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Phylogeographic divergences of nuclear ITS sequences in *Coprinus* species *sensu lato**

Kwan S. KO†, Young W. LIM‡, Yang H. KIM§ and Hack S. JUNG¶

School of Biological Sciences, Seoul National University, Seoul 151-742, Korea.
E-mail: minervas@snu.ac.kr

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Phylogeographic divergences of four coprinoid species, *Coprinus comatus*, *Coprinellus disseminatus*, *Coprinellus micaceus* and *Coprinopsis lagopus* were investigated using nuclear ITS sequences. Each taxon showed genetic variation that corresponds with the geographic origins of collections. Groupings produced from ITS1 and ITS2 sequences were similar together. In *C. comatus*, East Asian strains were well separated from New Zealand and North American strains. In *C. disseminatus*, Hawaiian strains formed an independent clade with that from Nepal, separating those from East Asia. And there were two distinct East Asian groups for *C. disseminatus*. East Asian *C. micaceus* strains constituted a distinct group from Hawaiian strains. In *C. lagopus*, Hawaiian and European strains were clearly separated from each other. There was a great genetic diversification in *C. disseminatus* and *C. micaceus* from East Asia, suggesting divergence process of these taxa in that region. However, Hawaiian strains lacked genetic variation, which indicated their recent origin in Hawaii.

INTRODUCTION

The genus *Coprinus s. lat.* has been characterized by dark spores possessing an apical germ pore, well-developed paraphyses, the deliquescent nature of lamellae, and inaequihymeniferous development of basidia. In most species, autolysis of lamellae and pilei can be detected, a characteristic responsible for the common name ink caps. Morphological and developmental characters based on the above-mentioned ones have been used to define coprinoid taxa (Singer 1986, Smith 1971). Several species have been well-known for a model system in the studies of mating compatibility, speciation, molecular biology and fruitbody development (Pukkila & Casselton 1991).

The phylogenetic relationships of coprinoid fungi have recently been investigated in some studies using molecular sequences (Hopple & Vilgalys 1994, 1999, Moncalvo *et al.* 2000, Park *et al.* 1999, Redhead *et al.* 2001). In these studies, *Coprinus s. lat.* proved not to be monophyletic. *Coprinus comatus*, the type species of *Coprinus* , and *C. sterquilinus* were separated at a different position from those of almost all other species. Most coprinoid species (90% of *Coprinus s. lat.*) were mixed up together with *Psathyrella* and *Lacrymaria* species (Hopple & Vilgalys 1999, Moncalvo *et al.* 2000, Redhead *et al.* 2001). For this reason, Redhead *et al.* (2001) recently subdivided the genus *Coprinus* into *Coprinus sstr.* (*Agaricaceae*), and *Coprinellus*, *Coprinopsis*, and *Parasola* in the new family *Psathyrellaceae*, making the necessary new combinations, and whose names are adopted in this study.

Ribosomal DNA sequences have been commonly used to study patterns of phylogenetic divergences in fungi (Hibbett *et al.* 1995, Hibbett, Hansen & Donoghue 1998, Isikhuemhen *et al.* 2000, O'Donnell, Cigelnik & Nirenberg 1998, Vilgalys & Sun 1994, Vilgalys *et al.* 1996). In earlier studies, phylogeographical approaches using ribosomal DNA sequences were proven to be useful for understanding of fungal speciation. In the studies of Hibbett *et al.* (1995, 1998), shiitake constituted five independent lineages, which did not correspond with their origins at all. Vilgalys & Sun (1994) revealed that phylogenetically based pattern of genetic divergence of *Pleurotus* was associated with allopatric speciation among populations from different continents. Recently, Isikhuemhen *et al.* (2000) investigated correlation between mating compatibility and phylogeography of *Pleurotus tuberregium* based on ITS sequences. And for *Fusarium*, protein encoding genes as well as ribosomal DNA were used

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† Present addresses: Department of Microbiology and Cancer Research Center, Seoul National University College of Medicine, Seoul 110-799, Korea;

§ Department of Dermatology, Yonsei University College of Medicine, Seoul 120-749, Korea; and

‡ Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Taejon 305-333, Korea.

¶ Corresponding author.

Table 1. Strains of coprinoid taxa examined in this study.

Species/Strain number[a]	Locality	DNA source[b]	GenBank Accession ITS1	GenBank Accession ITS2
Coprinus comatus				
DUKE-D252	USA	R	U85334	U85334
GBDS 536	Korea	R	AF059584	AF043656
GBDS 1035	Korea	R	AF059585	AF043657
IFO 30325	Japan	C	AF296768	AF296788
IFO 30480	Japan	C	AF296767	AF296787
PDD 63821	New Zealand	S	AF296769	AF296789
Coprinellus disseminatus				
DED 5969	Hawaii	S	AF296770	AF296790
DEH 1312	Hawaii	S	AF296772	AF296792
DEH 1832	Hawaii	S	AF296771	AF296791
GBDS 2221	Nepal	R	AF043665	AF059592
IFO 7550	Japan	C	AF296773	AF296793
IFO 30297	Japan	C	AF296775	AF296795
IFO 30972	Japan	C	AF296774	AF296794
SFC 950830-1	Korea	S	AF296776	AF296796
SFC 990623-5	Korea	S	AF296777	AF296797
Coprinellus micaceus				
DED 6373	Hawaii	S	AF296783	AF296803
DEH 627	Hawaii	S	AF296784	AF296804
DEH 953	Hawaii	S	AF296785	AF296805
GBDS 1004	Korea	R	AF059589	AF043662
GBDS 1388	Korea	R	AF059588	AF043661
GBDS 2056	Korea	R	AF059591	AF043664
GBDS 2112	Korea	R	AF059590	AF043663
TMI 6661	Japan	S	AF296786	AF296806
Coprinopsis lagopus				
CBS 145.47	France	C	AF296778	AF296798
DEH 1189	Hawaii	S	AF296779	AF296799
DEH 1801	Hawaii	S	AF296780	AF296800
DEH 1828	Hawaii	S	AF296781	AF296801
IFO 30119	England	C	AF296782	AF296802

[a] Abbreviations are as follows: CBS, Centraalbureau voor Schimmelcultures, Utrecht; DED, Dennis E. Desjardin, San Francisco State University, California; DEH, Don E. Hemmes, University of Hawaii at Hilo, Hawaii; DUKE, Duke Mycology Laboratory, Duke University, Durham, NC; GBDS, Gene Bank Dried Sample, Suwon, Korea; IFO, Institute of Fermentation, Osaka; PDD, Plant Diseases Division, Auckland; SFC, Seoul National University Fungus Collection, Seoul, Korea; TMI, Tottori Mycological Institute, Tottori, Japan.

[b] DNAs were extracted from cultures (C) or dried specimens (S) and sequences were retrieved (R) from GenBank.

Table 2. Sequence data and results of phylogenetic analyses.

Species/ITS Region	Sequence length	Similarity	Variable sites	Informative sites	MPT numbers	MPT lengths	CI[a]	RI[b]	RC[c]
Coprinus comatus (6)[d]									
ITS1	265	90.1–100	35	8	1	35	1.00	1.00	1.00
ITS2	271	96.8–100	13	7	2	14	0.93	0.88	0.81
Coprinellus disseminatus (9)									
ITS1	324	93.3–100	39	25	3	51	0.90	0.89	0.80
ITS2	267	94.6–100	20	8	8	23	0.94	0.94	0.80
Coprinellus micaceus (8)									
ITS1	302	92.2–100	33	19	3	41	0.90	0.90	0.82
ITS2	264	94.9–100	20	10	2	21	0.95	0.93	0.89
Coprinopsis lagopus (5)									
ITS1	298	95.2–100	15	12	1	15	1.00	1.00	1.00
ITS2	254	96.5–100	8	8	1	8	1.00	1.00	1.00

[a] Consistency index.

[b] Retention index.

[c] Rescaled consistency index.

[d] Numbers of examined samples.

for phylogeographical studies (O'Donnell *et al*. 1998, 2000). In this study, phylogeographic variations of four coprinoid taxa, *Coprinus comatus*, *Coprinellus disseminatus*, *C. micaceus* and *Coprinopsis lagopus* were examined using molecular data of nuclear ITS sequences, and their phylogenetic significance was discussed.

MATERIALS AND METHODS

Strains and DNA extraction

Strains of coprinoid taxa used in this study are listed in Table 1. Morphological species concept was applied to identify the strains used in this study. Total DNA was extracted from cultured mycelia or dried specimens by a rapid method for nucleic acid extraction (Ko & Jung 1999, Lecellier & Silar 1994, Lee, Ko & Jung 2000) with some modifications. Cultured mycelia or dried herbarium specimen pieces were recovered in an Eppendorf tube where 750 µl extraction buffer (100 mM Tris–HCl at pH 8.0, 1 mM EDTA at pH 8.0, 100 mM NaCl, 2% SDS) was added. The mixture was vortexed for 30 s, frozen in liquid nitrogen for 30 s and then warmed at 80 °C for 30 s to 1 min. This process was repeated several times until the complete breakdown of cells. DNA was purified through phenol and chloroform extractions, precipitated with 1 volume of isopropanol, and immediately centrifuged at 12000 rev min^{-1} for 10 min at room temperature. The supernatant was removed and the pellet was washed twice in 70% ethanol, dried in the air and then resuspended in 50 µl of sterile TE (pH 8.0) or deionized distilled water.

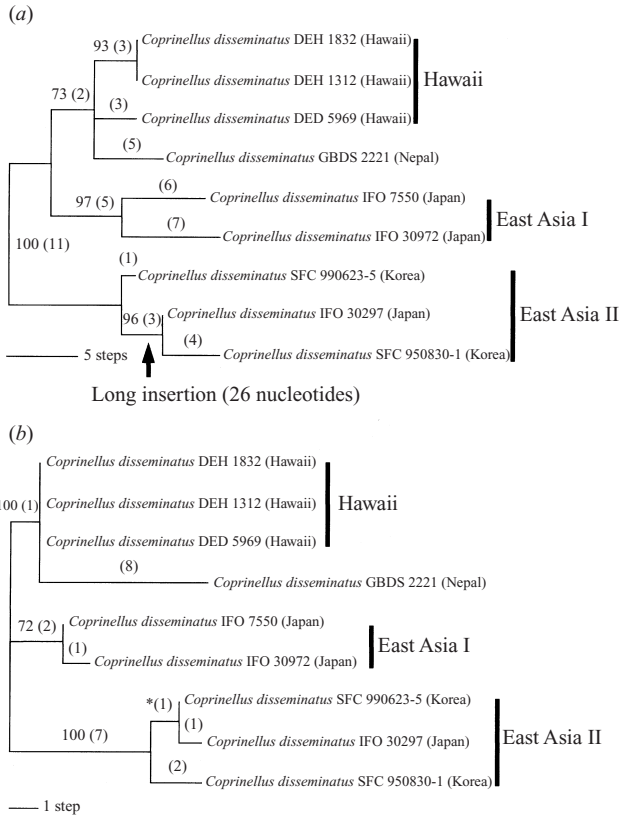

Fig. 2. The most parsimonious trees of *Coprinellus disseminatus* based on ITS sequences. (a) One of three equally most parsimonious ITS1 trees. (b) One of eight equally most parsimonious ITS2 trees. Other details are same as those of Fig. 1.

PCR and sequencing

PCR amplification was executed using primers, ITS5 and ITS4 (White *et al*. 1990), in a reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.1% BSA, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 1 µl of each primer (25 µ mol), and *ca* 200 ng of template DNA, with 2 units of DynaZyme DNA polymerase. Total volume was adjusted at 50 µl and the reaction was performed for 30–35 cycles in a PTC-100 Programmable Thermal Cycler (MJ Research). Each cycle consisted of 1 min at 94 ° for denaturation, 1 min at 50 to 55 ° for annealing, 1 min at 72 ° for extension, and final extension was executed for 10 min at 72 °. Amplified PCR products were detected on

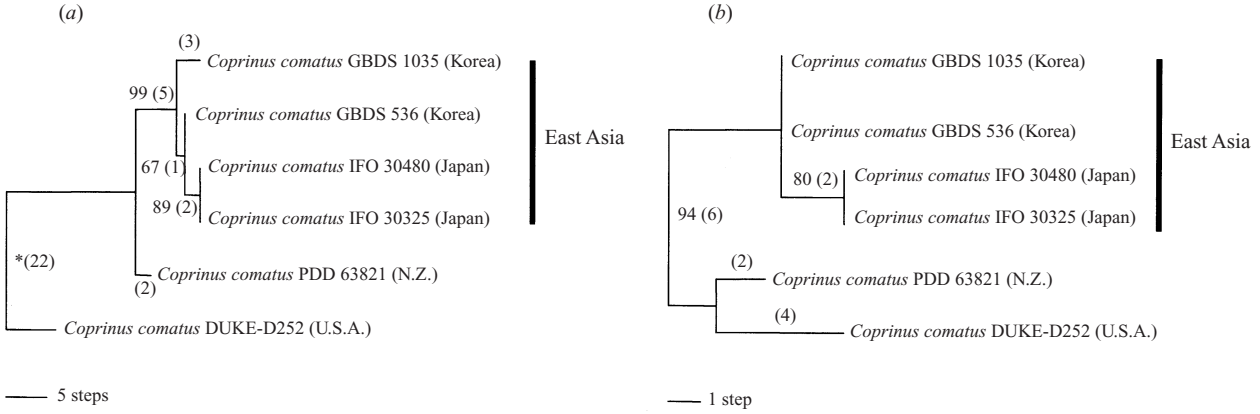

Fig. 1. The most parsimonious trees of *Coprinus comatus* based on ITS sequences. (a) A single most parsimonious ITS1 tree. (b) One of two equally most parsimonious ITS2 trees. Numbers on branches represent the bootstrap value based on 500 replications, and values more than 50% are shown. Numbers within parentheses indicate the length of branches calculated by the character-state optimization of ACCTRAN. The asterisk indicates the branch absent in bootstrap consensus trees.

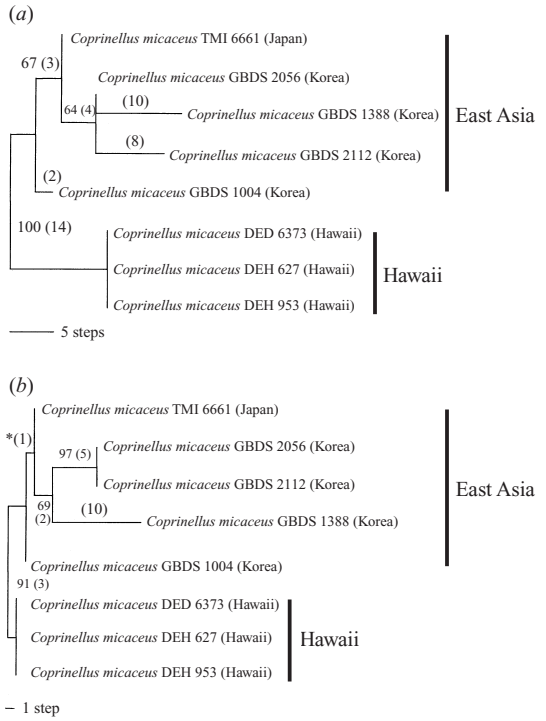

Fig. 3. The most parsimonious trees of *Coprinellus micaceus* based on ITS sequences. (a) One of three equally most parsimonious ITS1 trees. (b) One of two equally most parsimonious ITS2 trees. Other details are same as those of Fig. 1.

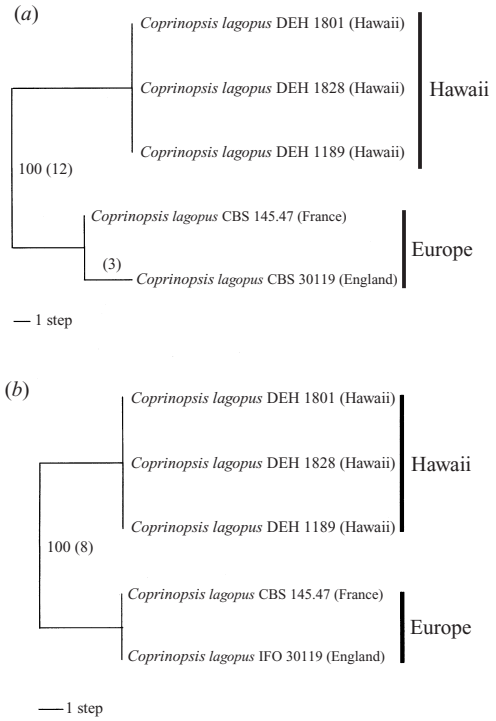

Fig. 4. The most parsimonious trees of *Coprinopsis lagopus* based on ITS sequences. (a) ITS1 tree. (b) ITS2 tree. Exhaustive search generated a single most parsimonious tree in each analysis. Other details are same as those of Fig. 1.

agarose gel stained by EtBr and purified for sequencing process by a PCR purification kit (I. J. BIO). Direct sequencing was done by the thermal cyclic termination method with ^{35}S-labeled dATP (Hillis, Moritz & Mable 1996) and ITS5, ITS2, ITS3 and ITS4 primers (White *et al.* 1990) using the Top^{TM} DNA sequencing kit (Bioneer) according to manufacturer's instructions. Sequencing reactions were run on 6% polyacrylamide gel by electrophoresis, and autoradiographs were read by eye.

Sequence analyses

Nuclear ITS1 and ITS2 sequences were aligned by the multiple alignment program CLUSTAL X (Thompson *et al.* 1997). Delimitations of the 5′-and 3′-ends of ITS1 and ITS2 regions were based on the studies of Hibbett *et al.* (1995, 1998). Lengths of the alignments submitted to phylogenetic analyses are listed in Table 2. Phylogenetic topologies were determined by the parsimony method in PAUP* 4.0b4a (Swofford 1999), with unambiguously aligned single gaps scored as fifth character states. Gaps forming insertions/deletions (indels) of two or more nucleotides (Fig. 5) were not used in the construction of phylogenetic trees. Exhaustive searches were conducted for all data sets. Due to lack of relevant outgroups, trees were rooted using the midpoint method in PAUP*. Each branch length was calculated using the character-state optimization of ACCTRAN. To decide the strength of support for the branches, 500 replications of bootstrap were performed (Felsenstein 1985, Hillis & Bull 1993).

(*a*) *Coprinellus disseminatus* (ITS1)

```
Sequence position          146                         171
                           ↓                           ↓
DED 5969 (Hawaii)    AGGTTTGCGT--------------------------TTCGCGT-G
DEH 1312 (Hawaii)    AGGTTTGCGT--------------------------TTCGCGT-G
DEH 1832 (Hawaii)    AGGTTTGCGT--------------------------TTCGCGT-G
GBDS 2221 (Nepal)    AGGTTTGCGT--------------------------TTCGCGT-G
IFO 7550 (Japan)     AGGTTTGCGT--------------------------T-CAAGT-G
IFO 30972 (Japan)    AGGTTTGCGT--------------------------T-CAAGT-G
IFO 30297 (Japan)    AGGTTTGCGTGTGCGTAGGCTGCGTGTTCGCGCGCGT-TGCGTTG
SFC 950830-1 (Korea) AGGTTTGCGTGTGCGTAGGCTGCGTGTTCGCGCGCGT-TGCGTTG
SFC 990623-5 (Korea) AGGTTTGCGT--------------------------T-TGCGTTG
```

(*b*) *Coprinellus micaceus* (ITS2)

```
Sequence position      118 120
                       ↓ ↓
DED 6373 (Hawaii)    GTTCATTAAACTGAGC
DEH 627 (Hawaii)     GTTCATTAAACTGAGC
DEH 953 (Hawaii)     GTTCATTAAACTGAGC
GBDS 1004 (Korea)    GTTCGT---ACTGAGC
GBDS 1388 (Korea)    GTTCGT---ACTGAGC
GBDS 2056 (Korea)    GTTCGT---ACTGAGC
GBDS 2112 (Korea)    GTTCGT---ACTGAGC
TMI 6661 (Japan)     GTTCGT---ACTGAGC
```

(*c*) *Coprinopsis lagopus* (ITS1)

```
Sequence position      155  160
                       ↓    ↓
CBS 145·47 (France)  CTCG------GGAGG
DEH 1189 (Hawaii)    TCCGAAAGGGGGAGG
DEH 1801 (Hawaii)    TCCGAAAGGGGGAGG
DEH 1828 (Hawaii)    TCCGAAAGGGGGAGG
IFO 30119 (England)  CTTG------GGAGG
```

Fig. 5. Indels of more than two nucleotides in ITS1 and ITS2 data sets. (a) ITS1 region of *Coprinellus disseminatus*. (b) ITS2 region of *Coprinellus micaceus*. (c) ITS1 region of *Coprinopsis lagopus*.

RESULTS AND DISCUSSION

ITS genealogies of four coprinoid species were inferred from ITS1 and ITS2 sequences respectively (Figs 1–4). Parsimony analyses of ITS data sets yielded one to eight most parsimonious trees (MPTs) (Table 2). In cases that several MPTs were generated, the best tree was selected by the method of Kishino-Hasegawa test (Kishino & Hasegawa 1989). Most of eight phylogenies produced from ITS1 and ITS2 sequences of the four species represented intraspecific separation according to their geographic origins (Figs 1–4). All data sets showed very high CI, RI and RC indices (Table 2), indicating that there are few homoplasies in those data (Forey *et al*. 1992).

Coprinus comatus

Fig. 1 presented a well-resolved grouping of four east Asian strains, compared to those from New Zealand and the USA. The east Asian clade was supported by strong bootstrap values of 99% in ITS1 and 94% in ITS2 phylogenies. There is one discrepant point between the ITS1 and ITS2 trees. In ITS1 phylogeny (Fig. 1A), PDD 63821 from New Zealand clustered with the east Asian clade, while it grouped with DUKE-D252 from the USA in ITS2 phylogeny (Fig. 1B). In the bootstrap analysis of ITS1 data, the grouping of PDD 63821 with the East Asian clade was not supported. On the other hand, the cluster of PDD 63821 and DUKE-D252 was strongly supported by a bootstrap value of 94% in ITS2 data. Moreover, the distribution of segregating sites, which might be signature sequences for each subgroup (Carbone, Anderson & Kohn 1999), in both ITS1 and ITS2 regions showed that PDD 63821 might have a closer relationship with DUKE-D252 (Table 3).

In the study of *Pleurotopsis longinqua* (Hughes, Toyohara & Petersen 1998) based on ITS sequences, only a few base pair differences were present among strains from southeastern Australia, Chile and the USA, which then revealed close phylogenetic relationships between those from Australia and North America. The result of Hughes *et al*. (1998) suggested a possibility that fungi from New Zealand and North America could have exchanged their genes reciprocally. However, because just only one material was included per local origin in this study, it is too early to judge that *C. comatus* strains from New Zealand and USA would form a single phylogeographical group.

Coprinellus disseminatus

The sampling for *Coprinellus disseminatus* includes collections from Hawaii, east Asia and Nepal. The phylogeography of *C. disseminatus* developed a somewhat complicated pattern. The Hawaiian strains clustered into the same group with that from Nepal in both ITS1 and ITS2 phylogenies (Fig. 2), which was supported by bootstrap values of 73 and 100% respectively. As Hawaii and Nepal are far apart geographically, it is not easy to explain the present result. The lengths of the terminal branches to three Hawaiian strains were short, especially in the ITS2 gene tree (Fig. 2B). The Nepalese strain differs from the Hawaiian collections by several nucleotide substitutions. When more strains from diverse localities are added to this study, such a relationship is likely to be evaluated again.

Interestingly, the five strains identified as *C. disseminatus* from east Asia did not cluster into a single clade in both ITS1 and ITS2 phylogenies (Fig. 2). Two strains from Japan (IFO 7550 and IFO 30972) formed a distinct clade (East Asia I), while two Korean and one Japanese strains (SFC 990623-5, SFC 950830-1 and IFO 30297) clustered into another group (East Asia II) (Fig. 2). Their separation was robust, judging from the result of bootstrap analyses. Also, the distribution pattern of the segregating sites also supported two separate groups (Table 4), showing a reduced gene flow between the two groups. This phenomenon suggests that at least two genetically isolated *C. disseminatus* groups exist in east Asia.

Moreover, a long insertion of 26 nucleotides was detected in the ITS1 region from two strains (IFO 30297 and SFC 950830-1) of the three in the East Asia II clade (Figs 2A and 5A). Those two strains clustered into a single clade in the ITS1 tree, which was strongly supported by bootstrap value of 96% (Fig. 2A), while they did not form a distinct group in ITS2 phylogeny (Fig. 2B). Also, based on the distribution of segregating sites of ITS2 region (Table 4), IFO 30297 and SFC 950830-1 were separated from SFC 990623-5 at nucleotide position 72.

Coprinellus micaceus

Samples of *Coprinellus micaceus* from east Asia and Hawaii were separated into two independent groups both in ITS1 and ITS2 trees (Fig. 3). Their division was fully supported by bootstrap analyses (100% in ITS1 and 91% in ITS2). Indels of three nucleotides in the ITS2 region (Fig. 5B) and the distribution pattern of segregating sites in ITS1 and ITS2

Table 3. Distribution of segregating sites in ITS1 and ITS2 regions of *Coprinus comatus*.

	ITS1							ITS2					
	5[a]	16	126	138	150	152	207	59	76	196	198	209	225
DUKE-D252 (USA)	N[b]	A	T	C	T	G	C	–	C	A	T	A	A
GBDS 536 (Korea)	A	A	A	A	C	A	T	G	T	G	A	G	–
GBDS 1035 (Korea)	A	A	A	A	C	A	T	G	T	G	A	G	–
IFO 30325 (Japan)	C	C	A	A	C	A	T	–	T	G	A	G	–
IFO 32480 (Japan)	C	C	A	A	C	A	T	–	T	G	A	G	–
PDD 63821 (New Zealand)	A	A	T	C	T	G	C	–	C	A	T	A	A

[a] Sequence position number.
[b] Undetermined site.

Table 4. Distribution of segregating sites in ITS1 and ITS2 regions of *Coprinellus disseminatus*.

	ITS1																		ITS2										
	34[a]	102	122	123	124	129	173	174	175	176	179	181	187	192	207	218	267	311	24	36	42	63	72	73	82	104	120	121	126
DED 5969 (Hawaii)	T	C	T	C	C	A	T	C	G	C	–	–	C	T	G	C	C	C	G	C	C	C	T	C	C	C	–	–	G
DEH 1312 (Hawaii)	T	C	T	A	T	A	T	C	G	C	–	–	C	T	G	C	C	C	G	C	C	C	T	C	C	C	–	–	G
DEH 1832 (Hawaii)	T	C	T	A	T	A	T	C	G	C	–	–	C	T	G	C	C	C	G	C	C	C	T	C	C	C	–	–	G
GBDS 2221 (Nepal)	T	C	T	T	C	–	T	C	G	C	–	–	C	T	G	C	C	C	G	C	C	C	T	C	C	C	–	–	G
IFO 7550 (Japan)	T	C	C	T	C	G	–	C	A	A	–	–	C	T	G	A	C	G	G	C	T	T	T	C	C	C	–	–	G
IFO 30972 (Japan)	T	C	C	T	C	G	–	C	A	A	–	–	C	T	G	A	C	G	G	C	T	T	T	C	C	C	–	–	G
IFO 30297 (Japan)	C	T	T	C	C	G	–	T	G	C	T	C	G	A	A	T	A	G	C	G	C	T	C	T	T	T	A	A	A
SFC 950830-1 (Korea)	C	T	T	C	C	G	–	T	G	C	T	C	G	A	A	T	A	G	C	G	C	T	C	T	C	T	A	A	A
SFC 990623-5 (Korea)	T	T	T	C	C	G	–	T	G	C	T	C	G	A	A	T	A	N	C	G	C	T	T	T	T	T	A	A	A

[a] Sequence position number.

Table 5. Distribution of segregating sites in ITS1 and ITS2 regions of *Coprinellus micaceus*.

	ITS1														ITS2			
	52[a]	62	122	126	127	128	142	153	154	155	158	193	214	216	42	88	116	165
DED 6373 (Hawaii)	G	–	A	C	T	C	G	C	T	T	A	G	C	T	C	T	A	G
DEH 627 (Hawaii)	G	–	A	C	T	C	G	C	T	T	A	G	C	T	C	T	A	G
DEH 953 (Hawaii)	G	–	A	C	T	C	G	C	T	T	A	G	C	T	C	T	A	G
GBDS 1004 (Korea)	G	C	G	T	C	T	A	T	C	A	T	G	A	C	T	T	G	A
GBDS 1388 (Korea)	C	C	G	T	C	T	A	T	C	A	T	T	A	C	C	C	G	A
GBDS 2056 (Korea)	C	C	G	T	C	T	A	T	C	A	T	T	A	C	C	C	G	A
GBDS 2112 (Korea)	C	C	G	T	C	C	A	T	C	A	T	T	A	G	C	C	G	A
TMI 6661 (Japan)	G	C	G	T	C	T	A	T	C	A	T	G	A	C	T	T	G	A

[a] Sequence position number.

Table 6. Distribution of segregating sites in ITS1 and ITS2 regions of *Coprinopsis lagopus*.

	ITS1											ITS2							
	51[a]	142	147	148	151	152	172	193	200	221	223	9	25	30	70	72	73	156	163
DEH 1189 (Hawaii)	G	T	A	G	T	C	A	C	C	A	G	A	A	–	C	C	G	A	C
DEH 1801 (Hawaii)	G	T	A	G	T	C	A	C	C	A	G	A	A	–	C	C	G	A	C
DEH 1828 (Hawaii)	G	T	A	G	T	C	A	C	C	A	G	A	A	–	C	C	G	A	C
CBS 145.47 (France)	A	C	C	A	C	T	T	T	T	T	A	T	G	T	T	T	A	T	T
IFO 30119 (England)	A	C	C	A	C	T	T	T	T	T	A	T	G	T	T	T	A	T	T

[a] Sequence position number.

regions (Table 5) again supported their segregation. The three Hawaiian strains showed no nucleotide variation in both ITS1 and ITS2 sequences, like in *Coprinopsis lagopus*.

On the other hand, the five *C. micaceus* strains from east Asia showed a remarkable variation. Relationships within the east Asian clade were congruent in both trees from ITS1 and ITS2 sequences (Fig. 3). Three Korean strains, GBDS 2056, GBDS 1388 and GBDS 2112, clustered into a single clade in both trees (bootstrap values of 64 and 69% respectively). The other Korean strain, GBDS 1004, was placed at a basal position of the east Asian group. Lengths of terminal branches to the east Asian strains were long, which indicated long divergence time.

Coprinopsis lagopus

ITS1 and ITS2 gene trees (Fig. 4) revealed a clear separation between Hawaiian and European clades. Especially, both ITS1 and ITS2 regions of three Hawaiian samples showed completely identical sequences (Table 6). And European strains from France and England also had identical ITS2 sequences (branch length = 0 in Fig. 4B). CI, RI and RC values of ITS1 and ITS2 data sets were all 1.00 (Table 2), which suggests that there are no homoplasy (Forey *et al*. 1992) and no gene flow between the two geographic groups. Six inserted sequences (155 to 160 of sequence position numbers) in the ITS1 region were restricted to the three Hawaiian strains (Fig. 5C). This complete segregation between two groups, potentially belonging to the same geographically isolated biological species, and little variation within groups indicate a great possibility that an intermediate group may exist. Unfortunately, however, *C. lagopus* samples from other regions were unavailable for the present study.

CONCLUSIONS

The four coprinoid species included in this study showed clear phylogeographic disjunctions based on molecular evidence. Besides the examined species, it is known that *Coprinopsis phlyctidospora* (syn. *Coprinus phlyctidosporus*) also shows genetic

divergence according to its geographic localities (A. Suzuki, Chiba University, pers. comm.). For *C. disseminatus* and *C. micaceus* examined in this study, the great diversification of the ITS regions and genetic variability in east Asian samples suggest that these fungi might have diverged and evolved originally from the east Asian region. Moreover, two distinct groups were present in *C. disseminatus* from east Asia and a long insertion (26 nucleotides) in the ITS1 region existed only in one group. These findings suggest that a genetic barrier could exist amongst east Asian *C. disseminatus* strains. This study indicates that more than one biological species may be hidden under this single species name. The morphological species concept was also applied to define the species, but didn't permit the identification of the biological (interbreeding) species from one another. Correlation between compatibility and genetic divergence has been reported (Isikhuemhen *et al*. 2000, Vilgalys & Sun 1994), and a mating compatibility test of collections could be helpful in drawing a solid conclusion.

It is notable that there was little ITS sequence variation in Hawaiian samples of *C. disseminatus*, *C. micaceus*, and *C. lagopus*. Coprinoid fungi have been known to be alien to Hawaii and introduced with foreign plants and their accompanying soil (D. Hemmes, pers. comm.). In the Hawaiian Islands, coprinoid species apparently have not had enough time to diverge genetically and the little base difference of the ITS regions in Hawaiian material indicates their recent arrival in such remotely isolated islands.

It has been accepted that the lack of gene flow among populations from different regions leads to speciation (Brooks & McLennan 1991). In *C. micaceus* and *C. lagopus*, there were definite segregations of ITS1 and ITS2 sequences depending on geographic populations (Tables 5–6). These patterns of genetic divergence in *C. micaceus* and *C. lagopus* might suggest that cryptic species exist with indistinguishable morphologies (Roy *et al*. 1998). Geographical separation could provide an opportunity for new species to evolve in the Hawaiian Islands. Lack of morphological change could be due to insufficient time to build up their own morphologies since the introduction into such isolated islands. However, it is too early to conclude that these two species are in the process of allopatric speciation because only one DNA region was examined for this study, and mating studies to verify species delimitation are still wanting. Inclusion of other gene-encoding regions could also reveal whether or not genetic isolation has occurred (Geiser, Pitt & Taylor 1998, Koufopanou, Burt & Taylor 1997, Taylor *et al*. 1999). And additional materials from diverse localities will also provide a better understanding of genetic variation and speciation between geographic populations in coprinoid species.

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REFERENCES

Brooks, D. R. & McLennan, D. A. (1991) *Phylogeny, Ecology, and Behavior: A Research Program in Comparative Biology*. University of Chicago Press, Chicago.

Carbone, I., Anderson, J. B. & Kohn, L. M. (1999) Patterns of descent in clonal lineages and their multilocus fingerprints are resolved with combined gene genealogies. *Evolution* **53**: 11–21.

Felsenstein, J. (1985) Confidence limits in phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Forey, P. L., Jumphries, C. J., Kitching, I. J., Scotland, R. W., Seibert, D. J. & Williams, D. M. (1992) *Cladistics: a practical course in systematics*. Clarendon Press, Oxford.

Geiser, D. M., Pitt, J. I. & Taylor, J. W. (1998) Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. *Proceedings of the National Academy of Sciences, USA* **95**: 388–393.

Hibbett, D. S., Fukumasa-Nakai, Y., Tsuneda, A. & Donoghue, M. J. (1995) Phylogenetic diversity in shiitake inferred from nuclear ribosomal DNA sequences. *Mycologia* **87**: 618–638.

Hibbett, D. S., Hansen, K. & Donoghue, M. J. (1998) Phylogeny and biogeography of *Lentinula* inferred from an expanded rDNA dataset. *Mycological Research* **102**: 1041–1049.

Hillis, D. M. & Bull, J. J. (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42**: 182–192.

Hillis, D. M., Moritz, C. & Mable, B. K. (1996) *Molecular Systematics*. 2nd edn. Sinauer Associates, Sunderland, MA.

Hopple, J. S. jr & Vilgalys, R. (1994) Phylogenetic relationships among coprinoid taxa and allies based on data from restriction site mapping of nuclear rDNA. *Mycologia* **86**: 96–107.

Hopple, jr, J. S. & Vilgalys, R. (1999) Phylogenetic relationships in the mushroom genus *Coprinus* and dark-spored allies based on sequence data from nuclear gene coding for the large ribosomal subunit RNA: divergent domains, outgroups, and monophyly. *Molecular Phylogenetics and Evolution* **13**: 1–19.

Hughes, K. W., Toyohara, T. L. & Petersen, R. H. (1998) DNA sequence and RFLP analysis of *Pleurotopsis longinqua* from three disjunct populations. *Mycologia* **90**: 595–600.

Isikhuemhen, O. S., Moncalvo, J.-M., Nerud, F. & Vilgalys, R. (2000) Mating compatibility and phylogeography in *Pleurotus tuberregium*. *Mycological Research* **104**: 732–737.

Kishino, H. & Hasegawa, M. (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *Journal of Molecular Evolution* **29**: 170–179.

Ko, K. S. & Jung, H. S. (1999) Molecular phylogeny of *Trametes* and related genera. *Antonie van Leeuwenhoek* **75**: 191–199.

Koufopanou, V., Burt, A. & Taylor, J. W. (1997) Concordance of gene genealogies reveals reproductive isolation in the pathogenic fungus *Coccidioides immitis*. *Proceedings of the National Academy of Sciences, USA* **94**: 5478–5482.

Lecellier, G. & Silar, P. (1994) Rapid methods for nucleic acids extraction from petri dish-grown mycelia. *Current Genetics* **25**: 122–123.

Lee, J. S., Ko, K. S. & Jung, H. S. (2000) Phylogenetic analysis of *Xylaria* based on nuclear ribosomal ITS1-5.8S-ITS2 sequences. *FEMS Microbiology Letters* **187**: 89–93.

Moncalvo, J.-M., Lutzoni, F. M., Rehner, S. A. & Vilgalys, R. (2000) Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Systematic Biology* **49**: 278–305.

O'Donnell, K., Cigelnik, E. & Nirenberg, H. I. (1998) Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* **90**: 465–493.

O'Donnell, K., Kistler, H. C., Tacke, B. K. & Casper, H. H. (2000) Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. *Proceedings of the National Academy of Sciences, USA* **97**: 7905–7910.

Park, D. S., Go, S. J., Kim, Y. S., Seok, S. J., Ryu, J. C. & Sung, J. M. (1999) Phylogenetic relationships of genera *Coprinus* and *Psathyrella* on the basis of ITS region sequences. *The Korean Journal of Mycology* **27**: 274–279.

Pukkila, P. J. & Casselton, L. A. (1991) Molecular genetics of the agaric *Coprinus cinereus*. In *More Gene Manipulations in Fungi* (J. W. Bennett & L. A. Lasure, eds): 126–150. Academic Press, San Diego.

Redhead, S. A., Vilgalys, R., Moncalvo, J.-M., Johnson, J. & Hopple, J. S. jr (2001) *Coprinus* Pers. and the disposition of *Coprinus* species *sensu lato*. *Taxon* **50**: 203–241.

Roy, B. A., Vogler, D. R., Bruns, T. D. & Szaro, T. M. (1998) Cryptic species in the *Puccinia monoica* complex. *Mycologia* **90**: 846–853.

Singer, R. (1986) *The Agaricales in Moderan Taxonomy*. 4th edn. Koeltz Scientific Books, Koenigstein.

Smith, A. H. (1971) The origin and evolution of the *Agaricales*. In *Evolution in the Higher Basidiomycetes* (R. H. Petersen, ed): 481–504. The University of Tennessee Press, Knoxville.

Swofford, D. L. (1999) *PAUP* : phylogenetic analysis using parsimony (* and other methods)* Version 4.0b4a. Sinauer Associates, Sunderland, MA.

Taylor, J. W., Geiser, D. M., Burt, A. & Koufopanou, V. (1999) The evolutionary biology and population genetics underlying fungal strain typing. *Clinical Microbiology Review* **12**: 126–146.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876–4882.

Vilgalys, R., Moncalvo, J.-M., Liou, S.-R., & Volvovsek, M. (1996) Recent advances in molecular systematics of the genus *Pleurotus*. In *Mushroom Biology and Mushroom Products* (D. J. Royse, ed): 91–101. Pennsylvania State University, University Park.

Vilgalys, R. & Sun, B. L. (1994) Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of ribosomal DNA sequences. *Proceedings of the National Academy of Sciences, USA* **91**: 4599–4063.

White, T. J., Bruns, T., Lee, S. & Taylor, J. W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenies. In *PCR Protocols: A Guide to Methods and Applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315–322. Academic Press, New York.

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