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Mycorrhizal Diversity Associated to *Liparis japonica* (Miq.) Maxim. in China

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SMN and QB designed the study. Authors WPP and GJJ performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author LZ managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Orchidaceae (Orchidaceae) is the second largest family of angiosperms. It's the "flagship" group in plant protection. The existence of orchid plant is closely related to mycorrhizal fungi. The relationship between orchids and their symbiotic mycorrhizal fungi is a benefit to the protection and population restoration of orchids.

Aims: The research was aimed to study molecular identification about 15 strains of mycorrhizal fungi from 6 plots by rDNA ITS technology in order to understand and utilise the mycorrhizal fungi of *Liparis japonica* (Miq.) Maxim.

Study Design: The mycorrhizal fungi collected from different geographical locations were isolated and purified from the mycorrhizal fungi symbiotic with *Liparis japonica* in Northeast China, which were identified by rDNA ITS, meanwhile computed evolutionary distance and constructed the phylogenetic tree.

Place and Duration of Study: In 2017, the root segments of *Liparis japonica* were collected separately from Qianshan, Changbaishan, Gaoguan, Guanmenshan, Dongling, Daqinggou.

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Methodology: Fifteen strains of mycorrhizal fungi collected from six plots were identified by rDNA ITS. Using DNAMAN software to analyse, the pairwise homology was compared by using the optimal global sequencing option. The evolutionary distances of fifteen strains were calculated by MEGA (Molecular Evolutionary Genetics Analysis) software package and their phylogenetic trees were constructed by neighbour-joining method.
Results: With primers ITS1 and ITS4, the 15 mycorrhizal fungi strains of rDNA ITS got about 600 bp length. ITS length was about 582-613 bp, in which ITS1 length was about 177-190 bp, and ITS2 length was 246-273 bp. The mycorrhizal fungi strains were highly homology separated from one plot, mostly above 90%. The plots from the south to the north were as follows: Qianshan, Guanmenshan, Gaoguan, Dongling, Changbaishan, Daqinggou in China. Fifteen strains after separated and purified were identified to be the *Epulorhiza* of Orchid *Rhizoctonia* blasted with Genbank. The homology of the strains gradually decreased affected by the difference of the north and the south, namely there was an increasing trend of diversity from south to north.
Conclusion: The homology of mycorrhizal fungi from one plot was higher because of the same soil environment and climate environment and so on, and strain type was single. Under the influence of microclimate in Northeast China, the homology of strains decreased gradually in the sample area, that is, the diversity gradually increased from the south to the north.

Keywords: Mycorrhizal fungus; diversity; Liparis japonica; rDNA ITS.

1. INTRODUCTION

Orchidaceae plants are not only the precious wild plants resources, but also the highly evolved mycorrhizal plants in the world [1]. Orchidaceae mycorhiza is the symbiont between orchidaceae plants and fungi, which plays an important role in the orchidaceae plants growth and life activities [2]. Mycorrhizal fungi can promote the seed germination of orchidaceae plants, enhance reproductive rate, speed up the growth of plant, strengthen the resistance of host and promote plants produce of many medicinal composition and new compounds [3]. Therefore, a deep research of the orchidaceae mycorhiza will contribute to explore adaptation mechanism of orchidaceae plants and strengthening effective protection of wild orchidaceae resources. On the other hand, separating and screening out the excellent fungi resources and applying them to orchidaceae plants' artificial cultivation and rapid propagation can accelerate the industrialisation of its production.

Liparis japonica (Miq.) Maxim. is one of the orchidaceae plants, which is harvested in summer and fall, and can be used as medicine with root.

In the Tujia pharmacy, the whole plant of *L. japonica* can invigorate the circulation of blood, regulate the menstruation and also has the effects of analgesia, cardiotonic, appeasement and so on [4]. However, its habitat has been great damaged, leading to the consequences of the smaller distribution range and threatened population by ecological destruction. So it has been protected by International Trade Conventions. In this study, we selected the Northeast China *L. japonica* as the experimental material to study the mycorrhizal fungi. The plants of *L. japonica* were collected from different geographical locations to separate and purify the mycorrhizal fungi, then to identify and find out the symbiotic fungus in order to rich the fungal resources and to serve the germination of seeds, providing reference strategy for orchidaceae plants protection.

ITS (internal transcribed spacer) includes ITS1, 5.8 S and ITS2. ITS1 and ITS2 are the moderate conservative area, and conservation maintains a relative intraspecific consistency and a obvious interspecific difference. Above characters are not only suitable for relationship analysis of historical development among flora of genus or intraspecies variation, but also for molecular identification of fungal species [5]. White et al. (1990) designed the universal primers for the fungi rDNA ITS segment amplification earliest [6]. ITS1 and ITS4 can amplify all ITS area effectively including 5.8 S rDNA. Many scholars use differences of ITS sequences to research fungi system development or classification of identification [7-10].

Therefore, this research which was aimed to study molecular identification about 15 strains of mycorrhizal fungi from 6 plots by rDNA ITS technology was of great significance of understanding and utilising the mycorrhizal fungi of *L. japonica* (Miq.) Maxim.

2. MATERIALS AND METHODS

2.1 The Strain Materials

Fifteen mycorrhizal fungi strains used for the analysis of rDNA ITS were stored in botany Ⅱ laboratory of biological technology institute of Shenyang Agriculture University. Six sample plots were Qianshan of Anshan, Gaoguan of Benxi, Changbaishan of Jilin, Guanmenshan of Benxi, Dongling of Shenyang and Daqinggou of Fuxin in China. The informations of strain numbers and sampling sites were shown in the Fig. 1 and Table 1. Fifteen strains after separated and purified, were identified to be the *Epulorhiza* of Orchid *Rhizoctonia*.

Fig. 1. Six collecting sites of mycorrhizae from *L. japonica* (Miq.) Maxim. in the location marked on the Google Earth

Table 1. Statistics of the mycorrhizae isolation from *L. japonica* (Miq.) Maxim.

No.	Producing area	Strain no.	Longitude	Latitude
1	Daqinggou of Fuxin	DQG-71	122°12′03″	42°43′55″
2	Daqinggou of Fuxin	DQG-72	122°12′03″	42°43′55″
3	Dongling of Shenyang	DL-68	123°34′39″	41°49′53″
4	Qianshan of Anshan	QS-3	123°07′48″	41°01′21″
5	Qianshan of Anshan	QS-11	123°07′48″	41°01′21″
6	Qianshan of Anshan	QS-82	123°07′48″	41°01′21″
7	Gaoguan of Benxi	GG-15	124°02′32″	41°20′19″
8	Gaoguan of Benxi	GG-91	124°02′32″	41°20′19″
9	Guanmenshan of Benxi	GMS-16	124°09′30″	41°07′41″
10	Guanmenshan of Benxi	GMS-17	124°09′30″	41°07′41″
11	Guanmenshan of Benxi	GMS-45	124°09′30″	41°07′41″
12	Guanmenshan of Benxi	GMS-69	124°09′30″	41°07′41″
13	Changbaishan of Jilin	HCZ-47	127°47′55″	42°30′35″
14	Changbaishan of Jilin	HCZ-89	127°47′55″	42°30′35″
15	Changbaishan of Jilin	HCZ-98	127°47′55″	42°30′35″

2.2 Isolation and Purification of Mycorrhizal Fungi

The methods of disinfection and separation were used from the root tissue surface in this experiment [5]. Firstly, taking healthy and fresh plant of *L. japonica*, its roots were washed by water, and removed surface appendages and contaminated bacteria. Secondly, one centimeter segment of rootwas taken from *L. japonica*, sterilised with 75% alcohol for 5 s, then washed with sterile water. Sterilised in 0.1% mercuric solution for 10 min, then washed with aseptic water twice. The cutting surface of the root segment was placed on PDA medium, 3 pieces per dish were made and incubated at 25 ℃ for constant temperature. The purified hyphae was transferred to the oblique surface of the PDA tube, cultured at 25 ℃ to grow to a certain size, and preserved in the refrigerator at 4 ℃.

2.3 DNA Obtained from Strains of Mycorrhizal Fungi

Mycorrhizal fungi strains separated from PDA(Potato Dextrose Agar) medium were incubated 6 days at 28℃. When the stains were going to cover petri dishes, taking strains out by sterilised slide about 0.02g to a 2mL centrifuge tube, then added 1mL CTAB (Cetyltrimethylammonium Ammonium Bromide) extraction buffer and two steel balls, shaking 30min on the Fast Mill oscillation instrument at strength 99, incubating them 1h in water bath at 65℃after fully oscillation. Then taking 700μL of supernatant fluid, added Tris-phenol and Chloroform-isoamyl alcohol (1:1) 350μL, concussing after blending by centrifugation 10min under 12000 $min.r^{-1}$. Then took the supernatant 500μL and added chloroform 500μL, centrifuged 10min with 12000 $min.r^{-1}$. Took the supernatant 300μL, then added 750μL anhydrous ethanol (precooling under -20℃), gently blending, standing under -20 ℃ by 30 min. Centrifuged 10min under 12000 $min.r^{-1}$ and removed supernatant, adding 500μL 70% ethanol (precooling under -20℃), centrifuging 5min under 12000 $min.r^{-1}$. Removed supernatant again and reserved the precipitation, drying 1h. The precipitation was the DNA. Adding 200μL 1×TE buffer, dissolving under 4℃, set aside.

DNA samples(3μL) was electrophoresised in 1×TBE buffer with 1% agarose gel (100v, 30min), dyeing with EB(Ethyl bromide), observed and taken pictures under Ultraviolet lamp to examine the DNA.

2.4 PCR Amplification and Sequencing for the Strains of rDNA ITS Segment

General primers of mycorrhizal fungi rDNA ITS were ITS-1(5'-TCCGTAGGTGAACCTG CGG-3') and ITS - 4 (5 '- TCCTCCGCTTATTGATATGC - 3').

PCR system: DNA moldboard 1μL, 10×buffer 5μL, dNTP (each 2.5 mM) 4μL, upstream primer ITS-1 (10μM) and downstream primer ITS - 4 (10μM) each 1μL, rTaq ($5u.\mu L^{-1}$) 0.5μL, ddH_2O 37.5μL, total 50μL.

PCR(Polymerase Chain Reaction) cycles: preliminary degeneration 5min at 94℃; degeneration 1min at 94℃; annealing 1min at 53℃; stretching 1min at 72℃; for 35 reaction cycles; stretching 5min at 72℃[9].

The PCR reaction product was sequenced by Shanghai company.

2.5 ITS Sequence and System Analysis

ITS1 and ITS4 sequences of start-stop range consulted ITS range of orchidaceae mycorrhizal fungi from GenBank. The systematic analysis of ITS sequence was by DNAMAN. The evolutionary distance was calculated according to Kimura two parameter method [11] with the MEGA4.0 (Molecular Evolutionary Genetics Analysis 4.0) software package. Evolutionary tree was built by the Neighbor-Joining method [12].

3. RESULTS AND DISCUSSION

3.1 PCR Amplification and Sequencing of rDNA ITS

The rDNA ITS fragments of 15 mycorrhizal fungi strains of *L. japonica* (Miq.) Maxim. were amplified by primers ITS1 and ITS4, and about 600 bp products were obtained (Fig. 2 for parts of the strains of rDNA ITS PCR product electrophoresis). After the sequencing, the whole sequences of ITS section were taken from 15 strains PCR products of rDNA ITS section. ITS length was about 582-613 bp, ITS1 length was about 177-190 bp, and ITS2 length was 246- 273 bp (Table 2).

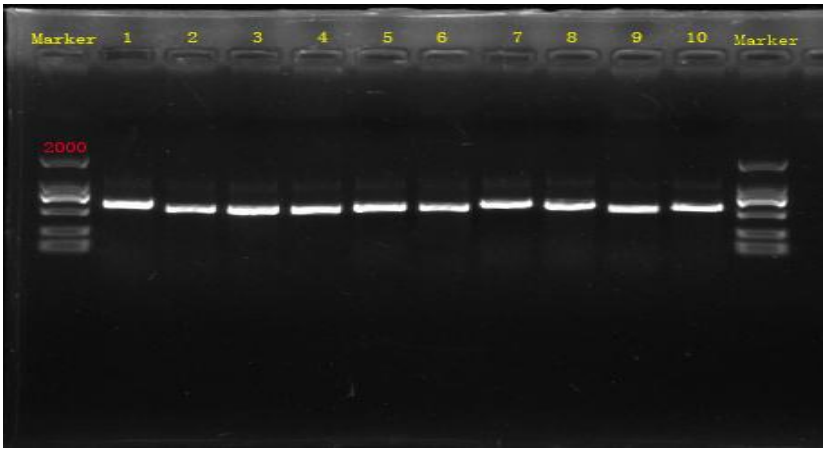

Fig. 2. Electrophoresis result about the PCR production of parts strains' rDNA ITS section
Note: 1-10 mean parts of the strains of rDNA ITS PCR product electrophoresis

Table 2. The length of ITS about mycorrhizae strains in *L. japonica* (Miq.) Maxim.

Strain no.	ITS1 length	ITS2 length	ITS length	5.8S length
QS-3	179	258	596	159
QS-11	189	246	601	166
QS-82	179	257	582	146
GG-15	178	255	599	166
GG-91	178	255	589	156
HCZ-47	190	259	597	148
HCZ-89	187	265	609	157
HCZ-98	187	265	609	157
GMS-16	189	265	594	140
GMS-17	186	265	612	161
GMS-45	186	273	613	154
GMS-69	186	265	609	158
DL-68	188	265	595	142
DQG-71	177	259	596	160
DQG-72	178	265	610	167

3.2 Blast Results about rDNA ITS Sequence

The rDNA ITS sequences of 15 isolates of mycorrhizal fungi from *L. japonica* were aligned in GenBank (Table 3). The identification results were confirmed to be *Epulorhiza*, which was consistent with the morphological identification results.

Blast results showed that *Epulorhiza* or *Tulasnella* fungi was highly homologous to *Epulorhiza* (96% - 99%) and belonged to the same genus. Some foreign scholars [13-15] have made a detailed description of the morphological identification criteria of fungi, similar to the Rhizobium fungi and glue membrane fungi, which appeared in this experiment, are considered to be two different forms of existence of the same fungi, namely, anamorphic Rhizobium fungi and sexual glue membrane fungi. Nodular mycorrhizal fungi are the most common mycorrhizal fungi in orchids in China.

3.3 Clustering Results of rDNA ITS Sequence

Clustering results of rDNA ITS sequence from 15 mycorrhizal fungi strains were shown as Fig. 3. By cluster analysis of rDNA ITS sequences of 15 strains in Fig. 1, strains QS-3 and QS-82 isolated from the roots of *L. japonica* collected from Qianshan in Anshan, were clustered together with strains QS-11 isolated from the roots of *L. japonica* from the same origin; strains GG-15 and GG-91 isolated from the roots of *L. japonica*

collected from senior officials were clustered together. The strains HCZ-89 and HCZ-98 separated from the roots of *L. japonica* collected from Changbai goats gathered together; the strains GMS-17, GMS-45 and GMS-69 separated from the roots of *L. japonica* from Benxi Guanmen gathered together, and then with the strains GMS-16 isolated from the roots of *L. japonica* from the same origin.

3.4 Discussion

There is a high specificity between orchids and their symbiotic mycorrhizal fungi [16]. At present, mycorrhizal fungi are divided into four groups according to their morphological structure and function, which are arbuscular mycorrhizal fungi, ectomycorrhizal fungi, orchid mycorrhizal fungi, and *Erica* mycorrhizal fungi. In the symbiotic relationship between these four mycorrhizal fungi and their hosts, the specificity of orchid plants and their mycorrhizal fungi is much higher than that of other mycorrhizal fungi [17]. In this symbiotic relationship, almost all orchids have to rely on the corresponding mycorrhizal fungi to survive, but the orchid mycorrhizal fungi do not need to rely on the orchid plants to survive well [18-19].

Table 3. Blast results of rDNA ITS sequence about mycorrhizae fungi in *L. japonica* (Miq.) Maxim.

Strains no.	Semblable clone in GB	Fungus of semblable clone in GB	Identities
QS-3	FJ594919	*Epulorhiza*	IDs=577/580 (99%) Gaps=1/580 (0%)
QS-11	FJ594919	*Epulorhiza*	IDs=577/581 (99%) Gaps=2/581 (0%)
QS-82	FJ594919	*Epulorhiza*	IDs=574/576 (99%) Gaps=0/576 (0%)
GG-15	FJ613149	*Epulorhiza*	IDs=578/580 (99%) Gaps=1/580 (0%)
GG-91	JF926501	*Tulasnella*	IDs=575/578 (99%) Gaps=1/578 (0%)
HCZ-47	FJ788887	*Tulasnella*	IDs=575/580 (99%) Gaps=0/580 (0%)
HCZ-89	FJ788887	*Tulasnella*	IDs=587/591 (99%) Gaps=0/591 (0%)
HCZ-98	FJ788887	*Tulasnella*	IDs=593/599 (99%) Gaps=2/599 (0%)
GMS-16	FJ788887	*Tulasnella*	IDs=569/578 (98%) Gaps=1/578 (0%)
GMS-17	FJ788887	*Tulasnella*	IDs=588/599 (98%) Gaps=2/599 (0%)
GMS-45	FJ788887	*Tulasnella*	IDs=588/601 (98%) Gaps=3/601 (0%)
GMS-69	FJ788887	*Tulasnella*	IDs=587/600 (98%) Gaps=3/600 (0%)
DL-68	JF926510	*Tulasnella*	IDs=561/574 (98%) Gaps=0/574 (0%)
DQG-71	JF926510	*Tulasnella*	IDs=545/570 (96%) Gaps=1/570 (0%)
DQG-72	FJ613228	*Epulorhiza*	IDs=594/608 (98%) Gaps=4/608 (1%)

Note: GB-GenBank; IDs-Identities

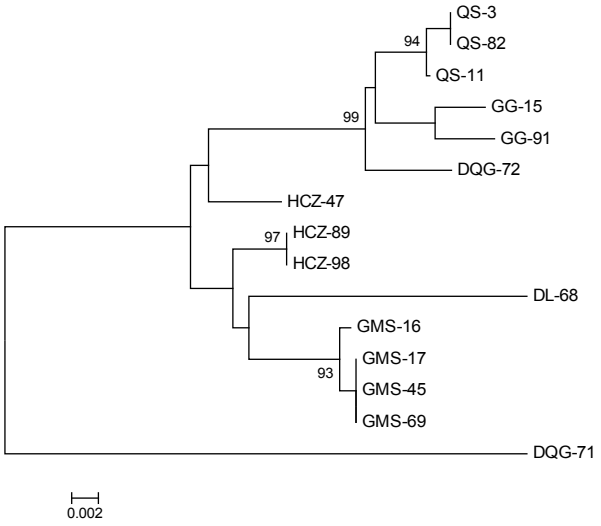

Fig. 3. Cluster analysis of rDNA ITS sequence about mycorrhizae fungi from *L. japonica*

Fifteen strains were determined to be *Epulorhiza* of *Orchid Rhizoctonia* after identified by morphology and molecular biology, and took the symbiotic fungus separated from *L. japonica* (Miq.) Maxim for ITS sequence analysis. According to the Fig. 2, the sizes of PCR products were roughly in the range of 500-750 bp, close to company sequencing results (~600bp). Submitted these strains ITS to the GenBank and compared with those published ITS sequences, found that the *Epulorhiza* and *Tulasnella* mycorrhizal fungi had high homology, reaching 96%-99% (Table 3). Some researchers had detailed instructions for the standard of mycorrhizal fungi morphological identification. Similar to this research, the *Epulorhiza* and *Tulasnella* were considered to be two different forms of same fungal existence, they were amorphous of *Epulorhiza* and morphometric of *Tulasnella*. According to research records, many scholars separated this mycorrhizal fungi from orchidaceae plants [20].

It was observed that the *Epulorhiza* was a general mycorrhizal fungi among orchidaceae plants. The results of this research indicated that sequence of blast comparison results was a strong support for morphology identification. The methods of morphology and molecular biology were used to identify unknown mycorrhizal fungi, increasing the identified results credibility and persuation, improving the scientificity and accuracy of fungal identification and provide enormous help for more fungal identification researches in the future.

Cluster analysis of rDNA ITS from 15 strains showed that QS-3 and QS-82 were gathered together, then clustered with QS-11 from Qianshan of Anshan city. GG-15 and GG-91 were gathered together which were selected from Gaoguan of Benxi city. HCZ-89 and HCZ-98 were gathered together which were selected from Changbaishan of Jilin province. GMS-17, GMS-45 and GMS-69 were gathered together, then with GMS-16 which were all selected from Guanmenshan of Benxi city. The mycorrhizal fungi strains were highly homology separated from one plot, mostly above 90%. For example, the homology of 4 strains collected from Guanmenshan was 93%. And the homology of 3 strains collected from Qianshan was 94%. This phenomenon showed that the homology of mycorrhizal fungi from one plot was higher because of the same soil environment and climate environment, and strain type was single. There were some exceptions, DQG-71 and DQG-72, HCZ-89 and HCZ-98, HCZ-47 had the low homology and they were on the two big branches. For this situation, the possible reasons were that natural areas of Daqinggou and Changbaishan were relatively larger and the internal community structure was original, so its internal structure was complex compared with other plots, resulting in the abundance of the species diversity. Combined with the data of Table 1 and Fig. 1, the plots from the south to the north were as follows: Qianshan, Guanmenshan, Gaoguan, Dongling, Changbaishan, Daqinggou in China. Affected by the difference of the north and the south, homology of the strains gradually decreased, namely diversity gradually increased from south to north. Geographical location is a leading factor affecting the diversity of mycorrhizal fungi of *L. japonica* (Miq.) Maxim., but it is not the only factor that can be determined. The diversity of mycorrhizal fungi is also affected by the growth environment, climate and soil during the evolution of host plants [21-23]. Therefore, the mechanism of host plant evolution on the diversity of mycorrhizal fungi will be further studied.

4. CONCLUSIONS

The rDNA ITS fragments of 15 mycorrhizal fungi strains of *L. japonica* (Miq.) Maxim were amplified by primers ITS1 and ITS4, and about 600 BP products were obtained. ITS length was about 582-613 bp, in which ITS1 length was about 177-190 bp, and ITS2 length was 246-273 bp. The mycorrhizal fungi strains were highly homology separated from one plot, mostly above 90%. The plots from the south to the north were as follows: Qianshan, Guanmenshan, Gaoguan, Dongling, Changbaishan, Daqinggou in China. The homology of the strains gradually decreased affected by the difference of the north and the south, namely there is a increasing trend of diversity from south to north.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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