Divergence, dispersal and recombination in *Lepiota cristata* from China

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The basidiomycete *Lepiota cristata* (Agaricales) is among the most widely distributed saprophytic mushrooms in the Northern Hemisphere. It's commonly found on humus-rich soil in a variety of terrestrial habitats, from roadsides to forest floors. In this study, we analyzed 47 isolates of the *L. cristata* species complex from China using DNA sequences from three loci (the internal transcribed spacer region or ITS, the inter-genic spacer or IGS, and the mitochondrial small ribosomal subunit RNA gene or mtSSU rRNA). Our analyses identified abundant sequence variation and potentially divergent lineages within *L. cristata* for each of the three loci. Interestingly, there were significant differences among the three gene genealogies and our linkage disequilibrium analyses identified unambiguous evidence of recombination, hybridization and/or incomplete lineage sorting. Furthermore, several sequence types were shared among isolates from diverse geographic locations for each of the three analyzed loci. Our population genetic analyses indicated limited evidence for genetic differentiation among geographic populations, suggesting that long-distance gene flow might be common among geographic populations of this species in China. We discuss the implications of our results with regard to the population biology and taxonomy of this widely distributed but under-studied species.

Key words: Basidiomycete, genetic diversity, gene flow, recombination, cryptic species

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Introduction

Basidiomycetous fungi are important constituents of almost all terrestrial ecosystems. They play important roles in forestry, agriculture, animal and human health, and the global nutrient cycling. Despite their ecological and economical importance, aside from a few representative pathogens or edible mushrooms, relatively little is known about the ecology, evolution and population biology of most basidiomycete species, including those in *Lepiota* (Pers.: Fr.) Gray (Agaricales), a genus containing over 400 species (Kirk *et al*., 2001).

Among the species in *Lepiota*, *L. cristata* (Bolton: Fr.) P. Kumm. is one of the most widely distributed. It can be found in most regions in the Northern Hemisphere, and can be distinguished from other species by its small caps (1-5 cm in diameter) covered with concentrically arranged pinkish to reddish brown or brownish scales made up of tightly packed clavate, narrowly clavate to cylindrical terminal elements of different lengths arranged in a hymeniform structure, by its wedge or triangular-shaped basidiospores, and by its unpleasant pungent smell (Vellinga, 2001a,b, 2004a). In nature, the fruiting bodies of *L. cristata* are found growing either scattered or in clusters and they typically appear in late summer or early fall. Unlike fungal pathogens of plants and animals, *L. cristata* is a saprophyte commonly found in disturbed areas like footpaths, ditches, lawns, trails, gardens, and roadsides (Vellinga, 2004a). Their natural distribution pattern suggests their potential roles as significant decomposers of lignin and cellulose materials and as indicators in environmental disturbances.

Despite their wide distribution and potentially significant ecological roles, relatively little is known about the populations of *L. cristata*. A previous study using sequences from the internal transcribed spacers 1 and 2 (ITS1 and ITS2) and the inter-genic spacer (IGS) regions identified that isolates of *L. cristata* from North America and Europe contained significant genetic variation (Vellinga, 2001b). Among the 17 morphologically indistinguishable mushroom isolates from five countries in the Northern Hemisphere, 15 distinct ITS sequences were found. Two pairs of isolates were found to share ITS sequences, with one pair from California and the second pair from Michigan. Both pairs of isolates from Michigan and California had different IGS sequences. Among the 15 ITS types, two were suggested as belonging to a separate cryptic species based on their high levels of sequence divergence from the other isolates and both isolates were collected from California (Vellinga, 2001b). Because only a few isolates from regions outside of the USA were analyzed, the extent of genetic variation from other regions remained largely unknown.

The objective of this study is to analyze isolates of *L. cristata* from various areas in China and compare the observed variation with those published from the US and European populations. Specifically, we were interested in the following questions. First, how variable is the Chinese population of *L. cristata* at the DNA sequence level? Is the level of variation among Chinese samples similar to those found outside of China? Second, are isolates from the same geographic region more similar to each other than to isolates from different regions? On the one hand, the white basidiospores in this species might render them highly susceptible to environmental stresses such as desiccation, extreme temperatures and UV radiation and therefore they might not be suitable for long distance dispersal. If this hypothesis is supported, we should observe significant population structure based on geographic distances. On the other hand, *L. cristata* is often found in disturbed areas, habitats conducive for dispersals by humans and their associated anthropogenic activities. Whether one or both processes operate in natural populations of this species are unknown at present. Third, how similar are genealogies from different genes? Congruent gene genealogies suggest possible on-going divergence and speciation. In contrast, incongruent gene genealogies are indicative of recombination, incomplete lineage sorting and/or hybridization in natural populations. To address these questions, we analyzed 47 isolates from various geographic areas in China for three loci. Our results suggest a high level genetic variation within the Chinese population of *L. cristata*, including evidence for divergence, gene flow and recombination.

Materials and methods

Isolates

The isolates analyzed here were from six provinces/administrative regions in China, three provinces/regions in northeastern China and three in southwestern China. The majority of the isolates (39/47) were from southwestern China (Fig. 1). The three sampled regions in northeastern China were: Jilin Province (5 isolates), Hebei Province (2 isolates), and a suburb of Beijing Municipality (1 isolate). The three regions in southwestern China were: Yunnan Province (23 isolates), Sichuan Province (10 isolates) and Tibet Autonomous Region (6 isolates). Based on the characteristics of basidiospores, five of the specimens were previously described as belonging to a variety of a closely related species *L. fraterna* var. *macrospora* Zhu L. Yang. These five specimens were included for comparisons.

A total of 47 isolates were collected from a variety of habitats between 2000 and 2007 and at different altitudes (between 220 m to 4800 m above sea level but mostly from regions over 1800 m above sea level) (Table 1). These isolates were identified as belonging to *L. cristata* based on macro- and microscopic features of mushroom fruiting bodies characteristic of this species. In addition, we included sequences from isolates of *L. cristata* or closely related species obtained in previous studies (Vellinga and Huijser, 1998; Johnson, 1999; Krueger *et al*., 2001; Vellinga, 2001b, 2003, 2004b). Each isolate had a unique herbarium code and the abbreviated codes followed those of Holmgren *et al*. (1990) with two exceptions that were not listed in the

Table 1. Isolates of *Lepiota* used in this study and their sequence accession numbers in the GenBank.

Name	Voucher	Location	Alt.	GenBank accession No.		
				ITS	IGS	mtSSU
L. castaneidisca	UC-ecv 2516	Mendocino Co., CA-USA[1]		AF391065**	AF391081**	
	UC-ecv 2410	Skyline Blvd, San Mateo Co., CA-USA		AF391064**	AF391082**	
	UC-ecv 2411	Skyline Blvd, San Mateo Co., CA-USA		AF391063**	AF391078**	
	UC-ecv 2311	Wunderlich County Park, San Mateo Co., CA-USA		AF391060**	AF391079**	
	UC-ecv 2395	San Francisco Watershed, San Mateo Co., CA-USA		AF391062**		
	UC-ecv 2312	Wunderlich County Park, San Mateo Co., CA-USA		AF391061**		
	UC-ecv 2591	San Francisco Watershed, San Mateo Co., CA-USA		AF391054**	AF391075**	
	UC-ecv 2308	Wunderlich County Park, San Mateo Co., CA-USA		AF391058**		
	UC-ecv 2300	San Mateo County Memorial Park, San Mateo Co., CA-USA		AF391057**	AF391074**	
	UC-ecv 2599	San Francisco Watershed, San Mateo Co., CA-USA		AF391056**	AF391077**	
	UC-ecv 2594	San Francisco Watershed, San Mateo Co., CA-USA		AF391055**	AF391076**	
	UC-ecv 2309	Wunderlich County Park, San Mateo Co., CA-USA		AF391059**	AF391080**	
L. cristata	HKAS 48786	Laojun Mountain, Jianchuan Co., YN-CHN[2]	2700 m	EU081973*		EU082000*
	HKAS 46370	Mt. Xiaoxue, Shangri-La Co., YN-CHN	3600 m	EU081939*		EU081984*
	HKAS 46333	Geza Village, Shangri-La Co., YN-CHN	3800 m	EU081944*	EU081915*	EU081993*
	HKAS 46344	Mt. Sanjiemei, Shangri-La Co., YN-CHN	3100 m	EU081947*	EU081929*	EU081978*
	HKAS 46345	Mt. Sanjiemei, Shangri-La Co., YN-CHN	3100 m	EU081943*	EU081917*	EU081994*
	HKAS 46348	Mt. Sanjiemei, Shangri-La Co., YN-CHN	3100 m	EU081957*	EU081922*	EU081996*
	HKAS 46349	Mt. Sanjiemei, Shangri-La Co., YN-CHN	3100 m	EU081971*		
	HKAS 11366	Mt. Sanjiemei, Shangri-La Co., YN-CHN	3100 m	EU081961*		
	HKAS 46360	Mt. Birong , Shangri-La Co., YN-CHN	3100 m	EU081967*		
	HKAS 45928	Mt. Xiagejin , Derong Co., SC-CHN[3]	3700 m	EU081950*	EU081934*	EU081979*
	HKAS 49258	Shadui Town, Xinlon Co., SC-CHN	3400 m	EU081937*	EU098285*	EU081992*
	HKAS 49286	Near Wenda, Seda Co., SC-CHN	3775 m	EU081955*	EU081926*	EU081980*
	HKAS 49160	Near Luoxu, Shiqu Co., SC-CHN	4800 m	EU081938*	EU081921*	EU082001*
	HKAS 49292	Near Wenda, Seda Co., SC-CHN	3090 m	EU081942*	EU081914*	EU081977*
	HKAS 49367	Huoxi, Seda Co., SC-CHN	3600 m	EU081960*	EU098286*	EU081988*
	HKAS 45830	Xiangcheng Co., SC-CHN	3200 m	EU081946*	EU081920*	EU081991*
	HKAS 45492	Xiangcheng Co., SC-CHN	3000 m	EU081935*	EU081925*	EU081987*
	HKAS 45662	Qingnidong, Jiangda Co., Tibet-CHN[4]	3800 m	EU081969*		
	HKAS 46103	Leiwuqi Co., Tibet-CHN	3900 m	EU081965*		
	HKAS 45651	Tongpu Town, Jiangda Co., Tibet-CHN	3300 m	EU081941*	EU081931*	EU081989*
	HKAS 45975	Near Qiuka Bridge, Changdu Co., Tibet-CHN	3350 m	EU081956*	EU081928*	EU081995*
	HKAS 45791	Yangda, Changdu Co., Tibet-CHN	4300 m	EU081966*		
	HKAS 46153	Yangda, Changdu Co., Tibet-CHN	4300 m	EU081940*	EU081932*	EU081986*

Table 1 (continued). Isolates of *Lepiota* used in this study and their sequence accession numbers in the GenBank.

Name	Voucher	Location	Alt.	GenBank accession No.		
				ITS	IGS	mtSSU
L. cristata	HKAS 5800	Lushui River, Fusong Co., JL-CHN[5]	625 m	EU081936*	EU081913*	EU081976*
	HKAS 7547	White River, Antu Co., JL-CHN	740 m	EU081958*	EU081933*	EU081983*
	HKAS 50915	Erdaobai River, Antu Co., JL-CHN		EU081953*	EU081923*	EU081997*
	HKAS 11210	Jinyuetan Park, Changchun City, JL-CHN	220 m	EU081974*		
	HMJAU 3430	Jinyuetan Park, Changchun City, JL-CHN	220 m	EU081972*		EU081999*
	HMJAU 0822	Bashang Grassland, Fengning Co., HB-CHN[6]		EU081963*		
	HMJAU 1029	Bashang Grassland, Fengning Co., HB-CHN		EU081962*		
	HMJAU 1135	Yunmenshan Park, Huairou Co., BJ-CHN[7]		EU081968*		
	RITF 301	Dali City, YN-CHN	2700 m	EU826489*	EU826495*	EU826501*
	RITF 308	Feilai Tample, Deqing Co., YN-CHN	3444 m	EU826490*	EU826496*	EU826505*
	RITF 311	Deqing Co., YN-CHN	4500 m	EU826491*	EU826498*	EU826502*
	RITF 312	Yulong Co., Lijiang City, YN-CHN	3287 m	EU826492*	EU826497*	EU826503*
	RITF 314	Yanyuan Co., SC-CHN	3234 m	EU826493*	EU826494*	EU826504*
	UC-ecv 2460	Ann Arbor, Washtenaw Co., MI-USA[10]		AF391047**	AF391067**	
	SFSU-ded 5658	Figuarora Campground, Santa Barbara Co., CA-USA		AF391050**		
	WTU-pbm 1958	Priest Lake, Bonner Co., ID-USA[8]		AF391051**		
	UC-ecv 2384	Berkeley, Alameda Co., CA-USA		AF391049**	AF391069**	
	L-ecv 2285	Leiden, Zuid-Holland Prov., NL[9]		AF391048**		
	UC-ecv 2401	San Francisco Watershed, San Mateo Co., CA-USA		AF391046**		
	UC-ecv 2611	San Francisco Watershed, San Mateo Co., CA-USA		AF391045**	AF391068**	
	HKAS 32250	Maxingou near Riying, Xiangcheng Co., SC-CHN	3600 m	AF391044**		
	L-hah 20IX1989	Philips de Jong park, Eindhoven, Noord-Brabant Prov., NL		AF391043**		
	L-hah 22IX1993	Stokhem, Wijlre, Limburg Prov., NL		AF391042**		
	UC-ecv 2449	Ann Arbor, Washtenaw Co., MI-USA		AF391041**	AF391070**	
	UC-ecv 2452	Ann Arbor, Washtenaw Co., MI-USA		AF391040**	AF391071**	
	DUKE 1582	MT-USA[11]		U85327****		
	L-ecv 1445	Hollenfels, L[12]		AF391027**		
	TENN 58042	Bavaria, Germany		AJ237628***		
L. fraterna var. *macrospora*	HKAS 45049	KBG[13], YN-CHN	1980 m	EU081954*	EU081927*	EU081981*
	HKAS 45053	KBG, YN-CHN	1980 m	EU081948*	EU081930*	EU081990*
	HKAS 48457	KBG, YN-CHN	1980 m	EU081949*	EU081916*	EU081982*
	HKAS 49449	KBG, YN-CHN	1980 m	EU081945*	EU081918*	EU081985*
	HKAS 51356	KBG, YN-CHN	1980 m	EU081959*	EU081924*	EU081998*
L. sp. 1	UC-ecv 2542	Mendocino Co., CA-USA		AF391053**	AF391073**	
	UC-ecv 2515	Hendy Woods State Park, Mendocino Co., CA-USA		AF391052**	AF391072**	
L. sp. 2	HKAS 50021	KBG, YN-CHN	1980 m	EU081952*		EU082003*

Table 1 (continued). Isolates of *Lepiota* used in this study and their sequence accession numbers in the GenBank.

Name	Voucher	Location	Alt.	GenBank accession No.		
				ITS	IGS	mtSSU
L. sp. 2	HKAS 50183	Qiongzhu Temple, Kunming City, YN-CHN	2100 m	EU081951*		EU082002*
	HKAS 43063	Shibao Mountain, Jianchuan Co., YN-CHN	2400 m	EU081970*		
	RITF 300	KBG, YN-CHN	1980 m	EU826488*	EU826499*	EU826500*

[1], CA-USA, California, USA; [2], YN-CHN, Yunnan Prov., China; [3], SC-CHN, Sichuan Prov., China; [4], Tibet-CHN, Tibet Autonomous Region, China; [5], JL-CHN, Jilin Prov., China; [6], HB-CHN, Hebei Prov., China; [7], BJ-CHN, Beijing City, China; [8], ID-USA, Idaho, USA; [9], NL, The Netherlands; [10], MI-USA, Michigan, USA; [11], MT-USA, Montana, USA; [12], L, Luxembourg; [13], KBG, Kunming Botanical Garden.

*, this study; **, Vellinga, 2001b, 2003, 2004b, and Vellinga and Huijser, 1998; ***, Krueger *et al*., 2001; ****, Johnson, 1999.

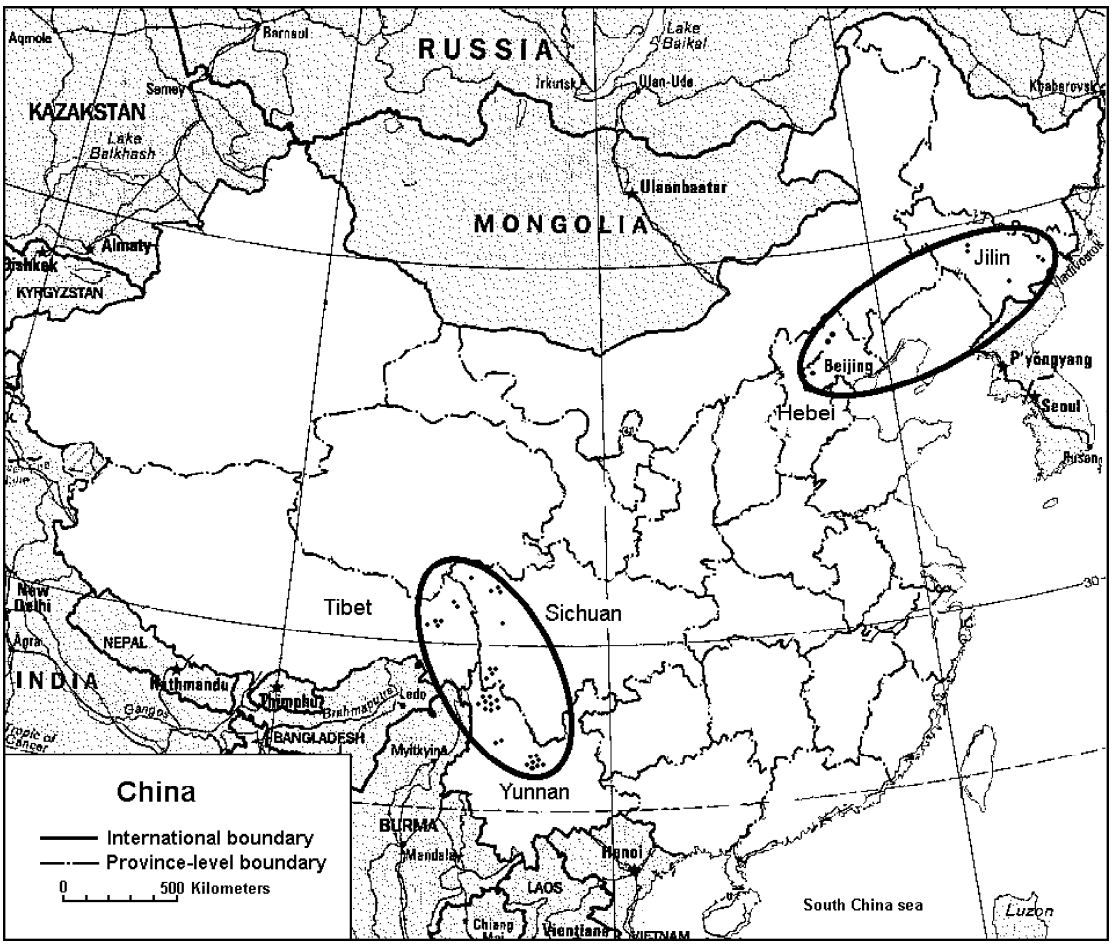

Fig. 1. Sampling locations of isolates of *L. cristata* analyzed in this study (black dots in circles).

Holmgren index. The first is HKAS that refers to the Herbarium of Cryptogams, Kunming Institute of Botany, the Chinese Academy of Sciences in Kunming, Yunnan. The second is RITF that refers to the Research Institute of Tropical Forestry, Chinese Academy of Forestry Sciences in Guangzhou, China.

DNA isolation, PCR amplification and DNA sequencing

The genomic DNA from each isolate was isolated from herbarium samples following the CTAB protocol described in Xu *et al.* (1994). For each sample, we analyzed three loci, two loci were located in the nuclear ribosomal RNA gene cluster [the internal transcribed spacer regions (ITS) and the inter-genic spacer (IGS) region] and the third locus was from the mitochondrial genome [part of the small ribosomal RNA subunit (mtSSU)]. The primer sequences used for amplifying the entire ITS regions were: ITS1F (5’ CTTGGTCATTTAG AGGAAGTAA 3’) and ITS4 (5’ TCCTCCGC TTATTGATATGC 3’) (White *et al*., 1990; Gardes and Bruns, 1993). For isolates that we failed to amplify the entire ITS regions using the above two primers, two primer pairs ITS1 (5’ TCCGTAGGTGAACCTGCGG 3’) (White *et al*., 1990) and ITS1-R: (5’ GAGAGCCAAG AGATCCGTTGC 3’), and ITS2-F (5’ GGTA TTCCGAGGAGCATGCCT 3’) and ITS4 were used to amplify the ITS1 and ITS2 regions respectively. The primers for amplifying the IGS region were: LR12R (5’ GAACGCCTTA AGTCAGAATCC 3’) and 5SRNA (5’ ATCA GACGGGATGCGGT 3’) (White *et al*., 1990). To amplify the mtSSU locus in the mitochondria, we used two primer pairs. The first pair was MS1 (5’ CAGCAGTCAAGAA TATTAGTCAATG 3’) and MS2 (5’ GCGGA TTATCGAATTAAATAAC 3’) (White *et al*., 1990). However, this primer pair amplified the sequence from only one isolate of *L. cristata*. Based on this sequence and those published from other *Lepiota* species, we designed an additional internal primer pair MS3 (5’ GTCAATGCTCGMAAGAGTGAACTAGCT 3’) and MS4 (5’ CAGTTTTGCAACCGTACT

CGCAAG 3'). This additional primer pair allowed us to amplify the sequences of 33 additional isolates used for the present study. We would like to point out that both the ITS and IGS are multicopy fragments. Though they are not next to each other, they are located within the same gene cluster and therefore may not be independent of each other and their variation might not be reflective of the variation in other parts of the genomes. However, ITS and IGS were chosen because of existing data for specimens from other parts of the world and because no other nuclear gene markers currently exist for this species complex. Indeed, we have attempted to amplify genes coding for three other commonly used markers for phylogenetic studies (beta-tubulin, translation elongation factor 1, and the second largest subunit of RNA polymerase II). However, using the primers utilized by the AFTOL project, we were unable to obtain any product from any of our isolates.

During amplification, our typical PCR reactions contained 20 ng genomic DNA, 5 µl of 10X PCR reaction buffer, 5 µl dNTP mix (0.2 mmol), 2 µl each of primers (5 µmol), and 1.5 U of the Taq DNA polymerase. The final volume was adjusted to 50 µl with sterile distilled H_2O. The amplification conditions were set as follows: denaturation at 95°C for 5 min, 35 cycles of 30s at 94°C, 30s at 50°C, 2 min at 72°C, and a final extension of 5min at 72°C.

The PCR products were purified using the Bioteke DNA Purification Kit (Bioteke Corporation, Beijing, China), and sequenced with an ABI 3730 DNA Analyzer and an ABI Bigdye3.1 terminator cycle sequencing kit (Sangon Co., Ltd, Shanghai, China). The same primers described above for PCR were used for the sequencing reactions. DNA sequences were edited and aligned with SeqMan (DNASTAR Package) and CLUSTAL_X (Thompson *et al*., 1997). The aligned sequences were visually inspected and manually adjusted.

Phylogenetic analyses

The analyses of DNA sequence variation within a gene and among genes were performed using the computer program PAUP 4.10b10 (Swofford, 2004). To eliminate ambiguity in our analysis, all gaps were treated as missing data. The maximum parsimonious trees were obtained using heuristic searches and the tree-bisection reconnection (TBR) branch swapping with 1000 starting trees obtained by a random sequential addition of taxa. Searches for maximum parsimonious trees were conducted for each of the three DNA fragments as well as for the combined sequences of all 3 fragments. When available, sequences from isolates of the closely related species *Lepiota castaneidisca* were used as outgroups for the presentation of all phylogenetic trees (Vellinga, 2001b). To examine the robustness of clades identified using maximum parsimony, tests using the maximum likelihood method were also used.

Genealogical comparisons among DNA fragments

To determine whether there were cryptic species within *L. cristata* as evidenced by divergent lineages, we examined the robustness of individual clades using bootstrap and tested the congruences between genealogies from different loci using the partition homogeneity (PH) test. Consistent divergence among groups of isolates across different genes would indicate the existence of cryptic species, with each clade constituting a phylogenetic species (Taylor *et al*., 2000). The PH test is also called the incongruence length difference (ILD) test (Farris *et al*., 1994). Specifically, when multiple genealogies are compared, the length of the shortest possible tree (i.e. in our case here, the simple sum of the lengths of the three individual gene trees) from the combined dataset is compared to that of observed data. If the tree length from the observed dataset is significantly longer than that of the shortest possible tree, the genealogies are considered incongruent. In contrast, if the tree length of the observed dataset is not significantly different from the shortest possible tree, these genealogies are considered statistically congruent. Statistical significance of this test was derived using 1000 randomizations. For this test, one hundred starting trees were obtained by random sequential addition of taxa and branch swapping was done using the tree-bisection-reconnection method. The PH test

was conducted between phylogenies from pairs of DNA fragments as well as among all three DNA fragments.

Relationships among geographic populations

To infer the relationships among geographic populations of *L. cristata* in China, we conducted three complementary tests. In the first, we coded each unique sequence at each locus as a unique sequence type and compared the frequencies of sequence types among geographic populations to examine the levels of genetic differentiation. The standard population genetic measure theta (θ), a modified measure of Wright's F_{ST} (Weir, 1996), was estimated among geographic populations using the computer program Multilocus version 1.0b (Agapow and Burt, 2001). Theta measures the relative contribution of geographic separation to the overall genetic variation in the total analyzed isolates (Weir, 1996). The test was conducted for each locus and averaged over all three loci. The null hypothesis of this test was no genetic differentiation between populations as defined by their geographical origin. Statistical significance for this test was derived by compairing the observed dataset to 10000 randomized datasets generated assuming no genetic differentiation. During randomization, any linkage disequilibria present in the observed data were maintained. The statistic θ ranges from 0 (for no population differentiation due to frequent gene flow) to 1 (total isolation of the populations from each other).

Due to the relatively large number of nucleotides analyzed for these isolates and the large number of unique sequence types for each locus identified within the analyzed sample (see below), we conducted a second test. In this test, we obtained the pair-wise nucleotide differences between each pair of isolates and compared the amount of genetic differences between isolates from within and between geographic regions. The pair-wise isolate sequence differences were calculated for all three genes, using the Hasegawa-Kishino-Yano-1985 (HKY85) distance measure. The HKY85 model was chosen because it treats transitions and transversions differently and uses observed base substitution patterns to derive the optimal weighing scheme among various types of transitions and transversions. In addition, HKY85 does not assume equal base frequencies in the analyzed sequences. The HKY85 distances were obtained through PAUP 4.0b10 but were then exported to Microsoft Excel for calculations of means and standard deviations. Statistical significance between samples in their mean pair-wise sequence divergence was assessed by t-test. If there is a significant geographic structuring, isolates from the same geographic region should be more similar to each other than to isolates from other areas. As a result, we should observe smaller mean pair-wise genetic distance for isolates from the same region than that between isolates from different regions. A lack of significant population structure would be consistent with significant gene flow between geographic populations.

The third test was the topology-dependent permutation tail probability (T-PTP) test. We used this test to examine whether isolates from the same regions were more similar to each other on the phylogenetic trees than to those from other regions by placing specific phylogenetic constraints. Specifically, in the T-PTP test, the length of the maximum parsimony (MP) tree with the monophyletic constraint was compared to the tree length that they were not constrained as a monophyletic group. Here the monophyletic constraint was that all isolates from the same geographic region belonged to the same monophyletic group. If the tree with a monophyletic constraint were significantly longer than that without, such a result would suggest that geographic origin did not correspond to the overall DNA sequence variation. The statistical significance of this test was derived from data permutation under the assumption of non-monophyly to generate a null distribution of tree lengths. Statistical support for non-monophyly was achieved when over 95% of all permuted data sets had tree lengths shorter than the MP tree generated with the constraint of monophyly. 1000 permuted data sets were generated and analyzed. The T-PTP test function was implemented in the phylogenetic program PAUP 4.0b10 (Faith, 1991; Swofford, 2004).

Clonality and recombination

Fruiting bodies of *L. cristata* can release a large number of meiotic spores and we expect that these spores should contribute to population variation. However, no definitive evidence has been reported that sexual recombination plays any role in natural populations of this species. The availability of gene sequences from three loci located in two distinct locations, one in the nuclear ribosomal gene cluster and one in the mitochondrial genome, offers us an opportunity to examine recombination between the mitochondrial and nuclear genes. To assess evidence for recombination, we tested the associations among sequence types at the three analyzed loci using two common statistics in population genetics: the index of association (I_A) (Maynard-Smith *et al*., 1993) and phylogenetic incompatibility tests. Both I_A and phylogenetic incompatibility were estimated using the program Multilocus, version 1.0b (Agapow and Burt, 2001). The basic principles and underlying statistics for these two tests were presented in the program manual. Briefly, the I_A test analyzes the variance of the distances between all possible pairs of multilocus genotypes. The null hypothesis for this test is that there is random association among alleles at different loci. Statistical significance of this test was derived by comparing the observed dataset to 1000 permuted datasets derived from artificial recombination of the observed sequence types among loci. During the process of generating the artificially recombined datasets, we permitted random shuffling of sequence types at each locus within a population but kept the proportions of sequence types at each locus constant. If there was linkage disequilibrium due to a lack of recombination, the observed I_A should be significantly higher than those in the randomized recombined datasets.

In the phylogenetic incompatability test, we calculated the proportion of phylogenetically compatible pairs of loci by looking at the allelic combinations in the samples. Two loci are deemed phylogenetically compatible if it is possible to account for all the observed genotypes by mutations without having to infer homoplasy (reversals, parallelisms, or convergences), or recombination. For example, if there are two alleles at each of two loci, then there are 4 possible haploid genotypes, and the loci are phylogenetically compatible if no more than 3 of them are observed in the sample. In contrast, the two loci are phylogenetically incompatible if all four possible genotypes are found. In a 'multi-allele per locus' situation as it is the case here in this study (see Results), the test performs in a similar way. Specifically, we list all the alleles that occur at one locus across the top of a rectangular matrix, and all the alleles that occur at a second locus down the side, and then mark every box in the matrix for which that combination of alleles is observed. The two loci are incompatible if it is possible to start at a marked box and then return to it by a series of horizontal and vertical moves through other marked boxes (we cannot go back to a box from which we have just come). Otherwise, the loci are considered phylogenetically compatible. The statistical significance for the observations in this test was inferred by comparing the number of incompatible pairs of loci in the observed dataset to those from a randomly recombined dataset, using the program Multilocus, version 1.0b (Agapow and Burt, 2001).

Results

DNA sequence variation within and among isolates of L. cristata

Using the above-mentioned primers, we successfully obtained the ITS sequences (or part of the ITS) from all 47 herbarium specimens of the *L. cristata* species complex isolated from various areas in China. In contrast, despite repeated tries and/or additional primers, we were able to amplify and sequence the IGS and mtSSU loci from only 29 and 34 of the specimens respectively. The GenBank accession numbers for our obtained sequences are presented in Table 1.

Among the three analyzed loci, we found no evidence for heterozygosity for the mitochondrial small subunit ribosomal RNA (mtSSU rRNA) gene locus in any of the 34 isolates that we successfully obtained sequences. In contrast, 29 isolates were found to contain between 1-12 heterozygous nucleotide sites for the ITS region. Similarly, 12 isolates were found to contain 1-5 heterozygous nucleotide sites for the IGS region. Among the above 29 isolates, ten were found to have

heterozygous nucleotide sites for both the ITS and IGS regions; 19 had heterozygous sites for only the ITS region; and 2 had heterozygous sites for only the IGS region.

Our ITS and IGS sequences were aligned along with those from the GenBank obtained previously from isolates of *L. cristata* and its closely related species, mostly from geographic areas outside of China. In total, the archived GenBank sequences included those from isolates of *L. cristata* (14 ITS and 5 IGS sequences), *Lepiota* sp. 1 (2 ITS and 2 IGS sequences), and *L. castaneidisca* (12 ITS and 9 IGS sequences). The aligned sequence lengths, the number of multilocus genotypes and the number of informative characters for each of the analyzed loci are presented in Table 2.

Sequence types and their geographic distributions

The sequenced fragment of 701bp for the ITS region revealed 27 distinct sequence types among the 47 isolates. Two ITS sequence types contained five isolates each; one contained four isolates, one contained three isolates, and seven contained two isolates each. The remaining 16 ITS sequence types were represented by one isolate each (Fig. 2). Of the two shared sequence types with five isolates each, one contained isolates exclusively from Yunnan Province (isolates HKAS45049, HKAS45053, HKAS48457, HKAS51356, and RITF312) while the other contained isolates from four provinces, two in northeastern China (isolate HMJAU3430 from Jilin and isolate HMJAU 0822 from Hebei) and two in southwestern China (isolates HKAS45830 and HKAS45492 from Sichuan and isolate RITF308 from Yunnan). The four isolates sharing one ITS sequence type were all from southwestern China, with three from Yunnan (isolates HKAS46370, RITF311 and RITF301) and one from Sichuan (HKAS32250). The three isolates sharing the same ITS sequence type were from Sichuan (HKAS49160), Yunnan (HKAS 46345), and Beijing (HMJAU1135). Of the seven ITS sequence types with two isolates each, five contained isolates from the same province while two contained isolates from different geographic provinces. It should be noted that none of our ITS sequences was identical to those from the USA and Europe (Fig. 2).

For the IGS DNA fragment, we found 21 distinct sequence types among the 29 isolates that we were able to obtain sequences (Fig. 3). Three sequence types were each shared by two or more isolates. One contained six isolates from two provinces in southwestern China, from Sichuan (2 isolates HKAS45830 and HKAS45928) and Yunnan (4 isolates RIFT301, RIFT308, HKAS46348 and HKAS46344). The second shared sequence type contained three isolates from Sichuan (2 isolates HKAS49367 and HKAS49160) and Yunnan (isolate HKAS 46345). The third shared type contained two isolates from Yunnan (RITF311 and RITF312). The remaining 18 IGS sequence types were represented by only one isolate each. Similar to that of the ITS fragment, none of the sequence types obtained here from the Chinese isolates were identical to those from the USA or Europe (Fig. 3).

For the mtSSU rRNA locus, we found 16 distinct sequence types among the 34 sequenced isolates (Fig. 4). The most common mtSSU rRNA sequence type contained 8 isolates. These 8 isolates were from four provinces/administrative regions, with six isolates from southwestern China (four from Sichuan, one each from Tibet and Yunnan) and two from northeastern China (both from Jilin). The second most common mtSSU rRNA sequence type contained six isolates. These six isolates were again from both northeastern China (one isolate from Jilin) and southwestern China (two isolates from Sichuan and three isolates from Yunnan). Two sequence types contained three isolates each and all six isolates were from Yunnan. One sequence type contained two isolates with one from Sichuan and another from Tibet. Because no mtSSU rRNA sequence is available for isolates of *L. cristata* from outside of China, the potential similarities and differences between the Chinese and USA/Europe isolates for this gene region cannot be assessed at present.

The combined analyses of all 2244 nucleotides identified that each of the 29 isolates with sequences from all three genes had a different multilocus genotype (data not shown).

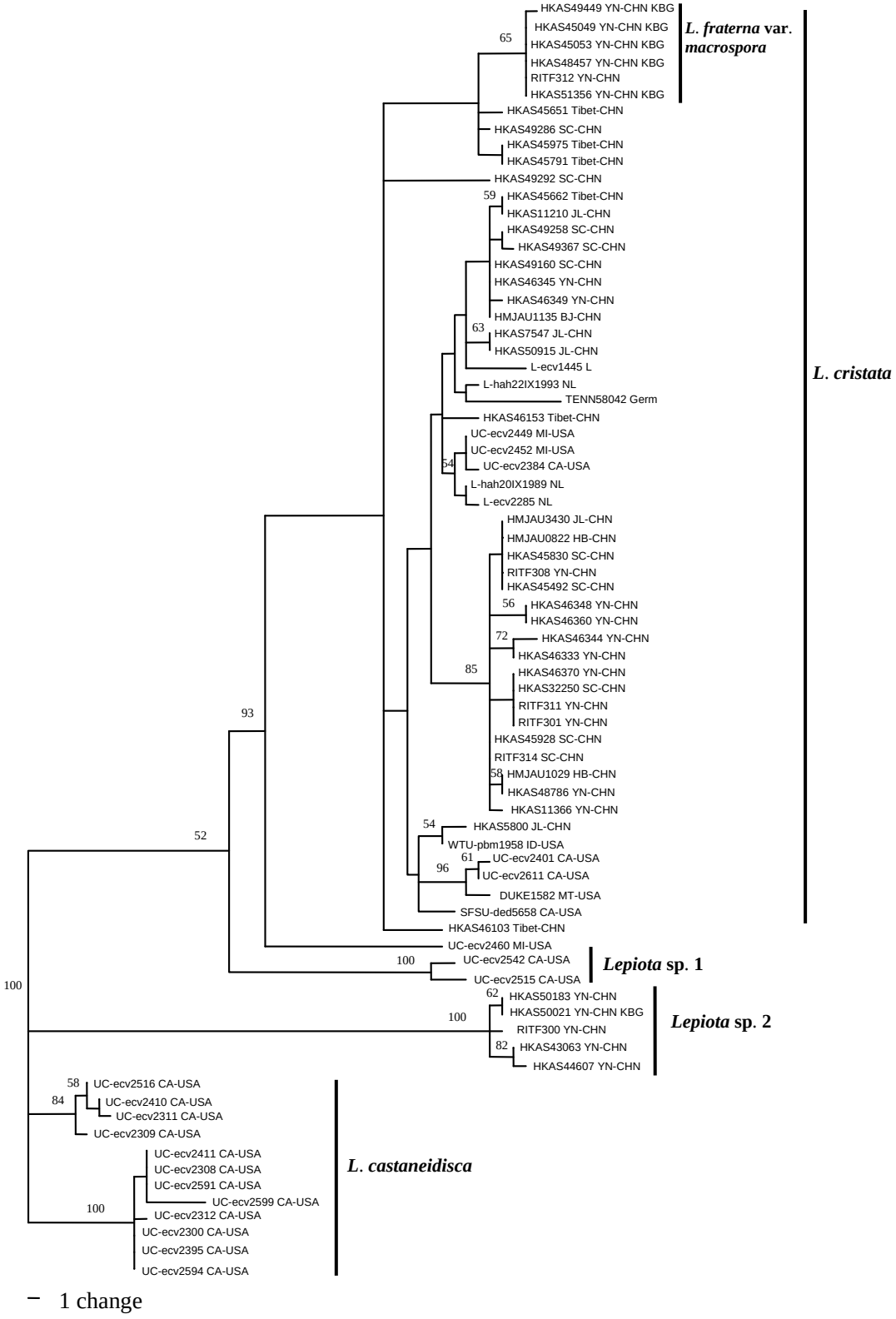

Fig. 2. Maximum-parsimony tree obtained using the ITS sequence data. Bootstrap values over 50% are indicated above the branches.

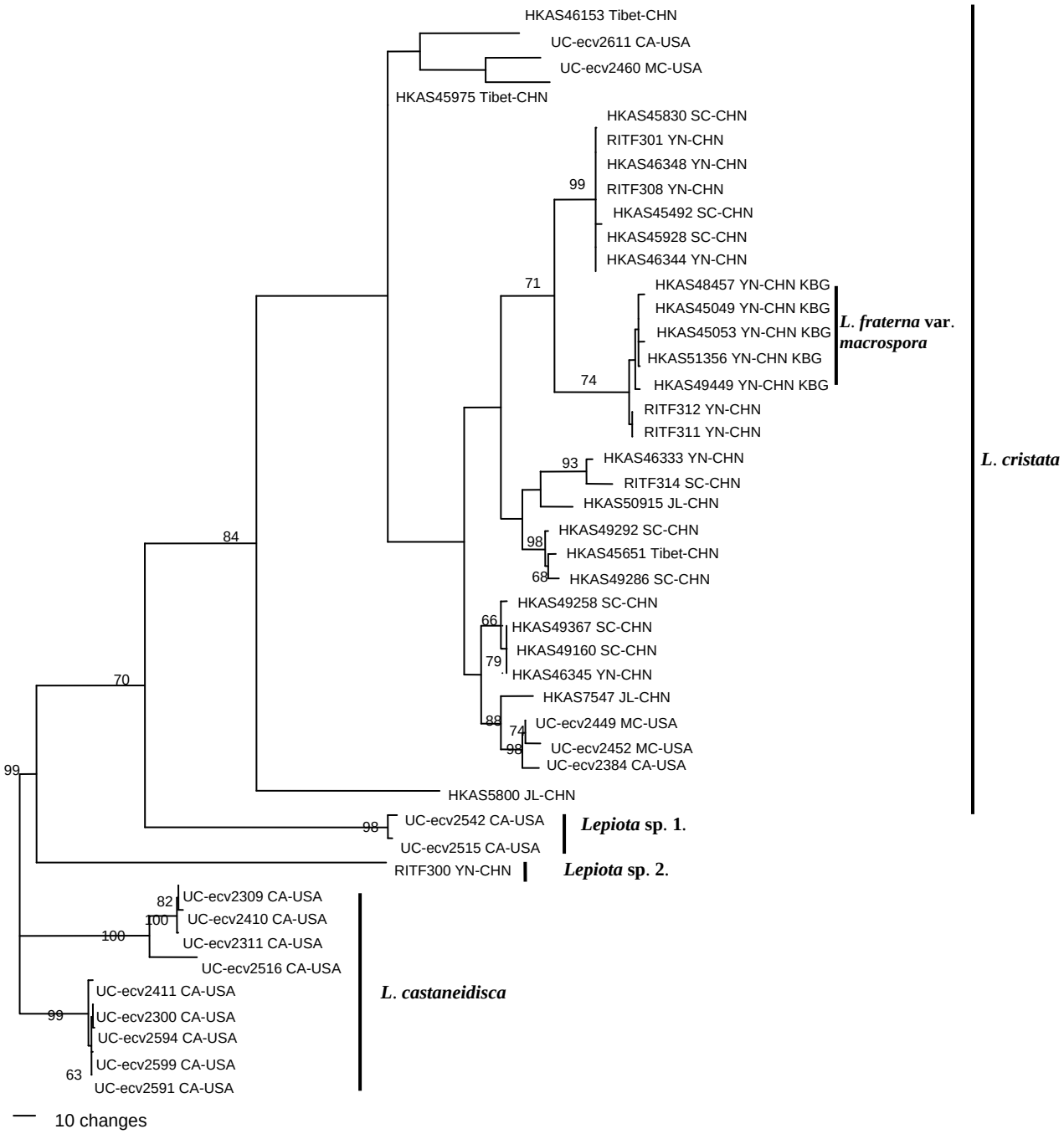

Fig. 3. Maximum-parsimony tree obtained using the IGS sequence data. Bootstrap values over 50% are indicated above the branches.

Phylogenetic divergence within L. cristata

Our phylogenetic analyses showed that each of the three analyzed DNA fragments revealed significantly divergent clusters. Specifically, for the ITS gene region, there were two clusters with more than five isolates each and with each cluster having bootstrap support values greater than 80%. One cluster contained five isolates from Yunnan (*Lepiota* sp. 2, 100% bootstrap support). The second cluster contained 18 isolates distributed in five of the sampled regions (except Tibet, 85% bootstrap support). Consistent with results from previous analyses of ITS sequences of the *L. cristata* species complex by Vellinga (2001b), several divergent clusters for isolates from Europe and North America were found, including *Lepiota* sp. 1 (100% bootstrap support) and the cluster with three isolates from the USA (UC-ecv2401, UC-ecv2611, and Duke1582; 96% bootstrap support). Similarly, the two divergent groups of *L. castaneidisca*

Table 2. Phylogenetic information about the three sequenced loci for the analyzed samples of *L. cristata* (excluding *L. castaneidisca*, *L.* sp. 1 and *L.* sp. 2)

Genes	Nucleotides analyzed (bp)	# Indels (Range of indel lengths, bp)	Polymorphic sites		Phylogenetic analysis			
			Parsimony informative sites (bp)	Parsimony uninform-ative sites (bp)	# of MP tree[1]	Tree length	CI[2]	RI[3]
ITS	701	10 (1-4)	39	42	1	103	0.816	0.937
IGS	1043	9 (1-2)	171	155	1	593	0.702	0.816
mtSSU	500	2 (1-5)	7	12	1	27	0.778	0.870

[1]MP, maximum parsimony
[2]CI, consistency index
[3]RI, retention index

Table 3. Partition homogeneity tests between gene genealogies for 29 isolates of *L. cristata* that contained sequence information for all three loci.

Gene combination	Sum of tree length of original partition	Range of summed tree length of 500 permuted datasets	P value
ITS/mtSSU	98	101-107	0.001
ITS/IGS	505	524-531	0.001
mtSSU/IGS	447	444-457	0.032
ITS/mtSSU/IGS	525	545-556	0.001

were well separated from isolates of *L. cristata* in our analyses.

For sequences from the IGS region, clades originally identified by Vellinga (2001b) for isolates from North America and Europe also showed up as distinct groups in the expanded analyses. In addition, similar to that for the ITS dataset, we identified several clades from the Chinese sample with high bootstrap support (Fig. 3). However, while some isolates had consistent patterns of clustering between ITS and IGS, clades identified using the IGS sequence information did not correspond exactly to those identified based on ITS sequences and vice versa. For example, isolate RITF311 was clustered with 17 other isolates based on the ITS gene sequences while it was clustered with the group containing five isolates of *L. fraterna* var. *macrospora* in the IGS genealogy (Fig. 3).

For sequences from the mtSSU rRNA gene, only one statistically well-supported branch was found for the Chinese samples of *L. cristata*. This gene genealogy separated the samples into two clades: one small group consists of four isolates (HKAS5800, HKAS 50021, HKAS50183, and RITF300) and the other group consists of the remaining 30 isolates that included the five specimens of *L. fraterna* var. *macrospora*. Because sequences from samples from North America and Europe were not obtained, they could not be analyzed here for comparison.

Genealogical incongruence

Our visual comparisons described above revealed obvious incongruences among genealogies of the three genes (Figs 2, 3, and 4). The statistical significances of their incongruences were further assessed using the partition homogeneity (PH) test. In this test, only the 29 isolates with sequences from all three loci were included. Our analysis identified that phylogenies from the three loci showed an overall significant incongruence (Table 3). Both the three pairwise gene genealogy comparisons and the combined analyses showed significant incongruence ($P < 0.05$, Table 3).

Geographic patterns of DNA sequence variation

As can be seen from the above analysis of sequence types for each locus, there is abundant evidence for long-distance gene flow between geographic populations. Indeed, our population genetic analyses identified no significant differentiation among the four geographic populations with sample sizes ≥ 5 (i.e. Jilin, Yunnan, Sichuan, and Tibet). The pairwise Theta values between populations and

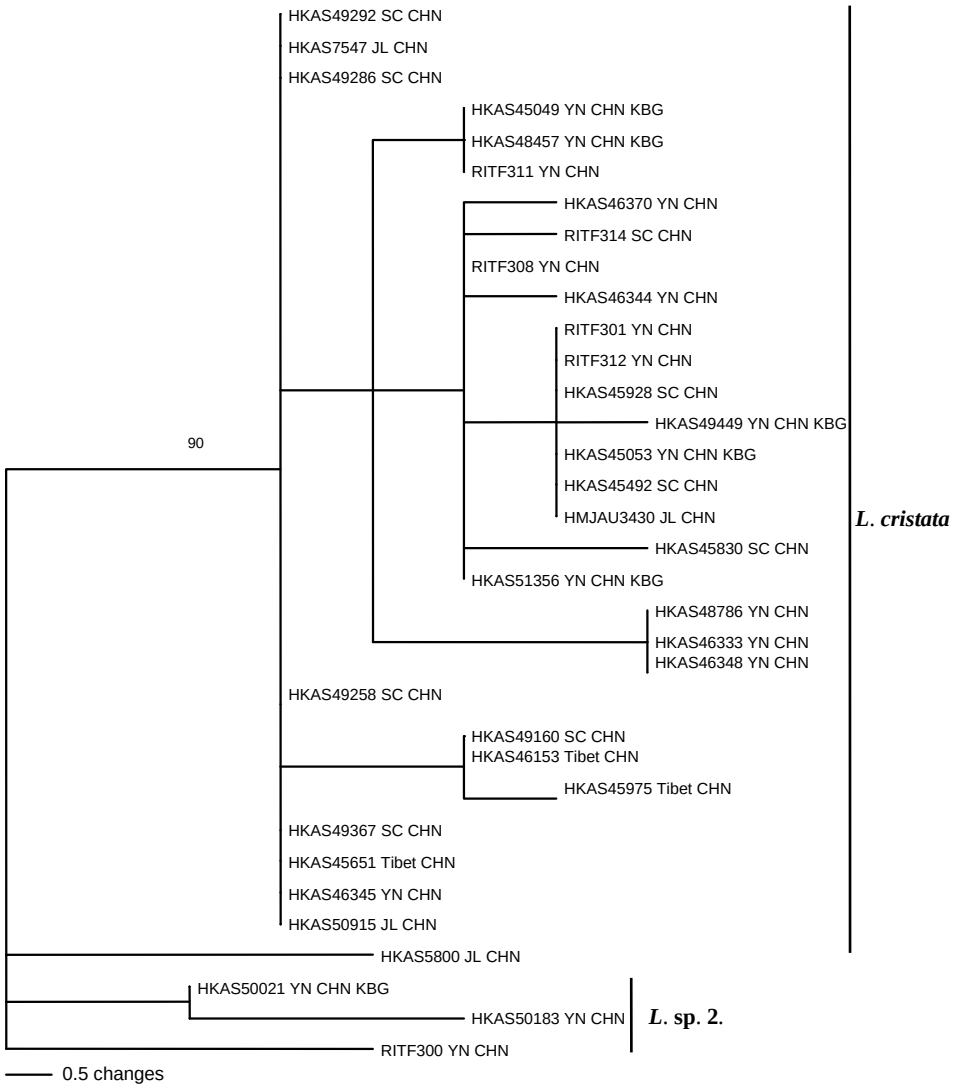

Fig. 4. Maximum-parsimony tree obtained using the mtSSU sequence data. Bootstrap values over 50% are indicated above the branches.

the statistical significance of these values are presented in Table 4. The lack of a strict geography-based population structure within the *L. cristata* complex in China is also supported by two additional analyses. Both the T-PTP test and the pairwise sequence comparisons showed a lack of an overall, strictly geographically structured pattern. In all our T-PTP analyses, the geographically constrained phylogenies were significantly longer than those without any constraints ($p < 0.01$ in all cases). The tested constraint criteria included within China vs. outside of China; between northeastern China and southwestern China; and among the six provinces / administrative regions (data not shown). Similarly, the analyses of the pairwise isolate HKY85 distances from within individual geographic regions in China were similar to those from between different geographic areas (Table 4).

Though there was no overall geographic pattern within the morphological species of *L. cristata*, our phylogenetic analyses did suggest a few potentially geographically specific clusters within China. One such cluster (*Lepiota* sp. 2 in Figs 2, 3, and 4) contained five isolates from Yunnan (isolates HKAS 44607, HKAS43063, HKAS50183, HKAS 50021 and RITF300) and might warrant the status of new species. This cluster was significantly divergent from all known isolates of *L. cristata* from either China or elsewhere (Figs 2, 3, and 4). Another cluster, labeled as "*L. fraterna* var. *macrospora*" in Figs. 2 and 3, included six and seven isolates respectively for the ITS and IGS gene trees from Yunnan

Table 4. Genetic differentiation between populations and the mean pairwise distances between isolates of *L. cristata* from the same or different geographic regions in China

Region[1]	Genetic differentiation between pairs of populations	Pairwise HKY85[2] distance:	
		Number of pairwise isolate comparisons	Mean ±SD
Within Yunnan	N/A	78	0.0215 ± 0.0135
Within Sichuan	N/A	36	0.0271 ± 0.0138
Within Tibet	N/A	3	0.0121 ± 0.0144
Within Jilin	N/A	3	0.0339 ± 0.0069
Between Yunnan and Sichuan	0.0274 (p=0.53)	123	0.0316 ± 0.0173
Between Yunnan and Tibet	0.0459 (p=0.24)	39	0.0245 ± 0.0131
Between Yunnan and Jilin	0.05088 (p=0.98)	39	0.0437 ± 0.0097
Between Sichuan and Jilin	0.0127 (p=0.38)	27	0.0351 ± 0.0109
Between Sichuan and Tibet	0.0091 (p=0.23)	27	0.0203 ± 0.0127
Between Tibet and Jilin	0.0092 (p=0.38)	9	0.0339 ± 0.0069
All isolates of *L. cristata*		378	0.0278 ± 0.0142
Between *L. sp*. 1 and all other isolates of *L. cristata*		28	0.0916 ± 0.0169

[1]Only regions with samples greater than 5 are included here.
[2]HKY85, Hasegawa-Kishino-Yano-1985 genetic distance. The distances here are calculated from only isolates with sequences from all three loci

(isolates HKAS49449, HKAS45049, HKAS 45053, HKAS48457, HKAS51356, RITF311, and RITF312). Our analyses here indicate that the former five isolates of *L. fraterna* var. *macrospora* should be classified into the *L. cristata* species complex.

Clonality and recombination

The PH tests presented above indicated signatures of recombination, hybridization or incomplete lineage sorting. To further confirm the results, we examined the associations of sequence types from the three different loci using two commonly used population genetic measures, the Index of Association (IA) and phylogenetic incompatibility. Indeed, both tests provided supporting evidence for recombination in natural populations of this species. Specifically, the observed value for the I_A test was 0.043 among the three loci for the total Chinese population. This value did not differ significantly from those calculated using randomly recombined datasets (i.e. re-shuffled using observed sequence type frequencies) (P = 0.80). In addition, a clear example of phylogenetic incompatibility was found between ITS and mtSSU rRNA loci (Figs 2 and 4) that involved isolates HKAS45049 (sequence types 1 and 1 for ITS and mtSS rRNA respectively), HKAS45053 (sequence types 2 and 1), HKAS 48457 (sequence types 1 and 1), RITF301 (sequence types 2 and 2), RITF311 (sequence types 1 and 2) and RITF312 (sequence types 2 and 1). The finding of all four combinations of sequence types for two loci with two sequence types each is consistent with recombination. All these six isolates were from Yunnan Province.

Discussion

Through the analyses of three loci for 47 isolates distributed in several regions in China, we identified abundant genetic variation both within and among regional populations of *L. cristata*. We found support for potentially multiple lineages within the Chinese samples of *L. cristata* in all three analyzed loci. Our analyses revealed significant gene flow among the analyzed geographic populations and that recombination must have played a role in generating the observed genetic diversity in nature.

The significant molecular divergence observed here suggests that there might be several cryptic species within the morphological species of *L. cristata*. For example, isolates from within the Kunming Botanical Garden (KBG) in the city of Kunming, Yunnan, were found to belong to two divergent lineages. One lineage from KBG included five isolates (i.e. HKAS49449, HKAS45049, HKAS45053, HKAS48457, and HKAS51356) from this garden that had been previously assigned to

L. fraterna var. *macrospora* due to the presence of lateral knobs in their basidiospores (Yang, 1994; see below). The other lineage included one isolate from KBG (HKAS50021) and four isolates from other parts of Yunnan (HKAS50183, HKAS43063, HKAS44607, and RITF300; Fig. 2). Further analysis using additional markers might reveal that the latter lineage indeed belonged to a different phylogenetic species. Specifically, genealogical concordance phylogenetic species recognition specifies that if groups of isolates show consistent divergence across several genes, they can be considered belonging to different phylogenetic species (Taylor *et al*., 2000, 2006). Using this approach, many phylogenetic species have been identified among human pathogenic fungi (Koufopanou *et al*., 1997; Kasuga *et al*., 1999, 2003), plant pathogenic fungi (O'Donnell, 2000; Steenkamp *et al*., 2002; Hirata *et al*., 2007; Alves *et al*., 2006, 2008), the lichenized fungi (Kroken and Taylor, 2001) and saprophytic fungi (Dettman *et al*., 2003).

Our phylogenetic results prompted us to further examine the microscopic features of the lineage with HKAS50021. Indeed, our analyses identified that the isolates within this lineage had significantly smaller basidiospores and basidia than those of other *L. cristata* specimens (detailed data not shown). Similarly, our phylogenetic analyses also suggest that the isolates previously assigned to *L. fraterna* var. *macrospora* from Yunnan (Yang, 1994) were likely mis-classified. Morphologically, basidiospores of *L. fraterna* contain a lateral knob on each side when observed through the ventral plane while basidiospores of *L. cristata* do not. The difference between *L. fraterna* var. *fraterna* E. Horak and *L. fraterna* var. *macrospora* is that the spores in var. *macrospora* are bigger than those in var. *fraterna* (Horak, 1980; Yang, 1994). However, the spores of *L. fraterna* var. *macrospora* are similar in size to those of *L. cristata* (Candusso and Lanzoni, 1990; Vel-linga, 2001a,b). It seems that spore size might be phylogenetically more informative than the presence/absence of the lateral knob in this group of fungi. Alternatively, a lineage of knobbed basidiospores in *L. cristata* complex has evolved in southwestern China. In all three gene genealogies, a variable number of isolates with features typical of basidiospores in *L. cristata* were intermingled with these five isolates from *L. fraterna* var. *macrospora*. Specifically, isolate RITF312 in the ITS genealogy, isolates RITF311 and RITF312 in the IGS genealogy, and several isolates in the mtSSU rRNA gene were identical or closely related to those five isolates previously assigned to *L. fraterna* var. *macrospora* (Figures 2, 3, and 4). The analyses of more loci might reveal a more significant separation of these five isolates from the majority of *L. cristata* isolates. At present, no DNA sequences of isolates from *L. fraterna* have been deposited in any public databases to allow us to evaluate the evolutionary relationships between *L. fraterna* and *L. cristata*.

While significant sequence variation was observed within the morphological species of *L. cristata*, even among isolates from the same geographic area, we also identified the sharing of sequence types and limited genetic differenttiation between geographic populations from distant regions in China (e.g. ITS sequence types between Jilin in northeastern China and Sichuan in southwestern China, the two locations about 2000 km from each other). The observed gene flow could be due to two possibilities. The first is that the lack of pigmentation in basidiospores of *L. cristata* might not be a significant impediment for long distance gene flow and that the dispersal occurred naturally without much human intervention. Long distance gene flow over hundreds to thousands of kilometers has been inferred in many fungi (James *et al*., 1999; Kuyper, 2003; Edman and Gustafsson, 2003). For example, spores of the pine pathogen *Heterobasidion annosum* can disperse over 300 km (Rishheth, 1959). Moyersoen *et al*. (2003) suggested that the high genetic diversity of *Pisolithus* spp. in New Zealand was likely due to multiple long-distance spore dispersals from Australia to New Zealand. Bergemann and Miller (2002) suggested that long distance spore dispersal events likely contributed to multiple colonizations in populations of *Russula brevipes*. The analyses of the diversity of trapped spores also indicated the existence of long distance spore dispersal in populations in *Schizophyllum commune* (James and Vilgalys, 2001). For

species lacking pigmented basidiospores, wind-aided dispersal could still occur, just like for species with pigmented spores. While the absence of pigmentation might reduce the viability of spores during dispersal, even if a small percentage of them survived environmental stresses such as high temperature and UV irradiation during the dispersal process, these spores could still contribute to the significant gene flow between populations.

The second hypothesis for the observed long-distance gene flow is that humans and anthropogenic activities might have contributed to the dispersal of *L. cristata*. Indeed, human activities have been found responsible for the long-distance dispersal of many fungi, including both pathogens (Fry *et al*., 1992; Roche *et al*., 1995; Milgroom *et al*., 1996; Zeng and Luo, 2006) and saprophytes such as the cultivated button mushroom *Agaricus bisporus* (Xu *et al*., 1997, 1998). The hypothesis that human activities might have contributed to ITS gene flow is also consistent with the preference of *L. cristata* for open and frequently human-disturbed habitats.

Given the significant observed sequence variations, the long-distance gene flow revealed here in *L. cristata* was a bit surprising. If the first hypothesis is correct that long-distance dispersal in *L. cristata* is a natural and ongoing event and has been happening over evolutionary time scale, we should probably not see significantly divergent lineages in this species, especially for those located geographically close to each other. Therefore, we propose that long-distance gene flow in *L. cristata* might be relatively limited and that there was ancient divergence to generate these distinct lineages. Because most of China, including the sampled regions in Yunnan, Sichuan and northeastern China, were not completely covered by ice during the last ice age, the populations of *L. cristata* analyzed here were unlikely influenced by population range expansion after the last ice age from a single Pleistocene refuge. Instead, human activities in the very recent history have likely played a role in the multiple introduction of overlapping lineages. To generate hybrids, mating and sexual compatibility between certain isolates from different lineages are required. Therefore, a complete reproductive isolation might not have been fully established between some of these lineages. Alternatively, incomplete lineage sorting for the repeated regions ITS and IGS during speciation could also have contributed to genealogical incongruence. At present, no methods have been established to germinate basidiospores of *L. cristata*, to conduct mating tests, or to artificially produce fruiting bodies of this species in the laboratory. The availability of such methods should allow us to critically examine the potential inter-fertility among isolates from divergent lineages and to understand the genetic basis for their morphological differences in basidiospores.

The incongruent genealogies observed here indicate that these three loci are informative for inferring evolutionary patterns of *L. cristata*. Indeed, due to their different patterns of inheritance, when analyzed jointly, the nuclear and mitochondrial loci have been found very informative for the studies in a variety of species. Specifically, during sexual mating, nuclear genes typically follow the Mendelian inheritance patterns of segregation, independent re-assortment and recombination while mitochondrial genes and genomes are often inherited uniparentally (Xu, 2005). In sexually reproducing populations, the different inheritance patterns should result in linkage equilibrium between markers from the two genomes. Therefore, the lack of a strict association between sequence types among the three loci is consistent with sexual reproduction, as was found here. Interestingly, the ITS and IGS loci also showed significantly different phylogenetic patterns. This result is surprising given that both the ITS and IGS are located within the same genomic region. Genes within this repeated genomic region have been found to undergo concerted evolution (Arnheim, 1983). While the mechanism for the observed differences between ITS and IGS is unknown at present, our data clearly shows that these two fragments do not reflect identical evolutionary patterns within *L. cristata*. Further cloning and sequencing of individual sequence types from isolates with heterozygous nucleotide sites at the ITS and IGS regions might allow us to dissect the complexity of

those heterozygous sites and to infer the potential mechanisms for the differences between these two loci.

The ITS regions are commonly used loci for fungal molecular systematics and population genetic studies (e.g. Chapela and Garbelotto, 2004; Vánky and Lutz, 2007; Wang *et al*., 2007; Alves *et al*., 2008; Buyck *et al*., 2008; Cui *et al*., 2008; Ge *et al*., 2008; Hiremath *et al*., 2008; Ortega *et al*., 2008; Li *et al*., 2009). Within the mycology community, there is a broad consensus to use this region for fungal barcoding. However, due to its multi-copy nature within individual cells, there are several potential problems of using this locus alone for systematics and phylogeographic inferences (Dubcovsky and Dvorák, 1995). For example, incomplete lineage sorting during speciation coupled with concerted evolution could have resulted the incongruent gene genealogies among the three loci analyzed here. To avoid such potential problems, Feliner and Rosselló (2007) suggested that the best approach is to use both ITS and additional markers from the organelle genomes and/or single-copy or low-copy number nuclear genes. Indeed, the combined use of genes located in different parts of the genomes have allowed the inferences of a variety of evolutionary, genetic, population and ecological phenomena. For example, the comparative analyses of sequences of single copy genes revealed multiple hybridization events and recombination in the human basidiomycete pathogen *Cryptococcus neoformans* (Xu *et al*., 2002; Xu and Mitchell, 2003). Unfortunately, there is no sequence available at present for single copy nuclear genes in *L. cristata* in any of the public databases. In addition, none of the primers for three single-copy genes (beta-tubulin, EF1-alpha and RBP2) designed based on sequences in other fungi yielded any PCR products in our samples of *L. cristata*.

While we have attempted to obtain as many samples from as many geographic areas as possible, compared to the size of China, our study sites are relatively limited. The majority of our samples were from southwestern China. Though this area is a world biodiversity hotspot and isolates of *L. cristata* from this region were genetically very diverse, this region might not be representative of other areas within China or outside of China. The analyses of additional samples from other areas as well as more genes might allow us to define precisely the number of cryptic species within the *L. cristata* species complex and their respective geographic distributions. We believe our study here represents an important step towards understanding the ecology and evolutionary biology of this fungus.

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