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# Effects of cultivating orchid *Gastrodia elata* with the introduced *Armillaria* in a local ecosystem

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**To characterize the potential effects of an introduced fungus in a local ecosystem, an experiment was performed in an abandoned farmland, where a traditional Chinese medicinal plant, *Gastrodia elata* had been continuously cultivated using the introduced *Armillaria* M2 and it had not been grown for the last 6 years. In this case, a newly introduced *Armillaria* strain M1 was used to cultivate orchid *G. elata* again. Inoculating strain M1 showed an infection rate of 95.56% on *G. elata*, much higher than that of without, 22.2%. Molecular evidence showed that all *Armillaria* re-isolated from *G. elata* in the farmland were genetically identical to the introduced ones. It suggested that the introduced *Armillaria* completely blocked the infection of natural ones on *G. elata*. Such an effect could persist several years even after stopping cultivation of *G. elata*. A total of 53 strains of *Armillaria* were obtained from a variety of isolation sources. Analysis of their intergenic spacer (IGS) sequences revealed that diverse and novel species of *Armillaria* existed in local forest. They are valuable resources for cultivating *G. elata* in future. Regarding ecological risk, utilization of the introduced *Armillaria* is not recommendable for local farmers.**

**Key words:** *Gastrodia elata*, *Armillaria*, the introduced microbe, orchid, ecological risk.

## INTRODUCTION

"Gastrodia", the tuber of the orchid *Gastrodia elata* Blume, is a valuable traditional Chinese medicinal plant and has been widely applied for treating a variety of disease, including headache, dizziness, hemiplegia, rheumatism and epilepsy (Tang and Eisenbrand, 1992; Chen and Sheen, 2011). In the wild, *G. elata* has to obtain the nutrients

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through symbiosis with *Armillaria* species, because *G. elata* is an achlorophyllous and aphyllous plant (Xu et al., 1989). Generally, it takes three years to obtain the mature tubers from seeds of *G. elata*. In the first year, the seeds are able to germinate through the symbiotic fungus *Mycena osmundicola* Lange and grow into protocorms. The protocorms could be used to obtain the immature tubers after one year of cultivation with *Armillaria*. Finally, the immature tubers could grow into mature ones after further one year of cultivation with *Armillaria* (Zhang and Yang, 2007).

Approximately 40 *Armillaria* species are distributed worldwide (Ota et al., 2011) and at least 19 biological species have been reported in Asia (Wang, 2010). However, only a few have been proved to be conducive to the growth of *G. elata* (Wang and Guo, 2002), including *Armillaria ostoyae*, *Armillaria gallica*, *Armillaria jezoensis*, *Armillaria sinapina*, *Armillaria singular* and *Armillaria nabsnona* (Cha and Igarashi, 1995; Sekizaki et al., 2008).

Currently, there is a trend in artificial inoculation of commercially grown *G. elata* to ensure colonization by effective *Armillaria* species and strains (Hua, 2004). From the ecological aspect, more and more concerns in recent years have been paid on the effects of the introduced fungus on local ecosystems (Litchman, 2010; Cowan et al., 2011). For example, an invasive mycorrhizal fungus originating in the Western United States has become a novel symbiont of endemic plants in California (Pringle et al., 2009a), and this novel association could affect not only the success of the plant species, but also nutrient cycling and other ecosystem properties (Pringle et al., 2009b).

It is well-known that *A. mellea* is a sapro-parasitic basidiomycete which is able to survive in the soil for a long time on wood and root debris without any living host. In forests, *Armillaria* genets can even spread across large areas up to 15 ha (Smith et al., 1992). Coetzee et al. (2001) reported that the *A. mellea* introduced into Cape Town, Africa, from Europe, could expand its colonization more than 300 years, with a colonized area at least 345 m in diameter. Therefore, people wonder if introducing exotic *Armillaria* spp. for increasing growth of comer-cially grown *G. elata* could have significant negative impacts on the native fungal community.

Xiaocaoba, located at Zhaotong, Yunnan Province, is a place famous for its high quality of *G. elata* in China (Yuan et al., 2002). The introduced *Armillaria* have been commonly applied for cultivating *G. elata* in the farmland (unpublished data). However, there is little knowledge on the effect of the non-local commercial species of *Armillaria* on the infection of *G. elata* by native *Armillaria* species. In this case, a newly introduced *Armillaria* strain M1 was used to cultivate *G. elata* in an abandoned farmland, where *G. elata* had been continuously cultivated using the introduced *Armillaria* M2 and it had not been grown for the last 6 years, to determine (1) whether the application of non-local *Armillaria* deters the infection of endemic *Armillaria*; (2) whether the activity of the introduced *Armillaria* persists in soil after stopping cultivation of *G. elata*.

## MATERIALS AND METHODS

### Description of experimental site

Cultivation of *G. elata* was carried out in Xiaocaoba, Zhaotong, at about N27°, E104°, with an average altitude of 1700 m. Xiaocaoba receives annually 960-1300 mm of rainfall and has relatively high humidity (ranging from 76 to 85%). The average temperature of the coldest month is above -1°C and the highest below 25°C. This kind of condition is suitable for the growth of *G. elata* (Zhang and Yang, 2007). An experimental plot (50 × 50 m) was selected to cultivate *G. elata*. Based on the investigation of local history of cultivating *G. elata*, this area had once grown *G. elata* with the inoculation of *Armillaria* M2. Since 2004, however, this farmland has been abandoned (unpublished data).

### The cultivation of *G. elata*

A standard cultivation of *G. elata* was carried out in September, 2010. Briefly, a total of 270 holes, each 0.5 (wide) × 1 (long) × 0.5 m (high), were dug on the experimental plot. Fresh tree branches were collected from forest, cut into nearly 50 cm length and were used as nutrients for the growth of *Armillaria*. In each hole, 5-10 fragments of fresh tree branches (about 10 kg) were placed as two layers. When necessary, a volume of solid culture of commercially produced *Armillaria* M1 (Zhaotong Shengnong Limited, China) was placed on the woods (see below). Then the holes were covered with soils to allow *Armillaria* to grow on the wood for two months. The inoculation volumes of *Armillaria* were arranged as zero, 250 and 500 g per hole, respectively, and each treatment has 90 repeats.

In November 2010, the top soils were uncovered and the immature tubers of *G. elata* were put on the bed of tree woods and then the top soils were put back into the hole again. In October 2011, at the end of cultivation of *G. elata*, the *Armillaria* rhizomorphs connected with mature tubers of *G. elata* were collected from the holes and were subject to re-isolate *Armillaria* strains.

### Isolation of *Armillaria*

Generally, 3-5 different rhizomorphs were collected from each hole in which there are mature tubers. To compare the genetic difference, *Armillaria* rhizomorphs were also obtained from four other farmlands (A, B, C and D), in which *G. elata* was traditionally cultivated through inoculating wild *Armillaria* collected from local forest (Table 1). In addition, wild *Armillaria* rhizomorphs associated with local tree barks were also collected from the mixed coniferous and broad-leaved forest far away from farmland of *G. elata*. A standard strain *Armillaria mellea* was bought from Yunnan Microbiology Institute, Kunming, China, and used as a reference.

The collected rhizomorphs were brought back to the laboratory. Rhizomorphs were cut into 1 cm fragments, washed with water and rinsed with 2% sodium hypochlorite solution for 1 min and 75% ethanol for 1 min. Then the disinfected rhizomorph was placed on Potato Dextrose Agar medium (PDA), containing streptomycin sulfate (40 µg/ml) and penicillin sodium (20 µg/ml) to inhibit bacterial

**Table 1.** Haplotype distribution of *Armillaria* in this study.

| Haplotype [a] | Strain [b] | Source |
|---|---|---|
| 1 (2) | SCH | Tree bark of *Dipentodon sinicus* |
| | S3c | *G. elata* tuber of farmland D |
| 2 (20) | 391a, 391b, 391c; 392b, 392c; 396a, 396b, 396d, 396e; 409a; 420a, 420b, 420c;1059a,1059b; 397a, 397b | *G. elata* tuber of our farmland [c] |
| | *Armillaria* M1 | A commercially obtained strain |
| | S4a, S4c | *G. elata* tuber of farmland A |
| 3 (2) | YQJ, YQJs | *G. elata* tuber of farmland B |
| 4 (25) | 828a, 828b; 824b; 532a, 532b, 532c; 227b, 227c; 533a; 33a, 33b; 46a, 46b; 219b, 219c, 219d; 44a; 218a, 218b, 218c; 220a, 220c; 211d; 829c | *G. elata* tuber of our farmland [d] |
| | *Armillaria* M2 | A commercially obtained strain |
| 5 (1) | 829a | *G. elata* tuber of our farmland [d] |
| 6 (5) | YYT | Tree bark of *Cerasus serrula* |
| | HSS | Tree bark of *Pinus armandii* |
| | SLZ | Tree bark of *Aleurites moluccana* |
| | S2a, S2b | *G. elata* tuber of farmland C |

[a] Number in parenthesis is no. of isolates in each haplotype; [b]The strains with the same number are those obtained from different tubers of *G. elata* in the same hole and are underlined; [c]This farmland was inoculated with *Armillaria* M1 when cultivating *G. elata*; [d]This farmland was not inoculated with *Armillaria* M1 in this study but had been inoculated with *Armillaria* M2 to cultivate *G. elata* 6 years ago.

proliferation. All plates were incubated in the dark at 28°C for 2-3 weeks and the pure mycelia were collected for phylogenetic analysis.

### Phylogenetic analysis

The genomic DNA of strains was extracted with CTAB method (Zolan and Pukkila, 1986). Because the ITS sequences are highly conserved and similar among *Armillaria* species (Dunne et al., 2002; Sekizaki et al., 2008), the IGS regions was amplified with primers LR12R (5'-CTG AAC GCC TCT AAG TCA GAA-3') (Veldman et al., 1981) and O-1(5'-AGT CCT ATG GCC GTG GAT CAG AA-3') (Duchesne and Anderson, 1990). The PCR reactions were performed with a PCR-Cycler (Biometra). PCR products were then purified using DNA gel purification kit (Shanghai Sangon Biological Engineering Technology & Services Co.,Ltd). Sequencing was performed by the Beijing Genomics Institute (BGI).

To determine the phylogenetic position of all isolates, the sequences were aligned using the DNAStar software and manually edited in SeqMan (from DNAStar software package). The edited sequences were uploaded on GenBank for BlastN search (http://www.ncbi.nlm.nih.gov). The sequences with the highest similarities were downloaded and were subjected to construct phylogenetic tree using the method of Neighbor-Joining tree by software PHYLIP3.65. The bootstrap analyses (1000 replicates) were preformed to calculate the confidence intervals at the branch nodes. The sequences in this report were deposited in GenBank as accession number KC844229 to KC844234.

### Data analysis

Unless *Armillaria* successfully infects, *G. elata* is not able to develop into a mature tuber. Therefore, the infection rate of *Armillaria* was defined as the percentage of the holes with mature tuber of *G. elata* at the end of experimental period. Statistical analysis was performed by using SPSS 13.0 for Windows (SPSS, Inc., 2004). Nonparametric test of two independent samples was used to evaluate differences of infection rate of *Armillaria* in artificial culture of *G. elata*. Mann-Whitney Test was used to determine significant variances between independent samples. Statistical significance was defined as $P < 0.05$, unless otherwise noted in the text.

## RESULTS

### Infection rate of introduced *Armillaria*

Among 90 holes without inoculation of *Armillaria* M1, only 20 holes were found to produce the mature tuber of *G. elata*, with an infection rate of 22.2%. However, among 180 holes being inoculated *Armillaria* M1 (half with an inoculation volume of 250 g and half with 500 g per hole), 172 holes were found to produce the mature tuber of *G. elata* and the infection rate of *Armillaria* was 95.56%. Therefore, artificial inoculation of *Armillaria* significantly

**Table 2.** Haplotype diversity of *Armillaria* strains obtained from different sources

| Isolation source | Sample size | IGS haplotype (no. of isolates in each haplotype) |
|---|---|---|
| Farmland inoculated with *Armillaria* M1[a] | 17 | 2(17) [d] |
| Farmland inoculated without *Armillaria* M1 [b] | 25 | 4(24) [e]; 5(1) |
| Farmland A, B, C, D[c] | 7 | 1(1); 2(2); 3(2); 6(2) |
| Tree barks | 4 | 1(1); 6(3); |
| Total | 53 | |

[a] This farmland was inoculated with commercial *Armillaria* M1 when cultivating *G. elata*. [b]This farmland was not inoculated with commercial *Armillaria* M1 in this study but had been inoculated with *Armillaria* M2 to cultivate *G. elata* 6 years ago; [c] These farmlands were inoculated with naturally infected *Armillaria*; [d] *Armillaria* M1 is included into this haplotype; [e] *Armillaria* M2 is included in this haplotype.

increased infection rate of *Armillaria* in the culture of *G. elata* (Mann-Whitney Test, p<0.001). However, inoculation volume of *Armillaria* (250 g/hole vs. 500 g/hole) has no influence on the infection of rate of *Armillaria* (94.4 vs. 96.7%, Mann-Whitney Test, p=0.471) but on the weight of *G. elata* tuber (data not shown).

### Phylogenetic analysis

A total of 53 strains of the *Armillaria* were isolated from the rhizomorphs and were divided into 6 haplotyes based on their IGS sequences (Tables 1 and 2). Seventeen isolates were obtained from 17 different tubers of *G. elata* collected from 7 holes inoculated with the commercial *Armillaria* M1. These strains were grouped into Haplotype 2, with the strain *Armillaria* M1. Similarly, twenty-five isolates from different tubers of *G. elata* collected from 13 holes without inoculation of *Armillaria* M1 were divided into 2 haplotypes. Among of them, 24 were grouped with *Armillaria* M2 into Haplotype 4 and remaining 1 isolate (829a) belonged to Haplotype 5.

Two isolates from farmland A were also grouped with M1 into Haplotype 2, but 2 from farmland B formed Haplotype 3. Isolate SCH, obtained from tree bark of *Dipentodon sinicus* was haplotype 1. Three strains YYT, HSS and SLZ, which were isolated from tree barks of *Cerasus serrula*, *Pinus armandii* and *Aleurites moluccana* in local forest, respectively, were grouped into haplotype 6. In addition, strains S3c (from farmland C), S2a and S2b (farmland D) were grouped with above wild strains (Table 2).

Phylogenetically, these strains were different from *A. mellea*. Haplotype 2 was close to *Armillaria cepistipes* Velen, and Haplotype 3 to *Armillaria calvescens* Bérubé & Dessureault and *Armillaria sinapina* Bérubé & Dessureault. However, Haplotype 1, 4, 5, and 6, were phylogenetically distinct from those reported *Armillaria* species (Figure 1).

## DISCUSSION

Currently, the *Armillaria* used in the artificial cultivation of *G. elata* is dependent on two sources. One is the naturally infected tree bark or branches of *Armillara.* This method benefits from low cost but is limited to low availability and production of wild *Armillaria.* The other one is the commercial *Armillaria*, in which the *Armillaria* were generally propagated in a laboratory and most of them are introduced into local farmland by a company. From the ecological perspective, potential risks for the introduced fungi are highly concerned in recent because the introduction of exotic fungi could have negative impacts on the native community (Litchman, 2010; Cowan et al., 2011). Our results showed that inoculating commercial *Armillaria* completely blocks the infection of natural genets of *Armillaria* on *G. elata* in soil, because the holes newly inoculated by commercial *Armillaria* M1 showed a very high infection rate (95.56%) and all of the strains isolated from different tubers of *G. elata* collected from different holes are grouped into one haplotype with strain M1 (Haplotype 2) (Tables 1 and 2). The holes that were not inoculated with the newly introduced *Armillaria* M1 also showed a certain degree of infection rate of *Armillaria* (22.2%). Interestingly, because all isolates obtained from these holes, with the exception of strain 829a, were phylogenetically identity to the *Armillaria* M2, a strain which had been used to inoculate *G. elata* in this experimental field but had not been applied for the last 6 years (Table 2), it further demonstrated that once the non-local *Armillaria* was introduced into an area, it was difficult for local wild *Armillaria* to infect *G. elata*. Moreover, this result indicated that the capacity of the introduced *Armillaria* for developing a symbiotic relationship with *G. elata* and blocking the infection of wild *Armillaria* on the *G. elata* could persist in soil several years even after stopping cultivation of *G. elata*.

Although many fungi are able to produce lots of spores, so as to easily disperse over a long distance by the wind, most fungi infect their hosts endemically (Taylor et al., 2006; Lumbsch et al., 2008), which may be partially due to dispersal barriers (Litchman, 2010; Peay et al., 2010). In nature, most of *Armillaria* species have been also considered to be endemic (Keča and Solheim, 2011). In this case, however, two strains (S4a and S4c) obtained

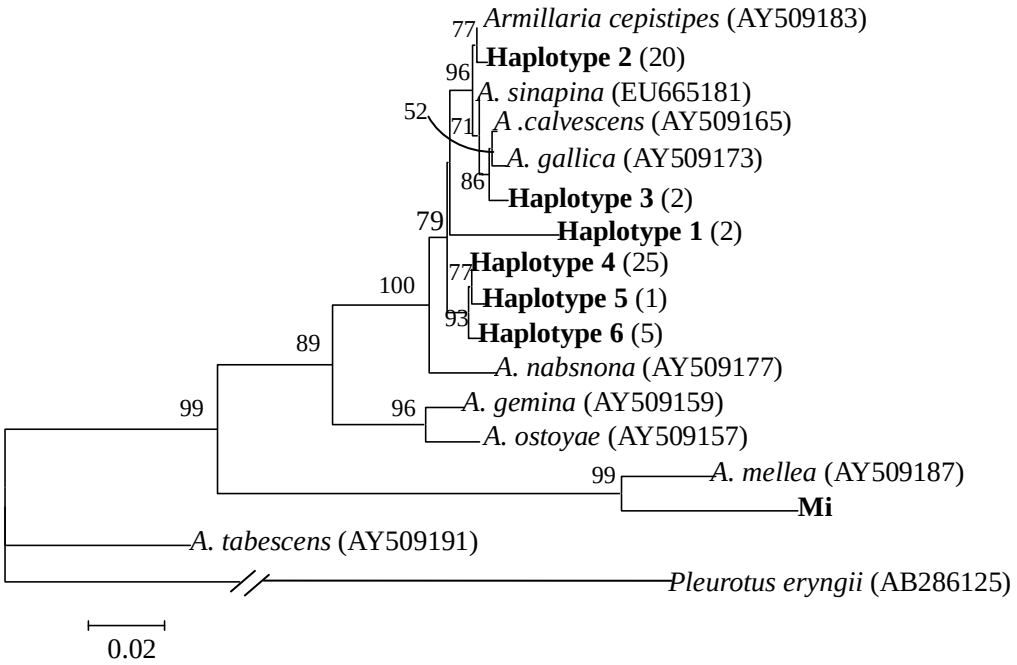


**Figure 1.** Neighbour-joining tree based on the IGS sequence of *Armillaria* in this case and highly similar sequences from GenBank. The occurrence time of each haplotype is indicated in parentheses. GenBank accession numbers of reference sequences are shown in parentheses. Bootstrap values are indicated on the tree branches (only those >50% are shown) and scale bar represented 2% of the genetic distance between the isolations. *Pleurotus eryngii* was used as an outgroup. Mi is a reference strain of *Armillaria mellea*.

from farmland A, in which cultivating *G. elata* was used to infect tree bark or branches of *Armillara* directly collected from local forest, were grouped with the introduced *Armillaria* M1 (Table 1). It suggested that those *Armillaria* being able to support the growth of *G. elata* should be easily dispersed into local forest through the facilitation of human force. Regarding potential risk on local ecosystem of exotic *Armillaria*, it is very important for local governments to supervise and control utilization of the introduced fungi.

On the other hand, *Armillaria* obtained from farmland B, C and D, and those from local tree species *Cerasus serrula*, *Pinus armandii* and *Aleurites moluccana*, were grouped into three different haplotypes which are distinct from *Armillaria* M1 as well as M2 (Tables 1 and 2). Interestingly, these strains are phylogenetically different from those *Armillaria* that were previously reported to be symbiotic with *G. elata* (Cha and Igarashi, 1995; Sekizaki et al., 2008), as well as those not (Figure 1). These data suggested that there are diverse and novel species of *Armillaria* in local forest and their application for cultivating *G. elata* should be encouraged for local organization and farmer. In addition, because different strains play a different role in the production of cultivating *G. elata* (Ning and Yu, 2008; Rong and Cai, 2010), it is necessary to explore their effects on the yield of *G. elata* in the Xiaocaoba areas in future.

## Conflict of Interests

The authors have not declared any conflict of interests.

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