Multiple genomically independent genotypes of *Ophiocordyceps sinensis* may not be targets of RIP mutagenesis causing cytosine-to-thymine transitions

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Short title:

O. sinensis genotypes aren't targets of RIP mutagenesis

Abstract

It has been hypothesized that AT-biased genotypes of *Ophiocordyceps sinensis* are generated through repeat-induced point mutation (RIP) and coexist as ITS pseudogenes in the *Hirsutella sinensis* genome. This study examined the *H. sinensis* genome, which contains multiple GC-biased repetitive ITS copies with multiple insertion/deletion and transversion alleles; these alleles are not generated through RIP, which causes cytosine-to-thymine transitions. The repetitive ITS copies are genetically and phylogenetically distinct from the AT-biased genotypes of *O. sinensis*, which possess multiple transition alleles. Genotypes #2–17 are absent from the *H. sinensis* genome; these genotypes belong to independent *O. sinensis* fungi and occur in various combinations in different compartments of natural *Cordyceps sinensis*. Their abundances are dynamically altered in an asynchronous and disproportional manner during *C. sinensis* maturation. Metatranscriptomic analyses of natural *C. sinensis* revealed the transcriptional silencing of 5.8S genes in all *C. sinensis*-colonizing fungi, including *H. sinensis*. The transcription assay reported by Li et al. (2013) has provided incomplete, controversial evidence to support the notion that the 5.8S genes of AT-biased *O. sinensis* genotypes are permanently nonfunctional pseudogenes. In parallel with GC-biased genotypes, AT-biased genotypes might have been generated from a common ancestor during a long evolutionary history.

KEY WORDS: Multiple genotypes of *Ophiocordyceps sinensis*; genomic independence; repeat-induced point mutations (RIP); insertion/deletion, transversion and transition point mutations; 5.8S gene transcript; transcriptional silencing.

I. Introduction

Natural *Cordyceps sinensis* is one of most valued therapeutic agents in traditional Chinese medicine and has a rich history of clinical use for health maintenance, disease amelioration, postdisease/surgery recovery, and antiaging therapy (Zhu et al. 1998a, 1998b; Li et al. 2011; Tan et al. 2011). The Chinese Pharmacopeia defines natural *C. sinensis* as an insect-fungal complex, which includes the remains of Hepialidae moth larva and >90 fungal species from >37 genera, with differential co-occurrence of some of 17 genotypes of *Ophiocordyceps sinensis*, *i.e.*, natural *C. sinensis* insect-fungal complex ≠ the entomopathogenic *O. sinensis* fungus (Engh 1999; Kinjo and Zang 2001; Chen et al. 2004, 2011; Stensrud et al. 2005, 2007; Wei et al. 2006, 2016; Xiao et al. 2009; Zhang et al. 2009, 2010; Zhu et al. 2010, 2012, 2018, 2019; Gao et al. 2011, 2012; Yao et al. 2011; Mao et al. 2013; Xia et al. 2015; Zhu and Wu 2015; Li et al. 2016b, 2020a, 2022a, 2022c, 2023b, 2023c; Zhu and Li 2017). However, the Latin name *C. sinensis* has been indiscriminately used for both the wild insect-fungal complex and the teleomorph/holomorph of the *C. sinensis* fungus since the 1840s (Yao & Zhu 2016; Li et al. 2022a). The fungus was renamed *Ophiocordyceps sinensis* using *Hirsutella sinensis* Strain EFCC 7287 (GC-biased Genotype #1 of *O. sinensis*) as the nomenclature reference (Sung et al. 2007), while the name of the wild and cultivated insect-fungal complex has remained unchanged. Since then, especially after the improper implementation of IMA's One Fungus=One name nomenclature rule in *O. sinensis* research (Hawksworth et al. 2011; Zhang et al. 2013b; Yao & Zhu 2016; Li et al. 2022a), the name *O. sinensis* has been used to refer to multiple teleomorphic and anamorphic fungi that have distinct genomes and has been indiscriminately applied to the natural insect-fungal complex (Zhu and Wu 2015; Yao & Zhu 2016; Li et al. 2016b, 2022a, 2022c; Zhu and Li 2017). The confusion surrounding Latin name use continues and has even spread from the scientific community to the public media and mass markets, causing a significant decrease in the wholesale and retail prices of natural *C. sinensis*. Because a consensus Latin name for the wild insect-fungal product has not been identified by taxonomists and multiple genotypes of *O. sinensis* with undetermined taxonomic positions are referred to by the same Latin name (Zhang et al. 2012; Ren et al. 2013; Yao & Zhu 2016; Li et al. 2022a, 2022c, 2023c), we temporarily refer to the multiple genotype fungi as *O. sinensis*, including Genotype #1 *H. sinensis*, and we continue to refer to the wild and cultivated insect-fungal complexes as *C. sinensis*; however, this practice only partially ameliorates the academic confusion arising from the indiscriminate use of Latin names and will likely be replaced

by the exclusive use of distinct Latin names in the future.

Wei et al. (2006) hypothesized that GC-biased Genotype #1 *H. sinensis* is the sole anamorph of *O. sinensis*. However, numerous studies have identified 17 mutant genotypes of *O. sinensis* in natural *C. sinensis*, which are indiscriminately referred to by the same Latin name (Zhu et al. 2010, 2018, 2019; Yao & Zhu 2016; Li et al. 2020a, 2022a, 2022c, 2023b, 2023c). Stensrud et al. (2007) and Zhang et al. (2013a) analyzed 71 and 397 ITS sequences, respectively, that were annotated as *C. sinensis* or *O. sinensis* in GenBank under taxid:72228 and confirmed the existence of 3 phylogenetic clades of mutant *O. sinensis* sequences: Group A (GC-biased Genotype #1) and Groups B□C (AT-biased Genotypes #4□5). Stensrud et al. (2007) reported that the variation in the 5.8S gene sequences of *O. sinensis* in Groups A□C "far exceeds what is normally observed in fungi … even at higher taxonomic levels (genera and family)" and that the genotypes "share a common ancestor" and originated from "accelerated nrDNA evolution". Kinjo and Zang (2001) and Mao et al. (2013) reported that the *O. sinensis* genotypes shared the same *H. sinensis*-like morphological and growth characteristics. Xiao et al. (2009) concluded that the genetically variable genotypic sequences likely belonged to independent *O. sinensis* fungi based on the differential coexistence of multiple *O. sinensis* mutants in the stroma and caterpillar body of natural *C. sinensis* in different maturational stages. In the debate regarding "cryptic species" and independent fungi of *O. sinensis*, Zhang et al. (2013a) supported the latter and disproved the former based on the results of K2P analysis. Wei et al. (2016) reported the existence of GC- and AT-biased genotypic teleomorphs of *O. sinensis* in natural and cultivated *C. sinensis*, respectively, and the species contradiction between anamorphic inoculants of GC-biased *H. sinensis* strains and a sole teleomorph of AT-biased Genotype #4 in cultivated *C. sinensis*. Li et al. (2023a) reported the differential occurrence of the mating-type genes of the *MAT1-1* and *MAT1-2* idiomorphs in the *H. sinensis* genome and the differential transcription and silencing of the mating-type genes, which invalidated the "self-fertilization" hypothesis under homothallism and pseudohomothallism (Hu et al. 2013; Bushley et al. 2013) and instead suggested the self-sterilization of *O. sinensis* requiring sexual partners to accomplish reproduction via physiological heterothallism or hybridization. In addition to the identification of molecular heterogeneity of genotypic *O. sinensis* fungi and the requirement of sexual partners for *O. sinensis* reproduction, Figure 3 of (Bushley et al. 2013) illustrates the multicellular heterokaryotic structure of *C. sinensis* hyphae and ascospores, containing multiple mono-, bi-, and tri-nucleated cells. Zhang and Zhang (2015) believed that the

various heterokaryotic cells of the *C. sinensis* hyphae and ascospores likely contain different genetic materials.

The molecular heterogeneity and self-sterilization features of *O. sinensis* and the multicellular heterokaryotic structure of natural *C. sinensis* challenge the sole anamorph hypothesis for *H. sinensis* (GC-biased Genotype #1 of *O. sinensis*). Responding to the academic challenge, Li et al. (2013) proposed the "ITS pseudogene" hypothesis for all AT-biased genotypes "in a single genome" of GC-biased Genotype #1 *H. sinensis*, based on (1) the identification of heterogeneous ITS sequences of both GC-biased Genotype #1 and AT-biased Genotype #5 of *O. sinensis* in 8 out of 15 clones following 25 days of liquid incubation of *C. sinensis* mono-ascospores and (2) the identification of the 5.8S gene cDNA of Genotype #1 but not Genotype #5 in a cDNA library of Strain 1220, one of 8 genetically heterogeneous clones. In this "ITS pseudogene" hypothesis, the simultaneous identification of homogenous Genotype #1 in 7 other clones derived from the same mono-ascospores was apparently ignored. The authors overgeneralized the "ITS pseudogene" hypothesis to all AT-biased Genotypes #4□6 and #15□17 of *O. sinensis* based on insufficient evidence. Li et al. (2020b) further postulated that all AT-biased *O. sinensis* genotypes "could have emerged either before or after" a new GC-biased "ITS haplotype was generated" through "repeat-induced point mutations (RIP)" that cause C-to-T (cytosine→thymine) transitions and cooccur as repetitive ITS copies in "a single genome" of GC-biased *H. sinensis*. These hypotheses proposed by Li et al. (2013, 2020b) attempted to justify the coexistence of AT-biased genotypes in natural *C. sinensis* with GC-biased *H. sinensis* under the sole *O. sinensis* anamorph hypothesis postulated by Wei et al. (2006).

The study presented in this paper examined relevant genomic and transcriptomic sequences in the GenBank database to examine the "ITS pseudogene" hypothesis and the extended hypothesis that AT-biased genotypes formed after a new GC-biased "ITS haplotype was generated" in "a single genome" of *H. sinensis* through "RIP" mutagenesis. We discuss the genetic and phylogenetic characteristics of multiple repetitive ITS copies in the genome of Genotype #1 of *O. sinensis*, the genomic independence of AT-biased genotypes, and whether sufficient evidence exists to reach a conclusion that the 5.8S genes of AT-biased genotypes are permanently nonfunctional pseudogenes.

II. Materials and Methods

II-1. Reagents

Common laboratory reagents such as agarose, electrophoresis reagents, nylon membranes, and blocking solution were purchased from Beijing Bioland Technology Company. The endonucleases *Ava*I, *Dra*I, and *Eco*RI are products of New England BioLabs, United States. Mercuric chloride (0.1%) for surface sterilization of freshly collected *C. sinensis* specimens was a gift from the Institute of Microbiology, Chinese Academy of Sciences. The Universal DNA Purification Kit was a product of TIANGEN BIOTECH Company, China. The DNeasy Plant Mini Kit was a product of Qiagen Company, Germany. The Gel Extraction Kit was a product of Omega Bio-Tek, United States. The *Taq* PCR Reagent Kit and Vector NTI Advance 9 software were purchased from Invitrogen, United States. All primers were synthesized by Invitrogen Beijing Lab. or Beijing Bomaide Technology Co. The DIG High Prime DNA Labeling and Detection Kit was purchased from Roche Diagnostics, Indiana, USA.

II-2. Information on fungal species in natural *C. sinensis*

Information regarding intrinsic fungal species in natural *C. sinensis* was summarized by Jiang and Yao (2013), and subsequent discoveries of additional species among natural *C. sinensis* specimens were reviewed by Li et al. (2016b, 2022a, 2022c). The sequences of 17 genotypes of *O. sinensis* have been obtained and uploaded to GenBank by many molecular mycology groups since 1999. A BLAST search of the GenBank database revealed 668 ITS sequences of *O. sinensis*, which were uploaded by many *O. sinensis* research groups worldwide. These genotypic sequences were analyzed genetically and phylogenetically by Stensrud et al. (2007), Zhu et al. (2010, 2012), Zhang et al. (2013a), Zhu & Wu (2015), Li et al. (2016b, 2022a. 2022c, 2023c), and Zhu & Li (2017).

II-3. Genetic, genomic, and transcriptomic sequences from GenBank

Five genome assemblies, ANOV00000000, JAAVMX000000000, LKHE00000000, LWBQ00000000, and NGJJ00000000, for *H. sinensis* Strains Co18, IOZ07, 1229, ZJB12195, and CC1406-203, respectively, were uploaded to GenBank (Hu et al. 2013; Shu et al. 2020; Li et al. 2016a; Jin et al. 2020; Liu et al. 2020) and used for the analysis of repetitive ITS copies and multiple GC- and AT-biased genotypes.

A Basic Local Alignment Search Tool (BLAST) search of the GenBank database revealed 668 *O. sinensis* ITS sequences under GenBank taxid:72228, 440 of which belong to GC-biased Genotypes #1□3 and #7□14 and 228 belong to AT-biased Genotypes #4□6 and #15□17 (Stensrud et al. 2005,2007; Zhang et al. 2013a; Li et al. 2016b, 2022c, 2023b, 2023c).

mRNA transcriptome assembly GCQL00000000 for *H. sinensis* Strain L0106 was uploaded to GenBank by Liu et al. (2015). Two metatranscriptome studies of natural *C. sinensis* have been reported. Xiang et al. (2014) uploaded the GAGW00000000 metatranscriptome assembly to GenBank, which was obtained from natural specimens (unknown maturational stages) collected from Kangding County of Sichuan Province, China. Xia et al. (2017) uploaded the unassembled SRR5428527 Shotgun Read Archive (SRA) to GenBank for mature *C. sinensis* specimens collected from Deqin County of Yunnan Province, China, but the assembled metatranscriptome sequences were uploaded to www.plantkingdomgdb.com/Ophiocordyceps_sinensis/data/cds/Ophiocordyceps_sinensis_CDS.fas, which is currently inaccessible, but a previously downloaded file is used for analysis.

II-4. Genetic and phylogenetic analyses

The ITS sequences of multiple genotypes of *O. sinensis* and multiple genomic repetitive ITS copies as well as metatranscriptomic/transcriptomic sequences were analyzed using the GenBank BLAST algorithms (https://blast.ncbi.nlm.nih.gov/blast/).

A Bayesian phylogenetic tree of the ITS sequences was inferred using MrBayes v3.2.7 software (Markov chain Monte Carlo [MCMC] algorithm) based on $3x10^4$ samples, with a sampling frequency of 10^3 iterations after discarding the first 25% of samples from a total of $4x10^6$ iterations (Huelsenbeck and Ronquist 2001; Li et al. 2022a, 2023c); this phylogenetic analysis was performed at Nanjing Genepioneer Biotechnologies Co.

II-5. Collection and on-site processing of fresh *C. sinensis*

Fresh *C. sinensis* specimens were purchased in a local market in Kangding County of Sichuan Province, China (3800□4600 m' altitude; latitude 30°049’N, longitude 101°959’E) (Zhu et al. 2010; Yao et al. 2014). Immature *C. sinensis* specimens were characterized by a plump caterpillar body and very short stroma (1.0□2.0 cm). Maturing *C. sinensis* specimens were characterized by a plump caterpillar body and stroma of 2.5□5.0 cm in length, without the formation of an expanded fertile portion close to the stromal tip. Mature *C. sinensis* specimens were characterized by a plump or slightly less plump caterpillar body and long stroma (>5.0 cm) and by the formation of an expanded fertile portion close to the stromal tip, which was densely covered with numerous ascocarps (Zhu et al. 2010; Li et al. 2023a). Governmental permission was not required for *C. sinensis* purchases in local markets, and the collections of *C. sinensis* specimens from sales by local farmers fall under

the governmental regulations for traditional Chinese herbal products.

Freshly collected *C. sinensis* specimens were washed thoroughly in running water with gentle brushing and surface sterilized in 0.1% mercuric chloride for 10 min, followed by washing 3 times with sterile water (Zhu et al. 2010; Li et al. 2023c). The thoroughly cleaned and sterilized *C. sinensis* specimens were immediately frozen in liquid nitrogen and kept frozen during transportation and storage prior to further processing.

II-6. Extraction of genomic DNA and preparation of *C. sinensis* DNA

Caterpillar bodies and stromata of sterilized, frozen *C. sinensis* were separately ground into powder in liquid nitrogen. Genomic DNA was individually extracted from the powder samples using a DNeasy Plant Mini Kit.

II-7. PCR amplification and amplicon sequencing

Genomic DNA of immature, maturing and mature stromata of natural *C. sinensis* was used as the template for PCR amplification. *H. sinensis*-specific primers were designed (a forward primer *Hsprp1*: 5' ATTATCGAGTCACCACTCCCAAACCCCC 3'; and a reverse primer *Hsprp3*: 5' AGGTTCTCAGCGAGCTA 3') based on alignment analysis of the ITS sequences (AB067721, AB067740, and AB067744 representing Genotypes #1 and #4□5 of *O. sinensis*, respectively).

A touch-down PCR protocol was used, in which the annealing temperature was initially set at 70 °C and was then decreased stepwise with a decrement of 0.3 °C every cycle, for a total of 36 cycles (Zhu et al. 2007, 2010). PCR amplicons were subjected to agarose gel electrophoresis, and DNA bands were recovered, purified using a gel extraction kit, and sequenced either directly or after cloning of the amplicons.

II-8. Preparation of fungus-specific ITS1 probe and 18S internal control probe

An *H. sinensis*-specific probe (158 bp) was designed based on the ITS1 sequence of GC-biased Genotype #1 *H. sinensis* and generated through PCR using the forward primer *Hsprp1* described above and the reverse primer *Hsprp2* (5' ATTTGCTTGCTTCTTGACTGAGAGATGCC 3').

A nonspecific 18S internal control probe was designed based on alignments of GenBank 18S rDNA sequences (DQ838795, AB187268, AB067735, AB067736, and AB080090) of several *C. sinensis*-associated fungi. A highly homologous region of 18S rDNA, located over 700 bp upstream

of the ITS1 regions of *C. sinensis*-associated fungi, was selected. The 18S control probe was generated through PCR using the forward primer *Icprp1* (5' ACCGTCGTAGTCTTAACCAT 3') and the reverse primer *Icprp2* (5' ATCGGCTTGAGCCGATAGTC 3').

Both amplicons were confirmed by sequencing, and the probes were labeled using the DIG High Prime DNA Labeling and Detection Kit.

II-9. Southern blotting analysis

The stromata and caterpillar bodies of immature and mature *C. sinensis* were used for Southern blotting. Genomic DNA was extracted from the samples and prepared by endonuclease digestion using *Ava*I, *Dra*I, and *Eco*RI. The digested genomic DNA (5 µg of each sample) was subjected to agarose gel electrophoresis and transferred onto a nylon membrane through capillary transfer, followed by baking the membrane at 58 °C for 4 hours for fixation, blocking the binding sites on the membrane in blocking solution, and hybridizing with the labeled *H. sinensis*-specific probe at 40 °C overnight. The hybridized transblotted membrane was viewed by colorimetric photography using a gel imaging system (Bio-Rad) and then stripped and re-probed with the labeled 18S internal control probe.

III. The results

III-1. Multiple GC-biased repetitive ITS copies in the *H. sinensis* genome

Generally, fungal genomes contain multiple repetitive copies of nrDNA ITS1-5.8S-ITS2 sequences. However, a single copy of the ITS1-5.8S-ITS2 sequences was identified in genome assemblies ANOV01021709, LKHE01000582, and LWBQ01000008 of *H. sinensis* Strains Co18, 1229, and ZJB12195, respectively (Hu et al. 2013; Li et al. 2016a; Jin et al. 2020), probably because the repetitive ITS copies were discarded during the assembly of the genome shotgun sequences. On the other hand, multiple repetitive ITS copies were identified in 2 additional genome assemblies, JAAVMX000000000 and NGJJ00000000, for *H. sinensis* Strains IOZ07 and CC1406-203, respectively (Liu et al. 2020; Shu et al. 2020) (Table 1), where 17 ITS1-5.8S-ITS2 copies within JAAVMX000000000 and 7 ITS1-5.8S-ITS2 copies were found, as well as 2 partial ITS segments within NGJJ00000000 that were not included in our analysis.

Most of the repetitive ITS copies listed in Table 1 were highly homologous (>97%) to the reference sequence AB067721 of GC-biased Genotype #1 *H. sinensis*. Two repetitive copies

(6233→6733 and 44729→45251) within JAAVMX010000019 showed 94.5% and 90.8% similarities to AB067721, respectively (Table 1). Two other copies, 18702095→18702586 within JAAVMX010000002 and 700→1186 within JAAVMX010000018, showed similarities of 97.4% and 97.0% to AB067721, respectively. All the repetitive ITS copies showed 80.1%□89.9% similarity to the sequences of AT-biased Genotypes #4□6 and #15□17 of *O. sinensis* (Table 1).

Table 1. *GC contents of the repetitive ITS1-5.8S-ITS2 sequences from the genome assemblies of 2 H. sinensis strains and percent similarities of the repetitive ITS sequences to the sequences of GC-biased Genotype #1 (AB067721) and AT-biased Genotypes #4□6 and #15□17 of O. sinensis.*

Genome segment	Sequence range & direction	GC content	% Similarity of the repetitive sequence	
			vs. Genotype #1 AB067721	*vs*. AT-biased Genotypes #4□6 and #15□17
JAAVMX010000002	18688917→18689407	64.8%	100%	86.7% □ 89.9%
	18702095→18702586	64.5%	97.4%	82.8% □ 88.0%
JAAVMX010000008	13823→14313	64.8%	100%	85.5% □ 89.9%
	1199→1687	64.8%	99.2%	85.2% □ 89.3%
JAAVMX010000017	9147←9637	64.8%	100%	85.5% □ 89.9%
	21791←22281	64.8%	100%	85.5% □ 89.9%
	34435←34925	64.8%	100%	85.5% □ 89.9%
	47079←47569	64.8%	100%	85.5% □ 89.9%
JAAVMX010000018	13381→13871	64.8%	100%	85.5% □ 89.9%
	26076→26566	64.8%	100%	85.5% □ 89.9%
	38771→39261	64.8%	100%	85.5% □ 89.9%
	51467→51958	64.6%	98.8%	85.5% □ 88.8%
	700→1186	64.5%	97.0%	82.6% □ 87.6%
JAAVMX010000019	19404→19894	64.8%	100%	85.5% □ 89.9%
	32048→32538	64.8%	100%	85.5% □ 89.9%
	6233→6733	65.3%	94.5%	81.3% □ 85.5%
	44729→45251	63.3%	90.8%	80.1% □ 84.6%
NGJJ01000573	13133←13623	64.8%	99.4%	85.2% □ 89.6%
	14624←15113	64.7%	99.4%	84.8% □ 89.6%
NGJJ01000582	10703←11192	64.7%	99.8%	85.5% □ 89.8%
NGJJ01000796	1514→2004	64.8%	100%	85.5% □ 89.9%
NGJJ01000798	1177→1665	64.6%	99.6%	84.8% □ 89.6%
NGJJ01000799	3295→3785	64.8%	100%	85.5% □ 89.9%
	16666→17156	64.8%	100%	85.5% □ 89.9%

III-2. Genetic characteristics of the multiple repetitive ITS sequences in the *H. sinensis* genome

The repetitive ITS copies within genome assemblies JAAVMX000000000 and NGJJ00000000 for *H. sinensis* Strains IOZ07 and CC1406-203, which are listed in Table 1, and the single genomic ITS copies ANOV01021709, LKHE01000582, and LWBQ01000008, for *H. sinensis* Strains Co18, 1229, and ZJB12195, respectively, which are listed in Table 2 (Hu et al. 2013; Jin et al. 2020; Li et al. 2016a; Liu et al. 2020; Shu et al. 2020), were GC biased; their average GC content was 64.7±0.33% (Table 1), similar to the GC content (64.8%) of the AB067721 Genotype #1 sequence. Apparently, these genetic features were distinct from those of AT-biased Genotypes #4□6 and #15□17 of *O. sinensis*, which included multiple scattered transition alleles and contained 46.9%□55.2% AT bases and 44.8%□53.1% GC bases.

Table 2. *Percent similarities between nrDNA ITS1-5.8S-ITS2 sequences of 5 genome assemblies of Genotype #1 H. sinensis and 17 O. sinensis genotype sequences. (Modified from Table 1 of Li et al. 2023c).*

Genotype # and GenBank accession #	% Similarity between the *H. sinensis* genomic ITS segments and 17 *O. sinensis* genotypes				
	ANOV01021709 838→1504	LKHE01000582 2074→2740	LWBQ01000008 991739→992405	JAAVMX010000017 9029←9695	NGJJ01000799 3237→3903
#1 AB067721	99.7%	100%	99.6%	100%	100%
#2 MG770309	94.4%	94.7%	94.7%	94.7%	94.7%
#3 HM595984	95.9%	96.1%	95.6%	95.3%	95.3%
#7 AJ488254	94.7%	95.0%	95.3%	95.0%	95.0%
#8 GU246286	89.5%	89.5%	89.2%	89.5%	89.5%
#9 GU246288	95.0%	95.2%	94.6%	95.2%	95.2%
#10 GU246287	83.0%	83.2%	83.0%	83.2%	83.2%
#11 JQ695935	78.9%	79.1%	78.5%	79.1%	79.1%
#12 GU246296	94.3%	94.5%	93.9%	94.5%	94.5%
#13 KT339190	87.5%	87.5%	87.2%	87.5%	87.5%
#14 KT339178	89.3%	89.6%	89.1%	89.6%	89.6%
#4 AB067744	89.1%	89.4%	89.3%	89.4%	89.4%
#5 AB067740	86.3%	86.7%	86.2%	86.7%	86.7%
#6 KJ720572	85.5%	85.5%	85.5%	85.5%	85.5%
#15 KT232017	89.6%	89.9%	89.4%	89.9%	89.9%
#16 KT232019	87.4%	87.7%	87.6%	87.7%	87.7%

#17 KT232010	87.7%	88.1%	87.5%	88.1%	88.1%

Note: "GT" represents genotype. GC-biased genotypes are listed in blue in the upper panel. AT-biased genotypes are listed in red in the lower panel.

The sequences of GC-biased repetitive ITS copies that were variable (94.5% and 90.8% similarities) and slightly less variable (97.4% and 97.0% similarities) in comparison to the reference sequence AB067721 of Genotype #1 predominantly contained insertion/deletion alleles and included less frequent transversion alleles, while no or only a few transition alleles were found. The variable ITS repeats 6233→6733 and 44729→45251 within JAAVMX010000019 (*cf.* Table 1) contained 22 and 32 insertion/deletion alleles and 4 and 11 transversion alleles, respectively (Table 3; in blue in Figure 1); they contained only 1 and 5 transition mutant alleles, having allelic ratios of insertion/deletion/transversion *vs*. transition of 26:1 and 43:5, respectively. The slightly less variable ITS repeats, 18702095→18702586 within JAAVMX010000002 and 700→1186 within JAAVMX010000018 (*cf.* Table 1), contained no transition alleles and predominantly included 13 and 15 insertion, deletion and transversion alleles, with allelic ratios of insertion/deletion/transversion *vs*. transition of 13:0 and 15:0, respectively (Table 3; in green in Figure 1). These genetic characteristics of the variable and slightly less variable repetitive ITS copies indicate that they might not have been generated through RIP mutagenesis, which theoretically causes C-to-T (cytosine→thymine) transition mutations. Other repetitive ITS copies with 98.1%□99.8% similarity to AB067721 listed in Table 1 contained only a few insertion/deletion alleles.

Table 3. *Types of mutations in the variable and slightly less variable genomic repetitive ITS sequences compared with GC-biased Genotype #1 (AB067721).*

	Sequence range & direction	% Similarity	Number of mutant alleles			Ins./Del./Transv. *vs*. Transit.
			Ins./Del.	Transv.	Transit.	
JAAVMX010000002	18702095→18702586	97.4%	13	0	0	13 : 0
JAAVMX010000018	700→1186	97.0%	10	5	0	15 : 0
JAAVMX010000019	6233→6733	94.5%	22	4	1	26 : 1
	44729→45251	90.8%	32	11	5	43 : 5

Note: "Ins./Del." indicates insertion/deletion point mutations. "Transv." denotes Transversion

point mutations. "Transit." refers to transition point mutations.

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GT#1  AB067721  59        TCGAGTCACCACTCCC AAACCCCCTGCGAACACCACAGCAGTTGCCTCGGCGGGACCGCCCCGGCG CCCCAGGGCCCG 136
JAAVMX010000019 19404     ---------------- ------------------------------------------------- ------------ 19481
JAAVMX010000019 32048     ---------------- ------------------------------------------------- ------------ 32125
JAAVMX010000002 18702095  ---------------- ----------------------- ---------------------------T------------ 18702172
JAAVMX010000018 700       --------- ------ ------------------------------------T------------ ------------T 776
JAAVMX010000019 6233      ----------------C-------------------------------------------------- ------------ 6311
JAAVMX010000019 44729     ---------------- ------------------------------------------------- ------------ 44806
GT#4  AB067744  55        ------T--------T --------------T--T------------------------------- ---T--------- 132
GT#5  AB067740  59        ------T--T----TT ---------ATA--T--T-T-----------TA------------TA--- ---T-A------ 136
GT#6  KJ720572  11                         -------A----T--T-T-----------TA------------TA--- --TT-A------ 70
GT#15 KT232017  37        ------T--------T --------------T--T------------------------------- ---T--------- 114
GT#16 KT232019  37        ------T--T-----T --------------T--T-T-----------TA------------TA--- ---T-A------ 114
GT#17 KT232010  37        ------T--T-----T --------------T--T-T-----------TA------------TA--- ---T-A------ 114

GT#1  AB067721  137       GACCAGG GCGCCC GCCGGAGGACCCCCAGAC CCTCCTGTCGCA GTGGCATCT CTCAGTCAAGAA GCAAGCAAAT 210
JAAVMX010000019 19482     ------- ------ ------------------ ------------ --------- ------------ ---------- 19555
JAAVMX010000019 32126     ------- ------ ------------------ ------------ --------- ------------ ---------- 32199
JAAVMX010000002 18702173  ------- ------A------------------ ------------A--------- ---- ------- ---------- 18702247
JAAVMX010000018 777       ------- ------ ------------------ ------------ --------- ---C-------- ---------- 850
JAAVMX010000019 6312      ------- ------ ------T-----------C------------ ---------C------------G---------- 6388
JAAVMX010000019 44807     -------C------ ------------------ ------------ --------- ------------ ---------- 44881
GT#4  AB067744  133       ---T--- ------ ------------TT---- ------A---T- --A------ --T---T----- ---------- 206
GT#5  AB067740  137       ---T-AA ------ ------------TT---- ------A-TAT- --A-T---- --T---T----- ---------- 213
GT#6  KJ720572  71        ---T--- ------ ------------TT---- ------A---T- --A-T---- --T---T----- ---------- 182
GT#15 KT232017  115       ---T--- ------ ------------TT---- ------A----- --A------ --T---T----- ---------- 188
GT#16 KT232019  115       ---T-AA ------ ------------TT---- ------A----- --A-T---- --T---T----- ---------- 188
GT#17 KT232010  115       ---T-AA ------ ------------TT---- ------A-TAT- --A-T---- --T---T----- ---------- 188

GT#1  AB067721  211       GAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATC GATG AAGAACGCAGCGAAATGCGATAAGTAATGTGAA 288
JAAVMX010000019 19556     -------------------------------------- ---- ---------------------------------- 19633
JAAVMX010000019 32200     -------------------------------------- ---- ---------------------------------- 32277
JAAVMX010000002 18702248  -- ------------------------------------C---- ---------------------------------- 18702325
JAAVMX010000018 851       ---------------------------------------- ----A----------------------- --------- 928
JAAVMX010000019 6389      -------------- ---CAC-------------------- ---- ---------------- --------- ------- 6463
JAAVMX010000019 44882     ---------------------------------------- ---- ---------------------------------- 44959
GT#4  AB067744  207       A---T--------T--T------------A---T-A----T A--A ----------------A-------------A-- 284
GT#5  AB067740  214       A---T--------T---------------A-----A----T A--A --A---------------A---------A-A-- 288
GT#6  KJ720572  183       A---T--------T---------------A-----A----- ---A -------T--T-----A-----------A-A-- 222
GT#15 KT232017  189       ----T--------T--T------------A---T-A----T A--A ----------------A-------------A-- 266
GT#16 KT232019  189       ----T--------T---------------A-----A----- ---A --A---------------A---------A-A-- 266
GT#17 KT232010  189       A---T--------T---------------A-----A----- ---A --A---------------A---------A-A-- 266

GT#1  AB067721  289       TTGCAGAATTCAGTGAACCA TCGAATCTTT GAACG CACATTG CGCCCGCCAGCACTCTGGCGGGCATGC  CTGTC 362
JAAVMX010000019 19634     -------------------- ---------- ----- ------- ---------------------------  ----- 19707
JAAVMX010000019 32278     -------------------- ---------- ----- ------- ---------------------------  ----- 32351
JAAVMX010000002 18702326  -------------------- ----------T----- ----- - ------- --------------------  ----- 18702398
JAAVMX010000018 929       -------------------- -----C---------- -----C-------C-------------------- -------  ----- 1000
JAAVMX010000019 6464      --------------------C---------- ----- -------C---------------------------  ----- 6539
JAAVMX010000019 44960     -------------------- ---------- ----- ------- ---------------------------CT----- 45035
GT#4  AB067744  285       ---T------T---A---T- ---------- A---- T-T-C-A -------T--------A----------  ----- 358
GT#5  AB067740  289       --A-------T---A---T- ---------- A---- --T---A -------T--------A----------  --A-- 362
GT#6  KJ720572  223       --AT------T---A----- ---------- A---- T-T---A -------T--------A----------  ----- 296
GT#15 KT232017  267       ---T------T---A---T- ---------- A---- T-T---A -------T--------A----------  ----- 340
GT#16 KT232019  267       --A-------T---A---T- ---------- A---- T-T---A -------T--------A----------  ----- 340
GT#17 KT232010  267       --A-------T---A---T- ---------- A---- --T---- -------T--------A----------  --A-- 340

GT#1  AB067721  363       CGAGCGTCATCTCAACCCTCGAG CCCCCCGCCTCGCGG  CGGCGGGGCCCGGCCTT GGGGGTCACGGCC CCGCGCC 437
JAAVMX010000019 19708     ------------------------  --------------  ----------------- ------------- ------- 19781
JAAVMX010000019 32352     ------------------------  --------------  ----------------- ------------- ------- 32425
JAAVMX010000002 18702399  ------------------------ ----- ---------  ----------------- ------------- ------- 18702472
JAAVMX010000018 1001      ------------------------ ------ ---------  -----------------G------------- ---- -- 1074
JAAVMX010000019 6540      ------------------------  --------------  ------------ ---- -------------C------- 6613
JAAVMX010000019 45036     ------------------------C----------A----TA--CG---C--------- ------TC-----AA------ 45119
GT#4  AB067744  359       -------T-C--T------TA-- ---------------  ----------------- A------------ --A---- 432
GT#5  AB067740  363       -------T----T---------- ---------------  ---------T------- A-----T------ ------- 436
GT#6  KJ720572  297       -------T-C--T----                                                                313
GT#15 KT232017  341       -------T----T---------- ---------------  ---------T------- A-----T------ ------- 414
GT#16 KT232019  341       -------T-C--T------TA-- ---------------  ----------------- A------------ --A---- 414
GT#17 KT232010  341       -------T----T---------- ---------------  ---------T------- A-----T------ ------- 414

GT#1  AB067721  438       G   CCCCCTAAACGC AG       TGGCGACC  CCGCCGCGGCTCC CCTGCGCAGTAGCT  CG  CTGAGAACCT 499
JAAVMX010000019 19782     -   ------------ --       --------  ------------- --------------  --  ---------- 19844
JAAVMX010000019 32426     -   ------------ --       --------  ------------- --------------  --  ---------- 32488
JAAVMX010000002 18702473  -   ------------ --       --------  ------------- --------------  --  ---------- 18702534
JAAVMX010000018 1075      -   ------------ --       --------  ------------- --------------  --  ---------- 1136
JAAVMX010000019 6614      -    -----------G--G      --------GC------------- --------------  --GCT--------- 6680
JAAVMX010000019 45120     -TCC-T---GT----- --CCTAAGTG-------  ----GA-------A----T-A-------CA--  ---------- 45189
GT#4  AB067744  433       -   ------------ --       -A-T----  ------------- ---A----A-----  TA  --A------- 495
GT#5  AB067740  437       -   ----T------T --       -A------  ------------- ---A--TG------  -A  -C-------- 529
GT#6  KJ720572  313
GT#15 KT232017  415       -   -----------T --       -A------  ------------- ---A--T-A-----  -A  -C-------- 477
GT#16 KT232019  415       -   ------------ --       -A------  ------------- ---A----A-----  TA  --A------- 477
GT#17 KT232010  415       -   -----------T --       -A------  ------------- ---A--T-A-----  -A  -C-------- 477

GT#1  AB067721  500        CGCACCGGGAGCGCGGA     GGCGG TCACGCC  TGG    AA ACCACC   ACACCCTCCA              549
JAAVMX010000019 19845      ------------------     ----- ----- -  ---    -- ------   ----------              19894
JAAVMX010000019 32489      ------------------     ----- ----- -  ---    -- ------   ----------              32537
JAAVMX010000002 18702535   ------------------     ----- -------GTGT-    -- ------   ----------              18702586
JAAVMX010000018 1137       ------------------     ----- ----- -  ---    -- ------   ----------              1186
JAAVMX010000019 6681      C------------------     ----- ----- -  ---    -- ------ACA----------              6733
JAAVMX010000019 45190      ------------------CCCAT-----C-------  ---GATG--C------ACC----------              45251
GT#4  AB067744  496        -----TA----------     ----- -------  --A    -- -----T   -T------              543
GT#5  AB067740  530        --------A--------     ----- -------  --A    -- -----T   --G--                 544
GT#6  KJ720572  313
GT#15 KT232017  478        --------A--------     ----- -------  --A    -- -----T   --G--                 522
GT#16 KT232019  478        -----TA----------     ----- -------  --A    -- -----T   -T------              525
GT#17 KT232010  478        --------A--------     ----- -------  --A    -- -----T   --G--                 522
```

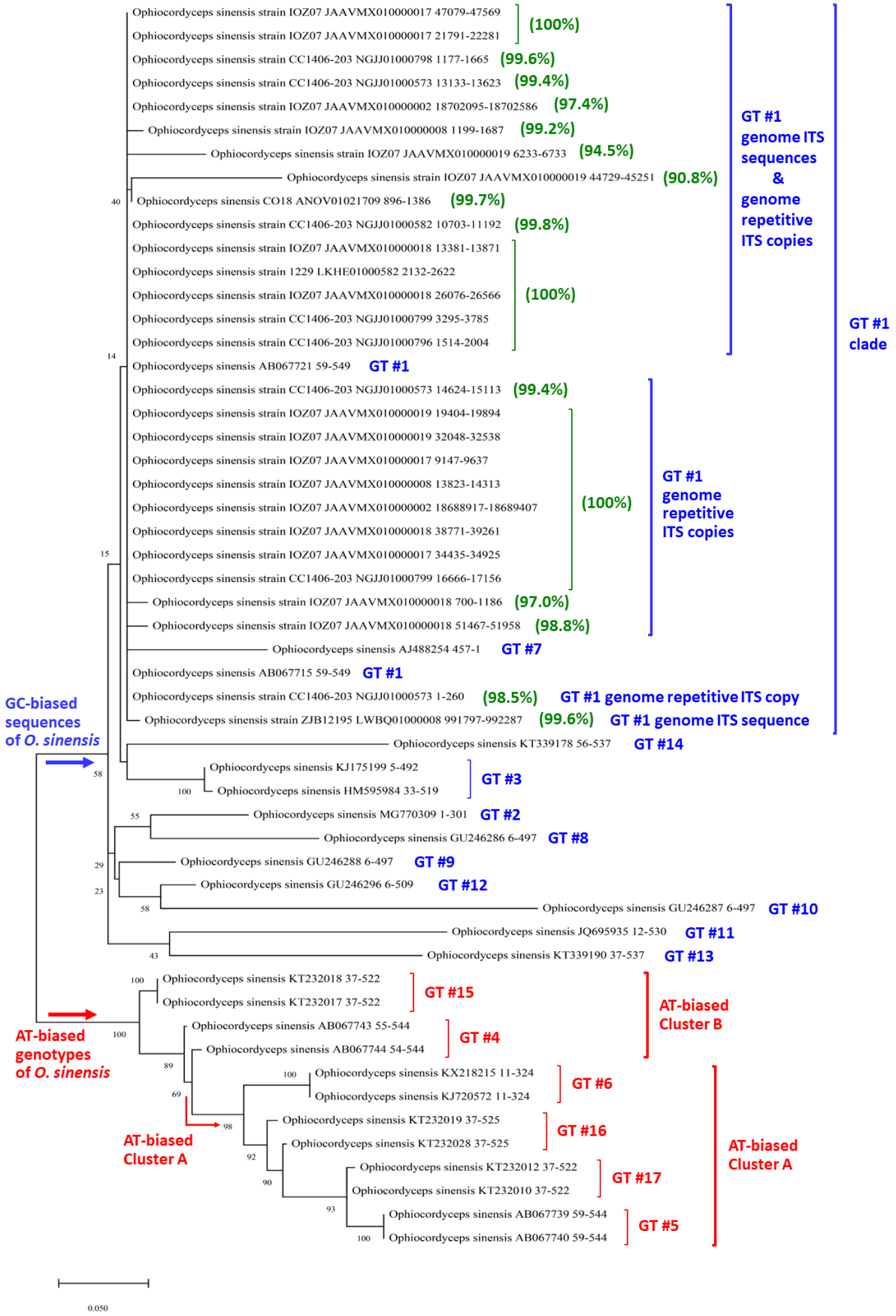
295

Figure 1. ITS sequence alignment of AB067721, repetitive ITS copies within genome JAAVMX000000000 of H. sinensis Strain IOZ07, and AT-biased genotypes of O. sinensis.

The ITS sequences contain complete or partial ITS1-5.8S-ITS2 nrDNA segments. "GT" denotes the O. sinensis genotype. The underlined sequence in black represents the 5.8S gene of GC-biased Genotype #1 H. sinensis. AB067721 is the ITS sequence of GC-biased Genotype #1 H. sinensis. Genome assemblies JAAVMX010000002, JAAVMX010000018, and JAAVMX010000019 were obtained from the H. sinensis Strain IOZ07 (Shu et al. *2020). One copy each within JAAVMX010000002 and JAAVMX010000018 indicated in* ***green*** *shows 97.4% or 97.0% similarity to AB067721. JAAVMX010000019 contains 4 repetitive ITS copies, including 2 copies in* ***black*** *(19404→19894 and 32048→32537), which are 100% identical to AB067721, and 2 other copies in* ***blue*** *(6233→6733 and 44729→45251), with 94.5% and 90.8% similarities to AB067721. Sequences in* ***red*** *(AB067744, AB067740, KJ720572, KT232017, KT232019, and KT232010) represent AT-biased Genotypes #4□6 and #15□17 of O. sinensis, respectively. The underlined "GAATTC" sequences in* ***purple*** *represent the Eco*RI *endonuclease cleavage sites in the sequences of GC-biased Genotype #1 and GC-biased genome assembly JAAVMX000000000. Eco*RI *endonuclease cleavage sites are absent in AT-biased ITS sequences due to a single-base C-to-T (cytosine→thymine) transition. Hyphens indicate identical bases, and spaces denote unmatched sequence gaps.*

III-3. Phylogenetic features of multiple repetitive ITS copies in the *H. sinensis* genome

As shown in Tables 1□2 and Figure 1, the sequences of GC-biased Genotypes #2–3 and #7–14 and of AT-biased Genotypes #4□6 and #15□17 of *O. sinensis* are absent in the genome assemblies ANOV00000000, JAAVMX000000000, LKHE00000000, LWBQ00000000, and NGJJ00000000 of *H. sinensis* Strains Co18, IOZ07, 1229, ZJB12195, and CC1406-203, respectively, and instead belong to the genomes of independent *O. sinensis* fungi (Stensrud et al. 2007; Xiao et al. 2009; Hu et al. 2013; Zhu & Wu 2015; Li et al. 2016a, 2016b, 2020a, 2022a, 2022c, 2023c; Zhu & Li 2017; Zhu et al. 2018, 2019; Jin et al. 2020; Liu et al. 2020; Shu et al. 2020). The genomic repetitive ITS copies were phylogenetically clustered into the GC-biased clades in blue alongside the Bayesian majority rule consensus tree in Figure 2 and were phylogenetically distant from the AT-biased clades containing Genotypes #4□6 and #15□17 of *O. sinensis* shown in red alongside in the tree, similar to what has previously been demonstrated in phylogenetic trees (Kinjo & Zang 2001; Stensrud et al. 2007; Zhang et al. 2013a; Zhu & Wu 2015; Li et al. 2016b, 2020a, 2022a, 2022c, 2023c; Wei et al. 2016; Zhu & Li 2017).


Ophiocordyceps sinensis strain IOZ07 JAAVMX010000017 47079-47569
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000017 21791-22281
(100%)
Ophiocordyceps sinensis strain CC1406-203 NGJJ01000798 1177-1665 (99.6%)
Ophiocordyceps sinensis strain CC1406-203 NGJJ01000573 13133-13623 (99.4%)
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000002 18702095-18702586 (97.4%)
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000008 1199-1687 (99.2%)
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000019 6233-6733 (94.5%)
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000019 44729-45251 (90.8%)
40
Ophiocordyceps sinensis CO18 ANOV01021709 896-1386 (99.7%)
Ophiocordyceps sinensis strain CC1406-203 NGJJ01000582 10703-11192 (99.8%)
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000018 13381-13871
Ophiocordyceps sinensis strain 1229 LKHE01000582 2132-2622
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000018 26076-26566
(100%)
Ophiocordyceps sinensis strain CC1406-203 NGJJ01000799 3295-3785
Ophiocordyceps sinensis strain CC1406-203 NGJJ01000796 1514-2004
GT #1 genome ITS sequences & genome repetitive ITS copies
GT #1 clade
14
Ophiocordyceps sinensis AB067721 59-549 GT #1
Ophiocordyceps sinensis strain CC1406-203 NGJJ01000573 14624-15113 (99.4%)
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000019 19404-19894
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000019 32048-32538
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000017 9147-9637
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000008 13823-14313
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000002 18688917-18689407
(100%)
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000018 38771-39261
15
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000017 34435-34925
Ophiocordyceps sinensis strain CC1406-203 NGJJ01000799 16666-17156
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000018 700-1186 (97.0%)
Ophiocordyceps sinensis strain IOZ07 JAAVMX010000018 51467-51958 (98.8%)
GT #1 genome repetitive ITS copies
Ophiocordyceps sinensis AJ488254 457-1 GT #7
Ophiocordyceps sinensis AB067715 59-549 GT #1
Ophiocordyceps sinensis strain CC1406-203 NGJJ01000573 1-260 (98.5%) GT #1 genome repetitive ITS copy
Ophiocordyceps sinensis strain ZJB12195 LWBQ01000008 991797-992287 (99.6%) GT #1 genome ITS sequence
GC-biased sequences of O. sinensis
Ophiocordyceps sinensis KT339178 56-537 GT #14
58
Ophiocordyceps sinensis KJ175199 5-492
100
Ophiocordyceps sinensis HM595984 33-519
GT #3
55
Ophiocordyceps sinensis MG770309 1-301 GT #2
Ophiocordyceps sinensis GU246286 6-497 GT #8
29
Ophiocordyceps sinensis GU246288 6-497 GT #9
23
Ophiocordyceps sinensis GU246296 6-509 GT #12
58
Ophiocordyceps sinensis GU246287 6-497 GT #10
Ophiocordyceps sinensis JQ695935 12-530 GT #11
43
Ophiocordyceps sinensis KT339190 37-537 GT #13
100
Ophiocordyceps sinensis KT232018 37-522
Ophiocordyceps sinensis KT232017 37-522
GT #15
AT-biased Cluster B
AT-biased genotypes of O. sinensis
100
Ophiocordyceps sinensis AB067743 55-544
Ophiocordyceps sinensis AB067744 54-544
GT #4
89
100
Ophiocordyceps sinensis KX218215 11-324
Ophiocordyceps sinensis KJ720572 11-324
GT #6
69
98
Ophiocordyceps sinensis KT232019 37-525
Ophiocordyceps sinensis KT232028 37-525
GT #16
AT-biased Cluster A
92
Ophiocordyceps sinensis KT232012 37-522
Ophiocordyceps sinensis KT232010 37-522
GT #17
90
93
Ophiocordyceps sinensis AB067739 59-544
Ophiocordyceps sinensis AB067740 59-544
GT #5
AT-biased Cluster A
100
0.050

327

Figure 2. A Bayesian majority rule consensus phylogenetic tree.
GT represents genotype. Twenty-eight ITS segments of the genome assemblies (ANOV01021709, LKHE01000582, LWBQ01000008, JAAVMX010000002, JAAVMX010000008, JAAVMX0100000017, JAAVMX0100000018, JAAVMX010000019, NGJJ01000573, NGJJ01000582, NGJJ01000796, NGJJ01000798, and NGJJ01000799) of H. sinensis strains (Co18, 1229, ZJB12195, IOZ07, and CC1406-203, respectively) and 25 ITS sequences of GC-biased Genotypes #1–3 and #7–14 (in blue alongside the tree) and AT-biased Genotypes #4–6 and #15–17 of O. sinensis (in red alongside the tree) were analyzed phylogenetically using MrBayes v3.2.7 software (cf. Methods Section II-4). Genome assemblies JAAVMX000000000 and NGJJ00000000 contain multiple repetitive ITS copies (cf. Table 1). The percent similarities of the genomic sequences of repetitive ITS copies with the representative Genotype #1 sequence (AB067721) are shown in green alongside the tree.

Figure 2 includes the 2 genomic repetitive ITS copies with low similarities (94.5% and 90.8%) and the 2 repetitive ITS copies with relatively low similarities (97.4% and 97.0%) that are shown in Tables 1 and 3. The ITS copies are individually scattered in the GC-biased clades. The 2 low-similarity genomic repetitive ITS copies, 6233→6733 (94.5% similarity) and 44729→45251 (90.8% similarity), within genome assembly JAAVMX010000019 (*cf.* Tables 1 and 3), showed greater phylogenetic distances (horizontal lines) from other GC-biased ITS copies within the GC-biased clades. GC-biased Genotypes #2□3 and #7□14 of *O. sinensis* showed greater phylogenetic distances from the sequences of GC-biased Genotype #1 and the repetitive ITS copies. These results indicate that genetic variations in the GC-biased repetitive ITS sequences shown in blue in Figure 1 had an impact on Bayesian phylogenetic clustering analysis.

III-4. Genetic characteristics of the multiple repetitive ITS sequences in the *H. sinensis* genome

The Bayesian tree in Figure 2 shows that GC-biased Genotype #7 was placed within the Genotype #1 clade with a great phylogenetic distance, although it showed 94.7%□95.3% similarity to Genotype #1 *H. sinensis* (*cf.* Table 1). The Genotype #7 sequence (AJ488254) was identified in the stroma of a *C. sinensis* specimen (H1023) collected from Qinghai Province and uploaded to GenBank by Chen et al. (2011). The authors identified GC-biased Genotype #1 ITS sequence (AJ488255) from the tail of the caterpillar body of the same specimen, giving rise to the question of whether the sequences of AJ488254 and AJ488255 coexisted in a single genome of GC-biased Genotype #1 *H. sinensis* as 2 of repetitive ITS copies or belonged to the genomes of independent *O. sinensis* fungi cooccurring in different compartments of the same *C. sinensis* specimen. The GC-biased genotype sequences were further analyzed, as shown in Figure 3, and compared with the

repetitive ITS copies in the genome.

```
GT#1  AB067721  59        TCGAGTC ACCACTCCC AAA CCCCCTGCGAACACCACAGCAGTTGCCTCGGCGGGACCGCCCCGGCG CCCCAGGGCCCG 136
JAAVMX010000019 19404     ------- --------- --- -------------------------------------------------- ------------ 19481
JAAVMX010000019 32048     ------- --------- --- -------------------------------------------------- ------------ 32125
JAAVMX010000002 18702095  ------- --------- --- ---------------------- --------------------------T------------ 18702172
JAAVMX010000018 700       ------- -- ------ --- ------------------------------------T------------ ------------T 776
JAAVMX010000019 6233      ------- ---------C--- -------------------------------------------------- ------------ 6311
JAAVMX010000019 44729     ------- --------- --- -------------------------------------------------- ------------ 44806
GT#2  MG770309
GT#3  HM595984  33        CT----T G-------- --- A----C-T-------------------------------------- ------------ 110
GT#7  AJ488254  464       ------- -A----T-- G--G--------------------------- -------------T-T---- -A-----TG--- 380
GT#8  GU246286  4         ------T --T------ --  ---------------------------------T-------T---- --G----T---- 83
GT#9  GU246288  5         ------TT--------- --- ----------------------C----G----------------------- ------------ 84
GT#10 GU246287  6         ------T -G ------ T-- ----------------------T----T-----TTTG------------ -----A------ 82
GT#11 JQ695935  12                 ------- --  ---------- ------------------------------------- ------------ 78
GT#12 GU246296  5         ------T --------- --- -------------------------------------------------- ------------ 83

GT#1  AB067721  137       GACCAGG GCGCCC GCCGGAGGACCCCCAGAC CCTCCTGTCGCA GTGGCATCT CTCAGTCAAGAA GCAAGCAAATGA 213
JAAVMX010000019 19482     ------- ------ ------------------- ------------ --------- ------------ ------------ 19557
JAAVMX010000019 32126     ------- ------ ------------------- ------------ --------- ------------ ------------ 32201
JAAVMX010000002 18702173  ------- ------A------------------- ------------A--------- ---- ------- ------------ 18702249
JAAVMX010000018 777       ------- ------ ------------------- ------------ --------- ---C-------- ------------ 852
JAAVMX010000019 6312      ------- ------ ------T------------C------------ ---------C------------G------------ 6390
JAAVMX010000019 44807     -------C------ ------------------- ------------ --------- ------------ ------------ 44883
GT#2  MG770309  1                 ----G AT--ATTATT-A----C- -----C-A---- -------T- ------------ ------------ 68
GT#3  HM595984  111       ---T--- ------ ------------------- ------------ -----G--- ------------ ------------ 186
GT#7  AJ488254  379       A------ ------ ------------------- ------------ --------- ------------ ------------ 295
GT#8  GU246286  84        -C-G--- -----T -GAAAT-TT--AA-T--- ------------ ----C---- ------------ ------------ 159
GT#9  GU246288  85        ------- ------ ------------------- ------------ --------- ----------G- -----G------ 160
GT#10 GU246287  83        ------- ------ -----G------------ -----------G  ----GT-T- T------------ AGG--G------ 157
GT#11 JQ695935  79        ------- ------ ------------------- ------------ --------- ------------ ------------ 154
GT#12 GU246296  84        ------- ------ ------------------- ------------ --------- ------------ ------------ 159

GT#1  AB067721  214       ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATC GATG AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC 292
JAAVMX010000019 19557     ----------------------------------------- ---- ---------------------------------------- 19637
JAAVMX010000019 32201     ----------------------------------------- ---- ---------------------------------------- 32281
JAAVMX010000002 18702249  ----------------------------------------C---- ---------------------------------------- 18702329
JAAVMX010000018 852       ----------------------------------------- ----A------------------------- ------------ 932
JAAVMX010000019 6390      ------------ ---CAC--------------------- ---- --------------- --------- ------------ 6467
JAAVMX010000019 44883     ----------------------------------------- ---- ---------------------------------------- 44963
GT#2  MG770309  68        ----------------------------------------- ---- ----GA---------C --------------------- 147
GT#3  HM595984  186       ----------------------------------------- ---- ---------------------------------------- 266
GT#7  AJ488254  295       ----------------------------------------- ---- -----------A---------------------------- 224
GT#8  GU246286  159       --------------T-----------------T-------- ---- -G---------------G--------------------- 239
GT#9  GU246288  161       -------------------------T--------------- ---- ---------------------------------------- 240
GT#10 GU246287  158       ------T-----------------T--------------- ---- -------C----C------------G------------ 237
GT#11 JQ695935  155       ----------------------------------------- ---- ---------------------------------------- 234
GT#12 GU246296  160       ----------------------------------------------------------------G---------------- 239

GT#1  AB067721  293       AGAATTCAGTGAACCA TCGAATCTTT GAACG CACATT G CGCCCGCCAGCACTCTGGCGGGCATGC  CTGTCCGAGC 367
JAAVMX010000019 19638     ---------------- ---------- ----- ------ - ---------------------------  ---------- 19712
JAAVMX010000019 32282     ---------------- ---------- ----- ------ - ---------------------------  ---------- 32356
JAAVMX010000002 18702330  ---------------- ----------T----- -----  - ------- --------------------  ---------- 18702403
JAAVMX010000018 933       --------------- C---------- -----C------ -C-------------------- -------  ---------- 1005
JAAVMX010000019 6468      ----------------C---------- ----- ------ -C---------------------------  ---------- 6544
JAAVMX010000019 44964     ---------------- ---------- ----- ------ - ----------------------------CT---------- 45040
GT#2  MG770309  148       ---------------- ---------- ----- ------ - ---------------------------  ---------- 222
GT#3  HM595984  267       ---------------- ---------- ----- ------ - -----------------A----------  ---------- 341
GT#7  AJ488254  223       ---------------- ---------- ----- A----- - ---------------------------  ---------- 147
GT#8  GU246286  240       ---------------- --T-T-T--- ----- ------ - ------------------------A----  ---------- 314
GT#9  GU246288  241       ---------------- ---------- ----- ------ - -----------C---------------  ---------- 315
GT#10 GU246287  238       ---------------- ---------- ----- ------G- G---G-----------------------  T--------- 313
GT#11 JQ695935  235       ---------------- ---------- ----- ------ - ---------------------------  ---------- 309
GT#12 GU246296  240       ---------------- ---------- ----- ------ - ---------------------------  ---------- 314

GT#1  AB067721  368       GTCATCTCAACCCTCGAG CCCCCCGCCTC GCGG  C GGCGGGG CCCGGCCTT GGGGGTCACGGCC CCGC  GCCG  438
JAAVMX010000019 19713     -------------------  ---------- ----  - ------- --------- ------------- ----  ----  19782
JAAVMX010000019 32357     -------------------  ---------- ----  - ------- --------- ------------- ----  ----  32426
JAAVMX010000002 18702404  ------------------- ----- ----- ----  - ------- --------- ------------- ----  ----  18702473
JAAVMX010000018 1006      ------------------- ------ ---- ----  - ------- ---------G------------- ----   ---  1075
JAAVMX010000019 6545      ------------------- ---------- ----  - ------- ---- ---- -------------C----  ----  6614
JAAVMX010000019 45041     -------------------C----------A ----TA- -CG---C --------- ------TC-----AA---  ----T 45123
GT#2  MG770309  223       ------------------- ----------- ----  - ------- --------- ------------- ----  ----  293
GT#3  HM595984  342       ------------------- ----------- ----  - -A----- --------- ------------- -T--  ----  412
GT#7  AJ488254  148       ------------------- ----A-A----T---A  -T------- --------- ------------- ----  ----  76
GT#8  GU246286  315       ------------------- ----------- ----  - -A----- --------- -------G----- ----  C--C  387
GT#9  GU246288  316       ------------------- ----------- -G--  - ------- --------- ------------- ----  ----  388
GT#10 GU246287  314       ----A--A----A------ ----------- T -G--  T ------- ------T-- T-----T--C--- --C-  C--C  384
GT#11 JQ695935  310       ------------------- ----------- ----  - ------- --TTCT--- --TT---CTTTAT TGAT  -A--  381
GT#12 GU246296  315       ------------------- ----------- ----  - -------C--------- ------------- --C-CGC---  388

GT#1  AB067721  439         CCCCC  T AAACGC AG       TGGCGACC  CC  GCCGCGGCTCC CC TGCGCAGTAGCT  CG  CTGAGAAC 497
GT#1  AB067721  439         CCCCC  T AAACGC AG       TGGCGACC  CC  GCCGCGGCTCC CC TGCGCAGTAGCT  CG  CTGAGAAC 497
JAAVMX010000019 19783       -----  - ------ --       --------  --  ----------- -- -------------  --  -------- 19842
JAAVMX010000019 32427       -----  - ------ --       --------  --  ----------- -- -------------  --  -------- 32486
JAAVMX010000002 18702474    -----  - ------ --       --------  --  ----------- -- -------------  --  -------- 18702532
JAAVMX010000018 1076        -----  - ------ --       --------  --  ----------- -- -------------  --  -------- 1134
JAAVMX010000019 6614        -----  - ------G--G      --------GC--  ----------- -- -------------  --GCT------- 6678
JAAVMX010000019 45125     CCT--- G - ----- --CCTAAGTG-------   --  --GA-------A-- --T-A-------CA--  -------- 45195
GT#2  MG770309  294         -----  - --                                                                      301
GT#3  HM595984  413         -----  - ---T-- --       --------  --  ----------- -- -------------  --  -C---G-- 479
GT#7  AJ488254  75          -----  - ------ --       --------  --  ----------- -T ------C-----  G-  -------- 18
GT#8  GU246286  388         ----T  - ------ --       G-------  --  ----- ---T- A- -------------  -A  -C------ 443
GT#9  GU246288  389         -----  - ------ --       G-------  --  ----------- -- --- -C-G-A--  -A  -C------ 452
GT#10 GU246287  385         -----CC-A------ G-       G--G-C--  --  C--C--C-C-- --T--GC-----TT-  AA  -C-G---- 417
GT#11 JQ695935  382        G-TTG   - -CTT-G --      G-C-T- --GG-T   -TCG- ----GG- - - -TC-GA--  T-  -ATTAGTG 445
GT#12 GU246296  361         -----  -A------ G-       G-------  --CG--G-------- -- -----G------  -AAC-GA----- 420

GT#1  AB067721  498       CT CGCACCGG  GA GCGCGGA     GGCGG TCACGCC GTG     AA ACCACC   ACACCC TCCA          549
JAAVMX010000019 19843     -- --------  -- -------     ----- ----- -  --G    -- ------   ------ ----          19894
JAAVMX010000019 32487     -- --------  -- -------     ----- ----- -  --G    -- ------   ------ ----          32537
JAAVMX010000002 18702533  -- --------  -- -------     ----- ------- ---TG   -- ------   ------ ----          18702586
JAAVMX010000018 1135      -- --------  -- -------     ----- ----- -  --G    -- ------   ------ ----          1186
JAAVMX010000019 6679      --C--------  -- -------     ----- ----- -  --G    -- ------ACA------ ----          6733
JAAVMX010000019 45196     -- --------  -- -------CCCAT-----C-------  --GGATG--C------ACC------ ----          45251
GT#2  MG770309  301
GT#3  HM595984  480       -C --------  A- ------G     --T-- -------   -C-   -- ------   ---     ---          519
GT#7  AJ488254  17        --T-----
GT#8  GU246286  444       T- -------A  A- ---- --     A---- -T----- ---     -- -A-T--   --G--T ----          494
GT#9  GU246288  453       -- --------  --G-------     ----- ------- -G-     -- - -----   --G--- - --          496
GT#10 GU246287  418       -C ---C----AA-- -G-G---     --G--GG- --                                          478
GT#11 JQ695935  446       -- AA-GT     T- C-- -CC     --TTCGC-GT-AT AAT    T-TCTG  --   TTG-   -G-G          485
GT#12 GU246296  421       -- --------  --G-G-----     ---G---------G---    A-- ------   --G---T----          480
```

Figure 3. Alignment of repetitive ITS copies within genome JAAVMX000000000 of H. sinensis Strain IOZ07 and ITS sequences of GC-biased genotypes of O. sinensis.

*Genome assemblies JAAVMX010000002, JAAVMX010000018, and JAAVMX010000019 were obtained from H. sinensis Strain IOZ07 (Shu et al. 2020). One copy within each of JAAVMX010000002 and JAAVMX010000018, indicated in **green**, shows 97.4% or 97.0% similarity to AB067721. JAAVMX010000019 contains 4 repetitive ITS copies, including 2 copies in **black** (19404→19894 and 32048→32537), which are 100% identical to AB067721, and 2 other copies in **blue** (6233→6733 and 44729→45251), with 94.5% and 90.8% similarities to AB067721. "GT" denotes the O. sinensis genotype. AB067721, MG770309, HM595984, AJ488254, GU246286, GU246288, GU246287, JQ695935, and GU246296 are the ITS sequences of GC-biased Genotypes #1□3 and #7□12 of O. sinensis in **pink**, respectively. The underlined sequence in black represents the 5.8S gene of GC-biased Genotype #1 H. sinensis. Hyphens indicate identical bases, and spaces denote unmatched sequence gaps.*

GC-biased Genotypes #13□14 were characterized by large DNA segment substitutions and recombination of genetic material between the genomes of the 2 parental fungi, Genotype #1 *H. sinensis* and an AB067719-type fungus, and are not included in the sequence analysis in Figure 3.

In addition to the 94.7%□95.3% sequence similarities between the Genotype #1 genomic repetitive ITS copies and Genotype #7 (*cf.* Table 2), the genetic features of Genotype #7, with numerous mutant alleles, are unlike any of the repetitive ITS copies. The study conducted by Chen et al. (2011) did not involve the purification of the *O. sinensis* fungus or fungi within the *C. sinensis* specimen. Thus, there is no evidence to date for determining whether the sequence of Genotype #7 indeed resides in the genome of Genotype #1 *H. sinensis* as a repetitive ITS copy, although Genotype #7 may be phylogenetically closer to Genotype #1 than other GC-biased genotypes.

The Bayesian tree in Figure 2 shows that GC-biased Genotypes #2□3 and #8□14 were placed outside of the Genotype #1 phylogenetic clade with great phylogenetic distances (horizontal bars) and were genetically distinct from the genomic repetitive ITS copies of Genotype #1 *H. sinensis* (*cf.* Figure 3). Notably, phylogenetically different Genotypes #1 and #2 were simultaneously detected in the stromata of the immature, maturing and mature *C. sinensis* collected from Kangding County of Sichuan Province. These 2 genotypes showed completely different developmental patterns in an asynchronous, disproportional manner in the *C. sinensis* stromata during maturation, indicating that the genotypic sequences do not reside in the single *O. sinensis* genome and are therefore not represented as repetitive ITS copies.

III-5. Transcriptional silencing of 5.8S genes

The above genetic, genomic, and phylogenetic analyses demonstrate GC-biased characteristics

of the multiple repetitive ITS copies in the *H. sinensis* genome and genomic independence of all GC- and AT-biased genotypes of *O. sinensis*. Li et al. (2013) identified ITS sequences for both Genotypes #1 and #5 from genomic DNA that was isolated from 8 (1206, 1208, 1209, 1214, 1220, 1224, 1227, and 1228) of 15 cultures of natural *C. sinensis* multicellular heterokaryotic mono-ascospores. However, the authors reported the identification of 5.8S cDNA only for Genotype #1 and not for Genotype #5 in a cDNA library constructed from total ascosporic RNA extracted from Clone 1220, one of the 8 genetically heterogeneous clones.

Li et al. (2013) did not detect the ITS sequences of AT-biased Genotypes #4, #6, and #15□17 in the genomic DNA isolated from any of the 15 ascosporic clones. Thus, there has been insufficient evidence to conclude that the 5.8S genes of all AT-biased genotypes of *O. sinensis* are permanently dysfunctional pseudogenic components coexisting in "a single genome" with a functional copy of GC-biased Genotype #1. Li et al. (2019) provided the following logical reasoning underlying the dysfunctional pseudogene hypothesis: "If AT-biased ITS sequences are not pseudogenes, 5.8S cDNA should have been detected because rRNAs are critical and essential for protein synthesis in all living organisms", which touches on 2 scientific issues. First, are the 5.8S genes of all genotypes of *O. sinensis* actively transcribed in *C. sinensis* ascospores (discussed in this section)? Second, is it correct to conclude that "5.8S cDNA should have been detected" by the techniques designed and used by Li et al. (2013) (discussed in Section III-6)?

Many research groups have reported differential coexistence of several AT-biased genotypes in various combinations in different compartments of natural *C. sinensis* (Table 4) (Xiao et al. 2009; Zhu et al. 2010, 2012; Gao et al. 2011, 2012; Li et al. 2023b, 2023c). Genotypes #4 and #15 of the AT-biased Cluster B of *O. sinensis* (*cf.* Figure 2) were reported to exist in the stroma and stromal fertile portion (densely covered with numerous ascocarps) of natural *C. sinensis* but not in the ascospores (Li et al. 2023b, 2023c), consistent with what Li et al. (2013) have reported. Thus, there has been no evidence to demonstrate that AT-biased Genotypes #4 and #15 occur in the *C. sinensis* ascospores, and there is no ground for claiming that the 5.8S genes of Genotypes #4 and #15 are dysfunctional pseudogenes in the genome of GC-biased Genotype #1 based on Genotypes #4 and #15 cDNA not being detected in the *C. sinensis* ascosporic cultures.

Table 4. *Differential occurrence of AT-biased genotypes of O. sinensis in the different compartments of natural C. sinensis.*

Genotype # and GenBank accession #	Phylogenetic AT-biased Cluster §	Stroma		Stromal Fertile Portion (covering with numerous ascocarps)	Ascospores
		Immature	Mature		
#4 AB067744	B	√	√	√	
#5 AB067740	A	√	√	√	√
#6 EU555436	A	√	√	√	√
#15 KT232017	B	√	√	√	
#16 KT232019	A	√			√
#17 KT232010	A	√			

Note: §, Phylogenetic AT-biased Clusters A and B are shown in Figure 2 (the Bayesian majority rule consensus phylogenetic tree) and are similarly shown in the phylogenetic trees generated by using the Bayesian, maximum likelihood, and minimum evolution algorithms by Stensrud et al. *(2007), Zhang* et al. *(2013a) and Li* et al. *(2016b, 2022a, 2022c, 2023b, 2023c).*

Xiang et al. (2014) reported a metatranscriptome study of natural *C. sinensis* specimens (unknown maturation stage) collected from Kangding County of Sichuan Province, China. A GenBank BLAST search confirmed that the GAGW00000000 metatranscriptome sequences do not contain any 5.8S gene transcripts of *C. sinensis*-associated fungi (including all genotypes of *O. sinensis*), indicating transcriptional silencing of 5.8S genes of all cocolonized fungi in natural settings.

Xia et al. (2017) reported another metatranscriptome study of mature natural *C. sinensis* specimens collected from Deqin County of Yunnan Province, China. The metatranscriptome assembly was not uploaded to GenBank but instead to another depository server www.plantkingdomgdb.com/Ophiocordyceps_sinensis/data/cds/Ophiocordyceps_sinensis_CDS.fas. Similarly, this assembled metatranscriptome contains no 5.8S gene transcripts. However, the authors uploaded the unassembled shotgun reads (SRR5428527) to GenBank. A BLAST search of the SRR5428527 Shotgun Read Archive (SRA) using the MegaBLAST algorithm identified 1894 unassembled metatranscriptome shotgun reads (100 bp each) that were 100% homologous to the 5.8S gene sequences (156 bp) of GC-biased Genotype #1 *H. sinensis*.

Fungal genomes contain multiple repetitive copies of rDNA ITS1-5.8S-ITS2 sequences (*cf.* Tables 1 and 3; Figures 1□3). Because the assembled metatranscriptome contained no 5.8S gene

transcripts (Xia et al. 2017) and no segments of the rDNA 18S and 28S gene of *H. sinensis* were homologous to the 5.8S gene, the 1894 unassembled shotgun reads (SRR5428527) of natural *C. sinensis* may likely be derived from highly homologous non-rRNA genes that evolved in a conservative manner within fungal metagenomes. Figure 4 shows the GenBank BLAST graphic summary of the alignment of the 5.8S gene (the query sequence; segment 218□373 of Genotype #1 *H. sinensis* AB067721) and the first 75 of the overlapping 1894 metatranscriptome shotgun reads (100 bp each) of mature natural *C. sinensis*.

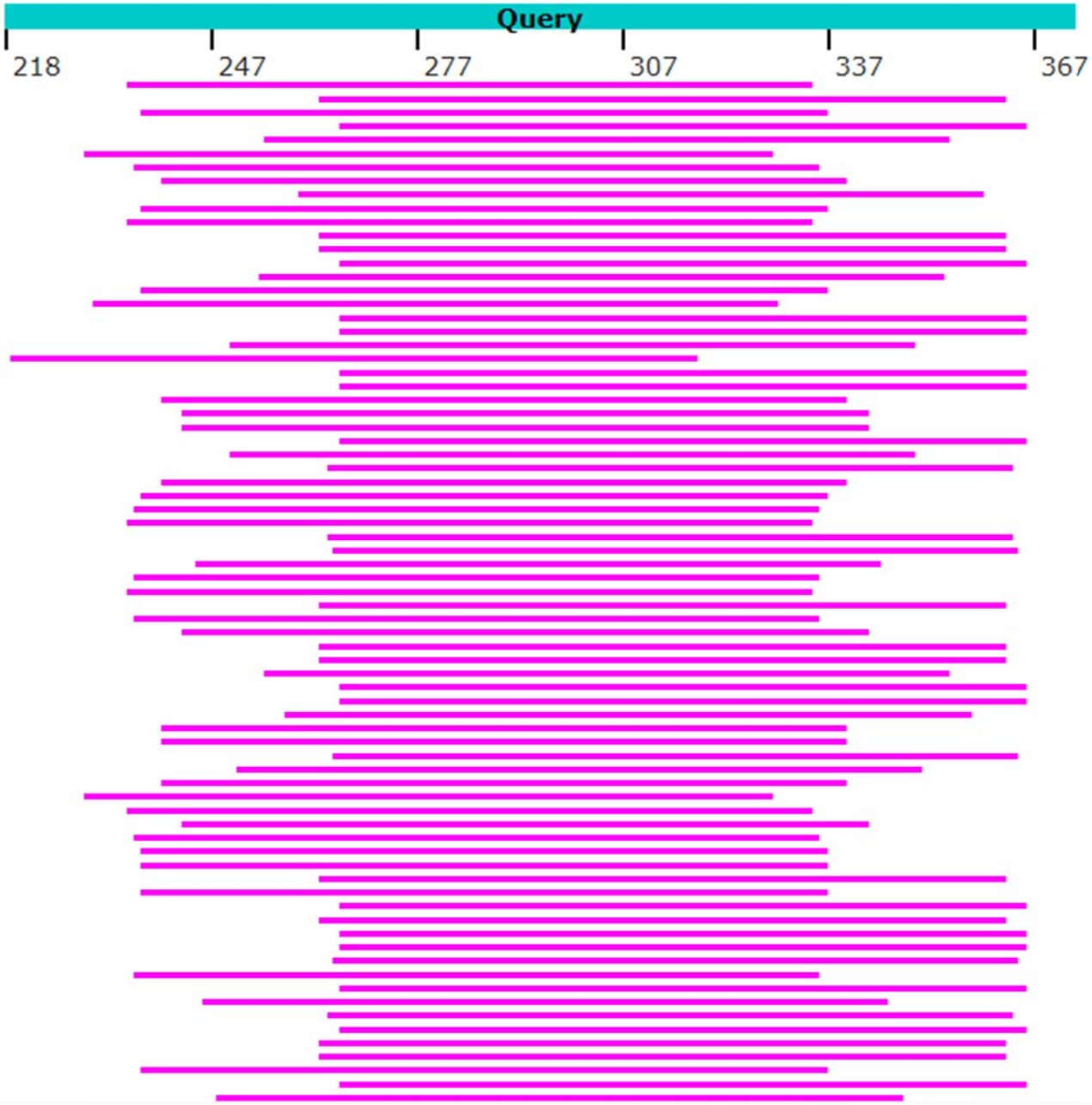


Figure 4. Graphic alignment summary of the query sequence (in blue□green), the 5.8S gene (218□373 segment of Genotype #1 H. sinensis AB067721) and the first 75 in the list of 1894 overlapping shotgun reads (pink bars; GenBank SRR5428527) of mature natural C. sinensis.

Various degrees of similarity were found when the 1894 unassembled shotgun reads were aligned with the 5.8S gene sequences of the 17 genotypes of *O. sinensis*. Table 5 shows 6 examples of shotgun reads of SRX2718557 aligned with the 5.8S sequences of *O. sinensis* genotypes, *Paecilomyces hepiali*, *Tolypocladium sinensis* (Group D), and an AB067719-type Group-E fungus (Stensrud et al. 2007). The high homology between the shotgun reads and the various 5.8S gene sequences of *O. sinensis* genotypes may not conclusively identify their fungal sources in natural *C. sinensis*, which contains >90 species spanning at least 37 fungal genera (Zhang et al. 2010, 2018; Xiang et al. 2014; Xia et al. 2015, 2017).

As shown in Table 5, the 5.8S gene sequence of Genotype #1 *H. sinensis* showed 98%□99% homology to the 5.8S gene sequence of *P. hepiali*. A GenBank BLAST search revealed the alignment of the 5.8S gene sequence (segment 218→373 of AB067721) of *H. sinensis* to 56□438 unassembled transcriptome shotgun sequence reads, which were identified in 5 RNA-Seq experimental data files (GenBank assessment #SRX3083435□SRX3083439) of *P. hepiali* Strain Feng (SRA Study: SRP115168; Pang et al. 2018) (Table 6). Pang et al. (2018) uploaded the 5 transcriptomic experimental datasets to GenBank for the total RNA samples extracted from the cultures of *P. hepiali* Strain Feng under different culture conditions and durations.

Table 5. *Percent similarity between the unassembled metatranscriptome shotgun reads (SRA: SRR5428527) of natural C. sinensis and the 5.8S genes of multiple genotypes of O. sinensis and P. hepiali*, T. sinensis *(Group D) and an AB067719-type fungus (Group-E).*

Geno-type	GenBank accession #	% Similarity between 5.8S genes and *C. sinensis* shotgun sequences					
		SRR5428527.17546327.1	SRR5428527.17554054.1	SRR5428527.17565575.2	SRR5428527.17679995.2	SRR5428527.17911498.2	SRR5428527.17929569.2
#1	AB067721	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**
#2	MG770309	96%	96%	**100%**	96%	**98%**	96%
#3	HM595984	**100%**	**99%**	**99%**	**100%**	**99%**	**100%**
#7	AJ488254	**98%**	**98%**	**99%**	**98%**	**98%**	**98%**
#8	GU246286	94%	96%	95%	94%	**97%**	94%
#9	GU246288	**99%**	**99%**	**99%**	**100%**	**99%**	**100%**
#10	GU246287	96%	94%	93%	95%	93%	**97%**
#11	JQ695935	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**
#12	GU246296	**99%**	**99%**	**99%**	**99%**	**99%**	**99%**
#13	KT339190	**98%**	93%	93%	**98%**	93%	**98%**
#14	KT339178	**98%**	93%	93%	**98%**	93%	**98%**
#4	AB067744	86%	87%	87%	83%	87%	83%
#5	AB067740	85%	87%	87%	84%	87%	84%
#6	KJ720572	85%	85%	87%	84%	86%	84%
#15	KT232017	85%	88%	88%	84%	88%	84%
#16	KT232019	86%	86%	87%	85%	87%	85%
#17	KT232010	87%	88%	88%	87%	88%	87%
P.h.	EF555097	**99%**	**98%**	**97%**	**99%**	**98%**	**99%**
T.s.	AB067720	**98%**	**97%**	**96%**	**98%**	**97%**	**98%**
	AB067719	**98%**	93%	93%	**98%**	93%	**98%**

Note: GC-biased genotypes are listed in blue, and AT-biased genotypes are listed in red. GenBank sequence ID SRA: SRR5428527 fragments (17546327.1, 17554054.1, 17565575.2, 17679995.2, 17911498.2, and 17929569.2) are the unassembled transcriptome shotgun sequences obtained from the mature natural C. sinensis specimens collected from Deqin County of Yunnan Province by Xia et al. *(2017). P.h. refers to P. hepiali; T.s. refers to Group-D* T. sinensis*, and AB067719 refers to an AB067719-type Group-E fungus (Stensrud* et al. *2007). Highly homologous percent similarities (97%□100%) are in bold* ***green*** *font.*

***Table 6**. Summary of BLAST searches of the transcriptome shotgun reads of P. hepiali Strain Feng against the 5.8S gene sequence (218→373 of AB067721) of Genotype #1 H. sinensis.*

P. hepiali sample	Culture duration (days)	Additive in culture medium	GenBank transcriptome data file	*vs*. the sequence of the *H. sinensis* 5.8S gene		
				# Of the total BLAST hits	(% Of total filtered reads)	% Similarity
P4	4	no	SRX3083438	219	(3.7% x 10^{-4})	93%□99%
P9	9	no	SRX3083437	56	(3.6% x 10^{-4})	95%□100%
P15	15	no	SRX3083436	438	(6.12% x 10^{-4})	96%□100%
Ps	4	Adenosine	SRX3083435	356	(4.13% x 10^{-4})	96%□100%
Pa	4	Adenine	SRX3083439	207	(6.12% x 10^{-4})	96%□99%

The extremely high similarity between the 5.8S gene sequences of *H. sinensis*, *P. hepiali* and *T. sinensis*, as shown in Table 5, indicates conservative evolution of the 5.8S gene in many fungal species. Table 6 shows significant differences in the number of BLAST hits and percent similarities of the 5.8S gene sequence of *H. sinensis* aligned with the transcriptome shotgun reads obtained from the culture experiments of *P. hepiali* Strain Feng under different culture conditions and durations (Pang et al. 2018). The results indicate the experimental switching on-or-off of *P. hepiali* genes, including the 5.8S gene and many other non-rRNA genes, in response to the different experimental settings.

Liu et al. (2015) reported the mRNA transcriptome assembly GCQL00000000 derived from *H. sinensis* Strain L0106, which contained no transcript of rRNA or tRNA. Continuous *in vitro* liquid fermentation performed in this study induced a nonlinear reduction in the total number of transcriptomic unigenes, first from 25,511 (after 3 days of fermentation) to 25,214 (after 6 days of fermentation) and then more sharply to 16,245 (after 9 days of fermentation). Table 7 summarizes the dynamic alterations in the total number of transcriptomic unigenes, average length and GC content of transcripts of *H. sinensis* Strain L0106, indicating the nonnatural switching on-or-off and differential transcriptional activation and silencing of numerous *H. sinensis* genes in response to continued liquid fermentation of *H. sinensis* Strain L0106. As shown in Table 7, more dramatic changes might conceivably occur after 9 days of fermentation. The prolonged 25-day liquid incubation period adopted by Li et al. (2013) might have a greater impact on the unbalanced transcriptional activation (switching on) and silencing (switching off) of long and short fungal genes with various GC content biases, in addition to the changes in the culture medium as an

important factor influencing the transcriptional activation and silencing of many fungal genes (*cf.* Table 6).

***Table 7**. Dynamic alterations of transcripts in the mRNA transcriptome of the H. sinensis Strain L0106 during continuous in vitro fermentation.*

Transcriptomic unigene	After 3 days of fermentation	After 6 days of fermentation (% change)	After 9 days of fermentation (% change)
Total number	25,511	25,214 (-1.2%)	16,245 (-36.3%)
Average length (nt)	681	682 (+0.1%)	994 (+46.0%)
GC content (%)	58.2%	57.9% (-0.5%)	57.0% (-2.1%)

III-6. 5.8S cDNA detected using 5.8S-F/R primers

In addition to the transcriptional silencing of 5.8S genes in natural settings and the significant impact of continuous *in vitro* culture and altered experimental settings on unbalanced gene transcription in experimental settings, as discussed above, the second issue related to the logic described by Li et al. (2019) is whether the methods designed by Li et al. (2013) were suitable for scientifically identifying 5.8S gene cDNAs of multiple *O. sinensis* genotypes (Li et al. 2020a).

For instance, Li et al. (2019) retrospectively explained the research design logic of 5.8S-F/R primers by Li et al. (2013): "The 5.8S-F/R primers … located in the most conserved region." No doubt such a design would largely reduce the primers' specificity for 5.8S cDNA because of the conservative evolution of the 5.8S gene and many other fungal genes. Table 8 and Figure 5 show similarities between the sequences of primers 5.8S-F (5' ACTTTTAACAACGGATCTCTT 3') and 5.8S-R (5' AAGATAACGCTCGGATAAGCAT 3') and the 5.8S gene of Genotypes #1, #4, and #5.

***Table 8**. Percent similarity of the 5.8S-F/R primers and the 5.8S gene sequences of Genotypes #1, #4 and #5 of O. sinensis.*

Genotype # and GenBank accession #	% Similarity of the primer *vs.* the 5.8S gene sequence	
	Primer 5.8S-F	Primer 5.8S-R[§]
#1 AB067721	95% (20/21 nt)	85% (17/20 nt)
#4 AB067744	95% (20/21 nt)	90% (18/20 nt)
#5 AB067740	100% (21/21 nt)	95% (19/20 nt)

Note: [§], Primer 5.8S-R has 2 flanking residues downstream in the ITS2 region.

The primers 5.8S-F/R are 100% and 95% homologous to the 5.8S gene sequence of AT-biased

Genotype #5, as shown in Table 8. This indicates that in contrast to the 95% and 85% similarities to the 5.8S gene sequence of GC-biased Genotype #1, the 5.8S-F/R primers may be favored for amplifying the 5.8S cDNA sequence of Genotype #5, rather than of Genotype #1. The primers showed variable similarity, ranging from 61.9%□100% and 31.8%□95.5%, respectively, among all 17 genotypes of *O. sinensis*, preferentially amplifying the most easily amplified cDNA sequence in the cDNA library considering the primary structure and secondary conformation of PCR templates and consequently causing false-negative results for other 5.8S cDNA sequences.

Section III-5 describes the 100% homologies between the 1894 unassembled metatranscriptome shotgun reads (100 bp) of mature natural *C. sinensis* and the 5.8S gene sequence (156 bp) of GC-biased Genotype #1 *H. sinensis*, including many non-rRNA transcripts among the highly homologous 1894 shotgun reads in natural settings. A BLAST search of *O. sinensis* transcriptomic sequences (taxid:72228) in the GenBank database showed that the 5.8S-F/R primers were 100% homologous to the mRNA transcriptome assemblies GCQL01001191, GCQL01006777, GCQL01007818, and GCQL01013443 of Genotype #1 *H. sinensis* Strain L0106 (Liu et al. 2015), which contain no rRNA or tRNA transcripts, and to assembled metatranscriptome sequences GAGW01003691, GAGW01007670, and GAGW01007671 of natural *C. sinensis* (Xiang et al. 2014). Figure 6 shows that metatranscriptome GAGW01003691 is 99.8% homologous to a segment of mRNA transcriptome GCQL01006777 of *H. sinensis* Strain L0106; metatranscriptomes GAGW01007670 and GAGW01007671 are 100% identical to mRNA transcriptome segments of GCQL01007818 and GCQL01001191, respectively (Li et al. 2020a). Because mRNA transcriptome assembly GCQL00000000 contains no rRNA or tRNA transcripts, the extremely high homology between the metatranscriptome and transcriptome sequences indicates that the 5.8S-F/R primers designed by Li et al. (2013) may be able to recognize and nonspecifically amplify non-rRNA components in the cDNA library derived from the genetically heterogeneous Clone 1220, one of the 8 heterogenous clones (1206, 1208, 1209, 1214, 1220, 1224, 1227, and 1228) of the *C. sinensis* mono-ascospore cultures obtained by Li et al. (2013).

```
GT1 AB067721 1     TAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTATC  60
GT4 AB067744 1         -------------------------------A--A---T-----------T------  56
GT5 AB067740 1     --A------------------T------------A--A---T-----------T------  60

GT1 AB067721 61    GAGTCACCACTCCCAAACCCCCTGCGAACACCACAGCAGTTGCCTCGGCGGGACCGCCCC  120
GT4 AB067744 57    ------------C--------------A--C-----------------------------  116
GT5 AB067740 61    ----T--T----TT---------ATA--T--T-T-----------TA------------T  120

GT1 AB067721 121   GGCGCCCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCCCAGACCCTCCTGTCGCAG  180
GT4 AB067744 117   -------T-----------T---------------------TT----------A---T--  176
GT5 AB067740 121   A------T-A---------T-AA------------------TT----------A-TAT--  180

GT1 AB067721 181   TGGCATCTCTCAGTCAAGAAGCAAGCAAATGAATCAAAACTTTCAACAACGGATCTCTTG  240
GT4 AB067744 177   -A--------T---T---------------A---T-A-ACTTTTAATAACGGATCTCTTA  236
GT5 AB067740 181   -A-T------T---T---------------A---T---ACTTTTAACAACGGATCTCTTA  240
Primer 5.8S-F                                             ACTTTTAACAACGGATCTCTT

GT1 AB067721 241   GTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA  300
GT4 AB067744 237   ---T-A----TA--A----------------A-------------A-----T------T-  296
GT5 AB067740 241   -----A----TA--A--A---------------A---------A-A----A-------T-  300

GT1 AB067721 301   GTGAACCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCACTCTGGCGGGCATGCCTG  360
GT4 AB067744 297   --A---T-----------A----T-T-C-A-------T--------A------ATGCCTG  356
GT5 AB067740 301   --A---T-----------A------T---A-------T--------A------ATGCCTA  360
Primer 5.8S-R                                                         ATGCTTA

GT1 AB067721 361   TCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTCGCGGCGGCGGGGCCCGGCCTTGGG  420
GT4 AB067744 357   TCCGAGCGTTACCTT------TA-----------------------------------A--  416
GT5 AB067740 361   TCCGAGCGTTATCTT-----------------------------------T-------A--  420
Primer 5.8S-R      TCCGAGCGTTATCTT

GT1 AB067721 421   GGTCACGGCCCCGCGCCGCCCCCTAAACGCAGTGGCGACCCCGCCGCGGCTCCCCTGCGC  480
GT4 AB067744 417   ------------A--------------------A-T--------------------A---  476
GT5 AB067740 421   ---T------------------T------T---A----------------------A--T  480

GT1 AB067721 481   AGTAGCTCGCTGAGAACCTCGCACCGGGAGCGCGGAGGCGGTCACGCCGTGAAACCACCA  540
GT4 AB067744 477   -A-----TA--A------------TA------------------------A-------T-  536
GT5 AB067740 481   G-------A-C----------------A----------------------A-------T-  540

GT1 AB067721 541   CACCCTCCAGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAA  600
GT4 AB067744 537   T------TG---A---------T-----------------A-------------------  596
GT5 AB067740 541   -G--T-TT----A---------T-----------------A-------------T-----  600

GT1 AB067721 601   GCGGAGGAAAAGAAACCAACAGGGATTGCCCCAGTAACGGCGAGTGAAGCGGCAACAGCT  660
GT4 AB067744 597   --------                                                       604
GT5 AB067740 601   ----------------T--T----------TT-------------A------T--T----  660
```

Figure 5. ITS sequence alignment of AB067721 of GC-biased Genotype #1, AB067744 and AB067740 of AT-biased Genotypes #4□5 of O. sinensis and the 5.8S-F/R primers.

"GT1", "GT4", and "GT5" represent Genotypes #1 and #4□5 of O. sinensis, respectively. The sequences in blue are 18S and 28S genes (5' and 3' end regions, respectively). Those in pink represent sequences of 5.8S genes. Those in black are ITS1 between the 18S and 5.8S genes and ITS2 between the 5.8S and 28S genes. The sequences underlined in green are for primers 5.8S-F/R, while primer 5.8S-R was converted to the sense chain sequence. Hyphens indicate identical bases.

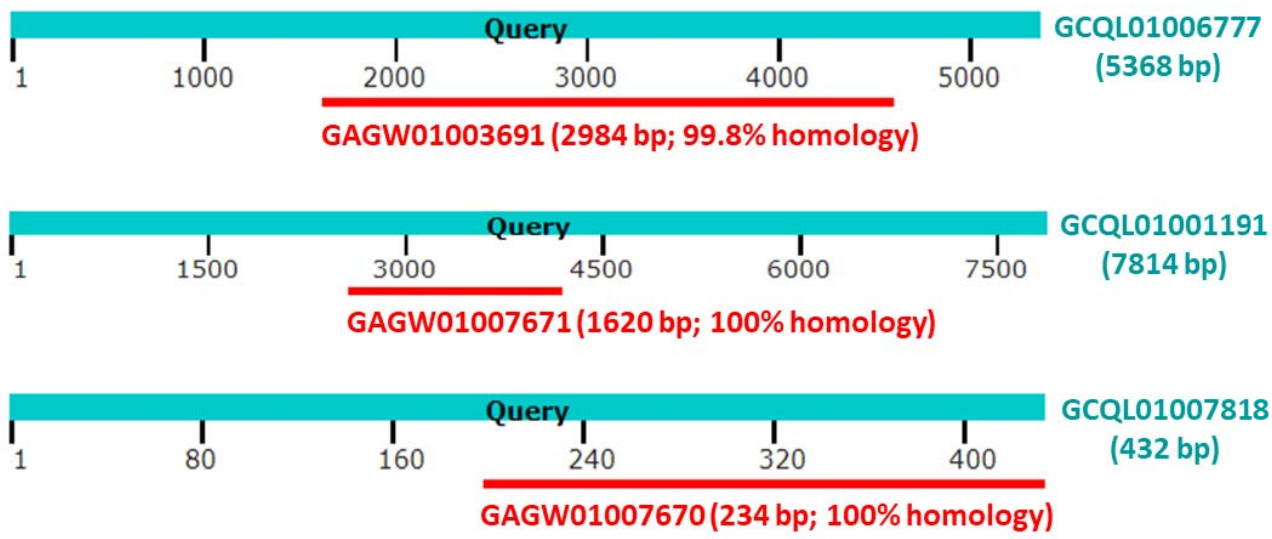


Figure 6. Graphic alignment summary of the mRNA transcriptome assembly sequences (GCQL00000000) of H. sinensis Strain L0106 and the metatranscriptome assembly sequences (GAGW00000000) of natural C. sinensis.

The graphic alignment summary was generated using GenBank BLAST. GCQL01001191, GCQL01006777, and GCQL01007818 are sequences of transcriptome segments that were obtained from the mRNA pool of H. sinensis Strain L0106 and contained no rRNA or tRNA transcripts. GAGW01003691, GAGW01007670, and GAGW01007671 are segment metatranscriptome sequences, which were obtained from the total RNA of natural C. sinensis specimens collected from Kangding County of Sichuan Province, China.

Unfortunately, Li et al. (2013) did not disclose the sequences of the identified 5.8S cDNA but instead reported that "Of these clones, 28 showed identical sequences (104 bp excluding the primers) to the most frequent sequence in group A (JQ900148). The remaining 14 sequences … had single substitutions at various positions", where group A = Genotype #1 *H. sinensis*. GenBank annotates the location of the 5.8S gene of JQ900148 as nucleotides 192→346, covering 155 nt, which is not a multiple of three and is not in accord with the open reading frame of the gene.

Alternatively, the 5.8S genes were annotated with nucleotide positions 218→373 in AB067721 of Genotype #1 and AB067740 of Genotype #5 and 214→369 in AB067744 of Genotype #4, covering 156 nt and 52 codons in the 3 cases (Zhu & Li 2017; Li et al. 2020a, 2022c). Excluding the 5.8S-F/R primer regions (*cf*. Figure 5), the remaining sequence of the 5.8S gene consists of 114 bp, with 10 additional nucleotides (mismatches) beyond the 104 bp of all cDNA clones in the 28 clones reported by Li et al. (2013). Adding "single substitutions at various positions" in 14 other clones, as reported by Li et al. (2013), the 42 clones presumably contained either 10 or 11 mismatches (8.8%□9.6%) in the 114-bp segment of the 5.8S gene cDNA, corresponding to

90.4%□91.2% sequence similarity to the 5.8S gene sequence of GC-biased Genotype #1 *H. sinensis*.

On the other hand, Li et al. (2013) did not provide any information about to the sequence alignment of the detected 5.8S-F/R PCR amplicons with the 5.8S gene sequence of AT-biased Genotype #5 of *O. sinensis*.

IV. Discussion

IV-1. The repetitive ITS copies in the genome of GC-biased Genotype #1 *H. sinensis* are unrelated to RIP mutagenesis

Selker and his colleague (1985, 1990, 1997, 2002) reported frequent C-to-T (cytosine→thymine) transitions and concomitant epigenetic methylation of nearly all remaining cytosine residues in fungal 5S RNA genes associated with transcriptional silencing. Gladyshev (2017) summarized that RIP "occurs specifically in haploid parental nuclei that continue to divide by mitosis in preparation for karyogamy and ensuing meiosis;" and "mutates cytosines on both strands of each DNA duplex in a pairwise fashion (e.g., preferentially affecting none or both repeated sequences)." Hane et al. (2017) stated that "the RIP process selectively mutated duplicated sequences in both DNA strands by inducing single-nucleotide point (SNP) mutations that converted C:G base pairs to T:A. This often led to the introduction of nonsense or missense mutations which affected the expression of these sequences." The sequence-specific targets of RIP mutations for de novo DNA methylation are under debate (Hood et al. 2005; Taranto et al. 2014; Hane et al. 2017).

Table 3 and Figure 1 demonstrate that the variable repetitive ITS copies were genetically characterized by multiple scattered insertion, deletion and transversion point mutations; these mutations might not be generated through RIP mutagenesis, which theoretically causes cytosine→thymine (C-to-T) transitions. On the other hand, the AT-biased genotypes of *O. sinensis* characterized by multiple scattered transition point mutations were generated in parallel with other GC-biased genotypes, through accelerated nuclear rDNA evolution from a common ancestor during a long evolutionary history (Stensrud et al. 2007), regardless of whether this occurred *via* RIP mutagenesis or other evolutionary mechanisms. Thus, Li et al. (2020b) reported that RIP mutagenesis induces the formation of mutant AT-biased repetitive ITS copies of *O. sinensis* within the single genome of GC-biased Genotype #1 *H. sinensis* lacking supporting evidence. Even if it were to be proven that AT-biased genotypes were indeed induced by RIP mutagenesis during a long

history of evolution, there has been no evidence that AT-biased genotypes are genomic components of the GC-biased Genotype #1 genome that emerged after a new GC-biased Genotype #1 "ITS haplotype was generated" as "ITS pseudogenes … in a single genome", as assumed by Li et al. (2013, 2019, 2020b).

Taranto et al. (2014) and Hane et al. (2015) reported that RIP "is observed only in certain fungal taxa; the Pezizomycotina (filamentous Ascomycota) and some species of the Basidiomycota." Tables 1 and 3 and Figures 1□3 show that all the multiple genomic repetitive ITS copies were genetically GC-biased, with no or only a few transition alleles, and were phylogenetically distinct from AT-biased genotypes, which possess numerous scattered transition alleles. It is then reasonable to question whether Genotype #1 *H. sinensis* is susceptible to epigenetic methylation attack through RIP mutagenesis.

IV-2. Genetic and phylogenetic differences of AT-biased genotypes of *O. sinensis* and GC-biased repetitive ITS copies in the genome of Genotype #1 *H. sinensis*

The sequences of AT-biased Genotypes #4□6 and #15□17 of *O. sinensis* shown in red in Figure 1 contain numerous scattered transition alleles. They are genetically distinct and phylogenetically distant from GC-biased repetitive ITS copies containing the predominant insertion, deletion, and transversion point mutations within the genome of GC-biased Genotype #1 *H. sinensis* (*cf*. Figures 1□2 and Tables 1□3). The sequences of AT-biased genotypes, as well as GC-biased Genotypes #2□3 and #7□14, of *O. sinensis* are absent in the genome assemblies ANOV00000000, JAAVMX000000000, LKHE00000000, LWBQ00000000, and NGJJ00000000 of *H. sinensis* Strains Co18, IOZ07, 1229, ZJB12195, and CC1406-203, respectively (*cf*. Tables 1□2 and Figures 1 and 3) but instead belong to the genomes of independent *O. sinensis* fungi (Stensrud et al. 2007; Xiao et al. 2009; Hu et al. 2013; Zhu and Wu 2015; Li et al. 2016a, 2016b; 2020a, 2022a, 2022c, 2023c; Zhu and Li 2017; Zhu et al. 2018, 2019; Jin et al. 2020; Liu et al. 2020; Shu et al. 2020). The nonresidency measure of AT-biased *O. sinensis* genotypes does not support the hypothesis that the AT-biased sequences are "ITS pseudogene" components of "a single genome" of GC-biased Genotype #1 *H. sinensis*. Stensrud et al. (2007) concluded that the variation in the 5.8S gene sequences of AT-biased genotypes "far exceeds what is normally observed in fungi … even at higher taxonomic levels (genera and family)". Xiao et al. (2009) concluded that the sequences of AT-biased genotypes likely belong to independent *O. sinensis* fungi.

IV-3. The previously proposed *O. sinensis* center of origin and the geography of *O. sinensis* evolution

GC-biased genotypes, including the GC-biased genomic repetitive ITS copies, might have evolved at accelerated rates in parallel with AT-biased genotypes from an ancient common ancestor during long-term evolution, as assumed by Stensrud et al. (2007). Zhang et al. (2009) detected GC-biased Genotype #3 in natural *C. sinensis* specimens collected from the Nyingchi District of Tibet and hypothesized that "Nyingchi might have acted as a center of origin of *O. sinensis* and *O. sinensis* might have been transmitted from Nyingchi to other locations". Accordingly, GC-biased Genotype #3 might represent a modern immediate ancestor of Genotype #1 *H. sinensis* that has been consistently identified in other production locations as the result of a relatively modern phase during long-term evolution. The authors described an evolution path from GC-biased Genotype #3 to Genotype #1 and transmission of *O. sinensis* from the "center of origin … spread eastward and westward first among southern regions on the Tibetan Plateau" and then "spread from south to north … at a later time".

Unfortunately, this evolutionary geography for *O. sinensis* is missing an evolutionary branch or branches related to AT-biased genotypes of *O. sinensis*. Perhaps the evolution of AT-biased genotypes of *O. sinensis* might have occurred in more ancient historical evolutionary phases, if not in modern evolutionary phases. Undoubtedly, the discovery of AT-biased genotypes of *O. sinensis* will trigger further research on the history of *O. sinensis* evolution and fungal-insect co-evolution [Zhang et al. 2014].

IV-4. Multicellular heterokaryons of natural *C. sinensis* hyphae and ascospores

Bushley et al. (2013) reported that *C. sinensis* ascospores are multicellular heterokaryons with mono-, bi-, and tri-nucleated cellular structures. Zhang and Zhang (2015) wondered whether the bi-nucleated cells (also tri-nucleated cells) of *C. sinensis* hyphae and ascospores contain homogenous or heterogeneous genetic materials, meaning that these polykaryotic cells may contain heterogeneous sets of chromosomes and genomes. Li et al. (2023b, 2023c) confirmed the genetic heterogeneity in the multicellular heterokaryotic ascospores of natural *C. sinensis*.

Li et al. (2013) identified heterogeneous ITS sequences of both GC-biased Genotype #1 and AT-biased Genotype #5 in 8 of 15 clones (1206, 1208, 1209, 1214, 1220, 1224, 1227, and 1228) and simultaneously identified 7 other clones (1207, 1218, 1219, 1221, 1222, 1225, and 1229) that contained only homogenous sequences of Genotype #1 after 25 days of inoculation with natural *C.*

sinensis mono-ascospores. These culture-dependent molecular mycological findings supported the morphological observations of multicellular heterokaryons discovered by their collaborators (Bushley et al. 2013). Apparently, the 2 groups of the reported ascosporic clones might be derived from different cells of the multicellular heterokaryotic ascospores of natural *C. sinensis*. In addition to the information for the 15 clones of the 2 groups, the authors did not disclose any information of other possible clones, namely, clones 1210, 1211, 1212, 1213, 1215, 1216, 1217, 1223, and 1226, for which the clone serial numbers are missing among those of the 2 groups (*cf*. Table S1 of Li et al. 2013), nor did the authors state whether these 9 possible clones were simultaneously obtained in the same study, why they were not published along with the 15 reported clones, and whether they contained different genetic materials, although these questions should have been asked during the journal's peer review process. Li et al. (2023b, 2023c), from a different research group, used culture-independent techniques and reported the cooccurrence of GC-biased Genotypes #1 and #13□14, AT-biased Genotypes #5□6 and #16 of *O. sinensis*, *P. hepiali*, and an AB067719-type fungus in *C. sinensis* ascospores, probably indicating that many of these fungi may not be culturable in the *in vitro* experimental setting set up by Li et al. (2013).

IV-5. Genomic variations of *H. sinensis* strains

By aggregating the information from molecular and genomic studies (Hu et al. 2013; Li et al. 2013, 2016a; Jin et al. 2020; Liu et al. 2020; Shu et al. 2020), different genomic DNA samples of "pure" *H. sinensis* strains were examined and published and can be grouped as follows.

(1) Group 1 of *H. sinensis* strains consisted of pure, homokaryotic anamorphic *H. sinensis* strains (1229, CC1406-203, Co18, IOZ07, and ZJB12195) (Hu et al. 2013; Li et al. 2016a; Jin et al. 2020; Liu et al. 2020; Shu et al. 2020). Total genomic DNA was isolated from these strains and subjected to genome-wide sequencing. The genomes contained no AT-biased genotype sequences.

(2) Group 2 of *H. sinensis* strains consisted of 7 clones (Strains 1207, 1218, 1219, 1221, 1222, 1225, and 1229) among 15 clones derived from 25 days of incubation of natural *C. sinensis* mono-ascospores (Li et al. 2013). Total genomic DNA was isolated from these strains and shown to contain the homogenous ITS sequence of Genotype #1 *H. sinensis* but included no AT-biased sequences of *O. sinensis* genotypes.

(3) Group 3 of "*H. sinensis*" strains consisted of 8 other clones (Strains 1206, 1208, 1209, 1214, 1220, 1224, 1227, and 1228) obtained from the incubation of *C. sinensis* mono-ascospores for

25 days (Li et al. 2013). Total genomic DNA was isolated from these strains and showed genetic heterogeneity, with the coexistence of GC-biased Genotype #1 and AT-biased Genotype #5 of *O. sinensis*.

The *H. sinensis* strains of Groups 1 and 2 were similar, with each strain possessing a homogenous GC-biased Genotype #1 genome, although multiple GC-biased repetitive ITS copies were identified in genome assemblies JAAVMX000000000 and NGJJ00000000, for *H. sinensis* Strains IOZ07 and CC1406-203, respectively (*cf*. Tables 1 and 3 and Figures 1–2) (Hu et al. 2013; Li et al. 2013, 2016a; Jin et al. 2020; Liu et al. 2020; Shu et al. 2020). Although a single copy of ITS sequences was identified in genome assemblies ANOV00000000, LKHE00000000, and LWBQ00000000 of *H. sinensis* Strains Co18, 1229, and ZJB12195, respectively (Hu et al. 2013; Li et al. 2016a; Jin et al. 2020), the genomes of these strains may theoretically contain more repetitive ITS copies that could have been discarded during the assembly of genome shotgun reads. Conceivably, reassembling the genome shotgun reads, if technically possible, may very likely identify additional repetitive ITS copies in the genomes of Group 1 Strains Co18, 1229, and ZJB12195, among which Strain 1229 and its genome assembly LKHE00000000 are also a Group-2 strain derived from mono-ascospores by Li et al. (2013, 2016a). Similarly, the genome sequencing of Group-2 strains may very likely identify multiple repetitive ITS copies.

The *H. sinensis* strains in Groups 2 and 3 were derived after 25 days of liquid culture of the same *C. sinensis* mono-ascospores (Li et al. 2013); however, they differed genetically. The Group-3 strains may likely have multicellular heterokaryotic mycelial structures, similar to the mycelia derived from microcycle conidiation of the *H. sinensis* ascospores initially cultured on the solid medium plate for 30 days and subsequently in liquid medium for 10□53 days, as shown in Figures 2□3 of (Xiao et al. 2011). Li et al. (2013) reported that the Group 3 strains included cocultured GC-biased Genotype #1 and AT-biased Genotype #5 of *O. sinensis* fungi, which were genomically independent and coexisted in heterokaryotic mycelial cells, indicating either diploid/polyploid mycelial cells containing heterogeneous sets of chromosomes/genomes, or impure mycelia, each containing a different chromosome/genome. However, the authors did not identify any haploid clones containing only AT-biased Genotype #5, indicating that this AT-biased *O. sinensis* fungus may not exhibit independent culturability under the *in vitro* experimental settings, although other studies have reported the identification of single AT-biased Genotype #4 or #5 of *O. sinensis*, without coculture with GC-biased Genotype #1, in cultures of natural and cultivated *C. sinensis*

739 under different experimental settings (Mao et al. 2013; Wei et al. 2016). Unfortunately, Li et al.
740 (2013, 2019, 2020b) neglected the differences in genetic material between the strains in Group 2
741 and Group 3, which were presumably derived from the same or different cells of multicellular
742 heterokaryotic ascospores (Bushley et al. 2013; Zhang and Zhang 2015; Li et al. 2020a, 2022c,
743 2023c).

744 In addition to the genome sequencing of the homogenous Group-2 Strain 1229 and the
745 generation of genome assembly LKHE00000000 by Li et al. (2016a), the genome sequencing of
746 one of the heterogeneous Group-3 strains will provide critical genomic evidence to validate the
747 hypothesis that AT-biased Genotype #5 of *O. sinensis* coexists as an "ITS pseudogene … in a single
748 genome" of GC-biased Genotype #1. Unfortunately, the authors elected not to do so since 2012
749 after obtaining the 8 Group-3 strains, or perhaps failed in such an endeavor due to the technical
750 difficulties in the assembly of shotgun reads because of the heterogeneous sequences from more
751 than one set of genomes of independent *O. sinensis* fungi having close evolutionary relations.
752 Consequently, they elected willingly or unwillingly to pass on the opportunity to experimentally
753 confirm their "ITS pseudogene" hypothesis that AT-biased Genotype #5 and GC-biased Genotype
754 #1 physically coexist "in a single genome". Studies from other research groups have confirmed that
755 the sequences of all AT-biased genotypes belong to the genomes of independent fungi (Stensrud et
756 al. 2007; Xiao et al. 2009; Zhu et al. 2010, 2012; Yao et al. 2011; Li et al. 2016, 2020a, 2022a,
757 2022c, 2023c) and that heterokaryotic ascospores and hyphae with multiple mono-, bi-, and tri-
758 nucleated cells contain heterogeneous sets of chromosomes and genomes (Bushley et al. 2013;
759 Zhang and Zhang 2015).

760 Of course, in addition to the 3 groups summarized above, many other *H. sinensis* strains have
761 been studied and presumably belong to Groups 1□3 or other groups. For instance, Li et al. (2016c)
762 isolated 2 *H. sinensis* strains, CH1 and CH2, from the intestine of healthy larvae of *Hepialus lagii*
763 Yan. These strains showed *H. sinensis*-like morphological and growth characteristics but contained
764 GC-biased Genotype #1, AT-biased Genotypes #4□5 and *P. hepiali*. The strains greatly enhanced
765 the inoculation potency of *H. sinensis* in the larvae (n=100 each experimental group) of *Hepialus*
766 *armoricanus* by 15- to 39-fold ($P<0.001$). Additionally, as mentioned above, Li et al. (2013) did not
767 discuss whether 9 other ascosporic clones (1210, 1211, 1212, 1213, 1215, 1216, 1217, 1223, and
768 1226) simultaneously obtained in the study constituted additional fungal group(s) containing
769 different genetic materials.

IV-6. Genomic independence of AT-biased genotypes of *O. sinensis*

IV-6-1 Differential occurrence of AT-biased genotypes in different compartments of natural *C. sinensis*

A BLAST search of the GenBank database revealed at least 668 ITS sequences of *O. sinensis* (GenBank taxid:72228), 228 of which belong to AT-biased Genotypes #4□6 and #15□17 (Li et al. 2016b, 2022c, 2023c; Zhu et al. 2018, 2019; Zhu & Li 2017). Stensrud et al. (2007) and Zhang et al. (2013a) analyzed the phylogeny of 71 and 397 ITS sequences of *O. sinensis*, respectively, and revealed 3 phylogenetic clades, *i.e.*, Groups A□C (GC-biased Genotype #1 and AT-biased Genotypes 4□5), which consisted of correct *O. sinensis* sequences. Notably, the Genotype #1 clade (Group A) in the phylogenetic tree constructed by Zhang et al. (2013a) using the minimum evolution algorithm consisted of the ITS sequences of Genotype #1 and some of the other GC-biased genotypes of *O. sinensis*. Additional GC- and AT-biased *O. sinensis* genotypes have been successively discovered thereafter (*cf.* Table 2) (Xiao et al. 2009; Zhang et al. 2009; Zhu et al. 2010, 2012; Chen et al. 2011; Zhu and Wu 2015; Li et al. 2013, 2016, 2022a, 2022c, 2023c; Zhu and Li 2017).

Many early studies conducted since 1999 used a single pair of "universal" primers (*e.g.*, *ITS1*□*ITS5*) for PCR and identified one of the *O. sinensis* genotypes in natural *C. sinensis* specimens collected from different production areas or cultivated *C. sinensis* (Engh 1999; Zhao et al. 1999; Kinjo and Zang 2001; Liu et al. 2001; Stensrud et al. 2005; Wei et al. 2006, 2016; Chen et al. 2011; Mao et al. 2013). Importantly, Wei et al. (2016) reported an industrial artificial cultivation project and identified a single teleomorphic AT-biased Genotype #4 of *O. sinensis* in cultivated *C. sinensis* and a single teleomorphic GC-biased Genotype #1 in natural *C. sinensis*, indicating the existence of at least 2 *O. sinensis* teleomorphs. Unfortunately, this study revealed a species contradiction between the anamorphic inoculants (reported to be 3 GC-biased Genotype #1 *H. sinensis* strains) and teleomorphic AT-biased Genotype #4 of *O. sinensis* in cultivated *C. sinensis*, inconsistent with the hypothesis of a sole anamorph of *O. sinensis* for *H. sinensis* proposed by the same key authors 10 years ago (Wei et al. 2006).

Other studies optimized and combined the use of a variety of molecular techniques, such as several pairs of genotype-specific primers, touchdown PCR, nested PCR, restriction fragment length polymorphism (RFLP), cloning-based amplicon sequencing, and single-nucleotide polymorphism (SNP) mass spectrometry genotyping techniques, etc. These research approaches

greatly improved the specificity and efficiency of detection and facilitated the identification of differential coexistence and dynamic alterations of AT-biased genotypes as well as GC-biased genotypes of *O. sinensis* in different combinations in the caterpillar body, stroma, stromal fertile portion with numerous ascocarps, and ascospores of natural *C. sinensis* during maturation (*cf*. Table 4) (Xiao et al. 2009; Zhu et al. 2010, 2012, 2018, 2019; Gao et al. 2011, 2012; Yao et al. 2011; Li et al. 2020a, 2022a, 2023b, 2023c).

Xiao et al. (2009) used pairs of genotype-specific primers for the first time and adopted a nested PCR approach to amplify mutant sequences of *O. sinensis* from the stroma and caterpillar body of immature and mature *C. sinensis* specimens collected from geographically remote production areas. The authors reported the consistent occurrence of GC-biased Genotype #1 and *P. hepiali* and the inconsistent occurrence of AT-biased Genotypes #4□5 of *O. sinensis*. The authors also detected the first sequence (EU555436) of AT-biased Genotype #6 and uploaded it to GenBank. The authors then concluded that the AT-biased genotypes likely belonged to independent fungal species.

Li et al. (2023b, 2023c) used several pairs of genotype-specific primers, touch-down PCR, cloning-based PCR amplicon sequencing and MassARRAY SNP mass spectrometry genotyping techniques and identified Genotypes #5□6 and #16 of AT-biased Cluster-A (*cf*. Figure 2) in the ascospores of natural *C. sinensis*, co-occurring with GC-biased Genotype #1, *P. hepiali*, and an AB067719-type fungus. The sequences of Genotypes #4 and #15 of AT-biased Cluster-B were identified in the stromal fertile portion that was densely covered with numerous ascocarps but not in ascospores obtained from the same *C. sinensis* specimens (*cf*. Table 4). Li et al. (2023c) also identified GC-biased Genotype #13 in semiejected ascospores and GC-biased Genotype #14 in fully ejected ascospores collected from the same samples of natural *C. sinensis*.

Li et al. (2013) made additional efforts to identify Genotype #4 of AT-biased Cluster B in 25-day cultures of mono-ascospores but failed, consistent with the finding shown in Table 4 (Li et al. 2023c). The authors did not manage to identify the ITS sequences of AT-biased Genotypes #4, #6, and #15□17 of *O. sinensis* in genomic DNA preparations extracted from the 15 ascosporic strains in both Groups 2 and 3 of *H. sinensis* (*cf*. Section IV-5). Thus, the expansion of the "ITS pseudogene" hypothesis from the identified Genotype #5 from the mono-ascospore cultures to all AT-biased genotypes was apparently an overly generalized interpretation with no supporting evidence. As shown in Tables 1□3 and Figures 1□2, genomic and phylogenetic analyses did not support the hypothesis that the "diverged ITS paralogs" (*i.e.*, AT-biased Genotypes #4□6 and

#15□17) as the "ITS pseudogenes" coexist "in a single genome" of Genotype #1 *H. sinensis*.

IV-6-2 Dynamic alterations of multiple genotypes of *O. sinensis* in different compartments of natural *C. sinensis*

Molecular physiological studies have revealed that the biomasses/abundances of genotypes are dynamically altered in an asynchronous, disproportional manner during *C. sinensis* maturation, indicating the genomic independence of multiple genotypes of *O. sinensis* from different perspectives (Zhu et al. 2010, 2012; Gao et al. 2011, 2012; Yao et al. 2011; Li et al. 2016b, 2016c, 2022a, 2022c, 2023c).

Zhu et al. (2010) and Gao et al. (2011, 2012) reported that Genotype #4 of AT-biased Cluster-B of *O. sinensis* predominated in the immature stroma of natural *C. sinensis*, which was in its asexual growth stage; however, its abundance dramatically declined in the mature stroma, which was in its sexual reproductive stage. The maturational decline in Genotype #4 abundance in the *C. sinensis* stroma was accompanied by a significant reciprocal elevation in the abundance of Genotype #5 of AT-biased Cluster-A of *O. sinensis*, indicating that the multiple AT-biased genotypes belong to independent *O. sinensis* fungi.

Li et al. (2023b) used the MassARRAY SNP mass spectrometry genotyping technique and found dynamic alterations of GC-biased Genotype #1 and several AT-biased genotypes of *O. sinensis* in the stromal fertile portion (densely covered with numerous ascocarps) before and after ejection of *C. sinensis* ascospores and in the fully and semiejected ascospores collected from the same *C. sinensis* specimens. A highly abundant SNP peak that contained both AT-biased Genotypes #6 and #15 was shown in the preejection stromal fertile portion of natural *C. sinensis*, but the abundance dramatically declined in the stromal fertile portion after ascospore ejection, while the coexisting AT-biased Genotype #5 maintained high abundance.

The ITS sequences of the GC-biased genotypes contained an *Eco*RI endonuclease cleavage site (G▾AATTC in purple in Figure 1) at the position of nucleotides 294→299 of AB067721 (a representative sequence of Genotype #1 *H. sinensis*). The *Eco*RI cleavage site was lost (GAATTT) in all AT-biased genotypes due to a single base C-to-T (cytosine→thymine) transition mutation. To examine dynamic alterations of the natural biomasses of GC- and AT-biased genotypes of *O. sinensis* without PCR manipulation that causes nonlinear, exponential amplification of DNA sequences, the Southern blotting technique was applied to examine genomic DNA after enzymolysis with endonucleases *Ava*I, *Dra*I, and *Eco*RI (Zhu et al. 2010). This non-PCR approach

identified a single *Eco*RI-sensitive DNA moiety in genomic DNA isolated from mycelia of *H. sinensis* (*cf*. Figure 4B of Zhu et al. 2010) and a doublet (both *Eco*RI-sensitive GC-biased Genotype #1 and *Eco*RI-resistant AT-biased genotypes) in genomic DNA isolated from natural *C. sinensis* (Figure 7). The lack of *Eco*RI-resistant AT-biased genotypes in pure *H. sinensis* shown in Figure 4B of (Zhu et al. 2010) indicates that the sequences of AT-biased genotypes of *O. sinensis* are absent in the genome of a GC-biased Genotype #1 *H. sinensis* strain, similar to the *H. sinensis* strains in Groups 1 and 2 summarized above in Section IV-5. Because Li et al. (2013) incorrectly explained biochemical findings obtained by Southern blotting (Zhu et al. 2010), we provide help to the mycologists by reproducing the results (with permission) below in Figure 7 with detailed explanations. The upper panel of Figure 7 shows the Southern blot using an *H. sinensis*-specific probe that was designed based on the ITS1 sequence of Genotype #1 *H. sinensis* and demonstrates the following:

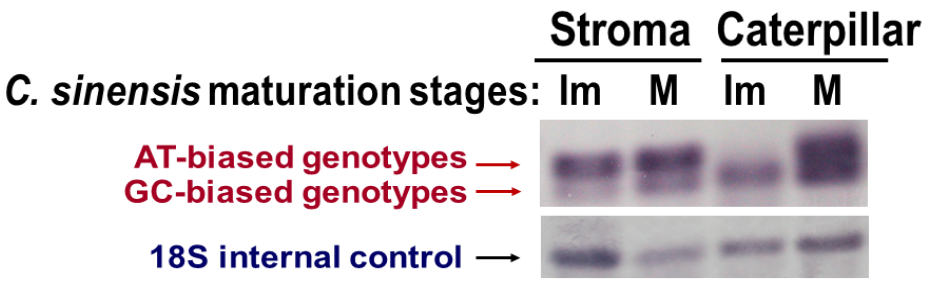


Figure 7. Southern blot of C. sinensis nrDNA in the stroma and caterpillar body of natural C. sinensis during maturation. *[Reproduced with permission from AJBMS (www.nwpii.com/ajbms) Am J Biomed Sci 2010; 2(3): 217-238] [Zhu* et al. *2010].*
*Caterpillar refers to caterpillar body. Genomic DNA templates were isolated from the stromata or caterpillar bodies of immature (Im) or mature (M) C. sinensis specimens collected from Sichuan Province in China and prepared using the restriction enzymes Ava*I*, Dra*I*, and Eco*RI*.* ***The upper panel*** *was probed with an H. sinensis-specific probe that was designed based on the ITS1 sequence of Genotype #1 H. sinensis.* ***The lower panel*** *was probed with a nonspecific 18S internal control probe that was designed based on the 18S gene sequence of Genotype #1 H. sinensis, which is >700 bp upstream of the ITS1 segment.*

(1) *O. sinensis* genotypes identified in the stromata: the left 2 lanes of the upper panel of Figure 7 show that the slower-migrating DNA moieties (*Eco*RI-resistant AT-biased genotypes) predominate naturally without PCR amplification in the stromata of immature (IM) and mature (M) *C. sinensis*. The faster-migrating moieties (*Eco*RI-sensitive GC-biased *H. sinensis*) are always minor moieties in the stromata during *C. sinensis* maturation.

(2) *O. sinensis* genotypes identified in the caterpillar bodies: although the second lane from the

right in the upper panel of Figure 7 seems to show a single *Eco*RI-sensitive moiety in the immature (IM) caterpillar body but a doublet in the mature (M) caterpillar body shown in the far right lane, PCR experiments using genotype-specific primers and touch-down and nested PCR protocols were able to identify *Eco*RI-resistant AT-biased genotypes in the immature caterpillar body. These results suggest that the natural quantity of *Eco*RI-resistant AT-biased genotypes in the immature caterpillar body was below the detection limit of the Southern blotting technique, which is much less sensitive than PCR-based techniques. The quantity of AT-biased genotypes was dramatically and disproportionally increased from a microquantity in the immature caterpillar body to a level comparable to that of *Eco*RI-sensitive GC-biased *H. sinensis* in the mature caterpillar body.

(3) *O. sinensis* genotypes identified in the stroma and caterpillar body of immature *C. sinensis*: the first and third lanes from the left in Figure 7 show that the abundances and density ratios of *Eco*RI-resistant AT-biased genotypes and *Eco*RI-sensitive GC-biased *H. sinensis* differed significantly in the stroma and caterpillar body of immature *C. sinensis*, which were sampled from the same specimens.

(4) *O. sinensis* genotypes identified in the stroma and caterpillar body of mature *C. sinensis*: the first and third lanes from the right in Figure 7 show that the abundances and density ratio of *Eco*RI-resistant and *Eco*RI-sensitive genotypes differed in the stroma and caterpillar body of mature *C. sinensis*, which were sampled from the same specimens.

The disproportional distribution of GC- and AT-biased *O. sinensis* genotypes in the stromata and caterpillar bodies of natural *C. sinensis* and their asynchronous, disproportional alterations of the natural biomasses without PCR amplification during *C. sinensis* maturation indicate that the sequences of AT-biased *O. sinensis* genotypes do not reside in the single genome of Genotype #1 *H. sinensis*. Instead, the genomically independent AT-biased *O. sinensis* genotypes are interindividually accompanied by GC-biased Genotype #1 *H. sinensis* in the stroma and caterpillar body of natural *C. sinensis*.

Zhu et al. (2010, 2012) also used the *Eco*RI enzymolysis approach and a restriction fragment length polymorphism (RFLP) technique and examined GC-biased Genotypes #1 and #2 of *O. sinensis* in the *C. sinensis* stromata. The authors demonstrated dynamic but asynchronous maturational alterations of the cooccurring Genotypes #1 and #2 in the stromata of immature, maturing, and mature *C. sinensis* specimens, indicating genomic independence of these 2 GC-

biased genotypes of *O. sinensis*. Figures 2 and 3 above show that Genotype #2 of *O. sinensis* is genetically and phylogenetically distinct from the repetitive ITS copies identified in the genome of Genotype #1 *H. sinensis*, further confirming the genomic independence of the 2 GC-biased genotypes and ruling out the possibility that they represent 2 of the repetitive ITS copies in the single *O. sinensis* genome.

If "Different types of ITS sequences were thus proved to coexist in a single genome" as assumed by Li et al. (2013), the distribution of GC- and AT-biased *O. sinensis* genotypes should be proportional in the *C. sinensis* stroma and caterpillar body and any biomass alterations of the co-occurring genotypes should be synchronous and proportional during *C. sinensis* maturation. Southern blotting, as well as RFLP after pretreatment with *Eco*RI enzymolysis and MassARRAY SNP mass spectrometry genotyping assays, clearly demonstrated asynchronous and disproportional alterations of the GC- and AT-biased *O. sinensis* genotypes (Zhu et al. 2010, 2012; Gao et al. 2011, 2012; Yao et al. 2011; Li et al. 2023b, 2023c), and Figures 1□3 and Tables 2 and 4 in this paper further confirmed the genomic independence of the multiple *O. sinensis* genotypes, which belong to independent *O. sinensis* fungi (Stensrud et al. 2007; Xiao et al. 2009).

After the hypothesis of "ITS pseudogenes … in a single genome" was published and subjected to academic discussion, Li et al. (2013) had sufficient time to validate their hypothesis by conducting genome sequencing on one of the Group 3 strains (*cf*. Section IV-5). However, scientific papers addressing this have yet to appear. Instead, Li et al. (2013, 2019, 2020b) stated repeatedly that "Different types of ITS sequences were thus proved to coexist in a single genome", "from a single genome, and not from different genomes", and "The coexistence of diverged ITS paralogs in a single genome". These scientists either did not conduct genome sequencing of one of the Group 3 strains (*cf*. Section IV-5) or may have failed the experiments due to high similarity of the genomic sequences of multiple genotypes of *O. sinensis* that evolved from a common ancestor during a long evolutionary history, which causes difficulties in assembly of shotgun reads to verify their hypothesis that Genotypes #1 and #5 coexist in "a single genome", although this genome sequencing strategy may not be able to confirm the coexistence of AT-biased Genotypes #4, #6, and #15□17 as the genomic components of "a single genome" of GC-biased Genotype #1 *H. sinensis*.

IV-7. Possible synergistic effects of several genotypic fungi in *O. sinensis* sexual reproduction

Hu et al. (2013) and Bushley et al. (2013) hypothesized self-fertilization as the reproductive

mode of Genotype #1 *H. sinensis* under homothallism or pseudohomothallism based on the identification of mating-type genes of both *MAT1-1* and *MAT1-2* idiomorphs in 2 *H. sinensis* strains. Li et al. (2020b) stated accordingly that "*O. sinensis* is one of the only known homothallic species in *Ophiocordycipitaceae* … the homothallic life-style may have increased the likelihood and frequency of sex in *O. sinensis*, leading to increased RIP in this species." However, Zhang and Zhang (2015) revealed differential occurrence of the mating-type genes of *MAT1-1* and *MAT1-2* idiomorphs in numerous *H. sinensis* strains, destabilizing the foundation of the self-fertilization hypothesis, and they alternatively hypothesized facultative hybridization as the mode of *O. sinensis* reproduction.

In addition to the analysis of general intraspecific genetic variations and differential expression of *H. sinensis* genes (Li et al. 2022b, 2022c), Li et al. (2021, 2023a) focused on the differential occurrence of mating-type genes of the *MAT1-1* and *MAT1-2* idiomorphs of 236 *H. sinensis* strains: 22 strains (9.3%) contained only MAT1-1-1 genes, 65 (27.5%) possessed only MAT1-2-1 genes, and 149 (63.1%) had both genes. The differential occurrence of mating-type genes underlies the genetic control of *O. sinensis* reproduction. The authors also identified differential but reciprocal transcription of mating-type genes in two *H. sinensis* strains. The MAT1-1-1 transcript was absent, but the MAT1-2-1 transcript was present in transcriptome assembly GCQL00000000 of *H. sinensis* Strain L0106 (Liu et al. 2015; Li et al. 2021, 2023a). The MAT1-1-1 transcript was present in *H. sinensis* Strain 1229, but the MAT1-2-1 transcript included an unspliced intron I, which contains 3 stop codons (Bushley et al. 2013; Li et al. 2021, 2023a). Bushley et al. (2013) specifically commented that a "comparison of DNA and cDNA sequences of MAT1-2-1 revealed only one spliced intron of 55 bp" in *H. sinensis* Strain 1229, which "did not result from contamination of genomic DNA". Thus, the results indicated the production of a largely truncated and dysfunctional MAT1-2-1 protein lacking the majority of the protein encoded by exons II and III. The transcriptional silencing of one of the mating-type genes and the MAT1-2-1 transcript with an unspliced intron I constitute *O. sinensis* reproduction controls at the transcriptional level and the coupled transcriptional-translational level.

Accordingly, Li et al. (2021, 2023a) demonstrated the self-sterility of GC-biased Genotype #1 *H. sinensis*, suggesting a requirement of mating partners for *O. sinensis* sexual reproduction. These partners could be either intraspecific species, such as *H. sinensis* Strains L0106 and 1229, for physiological heterothallism regardless of whether *H. sinensis* is monoecious or dioecious, or

heterospecific fungal species that may undergo hybridization if they are able to overcome interspecific reproductive barriers (Zhang and Zhang 2015; Li et al. 2021, 2022c, 2023a, 2023c). The taxonomic positions of Genotypes #2–17 of *O. sinensis* have not been determined, especially for AT-biased genotypes belonging to different *O. sinensis* fungi "even at higher taxonomic levels (genera and family)" (Stensrud et al. 2007) and GC-biased Genotypes #2 and #7 of *O. sinensis*, which cooccur with Genotype #1 in natural *C. sinensis* (Zhu et al. 2010, 2012; Chen et al. 2011). The coexistence of several AT-biased genotypes with GC-biased Genotype #1 in the ascocarps and ascospores of natural *C. sinensis* may constitute a genetic basis for sexual reproduction of *O. sinensis*, involving fungal hybridization (Li et al. 2023b, 2023c).

In addition, Barseghyan et al. (2012) reported the findings of a macro- and micromycology study and concluded that both *H. sinensis* and *T. sinensis* were the anamorphs of *O. sinensis*. Engh (1999) hypothesized that *Cordyceps* and *Tolypocladium* form a fungal complex in natural *C. sinensis*, and the *Cordyceps* AJ786590 sequence was reported and uploaded to the GenBank database by Stensrud et al. (2005). Stensrud et al. (2007) clustered AJ786590 and other sequences into Group-B of *O. sinensis*, which belong to the AT-biased Genotype #4 and are genetically and phylogenetically distinct from GC-biased Genotype #1 *H. sinensis* (*cf*. Figures 1□2).

Li et al. (2023c) discovered GC-biased Genotypes #13□14 of *O. sinensis*, which exhibit the genetic characteristics of large DNA segment reciprocal substitutions and genetic material recombination between the genomes of the 2 parental fungi, *H. sinensis* and an AB067719-type fungus. The discovery of Genotypes #13□14 of *O. sinensis* likely indicates the possibility of fungal hybridization or parasexuality between 2 heterospecific fungi.

IV-8. Transcription of the 5.8S gene and PCR amplicons of 5.8S-F/R primers

The analysis in Section III-5 indicates that Li et al. (2013, 2019) have neglected the evidence of transcriptional silencing of 5.8S genes demonstrated in metatranscriptome studies in natural settings and the evidence of dynamic and unbalanced transcriptional activation and silencing of numerous fungal genes during the continuous *in vitro* fermentation or liquid incubation of Genotype #1 *H. sinensis* (Xiang et al. 2014; Liu et al. 2015; Xia et al. 2017). Changes in culture conditions and durations have significant impacts on the transcription of fungal genes (*cf*. Tables 6□7), which involves the transcriptional activation and silencing of numerous fungal genes. Because of the conservative evolution of 5.8S genes and many other nonrDNA genes and the incomplete study design and controversial findings reported by Li et al. (2013), it needs to be verified using other

sophisticated transcriptional expression methods whether the cDNA identified by Li et al. (2013) was truly derived from the 5.8S gene of Genotype #1 *H. sinensis*, from mutant genotypes of *O. sinensis* or other colonized fungi, or even from non-rRNA genes before confidently declaring that the 5.8S genes of the multiple AT-biased genotypes of *O. sinensis* are truly permanently nonfunctional pseudogenes under the "ITS pseudogene … in a single genome" hypothesis.

Li et al. (2013) did not disclose the amplicon sequences amplified using 5.8SF/R primers and the PCR template of the cDNA library constructed through reverse transcription of total RNA derived from *H. sinensis* Clone 1220. Li et al. (2019) confirmed that Li et al. (2013) designed 5.8SF/R primers based on the sequence "in the most conserved region" of *H. sinensis* rRNA, meaning low specificity of the primers and resulting in uncertain genetic sources of the PCR amplicons. According to the sequence analysis in Section III-6, the amplicon sequences showed only 90.4%□91.2% similarity to the 5.8S gene sequence of Genotype #1 of *O. sinensis*. The 8.8□9.6% dissimilarity rates may not be convincingly attributed to "mismatches during reverse transcription or PCR amplification", as explained by Li et al. (2013). Thus, solid evidence is lacking to conclude that the amplicon sequences were truly derived from the 5.8S gene of Genotype #1 *H. sinensis* and to claim the *H. sinensis* 5.8S gene as the functional gene and the 5.8S gene of Genotype #5 of *O. sinensis* as the "ITS pseudogene".

Prior to declaring that the 5.8S genes of all AT-biased *O. sinensis* genotypes are permanently dysfunctional pseudogenes co-occurring with a functional 5.8S gene in the genome of GC-biased Genotype #1, several other concerns should be addressed.

(1) Selker and his colleague (1985, 1990, 1997, 2002), Taranto et al. (2014), Hane et al. (2015) and Gladyshev (2017) have repeatedly reported epigenetic methylation of nearly all cytosine residues in 5.8S RNA genes and subsequent transcriptional silencing of GC-biased genes, which occur only in certain fungal species. As shown in Table 3 and Figures 1 and 3 above, the multiple genomic repetitive ITS copies of GC-biased Genotype #1 *H. sinensis* contain mainly insertion, deletion and transversion alleles but no or only a few transition alleles. The repetitive ITS copies of Genotype #1 are genetically and phylogenetically distinct from the AT-biased genotypes of *O. sinensis* (Figures 1–3). Although the 5.8S gene of GC-biased Genotype #1 contains a large number of cytosine residues and is potentially more susceptible to epigenetic methylation attack, introducing nonsense or missense mutations that subsequently cause translational silencing of 5.8S genes, the evidence presented in this paper indicates that *H.*

sinensis species may not be the target of RIP mutagenesis and epigenetic methylation attack. It is unlikely that the AT-biased genotypes of *O. sinensis* emerged after the generation of a new *H. sinensis* genome; instead, they are likely genomically independent, existing in different *O. sinensis* fungi.

(2) Li et al. (2013) did not detect the ITS sequences of Genotypes #2–4 and #6–17 of *O. sinensis* in the genomic DNA pool of mono-ascosporic cultures. Thus, it is an apparent overgeneralization to infer that the 5.8S genes of these genotypes are dysfunctional "ITS pseudogene" components of the genome of Genotype #1 *H. sinensis*.

(3) The sequences of Genotypes #2–17 are absent in the genome assemblies ANOV00000000, JAAVMX000000000, LKHE00000000, LWBQ00000000, and NGJJ00000000 of *H. sinensis* Strains Co18, IOZ07, 1229, ZJB12195, and CC1406-2031229, respectively (Hu et al. 2013; Li et al. 2016a; Jin et al. 2020; Liu et al. 2020; Shu et al. 2020), indicating genomic independence of *O. sinensis* genotypes.

(4) The culture-dependent approach used by Li et al. (2013) might have missed some fungal species that are nonculturable or difficult to culture under the *in vitro* experimental settings of the study. The culture-dependent strategy might have a significant impact on the transcription of many fungal genes, with many genes being switched on-or-off nonnaturally, as evidenced by the nonlinear reduction in the total number of transcriptomic unigenes and increases in the average length but decreases in the GC content of transcripts during 3□9 days of liquid fermentation of *H. sinensis* Strain L0106 (*cf.* Table 7) (Liu et al. 2015). Table 6 also demonstrates that changes in the culture duration from 4□15 days and the use of culture medium with or without the addition of adenosine or adenine had dramatic impacts on fungal gene transcription (Pang et al. 2018). A much greater impact on differential transcriptional activation and silencing of many genes may be expected after the prolonged 25-day liquid incubation period adopted by Li et al. (2013).

(5) Three distinct types of secondary steric conformations of 5.8S rRNA were predicted for Groups A–C (*i.e.*, Genotypes #1 and #4–5) of *O. sinensis* by Li et al. (2013). The possibility of producing circular RNA through back splicing-exon circularization (Dong et al. 2014a) should be considered during study design because these distinct steric structures may have a considerable impact on reverse transcription PCR and cDNA sequencing. In addition, the ITS1-

5.8S-ITS2 sequences of Genotypes #2 and #6 may adopt different types of steric conformations (Zhu & Wu 2015; Li et al. 2016b, 2022c; Zhu & Li 2017); thus, the design of other genotype-specific primers and the combined use of other molecular techniques should be considered.

(6) Wei et al. (2016) reported the identification of a single teleomorph of AT-biased Genotype #4 of *O. sinensis* in cultivated *C. sinensis* and a single teleomorph of GC-biased Genotype #1 in natural *C. sinensis* (*cf.* Figure 6 of Wei et al. 2016). If AT-biased Genotype #4 represents a nonfunctional ITS pseudogene, as believed by Li et al. (2013, 2019, 2020b), the single *O. sinensis* teleomorph in cultivated *C. sinensis* might have disturbed teleomorphic functions, leading to reproductive sterility and an abnormal lifecycle of cultivated *C. sinensis*. Wei et al. (2016) did not share any information about ascospore production in cultivated *C. sinensis*, which is the critical sign of sexual reproduction of *O. sinensis* (Jiang and Yao 2003).

(7) Li et al. (2022c, 2023b) reported the identification of teleomorphic Genotypes #4 and #15 of *O. sinensis* of AT-biased Cluster-B in the stromal fertile portion densely covered with numerous ascocarps but not in ascospores collected from the same pieces of natural *C. sinensis*. AT-biased Genotypes #6 and #15 were found at high abundance in the stromal fertile portion prior to ascospore ejection, and their abundance drastically declined after ascospore ejection, while teleomorphic Genotype #5 maintained its high abundance in the stromal fertile portion before and after ascospore ejection (Li et al. 2022a, 2022c).

(8) The 5.8S genes of multiple *O. sinensis* genotypes may be transcriptionally activated or silenced in different developmental and maturational stages of natural *C. sinensis*. Significant changes in the proteomic expression profiles of the stroma and caterpillar body of natural *C. sinensis* during maturation constitute evidence of such dynamic alterations in epigenetic, transcriptional, translational, and posttranslational modifications of numerous genes (Dong et al. 2014b).

Apparently, Li et al. (2013) improperly designed the study and provided insufficient and nonconclusive evidence to support their "ITS pseudogene" hypothesis. The whole package of gene expression in *O. sinensis* fungi and natural *C. sinensis* needs to be further explored before the 5.8S genes of AT-biased genotypes of *O. sinensis* can be defined as permanently nonfunctional pseudogenes.

V. Conclusion

This study analyzes the multiple repetitive ITS copies in the genome of Genotype #1 *H.*

sinensis and multiple genotypes of *O. sinensis* fungi and discusses the "ITS pseudogene … in a single genome" hypothesis for all AT-biased *O. sinensis* genotypes (Li et al. 2013, 2019, 2020b). The repetitive ITS copies in the genome of Genotype #1 contain multiple scattered insertion, deletion and transversion point mutations and may not be generated through RIP mutagenesis because RIP mutagenesis theoretically causes C-to-T transitions. The GC-biased repetitive ITS copies are genetically and phylogenetically distinct from AT-biased *O. sinensis* genotypes that contain multiple scattered transition alleles. GC-biased Genotype #1 *H. sinensis* may not be the fungal species target of RIP mutagenesis and epigenetic methylation attack. The AT-biased genotypes are independent from the genome of Genotype #1 and might have been generated from a common ancestor through certain mutagenic and evolutionary mechanisms in the long history of evolution in response to the extremely harsh environment of the Qinghai-Tibet Plateau and become independent *O. sinensis* fungi with independent genomes coexisting in parallel with GC-biased genotypes in natural *C. sinensis*. Li et al. (2013) provided incomplete and controversial information to support their "ITS pseudogene … in a single genome" hypothesis, which needs to be further validated.

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