



## The Molecular Heterogeneity of Natural *Cordyceps sinensis* with Multiple *Ophiocordyceps sinensis* Fungi Challenges the Anamorph-Teleomorph Connection Hypotheses

Yu-Ling Li<sup>1</sup>, Yi-Sang Yao<sup>2</sup>, Wei-Dong Xie<sup>2</sup>, Jia-Shi Zhu<sup>2,3,\*</sup>

<sup>1</sup>Qinghai University, Institute of Grassland Research, Qinghai Academy of Animal Husbandry and Veterinary Sciences, Xining, Qinghai 810016 China.

<sup>2</sup>Division of Life Sciences and Health, Tsinghua University Graduate School at Shenzhen, Guangdong 518055, China.

<sup>3</sup>Department of Applied Biology and Chemistry Technology, The Hong Kong Polytechnic University, Hong Kong.

**\*Corresponding Author**

Jia-Shi Zhu

Division of Life Sciences and Health

Tsinghua University Graduate School at Shenzhen

Guangdong 518055, China

Department of Applied Biology and Chemistry Technology

The Hong Kong Polytechnic University

Hong Kong

Tel: +18587053789

Fax: +18587775435

E-mail: zhujosh@gmail.com

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### Abstract

Natural *Cordyceps sinensis* is a traditional Chinese medicine with a long history of use as a folk medicine in China. However, whether *Ophiocordyceps sinensis* is a single fungus or a collective name for multiple fungi remains controversial, as does the anamorph-teleomorph connection of *O. sinensis*. Although *Hirsutella sinensis* has been widely considered the sole anamorph of *O. sinensis*, direct evidence supporting this hypothesis is lacking. This paper reviews the molecular heterogeneity findings of *C. sinensis* studies and the issues with the hypotheses of the anamorph-teleomorph connection of *O. sinensis*. Molecular analyses have revealed the coexistence of multiple fungi and multiple genotypes of *O. sinensis* in natural *C. sinensis*. Mutant sequences from at least 11 *O. sinensis* genotypes are registered in GenBank and represent individual fungi distinct from the genome sequence of *H. sinensis*, which is inconsistent with the “sole anamorph” and “ITS pseudogenes” hypotheses. Together, the multicellular *C. sinensis* ascospores with mono-/bi-/trinucleate structures in each ascospore and the detection of at least 2 *O. sinensis* genotypes in the culture of heterokaryotic single-ascospore isolates challenge the study conclusions based on microcycle conidiation of *C. sinensis* ascospores. During *C. sinensis* maturation, the fungi that grow differentially in the caterpillar body and stroma of *C. sinensis* undergo asynchronous, dynamic alterations, supporting the integrated microecosystem hypothesis for natural *C. sinensis* proposed by Prof. Zongqi Liang.

**Keywords:** Molecular systematics of *Cordyceps sinensis*; mutant genotypes of *Ophiocordyceps sinensis*; insect-fungi complex; anamorph-teleomorph connection; binucleate; trinucleate; multicellular heterokaryons.

**Abbreviations used:** **1F1N**, One Fungus = One Name; **AFLP**, Amplified Fragment Length Polymorphism; **CAPS**, Cleaved Amplified Polymorphic Sequence; **DAF**, DNA Amplified Fingerprints; **gb**, GenBank; **ICTF**, International Commission on the Taxonomy of Fungi; **IMA**, International Mycology Association; **INSD**, International Nucleotide Sequence Databases; **ITS**, Internal Transcribed Spacer; **ISSR**, Inter-Simple Sequence Repeat; **MALDI-TOF**, Matrix-Assisted Laser Desorption/Ionization-Time Of Flight; **MS**, Mass Spectrum or Mass Spectrometry or Mass Spectrogram or Mass Spectrograph; **nrDNA**, nuclear ribosomal DNA; **OTUs**, Operational Taxonomic Units; **PCR**, Polymerase Chain Reaction; **qPCR**, quantitative Polymerase Chain Reaction; **RAPD**, Random Amplified Polymorphic DNA; **RFLP**, Restriction Fragment Length Polymorphism; **SCAR**, Sequence Characterized Amplified Regions; **SNP**, Single Nucleotide Polymorphism; **SSCP**, Single-Strand Conformation Polymorphism; **SSR**, Simple Sequence Repeat; **TCM**, Traditional Chinese Medicine; **UPGMA**, Unweighted Pair Group Method with Arithmetic Mean.

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Natural *Cordyceps sinensis* is the most expensive substance in traditional Chinese medicine (TCM), with a rich history of traditional use in China for “Yin-Yang” double invigoration, health maintenance, disease amelioration, post-disease recovery, and anti-aging therapy [1-2]. (Section 1, below, addresses the controversy surrounding the indiscriminate use of the Latin names for the natural wild product and the fungi.) Morphological and TCM descriptions of this medicinal substance in ancient TCM books as a unique therapeutic entity can be traced back to the 15<sup>th</sup> century [3]. Modern pharmacological examinations have validated the therapeutic profile of wild *C. sinensis* and its mycelial fermentation products [4-6]. We also demonstrated pharmacologically the lifespan-extending properties of a fermentation product of a *C. sinensis* isolate, *Paecilomyces hepiali* Cs-4, and its ability to reverse aging-induced changes in genome-wide gene expression patterns [7]. Natural *C. sinensis* grows only in alpine areas above 3,000-3,500 m on the Qinghai-Tibetan Plateau and has a complex life cycle [1,4,7-9]. The development-maturation stages of natural *C. sinensis* greatly impact its therapeutic efficacy and potency and are used as a market standard for grading the quality of natural *C. sinensis* [10-13].

The anamorph-teleomorph connection of *Ophiocordyceps sinensis* has been the subject of a decades-long academic debate. Based on the collection of indirect evidence obtained using several techniques, some mycologists agree that

*H. sinensis* is the sole anamorph of *O. sinensis* [14-15]. However, to date, no direct evidence strictly and fully satisfying Koch’s Postulates (association, isolation/purification, re-inoculation, and re-isolation) has been documented. The artificial production of sexual fruiting bodies and ascospores from postulated anamorphic fungal strains previously isolated from natural *C. sinensis* specimens, purified and cultured to inoculate the host (larvae of the family Hepialidae), has repeatedly proven unsuccessful [4-22]. Guo et al. [15] and Xiao et al. [20] reviewed indirect evidence for the “sole *H. sinensis* anamorph” hypothesis using multiple technologies, including the isolation and morphological examination of fungal strains, the microcycle conidiation of ascospores, and molecular systematics studies. Unfortunately, the majority of the fungal species in the natural world cannot be cultured, making it difficult to conduct studies using traditional techniques of fungal isolation, purification and morphological identification and microcycle conidiation [18,23-26]. Culture-independent molecular systematics studies of natural *C. sinensis* specimens have matured over the last 18 years and have generated indirect evidence for the assessment of the anamorph-teleomorph connection of *O. sinensis*. This review describes molecular biology studies of natural *C. sinensis* specimens and related scientific issues.

## 1. Indiscriminate use of the Latin name *C. sinensis* or *O. sinensis* for the wild product and the fungi

We begin this review with a discussion of the controversy concerning the Latin name *Cordyceps sinensis* (Berkeley) Saccardo, which has been used indiscriminately for both the wild product, which contains multiple fungi and the dead body of a larva of the family Hepialidae, and for the teleomorph and holomorph of *C. sinensis* fungus/fungi [3,14-16,27-30]. The indiscriminate use of the single Latin name can be traced back to the original literature published in 1843 and 1857, when British mycologist Miles Joseph Berkeley examined the fungal species in natural *C. sinensis* [3,30-34]. The fungus/fungi were re-named to the synonym *Ophiocordyceps sinensis* (Berkeley) Sung et al. [35], while the name of the wild product remains unchanged. Because of the indiscriminate practice that has created confusion among hundreds of publications, Ren et al. [36] proposed the use of “*Ophiocordyceps* & Hepialidae” to reflect the nature of the insect-fungi complex of the wild product. However, that proposal has not been generally accepted because the fungi currently under the name of *O. sinensis* may or may not belong to the genus *Ophiocordyceps* (see below for discussion). Zhang et al. [37], on the other hand, proposed the use of “Chinese cordyceps” for the wild product and *O. sinensis* for the fungus/fungi. However, the use of the non-Latin name Chinese cordyceps for the wild product never reached general consensus and was unfortunately rejected by taxonomists in TCM botany because of the general practice and governmental regulation that every TCM product must be given an exclusive Latin name. Due to this awkward situation, Lo et al. [4], Zhao et al. [38] and many other papers simply used the Chinese alphabetic “Dong Chong Xia Cao 冬虫夏草” for the wild product, and Zhou et al. [6] used its abbreviation “DCXC”. In this review, we temporarily refer to the fungi as “*Ophiocordyceps sinensis*” and continue the customary use of the name *Cordyceps sinensis* to refer to the wild product, although this practice will likely be

replaced by the discriminate use of unique Latin names.

In addition to the indiscriminate use of Latin names for the wild product and the teleomorph and holomorph of the fungus/fungi, the use of the teleomorphic name *O. sinensis* to replace the anamorphic name *H. sinensis*, the postulated anamorph of *O. sinensis*, has been proposed by Zhang et al. [28] following the Amsterdam Declaration (International Mycology Association, or IMA) of “One Fungus = One Name (1F1N)” [39-41]. This proposal, however, can only be accepted scientifically if *O. sinensis* is indeed “One Fungus” and if *H. sinensis* is truly the sole anamorph of *O. sinensis*.

## 2. Is *O. sinensis* the Latin name for a single fungal species?

As a prerequisite for implementing the 1F1N declaration [39-41] in natural *C. sinensis* research [28], *O. sinensis* must represent a single fungal species. For the *O. sinensis* fungus/fungi, however, the following 3 hypotheses from the literature require scientific validation:

### 2.1 *O. sinensis* is a single fungus, and *H. sinensis* is the sole anamorph of *O. sinensis* [4,14-15,28]

If this hypothesis is accurate, the anamorphic name *H. sinensis* could be replaced with the teleomorphic name *O. sinensis*. In a comparison of the sequence of *H. sinensis* that had been isolated and purified using currently available techniques with the database sequences of *O. sinensis*, Zhang et al. [29] stated that all *O. sinensis* sequences registered in the International Nucleotide Sequence Databases (INSD; GenBank is one member of the INSD), except for Group A (*H. sinensis*), should be “treated as incorrect sequences” under the hypothetical assumption that *H. sinensis* is the sole anamorph of *O. sinensis*, because these sequences were “reported from natural Chinese cordyceps samples rather than from isolated fungal cultures” (more discussion below). Despite the controversies (see 2.2 and 2.3 below), many scientific publications have followed this hypothesis.

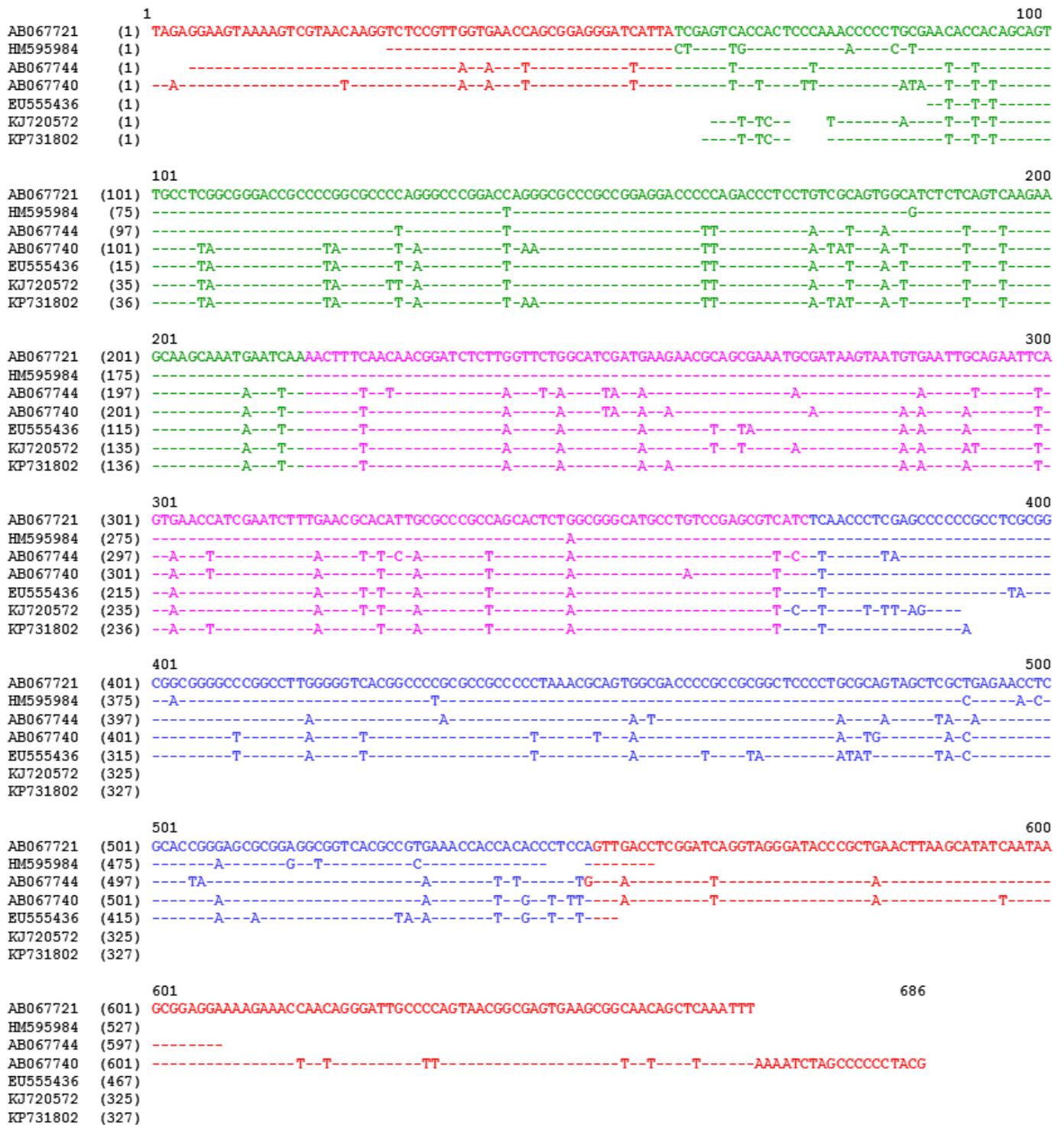
## 2.2 *O. sinensis* is the collective name for multiple fungi [6,10-13,15,17-18,25,27,42,44-51]

Jiang and Yao [17] summarized the isolation of 22 fungal species spanning 13 genera from natural *C. sinensis* specimens and reiterated the criteria for adequately confirming the correct anamorph of *O. sinensis*. According to these criteria [17-18], no report to date concerning any of the 22 fungal species has strictly and fully satisfied Koch's Postulates, leading to no direct evidence to prove or disprove any of the fungal species as the true anamorph of *O. sinensis*. Dong et al. [10] stated that "more than 20 anamorphic fungi were isolated from natural Chinese cordyceps and reported to be connected with the teleomorph of *O. sinensis*". Barseghyan et al. [27] confirmed that both *H. sinensis* and *Tolypocladium sinense* "were identified as the anamorphs of *Ophiocordyceps sinensis*". It is worth noting that Yang [21] previously speculated about hyperparasitism for *O. sinensis* and that Bushley et al. [52] reported the fluorescent-stained binucleate structure of hyphae and the mono-/bi-/trinucleate structures of ascospores of *C. sinensis* (Figure 3 of [52]). Xia et al. [48] used real-time qPCR and cloning-sequencing to profile the microbiota of natural *C. sinensis* samples collected from Nagqu in Tibet. They reported ITS sequences of 97 fungal clones and identified multiple fungi (gb KJ734995 - KJ735091), including dominant fungal species or Operational Taxonomic Units (OTUs) from the genera *Geomyces*, *Phoma*, and *Trichocladium* in the caterpillar body of *C. sinensis* and dominant species or OTUs from the genera *Geomyces* and *Cladosporium* in the stroma, in addition to the 16S sequences of 97 bacterial clones (gb KJ717845 - KJ717941). However, they did not detect *H. sinensis* ITS sequences from either the caterpillar body or the stroma of *C. sinensis* [48]. Similar findings were reported for both culture-dependent and culture-independent techniques by Zhang et al. [25,51], who detected different fungal dominance from the caterpillar body and the stroma of *C. sinensis* but no *H. sinensis* in either compartment.

## 2.3 *O. sinensis* is the collective name for multiple genotypes of fungi with multiple, scattered transition, transversion, and/or insertion/deletion point mutations, which probably evolved from the same genetic ancestor [12,13 26,60,49-50,53-59]

A BLAST search can identify hundreds of sequences registered in NCBI GenBank under the taxid 72228 and the name *C. sinensis* or *O. sinensis*. These sequences include at least 12 mutant genotypes of *O. sinensis* and can be grouped into 3 categories [13,50]: (1) 6 genotypes with multiple, scattered transition point mutations (Figures 1-3; Table 1); (2) 5 genotypes with large numbers of multiple, scattered point transversion mutations, in addition to some transition mutation bases (Figures 2 & 4; Tables 2-3); and (3) 3 *O. sinensis* sequences with multiple, scattered insertion and/or deletion mutations (ratio of Insertion/Deletion bases vs. Transversion/Transition mutant bases >1.0; cf. Figures 2 & 4, Tables 2-3).

The first 3 of the 6 transition mutant genotypes in Category 1 are GC rich (cf. Figures 1 & 2, Table 1): Genotype #1 (*H. sinensis*) is represented by AB067721 (AT=36.7%). Genotype #2 is a truncated form of Genotype #1 *H. sinensis* (the 320-bp amplicon shown in the left panel of Figure 3) with a distinct maturation pattern compared with Genotype #1 *H. sinensis*, shown as the 440(A)-bp amplicon in the left panel of Figure 3 [12]. Genotype #3 is a group of sequences represented by HM595984 (AT=36.7%) with 94.3% and 93.0% similarity with the ITS1 and ITS2 segments of Genotype #1 AB067721 (cf. Table 1), as well as 8 additional sequences as of July 2015: FJ654148, FJ654149, JQ286748, KM197540, KJ175197, KJ175199, KJ175203, KJ175205, and KJ175206 (98%-99% homology among the sequences in this group Genotype #3). Notably, the Genotype #3 sequences FJ654148, FJ654149, and JQ286748 were incorrectly included in Group A (Genotype #1) by Zhang S et al. [29].

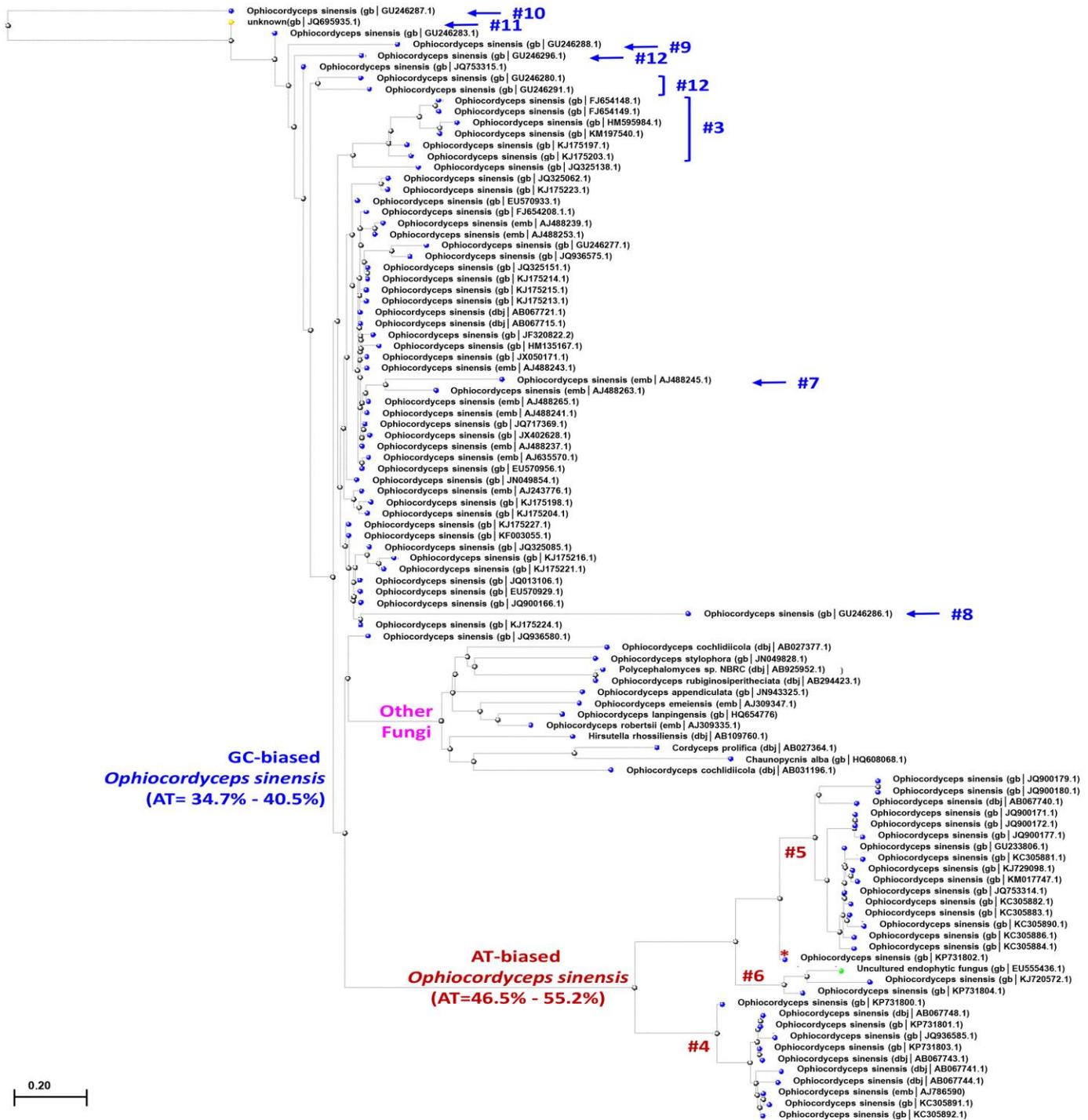


**Figure 1** ITS sequence alignment of 5 groups of transition point mutants of *O. sinensis*. AB067721 represents Genotype #1 of GC-biased *O. sinensis* fungi (including *H. sinensis*). HM595984 represents Genotype #3, a GC-biased mutant genotype. AB067744 (Genotype #4), AB067740 (Genotype #5), and EU555436 and KJ720572 (Genotype #6) represent 3 genotypes of AT-biased *O. sinensis* fungi, whereas KP731802 represents an AT-biased genotype with 98% homology to Genotype #5 and 97% homology to Genotype #6. The residues in red were annotated in GenBank as 18S (5' end region) or 28S (3' end region) rRNA; the residues in green were annotated as ITS1; the residues in pink were annotated as 5.8S rRNA; and the residues in blue were annotated as ITS2. Hyphens indicate identical bases, and spaces denote unmatched sequence gaps.

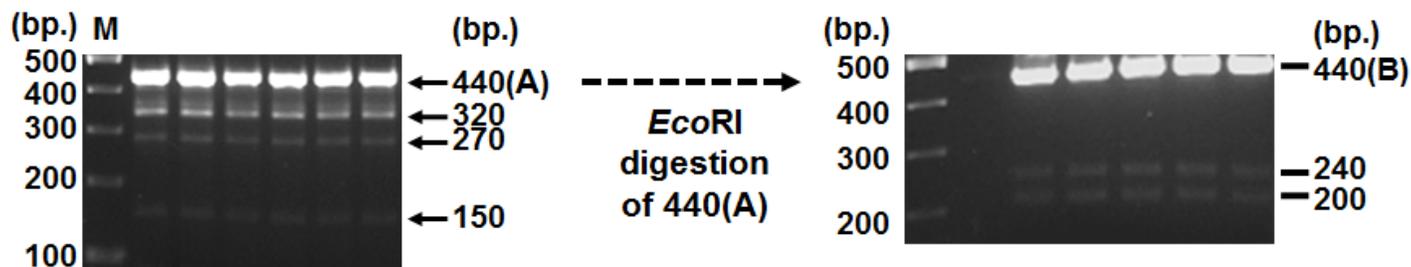
**Table 1. Segmented sequence similarities of the ITS1, 5.8S and ITS2 sequences of the GC-biased (Genotype #1 AB067721, Genotype #3 HM595984; cf. Figures 1&2) and AT-biased (Genotype #4 AB067744; Genotype #5 AB067740; and Genotype #6 EU555436) genotypes of *O. sinensis* fungi.**

	ITS1	5.8S	ITS2	ITS1-5.8S-ITS2 (excluding the 18S and 28S segments)
AB067721 vs. HM595984	94.3%	99.4%	93.0%	95.5%
AB067721 vs. AB067744	90.6%	85.3%	89.2%	88.4%
AB067721 vs. AB067740	80.5%	86.5%	89.2%	85.5%
AB067721 vs. EU555436	84.7%	87.8%	85.8%	86.0%
AB067721 vs. KP731802	82.8%	89.1%	—	86.5%
HM595984 vs. AB067744	87.4%	85.9%	83.1%	85.4%
HM595984 vs. AB067740	78.6%	87.2%	86.0%	84.0%
HM595984 vs. EU555436	84.5%	88.2%	80.8%	84.5%
HM595984 vs. KP731802	81.7%	89.7%	—	86.3%
AB067744 vs. AB067740	89.9%	92.3%	88.0%	91.4%
AB067744 vs. EU555436	94.7%	91.5%	84.4%	89.6%
AB067744 vs. KP731802	91.9%	92.9%	—	91.9%
AB067740 vs. EU555436	96.9%	93.6%	92.5%	94.1%
AB067740 vs. KP731802	97.9%	97.4%	—	97.8%
EU555436 vs. KP731802	96.9%	96.2%	—	96.7%

Note: The sequence alignment analyses for the entire ITS1-5.8S-ITS2 sequences and segmented sequences were performed using Vector NTI Advance 9 (Invitrogen) based on the *O. sinensis* sequences registered in GenBank, according to the segmentation annotations released by GenBank. “—” indicates inadequate sequence data for comparison because the ITS2 segment sequence of KP731802 is insufficiently long.



**Figure 2** Phylogenetic relationship of mutant *O. sinensis* genotype fungi constructed based on their ITS sequences using the Fast Minimum Evolution (pairwise alignment) algorithm in NCBI GenBank BLAST. The *O. sinensis* sequences were grouped into 12 genotypes: Genotypes #1-#3 and #7-#12 are GC-biased genotypes; Genotypes #4-#6 of Category 1 are AT-biased transition point mutation genotypes. Genotypes #1-#6 of Category 1 are transition point mutation genotypes (see Figure 3 for Genotype #2); Genotypes #7-#11 of Category 2 are transversion point mutation genotypes; and Genotype #12 of Category 3 features a large number of insertion/deletion point mutations. \*, KP731802 is highly homologous to Genotypes #5 (98%) and #6 (97%) and was placed between these 2 genotypes in this phylogenetic tree.



**Figure 3** Agarose gel electrophoresis of the PCR amplicons (Left Panel) and the *EcoRI* digestion of the 440(A)-bp amplicon (Right Panel) [Reproduced with permission from AJBMS (www.nwpjii.com/ajbms) Am J Biomed Sci 2010; 2(3): 217-238] [26].

**Left Panel:** Genomic DNA templates were prepared from the premature *C. sinensis* stroma (<1.5 cm in height). PCR was performed using the *H. sinensis*-specific primers Hsprp1/3 and a touchdown PCR protocol. The targeted 440(A)-bp amplicon shown in the left panel was recovered and sequenced, indicating 100% identity to that of Genotype #1, the GC-biased *H. sinensis*. A 320-bp amplicon was also recovered and sequenced, revealing a truncated, short sequence (Genotype #2) at the 5' and 3' ends and 100% identity to GC-biased *H. sinensis* sequences.

**Right Panel:** The recovered 440(A)-bp amplicon was subjected to overnight *EcoRI* digestion and analyzed by agarose gel electrophoresis. A trace amount of the 440(A)-bp amplicon was digested with *EcoRI* (200- and 240-bp DNA moieties), representing the GC-biased transition mutant *O. sinensis* species (Genotypes #1 & #3), the GC-biased transversion mutant *O. sinensis* species (Genotypes #7-#11), and the GC-biased deletion/insertion mutant *O. sinensis* species (Genotype #12) (cf. Figures 1 & 4). The majority of the 440(A)-bp amplicon was not digested with *EcoRI*, corresponding to the 440(B)-bp DNA moiety in the right panel. The 440(B) moiety represents Genotypes #4-#6, the AT-biased *O. sinensis* species with a single C-to-T mutation at the 299 allele site in the AB067721 sequence, which results in the loss of the *EcoRI* site (cf. Figure 1). The *EcoRI*-digestible component of the 440(A)-bp amplicon exhibited a development-maturation pattern completely different from that of the 320-bp amplicon (Genotype #2) (Left panel) during the maturation of natural *C. sinensis* [12].

The other 3 transition mutant genotypes in Category 1 are relatively AT rich (cf. Figures 1 & 2, Table 1): Genotype #4, represented by AB067744 (AT=47.8%; 103 entries in GenBank as of July 2015); Genotype #5, represented by AB067740 (AT=51.9%; 35 entries in GenBank as of July 2015); and Genotype #6, represented by KJ720572 (AT=55.2%), which shares 93% similarity with Genotype #4 AB067744, 94% similarity with Genotype #5 AB067740, and <86% similarity with the remaining *O. sinensis* genotypes, 2 other entries in this group (98% homology): EU555436 (AT=48.7%) and KP731804 (AT=50.4%) [12-13,26,29,30,49-50,53-59,61]. Notably, Genotypes #4 and #5 were labeled as Groups B and C, respectively, by Stensrud et al. [53]; the Genotype #6 sequence EU555436 was incorrectly included in Group C (Genotype #5) by Li Yi et al. [57]. KP731802 (AT=54.0%), one of the recently obtained AT-biased *O. sinensis* sequences in a cloning-

sequencing study of the ascocarps of natural *C. sinensis* specimens, shares 98% homology with Genotype #5 AB067740 and 97% with Genotype #6 EU555436 and KP731804 (cf. Figure 1, Table 1), residing between Genotypes #5 & #6 in the phylogenetic tree (cf. Figure 2). Further extending the ITS2 segment sequence of KP731802 towards its 28S nrDNA segment may help to determine whether KP731802 truly belongs to Genotype #5 or #6 or to a parental genotype of both. Mao et al. [58] reported that Genotypes #4 and #5 shared the same mycelial morphology as Genotype #1 *H. sinensis*, but the size of the conidia was significantly greater for Genotype #4 than for Genotype #5. Genotypes #5 and #6 were predominantly detected in the stroma of natural *C. sinensis* specimens in the late maturation stages and in the ascospores, while Genotype #4 is present in the ascocarp of natural *C. sinensis* [12,54,56-57,60].

**Table 2 Segmented sequence similarities of the ITS1, 5.8S and ITS2 sequences of the GC-biased AB067721 (Genotype #1 of Category 1) and transversion mutant *O. sinensis* genotypes: Genotype #7, AJ488254; Genotype #8, GU246286; Genotype #9, GU246288; Genotype #10, GU246287; Genotype #11, JQ695935 of Category 2; and insertion/deletion point mutant *O. sinensis* Genotype #12, GU246296 of Category 3.**

	ITS1	5.8S	ITS2	ITS1-5.8S-ITS2 (excluding the 18S and 28S segments)
AB067721 vs. AJ488254	93.2%	98.7%	89.4%	93.9%
AB067721 vs. GU246286	86.2%	94.8%	87.9%	89.6%
AB067721 vs. GU246288	96.3%	98.7%	91.5%	95.3%
AB067721 vs. GU246287	86.2%	92.9%	72.4%	83.2%
AB067721 vs. JQ695935	94.3%	100%	55.1%	81.6%
AB067721 vs. GU246296	99.4%	99.4%	87.0%	94.9%
AJ488254 vs. GU246286	82.0%	93.6%	80.7%	85.6%
AJ488254 vs. GU246288	89.4%	97.4%	84.5%	90.6%
AJ488254 vs. GU246287	81.4%	92.3%	67.8%	80.6%
AJ488254 vs. JQ695935	88.8%	98.7%	59.5%	82.8%
AJ488254 vs. GU246296	92.5%	98.1%	77.0%	89.5%
GU246286 vs. GU246288	83.8%	93.6%	85.2%	87.4%
GU246286 vs. GU246287	77.5%	87.8%	72.0%	78.7%
GU246286 vs. JQ695935	83.8%	94.9%	56.3%	77.5%
GU246286 vs. GU246296	86.9%	94.3%	82.3%	87.6%
GU246288 vs. GU246287	84.4%	92.9%	73.1%	82.8%
GU246288 vs. JQ695935	92.5%	98.7%	52.8%	79.4%
GU246288 vs. GU246296	96.9%	98.1%	87.4%	93.9%
GU246287 vs. JQ695935	83.9%	92.4%	48.1%	73.3%
GU246287 vs. GU246296	86.9%	91.7%	76.4%	84.6%
JQ695935 vs. GU246296	95.6%	99.4%	54.6%	81.7%

Note: The sequence alignment analyses for the entire ITS1-5.8S-ITS2 sequences and segmented sequences were performed using Vector NTI Advance 9 (Invitrogen) based on the *O. sinensis* sequences registered in GenBank, according to the segmentation annotations released by GenBank. GenBank did not provide segmentation information for nrDNA sequence JQ695935 (Genotype #11). The segmentations for JQ695935 are based on the alignment to the sequence segments of AB067721.

**Table 3 Percentages of AT residues in the ITS segments of Genotype #1 (the GC-biased AB067721) of Category 1, Genotypes #7-#11 of the *O. sinensis* transversion mutants (AJ488254, GU246286, GU246288, GU246287 and JQ695935) of Category 2, and Genotype-#12 of the Insertion/Deletion mutants (GU246280 and GU246296) of Category 3.**

Genotype	GenBank Accession #	Residues		AT (%) of total	# of mutant bases vs. AB067721			Ratio of	
		AT	GC		In./De.	Transv.	Transit.	Transv. vs. Transit.	In./De. vs. Transv./Transit.
#1	AB067721	264	413	39.0%					
#7	AJ488254	184	280	34.7%	4	9	10	0.90	0.21
#8	GU246286	216	318	40.5%	4	25	27	0.93	0.08
#9	GU246288	190	344	35.6%	8	11	7	1.57	0.44
#10	GU246287	211	314	40.2%	12	51	29	1.76	0.15
#11	JQ695935	272	350	43.7%	29	75	40	1.88	0.25
#12	GU246296	203	357	36.3%	17	8	5	1.60	1.31
	GU246280	205	262	36.2%	24	3	4	0.75	3.43

Note: Alignment analyses were performed using the “discontinuous megablast” algorithm in NCBI GenBank Blast. “In./De.” refers to the sum of insertion/deletion mutation bases; “Transv.” refers to transversion mutation bases; and “Transit.” refers to transition mutation bases.

A BLAST search of the GenBank database identified 5 GC-biased transversion mutant genotypes (AT=34.7%-43.7%) in Category 2 (*cf.* Table 2). In ascending order of the mutant base ratios of the transversion *vs.* transition point mutations, as shown in the upper panel of Table 3, they are Genotype #7, represented by AJ488254; Genotype #8, represented by GU246286; Genotype #9, represented by GU246288; Genotype #10, represented by GU246287; and Genotype #11, represented by JQ695935. In contrast with the multiple transition point mutations in Genotypes #3-#6, Genotypes #7-#11 contain multiple, scattered transversion point

mutations with some transition point mutations, and the ratios of transversion mutant alleles to transition mutant alleles were 0.90 (9:10), 0.93 (25:27), 1.57 (11:7), 1.76 (51:29), and 1.88 (75:40), respectively (*cf.* Table 3). Each genotype displays low overall similarity (81.6%-95.3%) with Genotype #1 ITS1-5.8S-ITS2 segments or 55.1%-91.5% similarity with the ITS2 segments of Genotype #1 (*cf.* Table 2). These transversion mutants have insertion/deletion *vs.* transversion/transition mutant base ratios of <0.5, distinct from Genotype #12, with a mutant base ratio >1.0 (*cf.* Table 3).



**Figure 4** ITS sequence alignment of the 5 groups of *O. sinensis* transversion mutants and 2 insertion/deletion mutants of *O. sinensis* compared with AB067721, using Vector NTI Advance 9 (Invitrogen). AB067721 represents Genotype #1, the GC-biased *O. sinensis*, as the reference sequence. AJ488254 (RC, reverse-complement), GU246286, GU246288, GU246287, and JQ695935 represent Genotypes #7, #8, #9, #10, & #11 of Category 2, the *O. sinensis* transversion mutants. GU246296 and GU246280 represent Genotype #12 of Category 3, the insertion/deletion mutants of *O. sinensis*. The residues in red were annotated as 18S (5' end) or 28S (3' end) rRNA; the residues in green were annotated as ITS1; the residues in pink were annotated as 5.8S rRNA; and the residues in blue were annotated as ITS2. Hyphens indicate identical bases, and spaces denote unmatched sequence gaps.

Genotype #12, Category 3, includes 3 GC-biased (AT=36.2%-36.3%) sequences (GU246296, GU246280, and GU246291) as of July 2015, and contains many insertion and deletion point mutations (17-24 bases) in the ITS1-5.8S-ITS2 segments (*cf.* Figure 4, Table 3). These sequences also contain a few transversion or transition point mutations. The insertion/deletion *vs.* transversion/transition mutant base ratios range from 1.31 to 3.43. This group of mutants shows high similarity to the ITS1 and 5.8S segment sequences of Genotype #1 *H. sinensis* but low similarity to the ITS2 sequence of Genotype #1 (e.g., 87% for GU246296 *vs.* AB067721 ITS2 sequence) (*cf.* Table 2).

In the scientific literature, a threshold of 97% is commonly accepted as indicative of homology [25]. The ITS1-5.8S-ITS2 segment sequences (excluding the partial 18S and 28S segment portions) exhibited overall similarities of 73.3% to 95.5% in pair-wise comparisons between Genotype #1 and Genotypes #3-#12 and among the mutant genotypes, except for sequence KP731802, whose position in relation to Genotypes #5 and #6 is unclear, as mentioned above (*cf.* Tables 1-2). The low similarities suggest that these genotypes belong to independent fungi that likely evolved from a common genetic ancestor [13,50,53-54]. An extended BLAST search revealed that these 12 mutant genotypes have high-scoring hits (80.6%-100%) with over 600 entries of *O. sinensis*, *C. sinensis*, and *H. sinensis* sequences, all under the taxid 72228 in GenBank, excluding those sequences registered with other taxa and indeterminate taxa, labeled “sp.”.

Tables 1-2 also provide the segmentation similarities (nrDNA ITS1, 5.8S, and ITS2) among the mutant genotypes. In contrast with the relatively high similarities (92.9%-100%) for the 5.8S ribosomal RNA genes when comparing Genotype #1 AB067721 with the GC-biased Genotypes #3, #7-#12, the similarities varied from 86.2% to 99.4% for the ITS1 segment and from 55.1% to 93.0% for the ITS2 segment (*cf.* Table 2). The low percentages of AT residues in the ITS sequences of Genotypes #3, #7-#12 are similar to that of Genotype #1 (*cf.* Table 3) but

are much lower than those (46.5%-55.2%) for the AT-biased Genotypes #4-#6. Alignment with the AB067721 sequence revealed that the insertion/deletion mutations comprise 4, 4, 8, 12, and 29 bases in the ITS segments of Genotypes #7-#11, respectively. However, Genotype #12 (GU246280, GU246291, and GU246296) features multiple scattered insertion and/or deletion mutations, resulting in insertion/deletion *vs.* transversion/transition point mutation ratios of 1.31-3.43 (*cf.* Table 3).

As shown in Figure 2, the phylogenetic tree branches into 2 major clades: (1) the GC-biased cluster (including Genotypes #1, #3, #7-#12 and other fungi) and (2) the AT-biased cluster (including Genotypes #4-#6). An “other fungi” clade is situated much closer in phylogenetic distance to the GC-biased *O. sinensis* cluster than the longer phylogenetic distances between the *O. sinensis* clusters for the GC-biases (Genotypes #1, #3, #7-#12) and AT-biases (Genotypes #4-#6). As of July 2015, when the GenBank BLAST search was expanded to 1000 maximum target sequences, the “other fungi” clade comprised at least 37 entries in GenBank, including *Chaunopycnis* sp., *Elaphocordyceps* sp., *Hirsutella* sp., *Metacordyceps* sp., *Ophiocordyceps* sp., *Polycephalomyces* sp., and some unknown fungal species, in addition to those fungal species or OTUs identified from natural *C. sinensis* specimens by Zhang YJ et al. [25,51], Zhang S et al. [29] and Xia et al. [48].

From the above sequence and phylogenetic analyses, none of the 3 hypotheses (*cf.* Sections 2.1, 2.2, and 2.3) regarding the definition of *O. sinensis* have been scientifically confirmed strictly according to Koch’s Postulates; therefore, they should be treated equally scientifically. Based on the complex fungal background of natural *C. sinensis* and the uncertain definition of *O. sinensis*, it is difficult at this time to implement the IMA Amsterdam Declaration 1F1N rule because *O. sinensis* may not be a single fungal species (see further discussion below) [4,13,28-30,39-41,50]. We hope that the International Commission on the Taxonomy of Fungi (ICTF) will soon establish mechanisms and procedures for handling academic disputes or a committee for fungi, as promised in the

Amsterdam Declaration signed by 88 IMA members, providing for fair exchange of opinion and a platform for disputing controversies [39-41]. In addition to the indiscriminate use of the term *O. sinensis* for both the wild product of the insect-fungi complex and the teleomorph-holomorph of the fungus/fungi, as discussed above, the implementation of the 1FIN rule in natural *C. sinensis* research at this time might create further confusion through the indiscriminate use of the term *O. sinensis* for multiple anamorphic fungi (cf. Sections 2.2 and 2.3 and Figure 2). For instance, Hu et al. [16] described their study material as *O. sinensis* and did not clarify in the manuscript whether the sample was the natural *C. sinensis* insect-fungi complex, teleomorphic *O. sinensis* fungi, or anamorphic *O. sinensis* fungi, nor was it clear which transition, transversion and insertion/deletion mutant genotypes of *O. sinensis* fungi were studied. It is critical for readers to understand this uncertainty in the study materials, results and conclusion, which was clarified by the sponsoring author of that study only upon questioning at a scientific conference. The genomic DNA sample isolated from the *H. sinensis* Co18 strain through the purification of single conidia (asexual form of spores) presented a feature of monokaryosis [16]; this monokaryotic genome differs completely from the genome isolated from the mycelial cultures derived from single ascospores (sexual form of spores); these cultures were likely heterokaryotic and mono-/bi-/trinucleate [52,57] (additional discussion below). In addition, Liu et al. [9] published a study entitled “Transcriptome sequencing and analysis of the entomopathogenic fungus *Hirsutella sinensis* isolated from *Ophiocordyceps sinensis*”, where the term *O. sinensis* was clearly referring not to the teleomorphic fungus but to the wild product and where the anamorphic name *H. sinensis* could not simply be replaced with the teleomorphic name *O. sinensis*, lest confusion be caused by incorrectly stating “*O. sinensis* isolated from *O. sinensis*”. Many other studies have used the same term, either *C. sinensis* or *O. sinensis*, to refer to the wild product, teleomorph/holomorph of *O. sinensis* fungus/fungi, and anamorph of *O.*

*sinensis* fungus/fungi in different contexts, resulting in different and incongruous understandings and interpretations. In addition to the academic confusion regarding the taxonomy, the enforcement of the 1FIN rule in *C. sinensis* research is currently supported only the first hypothesis (cf. Section 2.1) and is inconsistent with and therefore rejects the other 2 hypotheses (cf. Sections 2.2 and 2.3); however, this conclusion is unfortunately not based on experimental evidence. Therefore, the indiscriminate use of the same Latin name for anamorphic fungi, teleomorphic fungi and wild *C. sinensis* of the insect-fungi complex violates the principles of Latin nomenclature and the principle of academic fairness and will continue to cause confusion in academic publications, in annotations in the INSD (including GenBank), and even in governmental documents for regulation of marketed products.

### 3. Genetic heterogeneity of natural *C. sinensis*

The isolation of *C. sinensis*-related fungi from natural *C. sinensis* specimens collected from different geographical areas resulted in the identification of 22 fungal species spanning 13 genera [4,15,17-18]. Using both culture-dependent mycological and molecular approaches, Zhang et al. [25] reported the identification of more than 90 fungal species or OTUs from at least 37 genera from the stroma, sclerotia, or external mycelial cortices of natural *C. sinensis* collected from Tibet and Sichuan Provinces. To profile the microbiota of natural *C. sinensis* specimens using real-time PCR and cloning-sequencing techniques, Xia et al. [48] identified the dominant fungal species or OTUs from the genera *Geomyces*, *Phoma*, and *Trichocladium* in the caterpillar body and the dominant fungal species or OTUs from the genera *Geomyces* and *Cladosporium* in the stroma, in addition to the identification of multiple bacterial species. Microbiota profiling studies by Zhang et al. [25] and Xia et al. [48] using various techniques did not report the detection of Genotype #1 *H. sinensis* from the caterpillar body and stroma of natural *C. sinensis* specimens, instead suggesting different dominant fungi in the *C. sinensis*

compartments. Of the over 50 participants in a *C. sinensis* research conference on Oct 29, 2005, 19 scholars reached a hypothetical “consensus” that *H. sinensis* is the sole anamorph of *O. sinensis* based entirely on the compilation of indirect evidence obtained using several techniques [4,14-15,20]. However, no scientific reports have yet to fully satisfy Koch’s Postulates, and no successful artificial production of sexual fruiting bodies and ascospores derived from any of the absolutely pure, postulated anamorphic fungal strains under strict aseptic conditions has been documented [6,13,15-21,50]. Unfortunately, the majority of natural fungi still cannot be cultured [23-26], although culture-independent molecular techniques have enabled the identification of greater numbers of fungal taxa and genotypes in natural *C. sinensis* specimens [8,11-13,26,42,46-49,58-61]. Several studies have also reported dynamic changes in the abundance (reflecting the rates of fungal proliferation, degradation and other biological processes) of *H. sinensis*, other fungi and the mutant genotypes of *O. sinensis* in the different compartments of natural *C. sinensis* during maturation [12-13,26,49,54-57,59-60,62].

Using mycelia from the tissue cultures of the *C. sinensis* caterpillar body as the study material, Mao et al. [58] reported the detection of significantly larger conidia belonging to Genotype #4 AB067744 of the AT-biased mutants (*cf.* Figures 1 & 2) from *C. sinensis* specimens collected from Deqing in Yunnan Province, and significantly smaller conidia belonging to Genotype #5 AB067740 (GU233806) of the AT-biased mutants from *C. sinensis* specimens collected from Tibet, Qinghai and Sichuan Provinces. In contrast with the detection of the ITS sequence of Genotype #1 *H. sinensis* [14,63-69], the detection of AT-biased Genotypes #4 and #5 *O. sinensis* mutants, but not of Genotype #1 *H. sinensis*, by Mao et al. [58] contradicted the hypothesis that *H. sinensis* is the sole anamorph of *O. sinensis* and was inconsistent with the study conclusion of the microcycle conidiation of the *C. sinensis* ascospores [20,70-71] (more discussion below). Non-detection of Genotype #1 *H. sinensis* from the stroma and caterpillar body of *C. sinensis* has

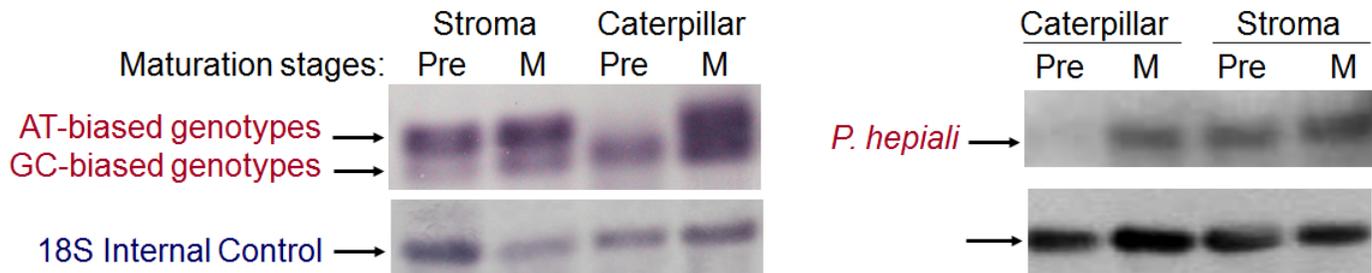
similarly been reported in other microbiota studies [25,48].

Can these multiple mutant *O. sinensis* fungi be detected from a single set of genomic samples isolated from *C. sinensis* specimens collected from a single production area? In other words, are the PCR ITS amplicons of a single *C. sinensis* genomic DNA sample homogeneous or heterogeneous? In an impure sample pool, such as the *C. sinensis* insect-fungi complex, the dominant DNA components in the pool of amplicons may represent the fungal DNA components with the highest amplification efficiency under particular PCR conditions and may or may not represent the actual dominance of the fungal species in natural *C. sinensis* specimens. The dominant and minor components of the PCR amplicons that can be sequenced may lack secondary structures or specific conformations that cause sequencing failures [26,55-57].

To specifically address the question of amplicon heterogeneity, a dual-step nested-PCR strategy was used, comprising an initial PCR using “universal” primers and a second PCR using inward, fungus-specific primers against the amplicon templates obtained from the first PCR, followed by molecular cloning-sequencing [49,54]. The results demonstrated the coexistence of the ITS sequences of the GC-biased *H. sinensis* (Genotype #1), AT-biased *O. sinensis* fungi (Genotypes #4 and #5), and *P. hepiali* in the stroma and caterpillar body of both premature and mature *C. sinensis* specimens collected from Sichuan and Qinghai Provinces [49]. Subsequent studies using the same strategy but different fungus-specific primers for the second PCR confirmed the coexistence of the GC- and AT-biased genotypes of *O. sinensis* fungi and *P. hepiali* and uncovered additional AT-biased genotypes of *O. sinensis* (Genotype #6, and sequence KP731802, which is highly homologous to both Genotypes #5 and #6; *cf.* Figures 1 & 2 and Table 1) from the caterpillar body, stroma, ascocarp, and ascospore samples of natural *C. sinensis* collected from Sichuan and Qinghai Provinces [11-12,26,50,59-60]. Another study of the cultured mycelia of single-ascospore isolates of *C. sinensis* collected from Qinghai

Province detected Genotypes #1 (GC-biased) and #5 (AT-biased) of culturable *O. sinensis* fungi using genotype-specific primers [57]. Southern blot analysis also confirmed the coexistence of

both GC- and AT-biased genotypes of *O. sinensis* fungi and *P. hepiali* (cf. Upper panels of Figure 5) [26,59].



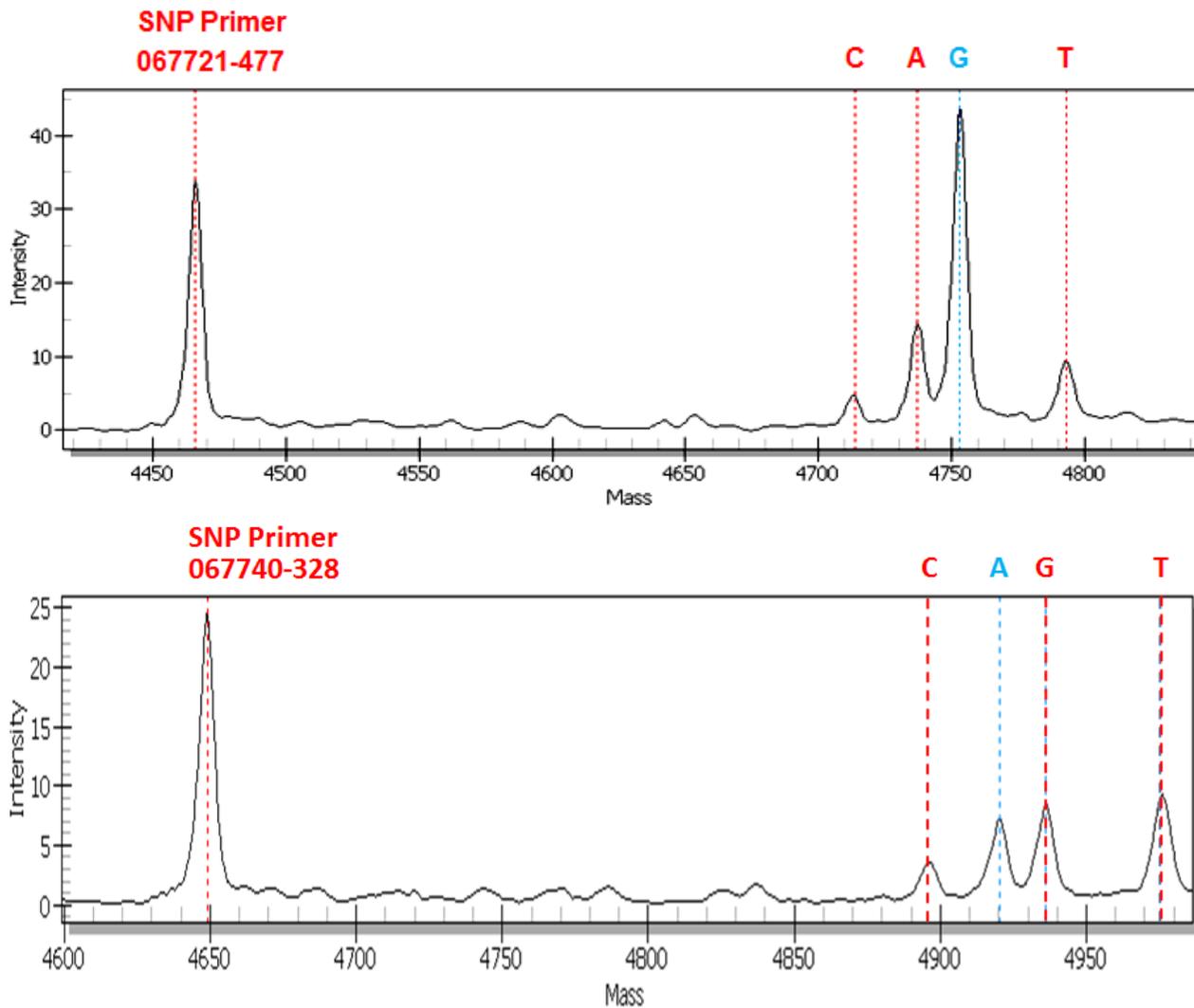
**Figure 5** Southern Blot of *C. sinensis* nrDNA in the stroma and caterpillar body of natural *C. sinensis* specimens during maturation. [Reproduced with permission from AJBMS ([www.nwpii.com/ajbms](http://www.nwpii.com/ajbms)) Am J Biomed Sci 2010; 2(3): 217-238] [26].

**Upper-Left Panel:** Using *H. sinensis*-specific probe for Southern blotting. **Upper-Right Panel:** Using *P. hepiali*-specific probe for blotting. **Both Lower Panels:** Using nonspecific 18S internal control probe for blotting. PreM, premature *C. sinensis*; M, mature *C. sinensis*.

To further confirm the coexistence of multiple genotypes of *O. sinensis* fungi, a biochip-based MassARRAY single nucleotide polymorphism (SNP) matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) technique was used to genotype the natural *C. sinensis* specimens using several extension primers specific to SNP alleles in the sequences of *O. sinensis* genotypes. When the premature stroma of *C. sinensis* collected from Sichuan Province were examined, the coexistence of 2-4 SNP alleles was reported, representing both transition and transversion point mutations, at each of several SNP sites [26,55]. As shown in the upper panel of Figure 6, using the extension primer 067721-477 [26], Allele G represents the GC-biased Genotypes #1-#3, #7-#9, and #11-#12; Allele A represents the AT-biased Genotypes #4-#6, with a single-base G→A transition mutation (cf. Figure 1); and Alleles C and T represent the single G→C and G→T transversion mutations, possibly in Genotype #11

JQ695935 and Genotype #10 GU246287, respectively.

As shown in the lower panel of Figure 6, using the extension primer 067740-328 [26], Allele A (reverse-complement sequence; “T” at nucleotide 328 in the AB067740 sequence shown in Figure 1) represents Genotypes #5 and #6 (the AB067740 and KJ720572 sequences); Allele G (reverse-complement nucleotide; “C” shown in Figure 1) represents Genotype #4 AB067744; and Alleles T and C represent 2 unknown transversion mutant genotypes. Additional SNP MS analysis (not shown in this review) identified a G→T transversion mutant SNP using extension primer 067721-531, corresponding to Genotype #10 GU246287; a C→A transversion mutant SNP using extension primer 067740-324, corresponding to Genotype #7 AJ488254; and a G→T transversion mutant SNP using extension primer 067740-360, corresponding to Genotype #10 GU246287 [12,26,55-56,60].



**Figure 6. MassARRAY SNP MALDI-TOF mass spectra [Reproduced with permission from AJBMS ([www.nwpii.com/ajbms](http://www.nwpii.com/ajbms)) Am J Biomed Sci 2010; 2(3): 217-238] [26].**

**Upper panel:** The SNP extension primer 067721-477 was used to distinguish between the GC- and AT-biased genotypes and the transversion mutation genotypes of *O. sinensis*. The extension reaction template was the amplicon from PCR with the “universal” primer pair ITS5/4. The 067721-477 primer was extended to the SNP at position 477 in the AB067721 sequence (*cf.* Figures 1 & 4 for the allele location). Allele “G” represents GC-biased Genotypes #1-#3, #7-#10, and #12 (*cf.* Figures 2 & 4) and is the result of a primer extension reaction with an extended guanine. Allele “A” indicates Genotypes #4-#6 (the AT-biased genotypes) and was the result of a primer extension reaction with an extended adenine. Alleles “C” and “T” are the results of primer extension reactions with an extended cytosine or thymine, representing Genotype #11 JQ695935 with a G→C transversion point mutation, and Genotype #10 GU246687 with a G→T transversion point mutation, respectively.

**Lower panel:** The SNP extension primer 067740-328 was used to distinguish between the AT-biased genotypes of *O. sinensis* and the unknown transversion mutation genotypes. The extension reaction template was the PCR amplicon obtained using the AT-biased *O. sinensis*-specific primer pair HsATp1/2. The SNP extension primer 067740-328 was extended to the SNP at position 328 in the reverse-complement sequence of AB067740 (*cf.* Figure 1 for the allele location). Allele “G” (reverse-complement; Allele “C” at the position 328 in the AB067744 sequence shown in Figure 1) represents Genotype #4 AB067744 and was the result of a primer extension reaction with an extended guanine. Allele “A” represents the AB067740 and KJ720572 sequences (Genotypes #5 and #6) and is the result of a primer extension reaction with an extended adenine. Allele “C” and “T” denote 2 transversion mutant genotypes of unknown sequences and are the results of primer extension reactions with an extended cytosine or thymine, respectively.

The coexistence of multiple transition, transversion and insertion/deletion mutant genotypes was observed under dynamic maturational alterations. Upon the maturation of natural *C. sinensis*, the MS peak heights of some alleles were higher or lower, and some alleles disappeared [12,55-56]. Restriction fragment length polymorphism (RFLP), cloning-sequencing, SNP MS genotyping, and Southern blotting revealed a potential predominance of Genotype #4 (AB067744) in the early development of *C. sinensis* stroma, as well as the *C. sinensis* ascocarps, whereas Genotypes #5 and #6 (AB067740 and KJ720572) of the AT-biased *O. sinensis* species dominated the mature stroma of *C. sinensis* and the ascospores [12,26,56,60]. Li Yi et al. [57] reported the successful detection of Group C (Genotype #5) ITS sequences and the unsuccessful detection of Group B (Genotype #4) ITS sequences in the mycelial culture of the wild-type ascospores of *C. sinensis*, in addition to the detection of Genotype #1 AB067721. The aforementioned dynamic maturational alterations were observed in studies of *C. sinensis* specimens collected from a single production area [12,56-57,60], whereas a geographical distribution pattern of the AT-biased genotypes, associated with conidia of various sizes, was observed using tissue cultures of the caterpillar bodies of *C. sinensis* (unfortunately, no clear descriptions of the maturation stages of the natural specimens were given) [58].

The heterogeneity of the PCR amplicons can also be viewed in an endonuclease digestion assay [12,26]. For instance, the GC-biased sequences (Genotypes #1-#3 and #7-#12; cf. Figure 1) contain an *EcoRI* site (GAATTC) at nucleotides 294-299 in the Genotype #1 AB067721 sequence (cf. Figures 1 & 4). This restriction site is lost in the AT-biased sequences (Genotypes #4-#6; cf. Figure 1), reflecting a single C→T transition mutation to GAATTT at the bases allelic to position 299 in the AB067721 sequence. Using a pair of Hsprp1/3 primers that are specific for Genotype #1 *H. sinensis*, the 4

PCR amplicons were amplified from the premature stroma of *C. sinensis* as shown in the left panel of Figure 3, in which the 440(A)-bp amplicon moiety predominates [26]. After overnight incubation with *EcoRI*, digestion of trace amounts of the recovered 440(A)-bp amplicons was observed, apparent as two faint fragments (200- and 240-bp) in the right panel of Figure 3, representing Genotypes #1-#3 and #7-#12. The vast majority of the recovered 440(A)-bp amplicons were *EcoRI*-resistant, apparent as the 440(B)-bp DNA moiety in the right panel of Figure 3, representing the AT-biased Genotypes #4-#6, which are the dominant components of the heterogeneous 440(A) amplicons [26]. Although Genotype #1 (the 200- and 240-bp fragments in the right panel of Figure 3) and Genotype #2 (the 320-bp amplicon in the left panel of Figure 3) are both GC-biased [26], these 2 genotype moieties display distinct development patterns during *C. sinensis* maturation [12]. The results of the *EcoRI* digestion RFLP assay (cf. Figure 3) and Southern blot assay (cf. Figure 5) demonstrated that GC-biased *H. sinensis* (Genotype #1) is a minor component of the heterogeneous genomic DNA pool in the stroma of *C. sinensis*, with or without using PCR amplification, although the relative quantity of the GC-biased genotypes, including Genotype #1 *H. sinensis*, showed a slight increase during *C. sinensis* maturation [12,26,59]. A study using real-time qPCR and amplicon cloning profiled the microbiota of natural *C. sinensis* specimens and detected ITS sequences of Genotype #1 in 2 amplicon clones out of a total of 43 clones from the external mycelial cortices of natural *C. sinensis* specimens, but none were detected from the specimens of the stroma (41 clones) or caterpillar body (40 clones) of *C. sinensis* [48]. Of the 97 sequences submitted to GenBank, the ITS sequences of the other 95 clones belong to other fungi or undetermined fungi, in addition to the 97 bacterial 16S sequences submitted to GenBank. To investigate the fungal infection pathways, Lei et al. [72] also used real-time qPCR to

quantify the fungal species existing in the tissues (including body wall, fat body, hemolymph and intestinal wall) of host *Thitarodes* larvae. However, the primers that they designed (IF2/IR2) had extremely high homology with the ITS1-5.8S segment sequences of Genotypes #1-#3 and #7-#12 of GC-biased *O. sinensis*, leading to non-conclusive results for the biomass quantifications of the multiple fungal species.

The conservation of 5.8S nrDNA in fungal species over evolutionary time scales has been hypothesized, and the large sequence variation (85%-89% similarity; *cf.* Table 1) in the 5.8S segment between the GC-biased Genotype #1 and the AT-biased genotypes “far exceeds what is normally observed in fungi, even at higher taxonomic levels (genera and families)” [26,53,55-56]. The 5.8S segment was 92%-97% similar among the AT-biased genotypes (*cf.* Table 1). Accelerated evolution (92.9% and 94.8%) in the 5.8S genes proposed by Stensrud et al. [53] can also be observed between Genotype #1 and the transversion mutation Genotypes #8 and #10, respectively (*cf.* Table 2), but the 5.8S sequences of Genotypes #7, #9, and #11-#12 are highly conserved (>98%). Taking advantage of the low similarity of the 5.8S segment amongst the AT-biased genotypes, as shown in the lower panel of Figure 6, SNP MS genotyping using extension primers 067740-328 distinguished between Genotype #4 AB067744 and Genotypes #5/#6 AB067740/KJ720572 from the heterogeneous PCR amplicon pool amplified from the genomic DNA samples of the *C. sinensis* stroma collected from Sichuan Province [12,26,55-56,60]. In addition, the 2 transversion mutant SNP alleles (Alleles T and C) shown in the lower panel of Figure 6 did not match any of the known transversion mutant genotype sequences described in this review, indicating the heterogeneous coexistence of at least 2 additional unknown transversion mutant genotypes in the PCR amplicon pool from a single *C. sinensis* genomic DNA sample. Using the extension primer 067740-324, in addition to the

detection of Allele T for Genotype #4 AB067744 and Allele C for Genotype #5 AB067740, Allele A was also detected with a high MS peak height [26], probably representing the C→A transversion mutation at nucleotide 273 in the Genotype #7 AJ488254 sequence (*cf.* Figure 4). A low MS peak height of Allele T was detected at position 360 in the Genotype #5 AB067740 sequence using the extension primer 067740-360 [56], probably representing the G→T transversion mutation at nucleotide 306 in the Genotype #10 GU246287 sequence; the MS peak height of this allele significantly increased in the stroma with *C. sinensis* maturation. Future studies will likely uncover additional heterogenetic information and identify more transversion mutants in natural *C. sinensis* specimens.

The above literature review clearly demonstrates the genetic heterogeneity of natural *C. sinensis* specimens and the molecular heterogeneity of the PCR amplicons using the “universal” primers and primers specific to GC-biased Genotype #1 or AT-biased genotypes, as well as the observation of heterokaryotic structures of the *C. sinensis* hyphae and ascospores [52,57]. Molecular systematic examination of genetic heterogeneity demonstrated that Genotype #1 *H. sinensis* is not the dominant fungal species in the compartments of natural *C. sinensis* specimens. Proving that the minor fungal species Genotype #1 *H. sinensis*, rather than the major AT-biased species, is the sole anamorph of *O. sinensis*, as hypothesized (*cf.* Section 2.1), must rely on direct evidence obtained from re-inoculation and re-isolation experiments strictly following Koch’s Postulates, using absolutely pure, postulated anamorphic fungus and obtaining the sexual fruiting bodies and ascospores of *C. sinensis* under strictly aseptic experimental conditions. Given the unavailability of the direct evidence to confirm the “*H. sinensis* sole anamorph” hypothesis for *O. sinensis* (*cf.* Section 2.1), the above literature analysis also indicates that treating the aforementioned mutant genotypes as “incorrect sequences”, as

suggested by Zhang et al. [51], may be neither convincing nor scientifically sound.

#### **4. Do the multiple *O. sinensis* sequences represent pseudogenes of the *H. sinensis* genome or mutant fungal species independent of the *H. sinensis* genome?**

Li Yi et al. [57] sequenced mycelia cultured from wild-type single-ascospore isolates and identified the coexistence of both the Group A (Genotype #1) GC-biased genotype and Group C (Genotype #5) of the AT-biased transition mutant genotypes of *O. sinensis* from the genomes of single-ascospore isolates; but they were unable to detect any of the 5.8S gene transcripts of the AT-biased genotypes. Therefore, these authors proposed that the highly divergent mutant ITS sequences were ITS pseudogene components of the genome of *H. sinensis*. This ITS pseudogene hypothesis, however, prompted some fundamental questions because the multiple heterogeneous ITS sequences of natural *C. sinensis* specimens were assumed to represent the descendants of a common genetic ancestor during long-term phylogenetic evolution [53]. Are these mutant ITS sequences intra-individual pseudogenic components of the *H. sinensis* genome [57], or do they belong to multiple inter-individual mutant fungal species [51,53-54]? Are the genotypes with multiple transition, transversion, and insertion/deletion point mutations (summarized above) persistently silenced during transcription in *C. sinensis* development under natural conditions, or can Genotype #5 be counter-silenced during some *C. sinensis* maturation stages? Goodhead and Darby [73] reviewed the identification and categorization of pseudogenes and suggested “taking the pseudo out of pseudogenes”. They defined pseudogenes as junk or relics of genomes and “fragments of once-functional genes that have been silenced by one or more nonsense, frame shift or missense mutations” in the functional homologs. Increasing evidence indicates that some persistent pseudogenes are transcriptionally “alive” and

play special roles, undergoing positive selection to persist during evolution, thereby raising questions regarding the ambiguous boundary between genes and pseudogenes and calling for superior nomenclature to replace the term “pseudogenes” and better describe their functions [73-80]. Given the controversy surrounding the concept of pseudogenes, the following conclusions regarding natural *C. sinensis* specimens and the genomes of *O. sinensis* fungi are notable.

#### **4.1 The functional genes and their “ITS pseudogene” counterparts belong to the genomes of independent fungal species**

Based on the findings of the coexistence of the postulated “ITS pseudogenes” (AT-biased Genotype #5) with functional copies (GC-biased Genotype #1) in the genomes of *O. sinensis* single-ascospore isolates, Li Yi et al. [57] commented that the hypotheses suggesting that AT-biased ITS sequences represent cryptic (phylogenetic) species [53], different fungal species [54], or different genotypes [26] were all “inaccurate”. In contrast, Li Yi et al. [57] reported the unsuccessful detection of ITS sequences of Genotype #4 AB067744 in the same genomic samples using a pair of fungus-specific primers, suggesting that this divergent mutant ITS sequences indeed belong to different fungi. Xiao et al. [54] reported that the mutant genotypes (Groups A, B, and C [53], i.e., Genotypes #1, #4, and #5 in this review) of *O. sinensis* likely belong to independent fungi in natural *C. sinensis*; this conclusion is consistent with the results of Zhang et al. [29], who conducted a bioinformatic analysis of the sequences of *O. sinensis* fungi registered in INSD and used the ITS sequences of the authentic anamorphic *H. sinensis* as an analytical reference. Using various tissue culture samples derived from the caterpillar body of natural *C. sinensis* specimens collected from different geographic areas, Mao et al. [58] reported the detection of sequences of either Genotype #4 or #5, but not of Genotype #1 *H. sinensis*. These mutant fungi with distinct conidia of significantly

different sizes most likely exhibited a unique geographic distribution pattern in *C. sinensis* production areas. Thus, the highly variable mutant ITS sequences were attributed to mutant fungal species or OTUs rather than to their coexistence as pseudogenes with functional counterparts in one anamorphic genome.

The existence of multiple inter-individual mutant fungal species is supported by several studies and by genome-wide sequencing of the authentic anamorphic fungus *H. sinensis* [12,16,26,29,49,53-54,58]. Although genome-wide sequencing of the authentic anamorphic *H. sinensis* Co18 strain has identified 3 types of pseudogenes, Hu et al. [16] did not report the presence of transition, transversion or insertion/deletion mutant ITS sequences in the whole-genome sequence ANOV00000000 of the *H. sinensis* Co18 strain. Alignment analysis of the *H. sinensis* genome sequence ANOV00000000 further revealed a DNA segment within the 2626-bp sequence KE659721 (=ANOV01021709) that was 99.7% homologous to Genotype #1 AB067721 of *O. sinensis*; no other segment sequences with high-similarity homologs were identified within ANOV00000000. The ITS sequences of *O. sinensis* Genotypes #3-#12 exhibited 83.0%-95.1% similarity to KE659721, suggesting that the mutant ITS sequences were clearly independent of the whole genome of the anamorphic *H. sinensis*. These results suggest that the multiple point mutations in the *O. sinensis* ITS sequences represent genomes of separate anamorphic fungal species [16,29,54,58], which is, unfortunately, inconsistent with the ITS pseudogene hypothesis of Li Yi et al. [57], who studied completely different genomes from that of the Co18 strain of anamorphic *H. sinensis* tested by Hu et al. [16]. In addition, cloning and sequencing of the ITS amplicons to examine several “pure” anamorphic *H. sinensis* strains revealed the coexistence of *P. hepiali* and *H. sinensis* (Genotype #1) ITS sequences, but other multiple mutant genotypes of *O. sinensis* (Genotypes #3-#12) were not detected [61]. Therefore, these

analyses confirmed the conclusion of Xiao et al. [54] that the highly variable mutant ITS sequences belong to different fungi.

Although Li Yi et al. [57] detected only the ITS sequences of Genotypes #1 AB067721 and #5 AB067740 and failed to detect the ITS sequences of Genotype #4 AB067744, no experiments have been designed to examine the other *O. sinensis* genotypes listed in the GenBank database: 3 transition mutant genotypes (Genotypes #2-#3 and #6) of Category 1, 5 transversion mutant genotypes (Genotypes #7-#11) of Category 2, and the 3 insertion/deletion mutants within Genotype #12 of Category 3. The inability to detect Genotype #4 AB067744 by Li Yi et al. [57] reflected the possibility that Genotype #4 AB067744 sequences might be associated with a fungus (which might or might not be culturable) in the different compartments of natural *C. sinensis* at different maturation stages [12,49,56,60] or in different geographic production areas [49,54,58]. The incomplete study design and findings of the report in question [57] therefore provide insufficient and contradictory evidence supporting the ITS pseudogene hypothesis.

The results of other studies also contradict the assumption of the genomic coexistence of “dysfunctional” ITS pseudogenes and functional counterparts in one genome of pure *O. sinensis*. The combined use of Southern blotting after *EcoRI* preparation and *EcoRI* digestion RFLP assays resulted in the detection of a single Southern blot moiety from genomic DNA isolated from a pure *H. sinensis* strain and of a doublet from genomic DNA isolated from the stroma and caterpillar body of natural *C. sinensis* [26]. These results indicate that the *EcoRI*-resistant AT-biased sequences did not exist in the genome of pure GC-biased *H. sinensis* but rather coexisted with the *EcoRI*-sensitive GC-biased sequences in the fungal genome pool of natural *C. sinensis*. As shown in the upper-left panel of Figure 5, Southern blot analysis of the *EcoRI*-digested genomic DNA demonstrated that the relative biomasses of the ITS segments of the mutant

*O. sinensis* genotypes (the faster-migrating GC-biased Genotypes #1, #3, and #7-#12 vs. the slower-migrating AT-biased Genotypes #4-#6 in gel electrophoresis) were dynamically altered in a non-synchronized manner during *C. sinensis* maturation over a short period of time (weeks or 1-2 months) in the stroma and caterpillar body [26,59], although Li Yi et al. [57] misinterpreted the maturational phenomenon of these asynchronous alterations in the biomasses of *O. sinensis* mutants as the "AT-biased genotypes are not found in the sclerotium of *O. sinensis*". Briefly, in premature *C. sinensis* (cf. upper-left panel of Figure 5), the fungi with the AT-biased genotypes, represented by the slower-migrating DNA moiety, were probably absent in the caterpillar body but highly predominant in the stroma; the biomass greatly increased in both compartments during *C. sinensis* maturation [26,59]. The biomass of the GC-biased genotypes, including Genotype #1 *H. sinensis* and Genotypes #3, #7-#12 (the faster-migrating DNA moiety in the upper-left panel of Figure 5), was extremely low in the stroma of premature *C. sinensis* and increased with *C. sinensis* maturation, but was never the predominant DNA species in the stroma. Consistent with the hypothesis of Liang et al. [19] that natural *C. sinensis* is an integrated micro-ecosystem, these asynchronous maturational changes in the biomasses of mutant *O. sinensis* genes do not support the coexistence of multiple *O. sinensis* genotype sequences in the genome of a purified anamorphic *H. sinensis* and are not consistent with the principle of genetic stability or the assumption of the genomic coexistence of the pseudogenic mutant sequences and functional gene counterparts in the genome of Genotype #1 *H. sinensis*. These asynchronous maturational alteration phenomena at the genomic DNA level were also confirmed in other experiments by *EcoRI* digestion RFLP and SNP MS genotyping assays [12,13,26,56].

In direct contrast with the coexistence of mutant "ITS pseudogenes" and functional gene counterparts in the same genome of an

anamorphic fungal species, the most reasonable interpretation of the inability to detect Genotype #4 and other mutant genotypes and of the phenomenon of dynamic, asynchronous changes in the biomasses of mutant genotype species during *C. sinensis* maturation is that these ITS sequences with multiple, scattered transition, transversion, and/or insertion/deletion point mutations belong to the genomes of independent fungi [13,26,50,53-54]. The asynchronous changes in fungal presences in the different compartments of *C. sinensis* during the spring-summer transition likely reflect maturation-related changes in the rates of proliferation, degradation and other biological processes of the different fungi, which may be psychrophilic (associated with the early development of *C. sinensis* in winter and early spring) or mesophilic (associated with the late maturation of *C. sinensis* in late spring and summer).

#### 4.2 Distinct genome samples tested in different studies

Previous studies have analyzed truly different genome samples of these organisms: (1) total genomic DNA isolated from a pure, authentic anamorphic *H. sinensis* Co18 strain that was used in the genome-wide sequencing study [16]; and (2) total genomic DNA isolated from *O. sinensis* mycelia derived from a 25-day incubation (in a liquid culture medium) of wild-type isolates from single ascospores of *C. sinensis*, whereas the multicellular teleomorph of *O. sinensis* showed structural features including mono-, bi-, and trinucleate cells in each ascospore [52,57]. Compared with the cultured mycelia of the anamorphic *H. sinensis* Co18 strain containing a homogeneous GC-biased Genotype #1 ITS1-5.8S-ITS2 segment in its genome, the cultured mycelia derived from the heterokaryotic wild-type *C. sinensis* ascospores comprised heterogeneous ITS components: both Group A (Genotype #1) GC-biased and Group C (Genotype #5) AT-biased mutant ITS sequences, whereas other mutant ITS sequences (of Genotypes #2-#4

and #6-#12) were not detected. Xiao et al. [81] confirmed the overall polymorphic differences between *H. sinensis* strains and the wild-type *C. sinensis* ascospore samples via ISSR molecular marker polymorphism analysis, although the authors incorrectly used density-unweighted algorithms for the similarity computations and phylogenetic tree construction. Do these results suggest that the ascospores of *C. sinensis* were the heterokaryotic meiotic organism if the genomes tested were isolated from the mycelial culture of truly pure single-ascospore isolates [2]? Unfortunately, Li Yi et al. [57] did not provide technical information regarding the collection, isolation and purification of their study materials, ascospores and genomes, instead guiding the readers to a Ph.D. dissertation [82] that is unavailable to the general public as a reference.

The distinct genotyping results from the cultured mycelia obtained from wild-type, mono-/bi-/trinucleate single-ascospore cultures [57] and the anamorphic mycelia obtained from a single conidia culture [16] raise a series of legitimate questions. (1) Were the wild-type multicellular, mono-/bi-/trinucleate *C. sinensis* single-ascospore samples from homokaryotic or heterokaryotic teleomorphic organisms [2,52]? (2) Was the study material used by Li Yi et al. [57] from an absolutely pure homokaryotic *O. sinensis* fungus or from a mycelial mixture of anamorphic haploid organisms derived from *in vitro* culture of wild-type polykaryotic, multicellular isolates? (3) Were there two or more sets of chromosomes in the test material examined by Li Yi et al. [57], as indicated by the fluorescent staining showing mono-/bi-/trinucleate structures in each multicellular ascospore reported by the same group of researchers [52]? (4) Were the phenotype(s) and genotype(s) of the conidia obtained from the microcycle conidiation of ascospores by Xiao et al. [20], Liu et al. [70] and Mo et al. [71] similar to those of the conidia and mycelia obtained by Li Yi et al. [57] from the culture of *C. sinensis* ascospores? (5) Do the

conidia of various sizes and shapes observed in the experimental microcycle conidiation of ascospores and conidia reported by Xiao et al. [20] have different genotypes, similar to those reported by Mao et al. [58]? (6) Which karyotype(s) of cells (mononucleate, binucleate, or trinucleate discovered by Bushley et al. [52]) of the multicellular ascospores are capable of conidiation? (7) Do the larger conidia derived from conidiation of ascospores have the same karyotype(s) and genotypes as their parental wide-type ascospores [52]? (8) Does the conidiation observed in the studies of microcycle conidiation of ascospores represent all natural conidiation processes of all mononucleate, binucleate and trinucleate cells of the wild-type, multicellular, heterokaryotic ascospores of *C. sinensis* [2,18,21,52,83]? As reported by Mao et al. [58], fungi with Genotypes #4 & #5 exhibit the same mycelial morphology as Genotype #1 *H. sinensis*, and the fungal conidia may be larger for Genotype #4 than for Genotype #5, suggesting the impossibility of distinguishing between the mutant fungi of various genotypes based solely on their microscopic morphology.

### 4.3 The silencing of 5.8S genes

In addition to obtaining both the Genotype #1 sequence and one (Genotype #5) of the AT-biased sequences from the genomes of the mycelial culture of the wild-type single-ascospore isolates, Li Yi et al. [57] also examined the transcripts of 5.8S genes derived from reverse transcription PCR, which resulted in the detection of the 5.8S cDNA only for Genotype #1 (Group A), but not for Genotype #5 (Group C) or other genotypes. This result provided critical evidence that the 5.8S gene of the GC-biased Genotype #1 was the functional copy of the *O. sinensis* genome, whereas Genotype #5 5.8S genes were the dysfunctional, pseudogenic components of the teleomorphic *O. sinensis* genome.

The use of fungal non-specific 5.8S-F/R primers in the study [57] makes it necessary to seriously consider the extremely high

similarity between the 5.8S genes of Genotypes #1-#3, #7-#9, & #11-12 (*cf.* Figures 1 & 4), *P. hepiali* and hundreds of different fungi [26] and the low similarity between the 5.8S genes of the GC-biased Genotype #1 and AT-biased Genotypes #4-6 (*cf.* Figure 1) before concluding that the detected 5.8S transcript truly belonged to Genotype #1 *H. sinensis* and thus that the 5.8S transcripts of the mutant genotypes were not detected. Thus, doubt regarding the functionality of the 5.8S gene of the GC-biased Genotype #1 needs to be further addressed if the detected 5.8S cDNA did truly belong to Genotype #1 of *O. sinensis*, as stated in [57], because the authors did not disclose either the experimental design or the results for the detection of other fungi and because the ITS sequences of *H. sinensis* and *P. hepiali* were detected using genomic DNA isolated from the ascospores of natural *C. sinensis* [60]. To confirm that the detected 5.8S cDNA truly belonged to Genotype #1 of *O. sinensis*, the combined use of other molecular techniques may be warranted.

Before concluding that the AT-biased 5.8S genes are non-functional based on the non-detection of AT-biased 5.8S cDNA [57], several other concerns should be addressed:

(1) The homology of the primers, 5.8S-F and 5.8S-R, for detecting 5.8S cDNA must be verified to ensure the same high primer-binding capability for all AT-biased mutant genotypes under the experimental conditions of the competitive binding of primers to the available templates and to eliminate the potential technical failure of reverse transcription PCR.

(2) Because Li Yi et al. [57] did not detect the ITS sequences for mutant genotypes other than Genotypes #1 and #5 from the genomic DNA pool of the single-ascospore cultures, it may be assumed that the 5.8S transcripts of Genotypes #3-#4 and #6-#12 cannot be detected from the total RNA pool after being cultured in a liquid culture medium at 18°C for 25 days. Claiming that the 5.8S genes of those mutant genotypes (Genotypes #3-#4 and #6-#12) are non-

functional pseudogenes would be an over-interpretation.

(3) To confirm the non-detection of the 5.8S transcripts of the mutant genotypes of *O. sinensis*, the 3 types of secondary steric conformations of 5.8S genes predicted by Li Yi et al. [57] for Groups A, B, and C (Genotypes #1, #4 & #5) of *O. sinensis* must be considered during the primer design because these secondary structures might have a considerable impact on reverse transcription PCR. Multiple sets of primers or other molecular techniques and functional assays may be required in designing a study to examine the 5.8S gene transcription.

(4) Li Yi et al. [57] disclosed that teleomorphic ascospores were subjected to liquid-phase rotary-shaker incubation (100 rpm) at 18°C in a particular culture medium (PDA with 5% wheat bran and 0.5% peptone) for 25 days prior to extraction of total genomic DNA and total RNA from the mycelia. These non-natural 25-day culture conditions might significantly impact the transcription of many functional genes, potentially non-physiologically switching on or off some genes, as was confirmed by the gradual nonlinear reduction in the total number of transcriptomic unigenes from 25,511 after 3 days of fermentation to 25,214 at Day 6 and then drastically down to 16,245 at Day 9 during continuous liquid fermentation [9].

(5) The findings of Li Yi et al. [57] are inconclusive if the 5.8S genes of the mutant *O. sinensis* genotypes are transcriptionally silenced during some physiological stages of the development and maturation of natural *C. sinensis*, for instance, becoming silenced after the ejection of ascospores. Evidence supporting such dynamic, physiological alterations of gene transcription came from proteomic profiling analysis, indicating significant changes in proteomic profiles in the different compartments of natural *C. sinensis* specimens during maturation [10]. Conditional and periodic silencing of the 5.8S genes of mutant genotype fungi must be

further explored before declaring some genes to be non-functional “pseudogenes”.

Natural *C. sinensis* is an insect-fungi complex with an extremely complex life cycle. Studies have reported that the presence/absence and quantities of multiple mutant fungi resulting from fungal proliferation, degradation and other biological processes are altered in different compartments (caterpillar body, stroma, ascocarp and ascospores) of natural *C. sinensis* during different developmental and maturation stages [11-13,19,26,29,49-50,54-56,59-60]. Notably, the transcription of the genomes of the intrinsic psychrophilic and mesophilic fungi might also be altered during various stages of *C. sinensis* formation, development, and maturation in the compartments of *C. sinensis* in response to seasonal changes in alpine environmental conditions. Transcriptomic alterations have been demonstrated in 3-, 6-, and 9-day mycelial cultures of a wild-type “*H. sinensis*” L0106 strain isolated from premature natural *C. sinensis* and cultured *in vitro* in 200-liter submerged stirred fermenters [9]. A proteomic polymorphism study demonstrated dramatic differences in proteome expression in the stroma and caterpillar body of natural *C. sinensis* between the premature and mature stages [10]. Additional studies are necessary to determine whether the so-called “ITS pseudogenes” of the multiple mutant genotype fungi are counter-silenced and actively transcribed to play specific biological roles in the *C. sinensis* lifecycle: (1) fungal infection of host larvae of the family Hepialidae and synergy in infection between the fungi with functional ITS genes and those with so-called “ITS pseudogenes”; (2) initial asexual growth of fungi inside the larva; (3) hibernation in the dead larva during the extremely cold winter on the Qinghai-Tibet Plateau; (4) stroma germination; (5) transition of initial anamorphic fungal growth in premature *C. sinensis* to teleomorphic growth in mature *C. sinensis*; (6) changes during the

courses of plasmogamy and karyogamy; (7) formation of polyploids and polykaryons; (8) scaffold construction for the *C. sinensis* ascocarps; (9) meiosis of teleomorphic fungal cells; (10) formation and maturation of the multicellular ascospores; (11) natural ejection and/or semi-ejection of the mature ascospores [60]; (12) post-ejection survival of the teleomorphic ascospores in the environment and the transition to the anamorphic stage(s), etc. The complexity of proving or disproving the non-functionality of the 5.8S genes of the AT-biased and other mutant genotypes and the ITS pseudogene hypothesis appears to be far beyond the current knowledge of natural *C. sinensis*, multiple mutant *O. sinensis* fungi, and their accompanying fungi.

The analyses and discussions described above suggest that these multiple, scattered transition, transversion, and insertion/deletion mutant genotype sequences are likely the genomic components not of GC-biased *H. sinensis* but of individual mutant fungi. The genetic heterogeneity of single-ascospore isolates may indicate that the ascospores of *C. sinensis* feature mixtures of the mono-, bi-, and trinucleate multicellular structures [2,13,50,52,57]. The reported detection of the 5.8S transcript through reverse transcription PCR using non-specific primers may or may not belong to the Genotype #1 *H. sinensis*, and the non-detection of the mutant 5.8S transcripts under the experimental conditions described in [57] provide insufficient evidence to determine functionality. Other techniques are needed to directly examine the functionality of the 5.8S genes of the multiple mutant *O. sinensis* fungi in the natural *C. sinensis* samples at different developmental and maturation stages to differentiate between the truly permanent dysfunction of so-called “ITS pseudogenes” and the temporary transcriptional silencing of the 5.8S genes in the caterpillar body, stroma, ascocarp, and ascospores of natural *C. sinensis* during development and maturation.

## 5. Heterogeneous ITS amplicons of the *C. sinensis* ascospores and microcycle conidiation

The ITS sequences of the GC-biased Genotype #1 (Group A) and the AT-biased Genotype #5 (Group C) were simultaneously detected in the heterogeneous amplicon pool from the genomic DNA of the mycelial culture of the wild-type ascospores of natural *C. sinensis* [57], suggesting heterokaryosis of the *C. sinensis* ascospores with mixed mono-/bi-/trinucleate structures in each multicellular ascospore [2,13,50,52]. Further genotyping analysis of the ascospores of *C. sinensis* using PCR amplicon cloning-sequencing and SNP MS genotyping techniques detected several fungi [60]: Genotype #1 *H. sinensis*, Genotype #5 AT-biased KJ729098 and KM017747 (98% homologous to AB067740), Genotype #6 AT-biased KJ720572 (98% homologous to EU555436), and *P. hepiali* (100% homologous to EF555097). These studies using PCR amplicon cloning-sequencing and SNP genotyping of the wild-type teleomorphic ascospores [60] or PCR amplicon sequencing after mycelial culture of the ascospores [57] revealed a complex genetic background for the *C. sinensis* ascospores, suggesting that the *C. sinensis* ascospores have a heterokaryotic multicellular structure [2,52].

As discussed above, the occurrence of heterogeneous mutant ITS sequences in the genomes of different fungi and the findings of the mixed mono-, bi-, and trinucleate structures in the single multicellular ascospore directed our interest to the microcycle conidiation of ascospores. Jiang and Yao [17] suggested that microcycle conidiation of the *C. sinensis* ascospores is an auxiliary technique and provides only indirect evidence for confirmation of the correct anamorph of *O. sinensis*. Scholars have doubted whether studies of microcycle conidiation of the ascospores of natural *C. sinensis* under specific experimental conditions can completely profile all conidiation of ascospores, particularly for

those anamorphic species for which experimental culture and conidiation are difficult to achieve in laboratory settings [18,21,23,83]. Studies of microcycle conidiation of *C. sinensis* ascospores reported the detection of only “*H. sinensis* conidia” through microscopic morphological examinations [20,70-71]. However, this morphological assumption of “*H. sinensis* conidia” might be challenged by the following: (1) the distinct morphology of conidia obtained either from the microcycle conidiation of the *C. sinensis* ascospores or of the anamorphic conidia of *H. sinensis* reported by Xiao et al. [20]; (2) the similar conidial morphology of AT-biased genotype fungi and the Genotype #1 *H. sinensis* according to Mao et al. [58]; (3) the overall polymorphic differences in molecular markers between pure *H. sinensis* strains and the wild-type *C. sinensis* ascospore samples via ISSR polymorphism analysis [81]; and (4) the simultaneous detection of the ITS sequences of the culturable fungi of Genotypes #1 and #5 [57] in addition to those of fungi of Genotype #6 and *P. hepiali* [60] from the genomes of the ascospores of natural *C. sinensis* with the mixed mono-, bi-, and trinucleate multicellular structures [52]. Unfortunately, none of these microcycle conidiation studies reported the karyotypes and genotypes of the conidia produced from the conidiation of the *C. sinensis* ascospores [20,70-71]. As discussed above, the mutant sequences of multiple *O. sinensis* genotypes are not likely to represent the pseudogenic components of one genome of the single GC-biased *H. sinensis* fungus but instead to belong to different anamorphic fungi that are closely associated with each other either in a fungal (species) complex formed under natural settings or within the mono-/bi-/trinucleate heterokaryotic teleomorph of *O. sinensis* [2,21,52]. Given the successful development of methods for culturing fungal species susceptible to difficulties in ascospore germination and experimental culture in laboratory settings [18,21,83], the combination of microcycle conidiation of

ascospores and proper molecular approaches in future studies may provide critical insight into the conidiation of the ascospores of natural *C. sinensis* and the profile of heterokaryotic fungal molecules.

## 6. Proteomic polymorphisms and protein-coding genes of natural *C. sinensis*

In a symposium on *C. sinensis* anamorphs on Oct 29, 2005, Prof. Liang ZQ suggested using omic technologies to study natural *C. sinensis* for confirmation of the anamorph-teleomorph connection for *O. sinensis* fungi. Despite the strong objection from his mycology colleagues at that conference 11 years ago, the omic approaches used in *C. sinensis* studies in recent years have uncovered a substantial amount of scientific information in several publications, ranging from whole genome, transcriptome, and proteome studies to metabolic/chemical fingerprinting and *C. sinensis* microbiota profiling [9-10,16,25,48,51,84-85], greatly enriching our knowledge of natural *C. sinensis* as a holistic insect-fungi complex and of the anamorph-teleomorph connection for *O. sinensis* fungi. For instance, a study of natural *C. sinensis* reported profound, dynamic, asynchronous changes in proteomic polymorphisms in the stroma and caterpillar body of *C. sinensis* during maturation [10]. In examining the whole genome protein-coding genes of a purified anamorphic *H. sinensis* strain, Hu et al. [16] reported 6,972 protein-coding genes and identified the genes encoding 2,229 protein families through InterProScan analysis, including both actively transcribed and silent genes. Through transcriptome sequencing of total RNA isolated from natural *C. sinensis* collected from Sichuan Province, however, Xiang et al. [85] reported a total of 34,289 unique sequences with or without gene annotations (including 17,230 singletons and 17,059 contigs) and identified 7,229 unique transcriptomic sequences with functional annotations through InterProScan analysis. Despite the potential maturation-dependent

silencing of gene transcription in natural *C. sinensis*, the numbers of actively transcribed genes identified from the natural *C. sinensis* insect-fungi complex [85] were several times greater than the number of genes (both actively expressed and silent) identified from the genome-wide sequencing of a purified anamorphic *H. sinensis* strain [16], which is inconsistent with the sole anamorph hypothesis for *H. sinensis* described above.

In contrast with a previous examination [85] of the transcriptome profile directly from total RNA isolated from natural *C. sinensis*, another transcriptome study [9] was conducted on cultures of a wild-type *H. sinensis* L0106 strain that was isolated from the tissue of the premature *C. sinensis* collected in May from Qinghai Province. The ITS sequences (KP090933) of this strain share 99.5% homology with Genotype #1 AB067721, although possible molecular heterogeneity of ITS sequences of this wild-type fungal strain was not reported under the metagenomic fosmid library construction and the observation of multicellular polykaryotic phenomena [9]. The L0106 strain was cultured in a medium favoring *H. sinensis* growth (containing a wide selection of carbon sources) at 16°C for 3, 6 or 9 days in 200-liter submerged stirred fermenters, followed by reverse transcription of pure mRNA isolated from the mycelia of the L0106 strain. Transcriptome profiling detected a total of 25,511 unigenes from the 3-day culture, 25,214 unigenes from the 6-day culture, and 16,245 from the 9-day culture. These authors also reported in a supplementary file that 71%-78% of the L0106 transcriptome reads could be mapped to the reference genome and that 31-33% of reads could be mapped to reference genes, indicating the heterokaryosis of the L0106 strain. (Note: the information provided in a different report [9] suggests that the genome survey may have been performed on the L0106 strain, probably through metagenomic fosmid library end-sequencing, and the heterokaryotic results were used as the reference for transcriptomic gene mapping. In addition, Bushley et al. [52] reported the

observation of two nuclei in hypha using fluorescent staining for mycelia.) All of these transcriptome data identified greater numbers of actively transcribed genes from the natural *C. sinensis* insect-fungi complex [85] or from the cultures of the wild-type fungal isolate [9] than the total number of genes (both actively expressed and silent) identified from the genome-wide sequencing of a purified anamorphic *H. sinensis* strain [16], thus confirming the coexistence of multiple fungi in natural *C. sinensis* and supporting the notion that the considerable therapeutic functions result from the symbiotic activities of multiple fungal sources and the dead body of the larva of the family Hepialidae. Although Xiang et al. [85] did not specify the maturation status of the specimens tested, and Liu et al. [9] profiled the transcriptome genes in the cultures of a fungal isolate, the significant changes in proteomic polymorphisms [10] indicate dynamic changes in the expression of transcriptomic genes in the different compartments of *C. sinensis* during maturation. All of the proteins that are differentially expressed in the different compartments of *C. sinensis* and altered with maturation, along with the varied profiles of their component chemicals (lipids, polysaccharides, nucleotides and other chemical constituents), contribute to the varied efficacy profiles and potency of the therapeutic activities of natural *C. sinensis*, for the initial discovery of this highly valued TCM product was based on its therapeutic efficacy and unique appearance [1-2,4,7,10,11,19,86-87].

## 7. Molecular systematics for the genetically heterogeneous *C. sinensis*

Microcosmic molecular systematic studies comparing nrDNA ITS sequences have been used to determine the taxonomic status of the examined specimens. The detection of *H. sinensis* ITS sequences in the genomic DNA of natural *C. sinensis* from 1999-2003 consistently demonstrated the molecular “homogeneity” of the PCR

amplicons, providing molecular systematic evidence for the “correct” anamorph and supporting the hypothesis that *H. sinensis* is the sole anamorph of *O. sinensis* [63-69]. The success of these molecular studies of natural *C. sinensis* provided inertia for the continuing assumptions accepted in later years [14,28,29,51,65], regardless of the documented controversies as to the isolation of *C. sinensis*-associated fungi (22 species spanning 13 genera) [17-18,27] and the subsequent identification of additional fungi [25,44-46,48,51].

Chen et al. [8] first reported the molecular heterogeneity of *C. sinensis*-associated fungi using a PCR amplicon cloning technique. However, insufficient attention was given to these “all-or-none” qualitative research findings, and instead, the disproportionate amplicon clones selected for examining the ITS sequences of multiple fungi were overemphasized, unfortunately leading to an improper conclusion. The observations of molecular heterogeneity have attracted scientific attention since 2005, and there have been reports of the ITS sequences of multiple *C. sinensis*-associated fungi, including *Cladosporium macrocarpum*, *Geomyces pannorum*, *Neosetophoma samarorum*, *Paecilomyces hepiali*, *Phaeosphaeria pontiformis*, *Pseudogymnoascus roseus*, and *Tolyocladium sinense* (in total, more than 90 species spanning at least 37 genera), in addition to the multiple *O. sinensis* genotypes described above [11,25,26,27,42,44-49,51,59-60]. Southern blotting, RFLP assay, SSCR assay, SNP MS genotyping, nested-PCR, qPCR, amplicon sequencing and amplicon cloning sequencing have been used to identify several genotypes of *O. sinensis* mutant fungi with multiple, scattered point mutations from natural *C. sinensis* [11-12,25-26,49,51,54-62]. Therefore, the notion that *H. sinensis* is the sole anamorph of natural *C. sinensis* faces substantial challenges based on both mycological and molecular systematic evidence and remains a hypothetical assumption requiring further analysis that

should be conducted strictly according to Koch's Postulates.

Although aforementioned microcosmic molecular examinations have revealed individual taxonomies through ITS sequencing, individual fungal ITS and other sequences do not represent natural *C. sinensis* due to its genetic heterogeneity. The microcosmic ITS sequence data have fueled speculations, hypotheses and non-conclusive debates, similar to blind men each touching a portion of an elephant. To address this shortcoming, macrocosmic holistic molecular marker polymorphism analysis has been used as a component of overall molecular systematics strategies to profile natural *C. sinensis* as a holistic entity and to compare the holistic polymorphic similarities of the systems without the requirement of precise examinations of the DNA sequences or the individual taxonomies of the component fungi. These macrocosmic molecular techniques include AFLP (Amplified Fragment Length Polymorphism), CAPS (Cleaved Amplified Polymorphic Sequence), DAF (DNA Amplified Fingerprints), ISSR (Inter-Simple Sequence Repeat), RAPD (Random Amplified Polymorphic DNA), RFLP, SCAR (Sequence Characterized Amplified Regions), SSCP (single-strand conformation polymorphism), and SSR (Simple Sequence Repeat) [88-89]. Among these methodologies, RAPD molecular marker polymorphism analysis is the most frequently used technique for comparing overall similarities or dissimilarities (genetic distances) and exploring the phylogenetic cluster relationship between the test systems [14,62,90-97], although it has been suggested that ISSR may be more sensitive than RAPD [81,98-99], and metagenomics approaches may demonstrate advances in qualitative studies of microbial genetic diversity and molecular ecology [100-102]. A few issues regarding holistic molecular marker polymorphism studies are reviewed below.

## 7.1 Selection of RAPD random primers

RAPD and ISSR molecular marker polymorphism analyses compare the migration and density (abundance) of PCR amplicons in non-denaturing agarose gel electrophoresis by computing integral similarity and constructing phylogenetic (cluster) trees [13-14,62,81,90-99]. Prior to the agarose gel electrophoresis, these approaches require the use of a plurality of primers for PCR amplification of the genomic DNA templates isolated from the examined systems. The selection of the type and quantity of random primers, therefore, becomes crucial for the unbiased design of RAPD and ISSR marker polymorphism studies to obtain unbiased profiles of the holistic differences between the tested samples [13,17,50,62,93].

After screening 20-65 random primers, as many as 8-29 primers have been selected for the *C. sinensis* studies based on the number and density of the DNA amplicon moieties in the gel images [90-97]. The use of only a few random primers without reporting the objectivity and representativeness of the selection could lead to bias in the data analysis and thus bias in the study conclusions [17,50,62,93] when *C. sinensis* samples and fungal strain samples were not profiled as a whole, resulting in the inaccuracy of holistic comparisons, interpretations and conclusions [14]. Thus, scientists have warned that the selection of the type and number of random primers is critical for the interpretations and conclusions drawn from RAPD and ISSR results [17,50,62,93].

## 7.2 Computational biology algorithms for polymorphism similarity analysis

Macrocosmic analysis of molecular marker polymorphisms relies upon similarity computation and phylogenetic (cluster) tree construction. Previous studies have consistently used the PCR amplicon density-unweighted algorithm known as the Nei-Li equation [103] for similarity computations. This or similar density-unweighted algorithms

have also been used in SSCP and ISSR studies [25,51,81,99].

The Nei-Li algorithm (or similar) was designed to analyze "all or none" data, to compare pure systems in pairs and, in particular, to analyze the loss of restriction sites due to mutations [103]. The proper use of this algorithm has 2 prerequisites: (1) all matched DNA pairs in the electrophoretic lanes being compared must have essentially the same densities; and (2) all DNA amplicons must be well separated from the adjacent DNA moieties with similar molecular weights and conformations by electrophoresis [62,93]. Clearly, this algorithm is unsuitable for RAPD or ISSR studies of *C. sinensis* as an insect-fungi complex containing multiple fungi, and a series of new ZUNIX arithmetic methods ([www.ebioland.com/ZUNIX.htm](http://www.ebioland.com/ZUNIX.htm); Beijing Bioland Technology, 2013) was developed for density-weighted similarity computation [62,93]. The ZUNIX equations arithmetically consider the following: (1) the unmatched DNA (or protein or other chemical) bands and their densities, (2) differences in the density of the matched DNA (or protein, or other chemical) bands (or peaks, or areas under the curves), and (3) the ability to compare multiple samples. The density-weighted ZUNIX equations define similarity as the total density of all common parts present in the matched DNA bands of the samples being compared divided by the total density of all bands across the samples [93]. The ZUNIX equations are mathematically general, with no specific prerequisites, and govern all conditions, including the special cases under the strict prerequisites set forth by the Nei-Li equation [103], and they accurately capture all of the molecular information buried in the amplicon DNA bands (both the density and the migration speed in gel electrophoresis) in the RAPD (or ISSR, SSCP, or similar techniques) gel images, which are partially lost or even significantly lost when incorrectly using the density-unweighted Nei-Li equation [103]. Consequently, misuse of the Nei-Li equation [103] in *C. sinensis*

holistic polymorphism studies when the sample systems do not meet the specific prerequisites may lead to inaccurate calculations of overall similarities and questionable conclusions [81,92]. The mathematically general, density-weighted ZUNIX equations can also accurately calculate the similarities of proteomic polymorphisms of multiple *C. sinensis* samples [10,93].

### 7.3 Density-weighted algorithms for phylogenetic (cluster) tree construction

Phylogenetic analysis in previous RAPD and ISSR studies of *C. sinensis* primarily used PCR amplicon density-unweighted UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithms to construct phylogenetic trees [14,81,90,94,97,99]. Consistent with the computation of similarity, constructing phylogenetic trees using density-unweighted algorithms in the holistic analysis negates the differences between high- and low-density DNA amplicons or between the complete and incomplete separation of DNA amplicon moieties on agarose gel electrophoresis and their impact on the weights in exploring phylogenetic similarity and dissimilarity, leading to errors in constructing phylogenetic trees. Ni et al. [93] demonstrated the inaccurate construction of a phylogenetic tree improperly using the density-unweighted algorithm, reflecting the inability to capture and analyze all molecular information buried in the DNA bands (both the density and the migration speed in agarose gel electrophoresis) in RAPD gel images, whereas the density-weighted algorithm corrected such analytical errors. Thus, the selection of different clustering algorithms with or without consideration of the densities and incomplete separations of the DNA moieties (or protein, or chemical moieties) greatly impacts the data analysis and study conclusions, and the density-unweighted algorithms are not suitable for studies of *C. sinensis*, which contains multiple intrinsic fungi.

As indicated by Ni et al. [93], PAUP 4.0B requires semi-quantitative scoring prior to phylogenetic tree construction, which may slightly reduce the sensitivity in handling fully quantitative density data. Therefore, Ni et al. [93] performed their clustering analysis using software with full quantitative capacity, such as Cluster3.0, JMP9, and SPSS, and they demonstrated that the fully quantitative algorithms placed *H. sinensis* in a separate clade from the main *C. sinensis* cluster at a large rescaled distance. Both the semi- and fully quantitative methods, however, exhibited advantages in capturing all molecular information and accurately constructing phylogenetic trees in the *C. sinensis* molecular and proteomic polymorphism studies [10,62,93]. Other advantages of software for fully quantitative clustering include ease of use and accurate quantitation, but the algorithms provided by the software do not include bootstrap value calculation, whereas the semi-quantitative clustering algorithm provided by PAUP 4.0B calculates the bootstrap value (usually Bootstrap=1000). The type(s) of software should be considered when designing RAPD, ISSR, SSCP and other holistic profile comparison studies, although both fully and semi-quantitative clustering algorithms can generally be used for *C. sinensis* molecular and proteomic polymorphism studies and chemical fingerprint studies.

## 8. Summary

This review summarizes the scientific debates in the molecular studies of *O. sinensis* fungi from natural *C. sinensis* specimens, the natural fungal (species) complex of multiple fungi and the dead bodies of larvae of the family Hepialidae. The genetic heterogeneity of natural *C. sinensis* and multiple fungi under the name *O. sinensis* indicate that the IMA Amsterdam Declaration 1F1N can be properly implemented in the *C. sinensis* research field only after careful confirmation of *O. sinensis* as truly “One Fungus” and of the identification of one of the fungi in natural

*C. sinensis* as the true anamorph of *O. sinensis* strictly following Koch’s Postulations [28,39-41]. Prior to such scientific confirmation, all 3 hypotheses relating to the *O. sinensis* fungi under Sections 2.1, 2.2, and 2.3 should be treated scientifically and equally to avoid academic unfairness. An academic consensus to end this decades-long debate needs to be reached only after such scientific confirmation by a committee of multidisciplinary experts. In addition to the problematic use of the name *O. sinensis* for the multiple homokaryotic anamorphic fungi and the multicellular heterokaryotic teleomorph ascospores, the natural insect-fungi complex should not use the same Latin name of *O. sinensis* to avoid academic confusion if this name is used for the fungi [30]. The multiple fungi that differentially exist in the *C. sinensis* compartments, possibly in the form of bi-/trinuclate heterokaryons or fungal (species) complexes or other wild-type symbiotic relationships, undergo asynchronous alterations during *C. sinensis* maturation, resulting in altered integral molecular marker polymorphisms and proteomic polymorphisms that represent a dynamically altered holistic entity of natural *C. sinensis*. The symbiosis of multiple *C. sinensis*-associated fungi in the entire course of the *C. sinensis* lifecycle should be carefully studied to address fundamental questions concerning the mystery of natural *C. sinensis* biology (including the anamorph-teleomorph connection of *O. sinensis*) and the mass production of artificial *C. sinensis* to supplement this scarce, precious natural resource that suffers from disproportionately high market demand [13,50].

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