Sparassis latifolia	**KFRI 1076**	-	**KOREA**	**JX566485**
Sparassis latifolia	**KFRI 1077**	-	**KOREA**	**JX566486**
Sparassis latifolia	**KFRI 1079**	-	**KOREA**	**JX566487**
Sparassis latifolia	**KFRI 1080**	-	**KOREA**	**JX566488**
Sparassis latifolia	**KFRI 1081**	-	**KOREA**	**JX566489**
Sparassis latifolia	**KFRI 1082**	-	**KOREA**	**JX566490**
Sparassis latifolia	**KFRI 1083**	-	**KOREA**	**JX566491**
Sparassis latifolia	**KFRI 1084**	-	**KOREA**	**JX566492**
Sparassis latifolia	**KFRI 1121**	-	**KOREA**	**JX566493**
Sparassis latifolia	**KFRI 1122**	-	**KOREA**	**JX566494**

Table 1. Continued

Species	Isolate Number	Specimen Number	Locality	GenBank accession no. ITS
Sparassis latifolia	**KFRI 1178**	-	**KOREA**	**JX566495**
Sparassis latifolia	**KFRI 1306**	-	**KOREA**	**JX566496**
Sparassis latifolia	**KFRI 1406**	-	**KOREA**	**JX566497**
Sparassis latifolia	**KFRI 1515**	-	**KOREA**	**JX566498**
Sparassis latifolia	**KFRI 1516**	-	**KOREA**	**JX566499**
Sparassis latifolia	**KFRI 1787**	**KFRI 1787**	**KOREA**	**JX566500**
Sparassis latifolia	**KFRI 1788**	**KFRI 1788**	**KOREA**	**JX566501**
Sparassis miniensis	SPAIN	Lou-Fungi 18390	SPAIN	DQ270675
Sparassis nemecii	**CZ2**	**BRMN 714903**	**CZECH REPUBLIC**	**JQ586254**
Sparassis radicata	AME29	TENN56253	USA/CA	AY218446
Sparassis radicata	AME32	TENN50232	USA/TN	AY218449
Sparassis radicata	AME33	TENN52558	USA/WA	AY218450
Sparassis radicata	CAN26	UBC-F12464	CANADA	AY218443
Sparassis radicata	**KFRI 0692**	-	**CANADA**	**JX566471**
Sparassis spathulata	AME11	ZW-Clarku004	USA/MA	AY218432
Sparassis spathulata	AME7	ZW-Claru001	USA/MA	AY218428
Sparassis spathulata	AME8	ZW-Clarku002	USA/NH	AY218429
Polyporus squamosus	-	-	-	AY218421
Polyporus tuberaster	-	-	-	AF516597

at 56°C, and extension for 1 min at 72°C. Thirty-five cycles were run with the first denaturation and last extension times extended to 5 min at 72°C. Purified DNA was directly sequenced on an automatic sequencer (ABI Prism TM 377 DNA Sequencer) with the primers ITS1 and ITS4.

Phylogenetic analysis

The phylogenetic tree was generated using Bayesian (Markov Chain Monte Carlo [MCMC]) methods. An alignment of the sequences was performed using the CLUSTAL_X software package (Thompson *et al*., 1997). MCMC analysis was carried out using MRBAYES version 3.1 (Ronquist and Huelsenbeck, 2003). For a given data set, the general time reversible (GTR) model was employed with gamma-distributed substitution rates. Markov chains were run for 2,000,000 generations, saving a tree every 100th generation. Among a total of 2001 trees, the first 50 trees were discarded. The MRBAYES software was used to compute a 50% majority rule consensus of the remaining trees to obtain estimates for the posterior probabilities (PPs) of the groups. The sequences of *Polyporus squamosus* and *P. tuberaster* were selected as outgroups according to a previous report by Wang *et al.* (2004). Information on specimens and sequences, along with the GenBank accession numbers, used in this study are indicated in Table 1.

Results

Taxonomy

Sparassis latifolia Y.C. Dai & Zheng Wang, in Dai, Wang, Binder & Hibbett, Mycologia 98: 584–592, 2006. (Figs. 1–2)

Basidiomata Single, up to 400 mm diam, white to yellowish or cream, azonate, flabellae arising from a branched base. *Flabellae* broad, dissected and slightly contorted, with broadly rugulose and wavy, sometimes with slightly laciniate margin, white to cream concolorous with stipe when fresh, yellowish brown or pale brown when dry. *Stipe* 15–20 mm diam at base, white to cream.

Basidiospores (4.1–)5.0–5.2(–5.9) × (3.8–)4.0–4.1(–4.2) µm, L = 5.04 µm, W = 4.02 µm, E = 1.23–1.28, Q = 1.26, ellipsoid, thick-walled, hyaline. *Basidioles* 29–51 × 4.5–6.0 µm, (narrowly) clavate. *Basidia* 39–51 × 5.5–8.0 µm, 4-spored,

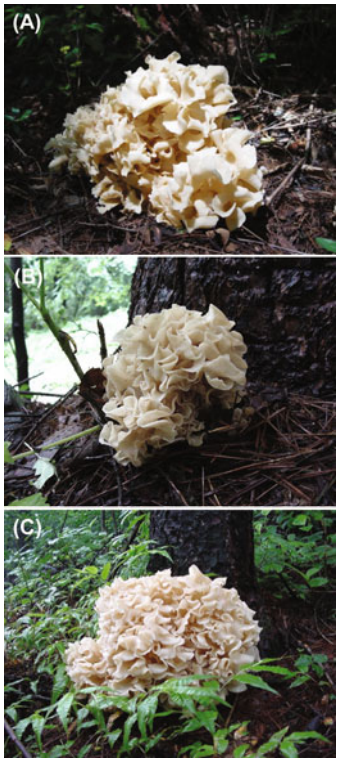

Fig. 1. *S. latifolia* collected from Korea. (A) *S. latifolia* (KFRI 0700). (B) *S. latifolia* (KFRI 0723). (C) *S. latifolia* (KFRI 0923). Habitat, the base of *Larix kaempferi* (KFRI 0700 and 0923) and *Pinus koraiensis* (KFRI 0723).

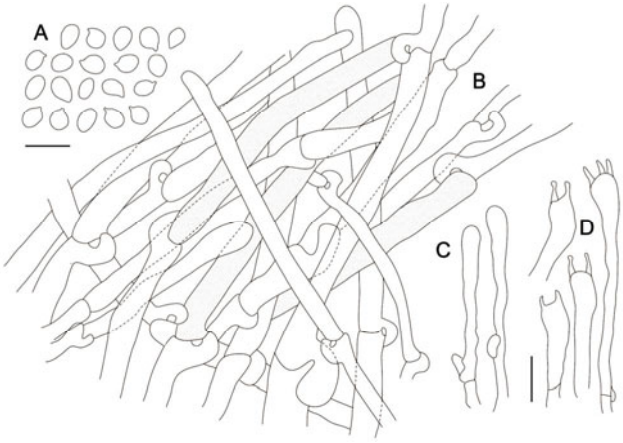

Fig. 2. Microscopic characteristics of Korean *S. latifolia*. (A) Basidiospores. (B) Subhymenial hyphae. (C) Basidiole. (D) Basidia. Bar=10 μm.

(broadly) clavate with sterigmata 4.5–5.1 μm. *Subhymenial hyphae* (2.5–) 3.0–7.0 (–7.5) μm diam, cylindrical, thin-walled, hyaline, tortuous, interwoven. *Context hyphae* 3.0–8.0 (–11.0) μm diam, slightly branched, thin- to slightly thick-walled, smooth, hyaline, interwoven. *Clamp connections* present in all tissues.

Habit and habitat. At the base of *Larix kaempferi* (KFRI 700 and 923) and *Pinus koraiensis* (KFRI 723)

Chemical reactions. Negative reaction from KOH and Melzer's reagent (in amyloid).

Material examined. THE REPUBLIC OF KOREA: Pocheon, Korea National Arboretum, Gwangneung forest, 18 July 2005, *H. Park* (KFRI 700) & 21 July 2006 *K.-H. Ka* (KFRI 723); Gurye, Seongsamjae, Jirisan National park, 18 July 2007 *K.-H. Ka* (KFRI 923).

Other material examined. *Sparassis crispa:* THE CZECH REPUBLIC, Labské pískovce, Jetřichovice u Děčína, N 50° 52′, E 14° 22′, ± 300–350 m, *Pinus sylvestris*, 16 Oct 2003 *A.*

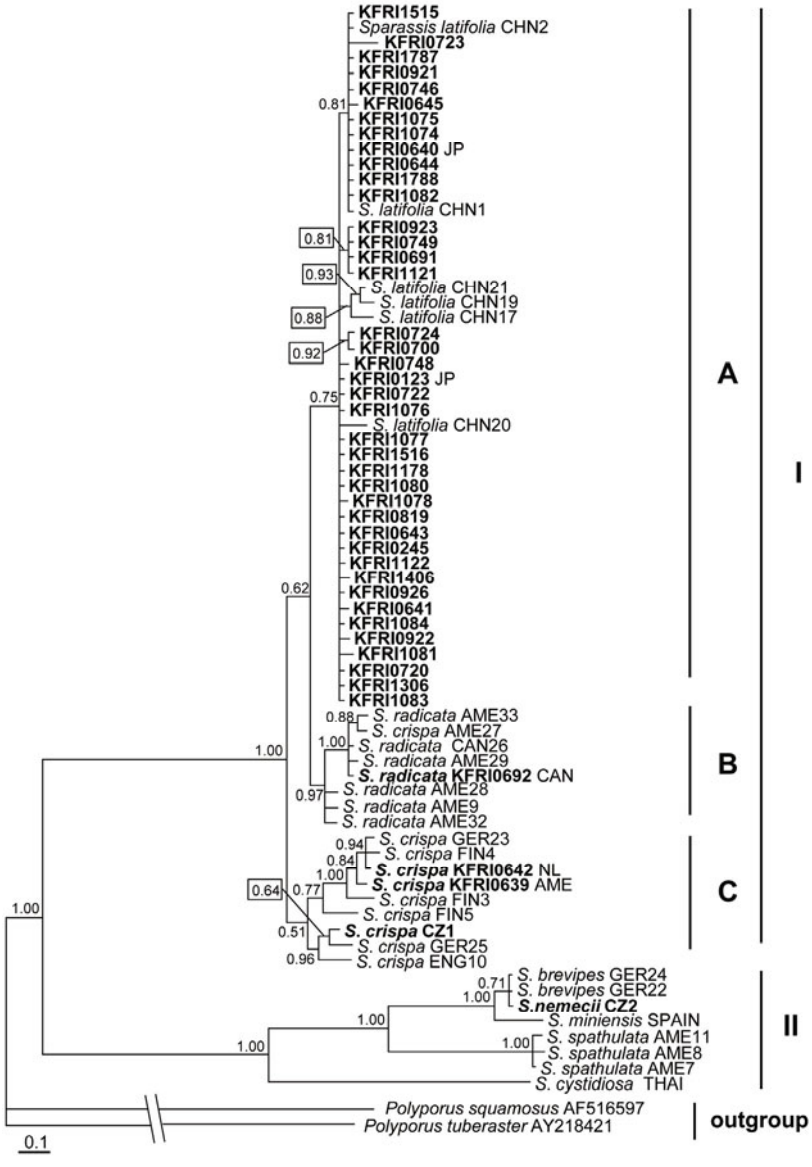

Fig. 3. Phylogenetic trees for Asian *S. latifolia* species based on the internal transcribed spacer (ITS) region (ITS1, 5.8S rDNA, and ITS2). Markov Chain Monte Carlo (MCMC) posterior probability (PP) values are given above the supported node. Bayesian analysis showing mean branch lengths of a 50% majority-rule consensus tree calculated from 2001 trees, revealed during a MCMC analysis of 200,000 length. Maximum parsimony analysis showing a 50% majority-rule consensus tree. Species names indicated in bold type were sequenced newly by the authors in this study. Abbreviations: AME, the USA; CAN, Canada; CHN, China; CZ, Czech Republic; ENG, England; FIN, Finland; GER, Germany; JP, Japan; KOR, Korea;, NL, Netherlands; SPAIN, Spain; THAI, Thailand.

Vágner (BRNM 686439). *Sparassis nemecii*: THE CZECH REPUBLIC, Javorníky, Velké Karlovice, NPR Razula, Abieto-agetum, N 49° 21′ 33″, E 18° 22′ 56″, ± 600–810 m, *Abies alba*, 4 Aug 2009, *V. Antonín* & *D. Janda* (BRNM 714903).

Notes: The authors' collections are characterized by having azonate and broad flabellae and clamp connections, growing in conifers such as *Larix* and *Pinus*. Most morphological characteristics of our collection agree with those of the original description (Dai *et al*., 2006). The present species are especially similar to the species collected from northern China in the following characteristics: (1) the size and Q value of basidiospores (5.0–5.2 × 4.0–4.1 µm, Q = 1.26); (2) large and broad flabellae with sometimes slightly tooth-like margins. They have, however, a difference size of basidia and basidioles. The Korean species have longer basidia or basidioles, up to 51 µm in length. *Sparassis crispa* and *S. nemecii* collected from the Czech Republic (BRNM 686439 and BRNM 714903) have lager basidiospores, of 6.0–7.0 × 4.0–5.0 µm (Q = 1.37) and 6.0–6.5 × 4.5–5.0 µm (Q = 1.33), respectively, but only the former presents clamp connections of basidiomata.

Phylogenetic analysis

The phylogenetic relationships of the seventy-four sequences of the genus *Sparassis*, obtained in this study and retrieved from GenBank were inferred from MCMC analysis based on internal transcribed spacer region (ITS) rDNA. The 50% consensus tree was obtained by Bayesian analysis (Fig. 3).

The combined *Sparassis* ITS rDNA sequences formed a monophyletic group, supported by the high posterior probability (1.00 PP) based on MCMC analysis. According to morphological characteristics, this monophyletic clade was divided into two groups. Clade I consisted of species that formed clamp connections and the clade II grouped the species which produced simple septates, without clamp connections. Three members from different geographical origins (0.62 PP) were designated as clade I. The subclade A consisted only of *S. latifolia* of eastern Asian species, containing 39 isolates collected from South Korea and 2 isolates obtained from Japan (0.75 PP). They formed one clade regardless of the host specificity. A second subgroup, subclade B, was a monophyletic clade of *Sparassis radicata* (or *S. crispa*) collected from North America, supported by strong probability values (0.97 PP). This subclade was divided into two groups according to the geographical distribution site. Isolates collected from western North America and Canada formed an independent and monophyletic subgroup, as supported by a significant posterior probability (1.00 PP). Isolates from eastern North America were in sister branches with the subgroup of isolates from western North America. A third subgroup, subclade C, included European isolates (0.51 PP). The clade II formed three subclades, which consisted of the European isolates subclade (1.00 PP) of *Sparassis nemecii*, *S. miniensis*, and *S. brevipes* collected from Czech, Germany, Spain, a subclade (1.00 PP) of *S. spathulata* from eastern North America, and *S. cystidiosa* from Thailand of eastern Asia, based on the geographical distribution.

Discussion

An Asian taxon of *Sparassis* was described as a new species and named *S. latifolia*, by Dai *et al.* (2006). Nevertheless, in the Republic of Korea, only collections under the name *S. crispa* have been recorded to date in several reports that addressed their cultivation method (Park *et al*., 2005; Ryu *et al*., 2009). It is necessary to have a taxonomically useful description for Korean *Sparassis crispa*. In this work, we studied 40 isolates of *S. latifolia* collected in the Republic of Korea. Their characteristics were not different from the original descriptions in previously published reports (Dai *et al*., 2006). Therefore, this work suggests that the Korean cauliflower mushroom used commercially and in academic research, probably includes both *S. latifolia* and *S. crispa*. It is possible that almost all the *Sparassis* isolates collected from Korea are *S. latifolia*. The results of this study have provided DNA sequence data for *S. latifolia* from Korea, for *S. crispa* and *S. radicata* from Canada and USA, and for *S. crispa* and *S. nemecii* collected from the Czech Republic and Netherlands. In addition, the DNA-based analysis of *Sparassis* species has clarified the answers to some of the taxonomic questions regarding the complex species in the genus. Based on the molecular data presented in this study, it is apparent that the phylogenetic relationships derived from the geographical distribution of *Sparassis* species are not entirely accurate.

The infrageneric taxonomy of the genus *Sparassis* was estimated through the mating systems as well as molecular analysis, using nuclear and mitochondrial rDNA (nuc-ssu rDNA, nuc-lsu rDNA, mt-ssu rDNA, and mt-lsu rDNA) and the gene encoding RNA polymerase subunit II (*RPB2*) (Wang *et al*., 2004). In this report, the authors modified the concept of *Sparassis* species as consisting of species with overlapping biogeographical distributions, with mainly the basidiospore size and clamp connection as additional considerations. Recently, based on geographical differences, Dai *et al.* (2006) also inferred polyphyletic subgroups within the genus *Sparassis*. The results of our study strongly support the conclusion in previous reports that species of the genus *Sparassis* conform to a relatively close geographic relationship in Europe, western North America, and eastern Asia. The molecular data of *Sparassis* groups have been found to correspond well with the morphological concept and topographical pattern.

The bayesian analysis of ITS rDNA sequence data indicated that clade I contains members producing clamp connections, and clade II includes members producing simple septates. Clade I was grouped to contain species with *S. crispa*, *S. latifolia*, and *S. radicata*. Subclade A consisted of Eastern Asian isolates collected from China, Korea, and Japan. Among the 39 Korean isolates, three isolates of KFRI 0700, 0723, and 0923 were selected for further morphological characterizations. These isolates had a sequence divergence of about 1.07%; 7 out of 650 nucleotides sequenced and analysed were different. These sequences showed no more than 5 base substitutions, compared with sequences of isolates from China (CHN 2; the paratype of *S. latifolia*) and Japan (KFRI 0123 and 0640). In the current study, *S. radicata* was designated monophyletic according to geographical relation-

ships. These isolates from North America, irrespective of whether they were western or eastern, were found to form the same clade. In subclade B, *S. radicata* (or *S. crispa*) from western North America made up one clade with a moderately high posterior probability value (0.97 PP) and the species from eastern North America were arranged monophyletically, despite the lack of a strong posterior probability value supporting this arrangement. In this study, North American species were independent from European species, although a close relationship was found between isolates of western North America and isolates of Europe. It was recently reported (Dai *et al*., 2006) that *S. radicata* (or *S. crispa*) of western North America and *S. crispa* of Europe are intertwined in one clade. Western North American *S. radicata* appeared to be closely related to Asian *S. latifolia* morphologically. Based on the results of the molecular systematic data, however, these two species did not form one clade; rather, western North American *S. radicata* seemed to be in the same clade with European *S. crispa* (Desjardin *et al*., 2004; Blanco-Dios *et al*., 2006; Dai *et al*., 2006). In previous research, *S. radicata* of western North America and *S. latifolia* (or *S.* cf. *crispa*) of Asia were monophyletic, supported by a low 50% posterior probability value (Wang *et al*., 2004). The data in the present study suggest that the interspecific ITS variation appeared to correspond to, or vary greatly with, these morphologically specific characteristics of the species within the genus *Sparassis*. The taxonomic positions of these species were compared and analysed between morphology-based systems and molecular systematic data. The results in this study suggest that some of the systematic divisions of *Sparassis* need careful re-evaluation, especially at the subgenus level. The analysis of molecular data may help accomplish this task by proposing new phylogenetic hypotheses that can subsequently be evaluated by integrating a broad range of morphological characteristics of the genus *Sparassis*.

These authors' analysis on *Sparassis*-informative characteristics is still contestable. Clearly, there is a need for thorough studies on the chemical and cultural characteristics of the genus *Sparassis*. It is likely that taxonomically important data will be found by conducting biochemical tests on basidiomata and vegetative cultures, and genetic variation analysis of hybridized isolates will continue to be used to define *Sparassis* subgenuses.

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