

Research Article

Molecular Characterization of Mycobiota and *Aspergillus* Species from *Eupolyphaga sinensis* Walker Based on High-Throughput Sequencing of ITS1 and CaM

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Eupolyphaga sinensis Walker is a valuable traditional Chinese animal medicine first recorded in *Shennong Bencao*. Previous research has shown that *E. sinensis* is easily contaminated by aflatoxins (AFs), which are highly toxic mycotoxins, during harvest, storage, and transport, thereby posing a considerable threat to consumer health. Most often, these AFs are produced by *Aspergillus* species. In this study, we contrast the traditional culture-based dilution plating method to the high-throughput sequencing (HTS) technology for fungal identification in TCM *E. sinensis*. Both of the methods used internal transcribed spacer 1 (ITS1) and calmodulin (CaM) sequencing for fungal molecular identification. The new CaM primer we designed in the study is suitable for MiSeq PE300 sequencing used for identification of *Aspergillus* species in community DNA samples. More fungal species were found in the *E. sinensis* samples based on HTS than those found using the culture-based dilution plating method. Overall, combining the sequencing power of ITS1 and CaM is an effective method for the detection and monitoring of potential toxigenic *Aspergillus* species in *E. sinensis*. In conclusion, HTS can be used to obtain a large amount of sequencing data about fungi contaminating animal medicine, allowing earlier detection of potential toxigenic fungi and ensuring the efficient production and safety of *E. sinensis*.

1. Introduction

The dried body of the female ground beetle *Eupolyphaga sinensis* Walker is a valuable traditional Chinese animal medicine first recorded in *Shennong Bencao* and has been used as a traditional Chinese medicine (TCM) for hundreds of years. The insect is collected, scalded to death by boiling water, and dried either in the sun or by baking. Due to its high protein content [1] and unique taste, it is also regarded as a high-class dish by some restaurants and customers. Therefore, with the demand increasing for *E. sinensis*, it has a substantial market potential in China as medicine and food. Currently, *E. sinensis* is raised through artificial breeding.

Because artificially cultivated *E. sinensis* is usually fed organic humus, such as wheat bran and decaying leaves, the

feed remaining in the intestinal tract of the ground beetle is highly susceptible to mould and fungus contamination during the process of harvesting, processing, transporting, and storing the insect. According to market research, *E. sinensis* can easily be contaminated by aflatoxins [2–4], which affect the safety of the medicine and threaten the health of consumers [5, 6]. Aflatoxin is produced mainly by *Aspergillus* fungi [7]. The early detection of *Aspergillus* contaminants in *E. sinensis* can prevent mycotoxins from entering the consumer market. The rapid discovery of *Aspergillus* fungi is of great significance for ensuring the efficient production and safety of TCM.

The identification of *Aspergillus* sp. is based on morphology and molecular species recognition. DNA barcoding has become increasingly important for molecular

identification of fungi and fungal ecological research. The nuclear rDNA internal transcribed spacer (ITS) region is the official DNA barcode for exploring fungal diversity and community in environmental samples because the ITS region can be readily amplified from most DNA samples with universal primers [8] and has the highest probability of identifying the broadest range of fungi [9]. However, ITS sequences lack sufficient variation to distinguish some *Aspergillus* clades [10]. Samson et al. recommend calmodulin (CaM) as a secondary identification marker for *Aspergillus* sp. [11]. So far, there has been limited research on CaM use for *Aspergillus* species identification in community DNA samples.

With the development of high-throughput sequencing (HTS) techniques, the number of large-scale studies of fungi occurring in association with plants [12, 13], animals [14], and human beings [15] has increased. Here, we designed a new primer CaMr1 paired with CMD5 [16] to acquire a CaM amplicon length of no more than 300 bp. The amplicon could then be used for MiSeq PE300 analysis of the *Aspergillus* species. We extracted fungal community DNA occurring throughout TCM *E. sinensis* samples and used HTS technology based on ITS1 and CaM amplicons to examine the fungal microbiome and *Aspergillus* sp. We also used culture-based dilution plating method to isolate fungi from *E. sinensis*. Compared with the traditional method, the HTS results for the two markers show that combining ITS1 and CaM for identifying fungi in TCM is very efficient for the discovery of potential toxigenic *Aspergillus* species.

2. Materials and Methods

2.1. Sample Preparation. *E. sinensis* was purchased from Cao County (Shandong, China) and authenticated by Prof. Yulin Lin (Institute of Medicinal Plant Development, CAMS). Three samples of *E. sinensis* (ES1, ES2, and ES3) were used in both culture-based dilution plating method and HTS.

2.2. Culture-Based Dilution Plating Method. Each of the three *E. sinensis* samples (4 g) was crushed in a flask containing 40 mL of sterile water and 20 glass beads; the flask was shaken at 120 r.min⁻¹ for 5 min and allowed to stand for 5 min. The suspension was diluted to 10⁻¹~10⁻⁶, and 1 mL of each sample was applied to malt extract agar (MEA) medium containing 100 ppm chloramphenicol, with three repeats for each dilute concentration. The plates were cultured at 25°C, and colonies were counted and separated into single spores after seven days. The hyphae were collected and ground with liquid nitrogen in a mortar. The DNA was extracted with the M5 Fungal Genomic DNA Kit (Mei5 Biotech, China), and PCR was conducted using ITS primers (ITS1: TCCGTAGGTGAACCTGCGG and ITS4: TCCTCCGCTTATTGATATGC) [8] and CaM primers (CMD5: CCGAGTACAAGGARGCCTTC and CMD6: CCGATRGAGGTCATRACGTGG) [16], respectively. The PCR products were sequenced using Sanger sequencing.

2.3. HTS Method

2.3.1. DNA Extraction. The three *E. sinensis* samples, each weighing 40 g, were ground with liquid nitrogen in a mortar. The total community DNA was extracted from 4 g of the resulting powder with the M5 Fungal Genomic DNA Kit (Mei5, China). The DNA concentration was measured with NanoDrop 2000 (Thermo Scientific, USA) and was ≥ 20 ng· μ L⁻¹. The OD₂₆₀/OD₂₈₀ was between 1.8 and 2.0. The extracted DNA was stored at -20°C for subsequent analyses.

2.3.2. ITS1 and CaM Primer Amplification. The DNA extracted from each sample was used as template DNA at 10 ng. Because the entire ITS region ranges between 450 and 700 bp which is too long for HTS, a partial sequence was used. The primers ITS1-F (5'-CTGGTCATTTAGAGGAAGTAA-3') [17] and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') [8, 18] were used for amplifying the ITS1 region. ITS1-F and ITS2 have been used to survey the diversity and composition of the fungal communities from environmental sample frequently during the last 10 years [19–21]. The primers CMD5 and CaMr1 (5'-CCTTRGTRG TGA TYT GRC CT-3', this study) were used for amplifying a segment of CaM. PCRs were performed with Trans Start Fast Pfu DNA Polymerase (TransGen, China). The reaction system contained 4 μ L 5× Fast Pfu Buffer, 2 μ L dNTP (2.5 mM), 0.8 μ L each of the forward and reverse primers, 10 ng of template DNA, 0.4 μ L Fast Pfu Polymerase, and ultrapure water to a final volume of 20 μ L. PCR amplification was performed under the following conditions: 95°C for 5 min; 95°C for 30 s, 55°C for 30 s, and 27 cycles of 72°C for 45 s; and 72°C for 10 min. All samples were used in the experiment, and each sample was amplified three times. The PCR products of the same samples were mixed and detected by 2% agarose gel electrophoresis, and the PCR was recovered with the Axy Prep DNA Gel Recovery Kit (Axygen, USA).

2.3.3. Illumina PE300 Sequencing and Data Analysis. The amplicons were sequenced using the Illumina MiSeq PE300 platform (Illumina, USA), Miseq Reagent kit v3 (600-cycle) (Illumina, USA), by Shanghai Biozeron Biotech Co., Ltd (Shanghai, China).

The Illumina PE300 sequencing data first included the effective sequences of all samples. Based on the overlap between paired-end (PE) reads, the paired reads were spliced into a sequence. Finally, according to the barcode and primer sequences, the high-quality sequences of each sample were obtained by splicing, and the sequence direction was corrected according to the forward and reverse barcodes, and the direction of the primer was used in the process. Chimeras were removed, and finally, a valid sequence file was obtained. Statistical analysis of the bioinformation of each sample's OTUs was performed using Usearch (version10: <http://drive5.com/uparse/>) software at a similarity level of 97% by categorization.

To obtain the species classification corresponding to each OTU, the RDP classifier [22] was used to classify the 97% similarity level of representative OTU sequences, and the community composition of each sample was calculated at

TABLE 1: Summary of HTS of the ITS1 amplicon for *E. sinensis*.

	ES1	ES2	ES3
Sequence	131,405	143,763	119,127
Average length (bp)	225	237	218
Bases (bp)	29,564,446	34,174,249	26,001,838
No. of OTUs	92	286	23
No. of genera	48	140	11

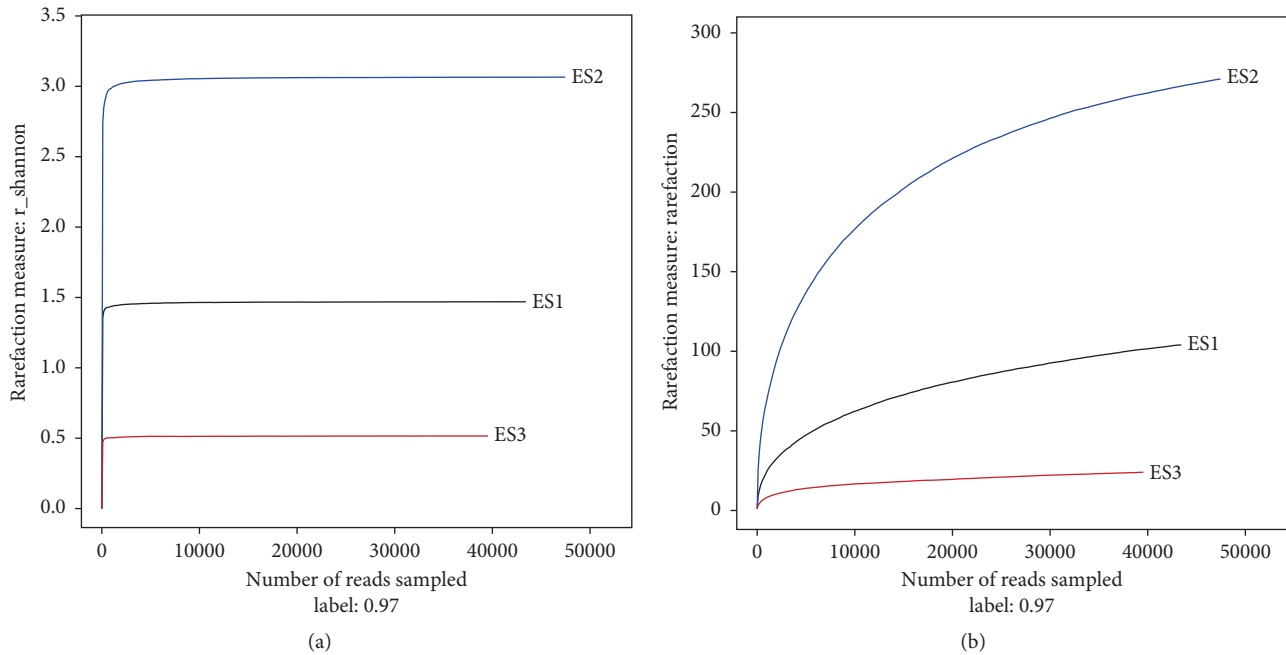


FIGURE 1: Evaluation of HTS data based on the ITS1 amplicon. Shannon–Wiener curves show that the fungal diversity levels off with deeper sequencing in all three *E. sinensis* samples (a). Rarefaction curves show that the abundance of fungal species increased with the number of reads (b).

each classification level (family, genus, and species). Alpha diversity values of the Shannon index [23] (<http://www.mothur.org/wiki/Shannon>) and the Simpson diversity index (<http://www.mothur.org/wiki/Simpson>) were calculated using mothur (version v.1.30.1 http://www.mothur.org/wiki/Schloss_SOP#Alpha_diversity) software [24]. The richness of the species in the samples with different sequencing data was compared by rarefaction curve [25].

3. Results and Discussion

The culture-based dilution plating method was used for the separation of fungi in the three *E. sinensis* samples (ES1, ES2, and ES3). A total of 32 fungal clones was acquired from all samples. We extracted genomic DNA from each of the 32 clones and sequenced ITS and CaM PCR products using Sanger sequencing. By comparing ITS and CaM sequences against the NCBI database with the BLAST tool, a total of six fungal species were identified, including *Aspergillus candidus*, *A. pseudoglaucus*, *A. ruber*, *A. tubingensis*, *Penicillium chrysogenum*, and *Hyphopichia burtonii*.

For HTS analysis, community DNA was extracted from each of the three *E. sinensis* samples in triplicate. A total of nine DNA samples were analysed using Illumina MiSeq PE300 based on the amplicon sequencing of the fungal ITS1 region. The average length of the amplicon is about 227 bp. A total of 394,295 sequences were obtained (Table 1). After PE alignments, quality filtering, and deletion of chimeras and singletons, more than 100,000 ITS1 sequences from each sample were recovered. Shannon rarefaction analysis shows that the sequence data for each sample are saturated (Figure 1). A total of 286 OTUs were found in *E. sinensis*. The taxonomically and functionally annotated sequence database NCBI nucleotide (Nt) was used to identify 140 fungal genera. In samples ES1 and ES3, *Aspergillus* was the majority fungal genus, representing 96.9% and 99.3% of genera, respectively. However, species-level taxonomy showed no *A. ruber* or *A. tubingensis*, which were found using the culture-based diluting plate method. In sample ES1, 25% of sequences were annotated to *Aspergillus* section *Aspergillus* (formerly the genus *Eurotium*) but cannot be identified to species (Figure 2).

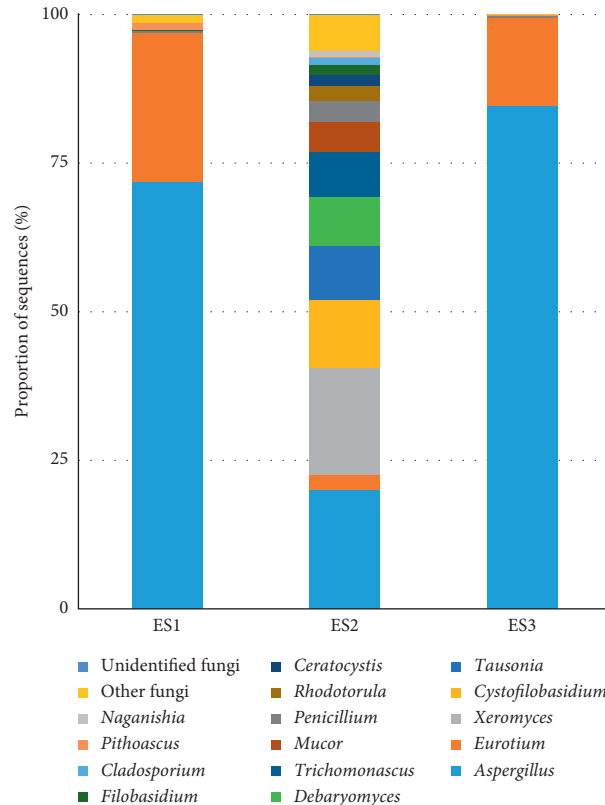


FIGURE 2: Genus-level percentage of fungal composition based on HTS of the ITS1 amplicon for all three *E. sinensis* samples.

Using the HTS platform, a portion of the CaM gene sequence was used to identify undefined *Aspergillus* sp. in *E. sinensis* samples. In *Aspergillus* sp., the CaM PCR product length obtained with previously described primers was about 600 bp [16], which is too long for HTS. The three *E. sinensis* samples were sequenced in triplicate using the two primers CMD5 and CaMr1. The average length of the amplicon was 249 bp. A total of 1,026,856 sequences were obtained (Table 2). The Shannon rarefaction analysis shows that the sequencing data for each sample are saturated (Figure 3). A total of 193 OTUs were found. When the taxonomically and functionally annotated sequence database Nt was used, only 13 fungal genera were found. The number of fungal genera identified by sequencing CaM was far fewer than the number of fungal genera identified by sequencing ITS1. This is because CaM sequences are abundant in *Aspergillus* sp., while being rarer in other fungi. Nineteen *Aspergillus* species were annotated based on the HTS of CaM (Figure 4). All four *Aspergillus* species (*A. candidus*, *A. pseudoglaucus*, *A. ruber*, and *A. tubingensis*) found using the culture-based diluting plate method were also found by HTS based on the CaM amplicon. In addition, another 15 *Aspergillus* species were found in *E. sinensis* samples, including potential toxigenic fungi *A. flavus*.

ITS is the primary fungal barcode marker used to identify individual strains and analyse fungal diversity in environmental samples [26]. In addition to the universal ITS1 and ITS4 primers, most primers targeting the ITS region were published in the 1990s [8, 17]. Other ITS primers targeting ITS1 or ITS2 region were more recently

TABLE 2: Summary of the HTS of CaM amplicon for *E. sinensis*.

	ES1	ES2	ES3
Sequence	386,069	423,179	217,608
Average length (bp)	247	250	250
Bases (bp)	95,284,351	105,669,488	54,406,592
No. of OTUs	169	193	60
No. of genera	10	13	2

designed to make better use of HTS [18, 27–29]. In this study, we used the ITS1-F and ITS2 primers, which generate PCR products no more than 300 bp in size and are suitable for HTS. The amplicon is efficient at distinguishing fungal genera in *E. sinensis*. However, some studies have indicated that the ITS sequence is highly conserved in the *Aspergillus* section *Aspergillus* (formerly the genus *Eurotium*) and cannot be used to distinguish different species [10]. CaM is a Ca^{2+} sensor involved in a large number of biological processes. In a previous study, the CaM sequence was used to differentiate *Aspergillus* species. In order to identify the potential toxigenic *Aspergillus* species using HTS in *E. sinensis*, we designed a new CaM primer which could be used in HTS analysis. Thus, CaM HTS analysis can be used to identify *Aspergillus* species in community DNA samples, making up for ITS1 deficiencies.

In the ES1 sample, 10.9% sequences were annotated to *A. flavus*; however, it was not detected through the

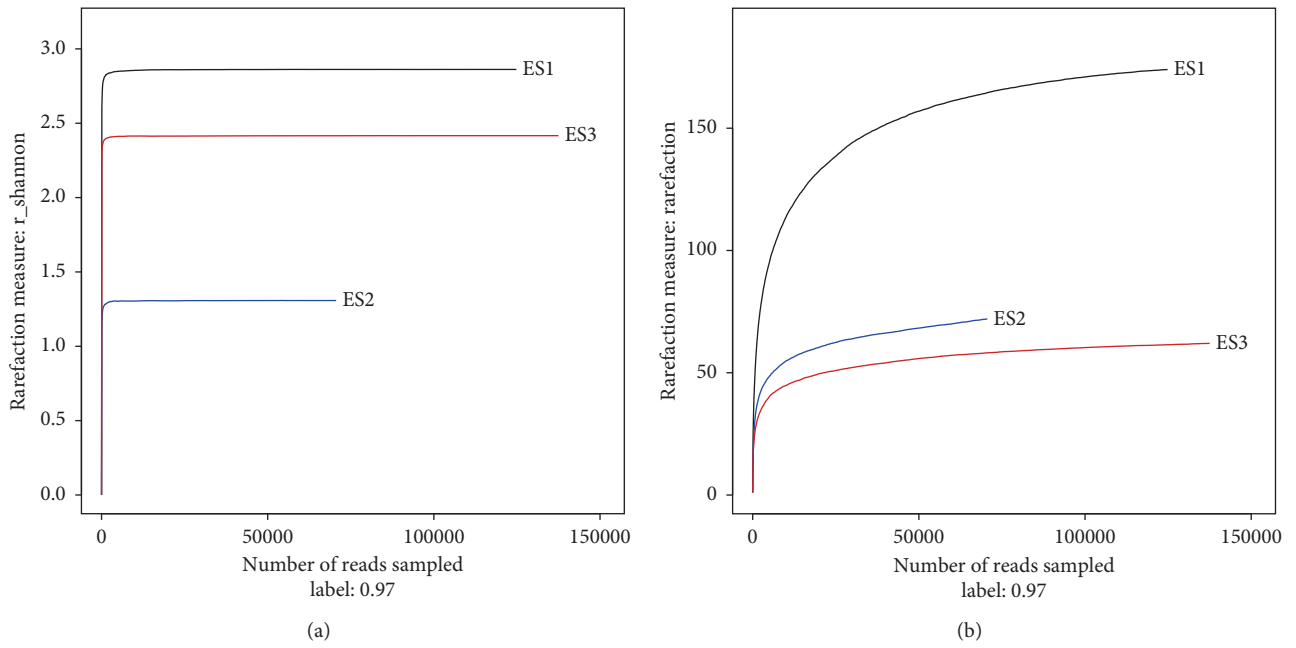


FIGURE 3: Evaluation of HTS data based on the CaM amplicon. Shannon–Wiener curves show that the fungal diversity levels off with deeper sequencing in all three *E. sinensis* samples (a). Rarefaction curves show that the abundance of fungal species increased with the number of reads (b).

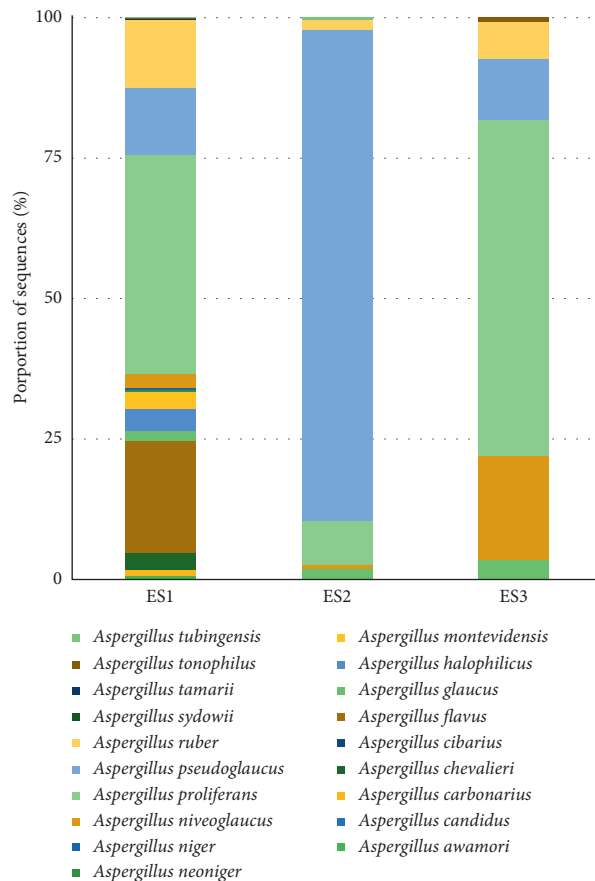


FIGURE 4: The percentage of *Aspergillus* species based on HTS of the CaM amplicon for all three *E. sinensis* samples.

culture-based dilution plating method. It is known that a high proportion of fungi cannot currently be cultured under available artificial conditions [30]. Although we crushed the *E. sinensis* samples, fungi on residual feed in the gut are difficult to isolate. The HTS can much more effectively identify the internal fungi.

In the ES3 sample, there were only two sequences annotated to *A. flavus* through HTS, while the culture-based dilution plating method could not separate *A. flavus* from the sample. The trace amount of toxigenic *A. flavus* in *E. sinensis* is difficult to isolate through the culture-based dilution plating method. Under improper storage conditions, the potential toxigenic fungi may bloom in *E. sinensis*. In addition, HTS technology only requires several hours, whereas the culture-based dilution plating method takes nearly half a month. Overall, HTS analysis of the fungal microbiome is better than the culture-based method for early detection of potential toxigenic fungi in *E. sinensis*. Our results show that HTS is highly effective for identifying toxigenic fungi in the animal medicines *E. sinensis*.

It is important to study the *Aspergillus* sp. in the foods and the medicines in order to stay away from mycotoxins. The next step in the development of HTS as a screen for mycotoxins may be detection of the aflatoxin genes themselves. The biosynthetic pathway of aflatoxin has been completely elucidated, and the expression level of two genes, *aflS* and *aflR*, regulates the transcription of the structural genes in the aflatoxin cluster [31, 32]. The expression level of the two genes may reflect the level of aflatoxin production. Next, we may detect the expression of *aflS* or *aflR* based on HTS to predict whether there is potential toxigenic fungi in TCM.

4. Conclusions

We used the culture-based dilution plating method and HTS to study the fungal composition of three *E. sinensis* samples. The number of contaminant fungal genera and species identified by HTS was clearly much higher than that obtained by using the culture-based dilution plating method. Based on HTS of the ITS1 amplicon, 286 OTUs annotated to 140 fungal genera were found. *Aspergillus* was the dominant fungal genus in all three *E. sinensis* samples. A total of 19 *Aspergillus* species were found based on HTS of the CaM amplicon. Among them, only four *Aspergillus* species were found based on the culture-based dilution plating method. There were some sequences of *A. flavus* found in ES1 and ES3 through HTS. Combined HTS of ITS1 and CaM is an effective early warning method for monitoring potential toxigenic fungi and mycotoxins in TCM.

Data Availability

Raw sequences are available in the Sequence Read Archive of the NCBI under the accession number PRJNA598990.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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