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Full Length Article

Methods of *Dendrobium* Rust Detection and Analysis on the Genetic Structure of *Dendrobium* Rust Populations

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Abstract

Dendrobium, as a rare and precious medicinal material in China, has high medicinal value. With the extension of the cultivating years, one of the main biological factors to affect and restrict its safe production is *Dendrobium* rust, on which there are very few studies involved currently. Our research has collected a total of 150 rust fungi samples bred from *Dendrobium candidum*, *D. aphyllum*, *D. crystallinum* and *D. devonianum* from its main producing areas (Honghe and Dehong), and had performed the symptoms observation, pathogenetic identification and ITS sequence analysis, with the results indicated that samples of the all rust fungi of *Dendrobium* spp. were belonged toBasidiomycetes, Teliomycetes, Uredinales, Coleosporaceae, and *Coleosporium*. The species will be confirmed in the future. In addition, we had analyzed on the *D. candidum* population structure in different main producing areas and different species of *Dendrobium* in the same area by taking advantage of ISSR (Inter-simple sequence repeated, ISSR) molecular markers, with the results showed that the *Cladosporium* causing the *Dendrobium* rust in Honghe and Dehong was mainly aggregated regionally, and a small amount of rusts in Honghe was found to be aggregated with that in Dehong, and the *Cladosporium* bred from different varieties of *Dendrobium* causing the *Dendrobium* rust was aggregated individually, which indicated that the rust fungi of *Dendrobium* had presented a stronger specialization.

Keywords: *Coleosporium*; *Dendrobium*; Molecular markers*;* Population genetic structure

Introduction

Dendrobium, is considered as the largest genus of the Orchidaceae family with about 1,100 species of plants, and is mainly distributed in tropical Asia and Pacific Islands (Chun, 2005). There are more than 60 species of *Dendrobium* plants in China (Fan *et al*., 2009), 39 of which have medicinal values and many of them are valuable medicinal materials (Xi *et al.*, 2011). These are mainly distributed in the Qinling Mountains, south of the Huaihe River, and most of the species are distributed between 15°30′ and 25°12′. Yunnan, Guangxi, Guangdong, Guizhou, and Taiwan are considered as the distribution centers of *Dendrobium* plants in China (Ji, 1980). *Dendrobium*, as the rare and precious medicinal materials in China, have the function of not only eliminating inflammation and heat but also equaling to a supplement to promote body fluids generation and improve the quality of life (Fan *et al*., 2009). Previous studies have confirmed that its main active ingredients are alkaloids and polysaccharides, having obvious immunity enhancement activity and anti-cancer activity with many functions such as immunity improvement (Wu *et al.*, 2012), anti-oxidation (Huang *et al.*, 2016) and anti-tumor (Bao, 2007). Moreover, it was also evaluated as having such functions as strengthening Yin and supplementing vital essence, nourishing internal deficiency, smoothing flatulence, muscles growth, skin growth promotion, stabilizing wisdom and quelling fears, body gracefulness and life-span extension, smoothing vital essence and dispelling heat, strengthening Yang, curing for bone cold and kidney invigoration and benefit for physical strength by Li Shizhen in his medical works "Compendium of Materia Medica".

With the continuous expansion of planting area of *Dendrobium*, the disease types and damage degree of *Dendrobium* are increasing year by year, and the *Dendrobium* diseases have spread from such old production areas as Zhejiang to many new production ones with diseases occurrence reported in most *Dendrobium* producing areas (Li *et al.*, 2013). In, Li *et al*. (2008) discovered the disease of *D. officinalis* in Zhejiang Province for the first time (Li *et al.*, 2008). Moreover, in 2003, Liang

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Table 1: Samples of *Dendrobium* rust in different species collected in the different area

Dendrobium species	Collecting date	Number of samples	Collecting site	No.
D. candidum	2017-5-3	30	Honghe-Honghe county	HH-SH
D. candidum	2017-6-1	30	Dehong-Lianghe county	LH-SH
D. candidum	2017-6-1	30	Dehong-Mang city	MS-SH
D. candidum	2017-10-25	15	Dehong-Ruili city	TP-SH
D. aphyllum	2017-10-25	15	Dehong-Ruili city	DC-SH
D. devonianum	2017-10-25	15	Dehong-Ruili city	CB-SH
D. crystallinum	2017-10-25	15	Dehong-Ruili city	JM-SH
Total		150		

Table 2: The primers used in genetic analysis

Primer name	Primer sequence	Tm/℃
ISSR1	AGAGAGAGAGAGAGTC	50
ISSR2	AGAGAGAGAGAGAGKG	48
ISSR3	ACACACACACACACTG	50
ISSR4	ACACACACACACACWC	52
ISSR5	ACACACACACACACYA	50
ISSR6	AGAGAGAGAGAGAGTC	50

Zhongji discovered and reported the anthracnose in the wild, domesticated *D. candidum* in Guangxi Province, and put forward some effective control measures against the anthracnose of *D. candidum* (Liang, 2003). In, Zhang and Zheng (2004) reported on *D. candidum* spot in detail (Zhang and Zheng, 2004). In, Li *et al*. (2013) discovered and reported the stem base rot of *Dendrobium* in *D. candidum* and *D. nobile* in the Xishuangbanna region of Yunnan (Li *et al.*, 2013). In addition, leaf spot, blossom blight, myxomatosis, root disease, soft stem rot and other diseases have been reported one by one (Liu *et al.*, 2015).

The current research on *Dendrobium* rust has been still staying in its primary stage. In 2013, Zhao Guihua and others had performed the preliminary identification on the pathogen of *D. Devonianum* rust with confirmation that Puccinia fungi of Uredinales of teliomycetes of basidiomycetes were the pathogen (Hu *et al.*, 2013). Zhao Guihua *et al.* (2016) reported for the first time the purple *Dendrobium* rust caused by *Puccinia gallinalis* (Zhao *et al*., 2016). Except for what hasbeen referred to above, there was no report on the pathogen of *Dendrobium* rust. In our research, the morphological character and analysis of the internal transcribed spacer (ITS) sequence of rust fungi of *Dendrobium* in different *Dendrobium* for proving *Dendrobium* rust. The molecular marker (inter-simple sequence repeated, ISSR) analysis has been used analysis of the genetic structure of *Dendrobium* rust population in Yunnan Province. It is established the theoretical basis for effective prevention and control of *Dendrobium* rust.

Materials and Methods

Collection of Rust Samples

All samples of rust fungi of *Dendrobium* in this test were collected from such regions as Dehong and Honghe in Yunnan Province live up to the standards for relatively clean leaves, plump spores, andsingle spots. After that, all samples were respectively packed and dried with a self-made sample collecting bag in order to prevent from mildew and cross-contamination between samples. And the details for the sample collecting of *Dendrobium* were seen as Table 1.

Primers

The primers used for sequence analysis of ITS for fungi were ITS1-TCCGTAGGTGAACCTGCGG and ITS4-TCCTCCGCTTATTGATATGC (Chen and Zheng, 2007). Six ISSR primers used for analysis on the population genetic structure among the rust fungus were developed and designed by our research group and the sequences were synthesized by the Company. The detailed information for the primers was shown in Table 2.

Apparatus and Equipment used in the Experiment

The main instruments used in this experiment: ultra clean workbench, high pressure steam sterilizer,oven,electronic balance, HH-4 digital display water bath pot with constant temperature, microfuge 18 centrifuge, Blue shield 522 transmission instrument in gel electrophores with visible light, ABI-9700 PCR instrument, 78HW-1 magnetic agitator heated with constant temperature, GeneQuant pro protein-nucleic acid analyzer, gel imaging system, ABI-3730XL genetic analysis system, Leica fluorescence microscope and stereoscopic microscopes and etc.

DNA Extraction

The chelex-100 method has been adopted to extract the genomic DNA of the rust fungi of *Dendrobium* with specific steps as follows: to extract 150 μL solution of Chelex-100 (concentration of 20% with 1/3 of chelex remains when extracting noted) with a pipettor into the centrifuge tube of 1.5 mL, then extract the Chelex-100 supernatant of 5uL with the pipettor of 10 μL into the spot with rust spores,and washed them repeatedly, eluting as far as possible, then extract all the spores eluted with the pipettor of 10 μL into the centrifuge tube with 150 μL of Chelex-100 in 1.5 mL, put the centrifuge tube into boiling water for bathing for 2 min and oscillate on the vortex finder for 20 s, and centrifuge for 30 s, what the supernatant got was the DNA extracted that should be kept in refrigerator under -20℃ for later use.

Sequence Analysis of ITS for *D. candidum* Rust

50 μL PCR reaction of the total volume was used in the sequence analysis on ITS for rust fungi, including 2 × phanta max Buffer 25 μL, dNTPs 1.0 μL, phanta 1.0 μL, ddH2O 18 μL,TTS1 and ITS4 primer 2 μL and templated DNA (25 ng/μL) 1 μL. ddH_2O negative control should be

established for each reaction. The amplification was carried out on ABI-PCR. The reaction procedure was: pre-denaturation at 95°C for 3 min; denaturation at 95°C for 15 s, annealing at 57°C for 15 s, extension at 72°C for the 30s, which should be performed for 35 cycles. Then secondary extension at 72°C for 5 min and stored at 4°C for 5 min. After PCR was finished, the 5 μL PCR products were mixed well with the 2 μL Loading-buffer (1 mL Loading buffer + 10 μL anthocyanin) with anthocyanins. The gel electrophoresis was carried out at 120 V for 45 min with 1.5% agarose gel, and take images by gel imaging system to do a preliminary analysis on PCR products. And the standard fragment size of PCR products was preformed the clone sequencing after gel recovery. The ITS sequence measured was made an analysis on sequence alignment in the NCBI website, and The ITS sequence of rust fungi with different symptoms of different *Dendrobium* was made cluster analysis by MAGE 5.1 software.

Analysis of the Genetic Structure of *Dendrobium* Rust Populations

Fifty micro liters of ISSR-PCR reaction of the total volume was used in the genetic structure of the *Dendrobium* rust populations, including 10×Easy Buffer (with Mg^{2+} content) of 3.0 μL, 2.5 m*M* dNTPs of 2.4 μL, 1.2 μL ITS1 primer and 0.3 μL TaqE primer, and templated DNA (25 ng/μL) 1 μL, ddH_2O 22.1 μL. The positive control should be established for the wheat stripe rust with ddH_2O negative control. The reaction procedure was: pre-denaturation at 95°C for 2.5 min; denaturation at 94°C for the 30 s, annealing at 50°C (the specific annealing temperature of each primer was shown in Table 2) for 15 min, extension at 72°C for 1 min, which should be performed for 35 cycles. Extension at 72°C for the 30 s, which should be performed for 35 cycles. Then secondary extension at 72°C for 5 min and stored at 4°C. After PCR was finished, the 5 μL PCR products were mixed well with the 2 μL Loading-buffer (with anthocyanin content of 10 μL) with anthocyanins. The gel electrophoresis was carried out at 120 V for 45 min with 1.5% agarose gel, and take images by gel imaging system to do a preliminary analysis on the strips. Samples with PCR products were sent to Kunming Shuoqing Biological Co., Ltd. for further analysis by the ABI-3730XL genetic analysis system.

Data Analysis

The ISSR-PCR amplified fragments were analyzed by genetic analysis system with their sizes between 100–700 bp (internal standard for ABI-LIZ1200). The fragments of PCR products obtained from genetic analysis system were converted into 01 matrix with a packing analysis on the PCR products (container width of 10 BP). And the population structure analysis was made by Pop gene and NTs is software.

Results

Symptoms and Pathogen Morphology of *Dendrobium* Rust Fungus

The rust samples, gathered from different species of *Dendrobium* as *D. candidum*, *D. devonianum*, *D. aphyllum* and *D. crystallinum*, were various symptoms present for infected *Dendrobium* by *Coleosporium* spp. fungi (Fig. 1). In the initial stage of the rust fungi, the leaves of *Dendrobium* degreens with one or more different disease spots formed on it, these spots are relatively small with an average size of 1–3 mm in yellow or dark red. In the later stage, the disease spots were enlarged gradually with a circle of crimson ones (diameter of 4–10 mm) formed around the initial disease spot. Some of the crimson spots were connected with the intermediate ones, and the others were distributed in annularity with the intermediate spots. In addition, itwas also found that there were not the same symptoms of rust fungi of different species of *Dendrobium*, with presumption that it was different rust fungi in different species of *Dendrobium*.

It was found that the morphology of urediosporespores and teliospores of the rust fungi of *Dendrobium* were relatively complicated based on observation on slices and pathogens pick-ups. The urediosporespores were present in gray at first and then turning into yellow. And dark red substance (protoplasm) was formed in some cells in the shape of suborbicular or irregularly elliptic with diameters between 20–30 μm (Fig. 2 a, b and c). While the winter spores in bright yellow has two cells in yellow before maturity in subcircular (Fig. 2d) at first, and there were 3 cells in light yellow or dark yellow with petiolates in short stick form or irregular shape after maturity, spores length of 50–80 μm, petiolates length of 15–25 μm (Fig. 2 d–f; Fig. 3).

Sequence Analysis of ITS for Rust Fungi of *Dendrobium*

Our research has made homology comparison on ITS sequence of the rust fungi of *Dendrobium* by NCBI website and found that the rust fungi collected from the *D. candidum*, *D. devonianum* and *D. aphyllum* was identified as Basidiomycotina, Teliomycetes, Uredinales *Coleosporaceae* fungus (seen as Table 3). Andin addition, a very close genetic relationship between the rust fungi samples collected from different varieties of *Dendrobium* can be discovered, and these rust fungiwere aggregated with the sphingopuccinia with *puccinia graminis* a single branch (Fig. 4).

Cluster Analysis of *Dendrobium* Rust Populations in different Places

This paper has made analysis on the rust fungi of *Dendrobium* population relationship in Lianghe County, Mango City, and Honghe region by taking advantage of

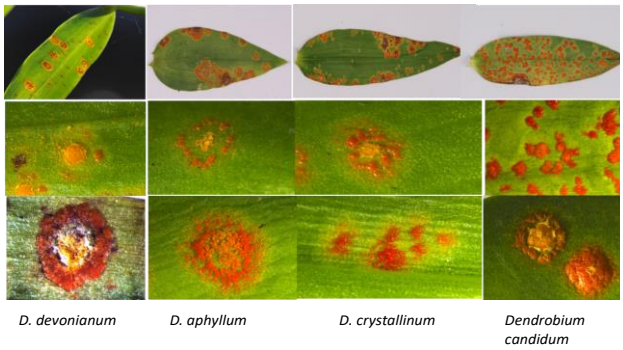

Fig. 1: Symptoms of *Dendrobium* infected by rust fungi

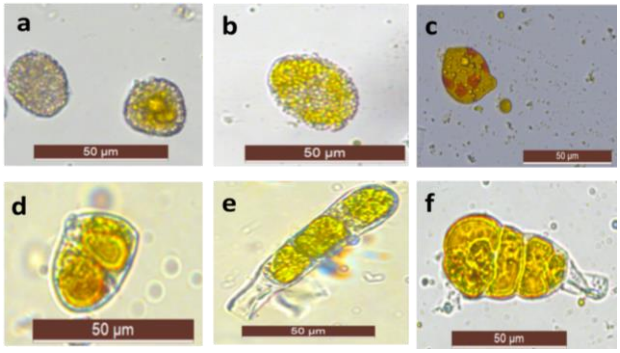

Fig. 2: Spores of rust *Coleosporium*
Note: a, b and c are of urediosporespores; d, e and f are of teliospores

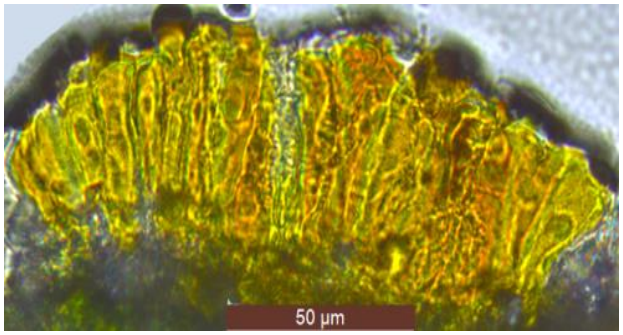

Fig. 3: Telium of *Dendrobium*

ISSR molecular markers, indicating that *Coleosporium* causing the disease of *Dendrobium* rust in Honge and Dehong was mainly aggregated regionally,and a small amount of rust in Honghe was found to be aggregated with that in Dehong, and more surprised, there was a high similarity coefficient between 0.62 and 0.98 for the rust fungi of *Dendrobium* in different places. The bacterial strains of Mangshi and Lianghe County were clustered in a relative scatter way and multiple bacterial strains mixed were aggregated in different subpopulations, except that most of the bacterial strains of Honghe with similarity coefficient at 0.76 were clustered into a single

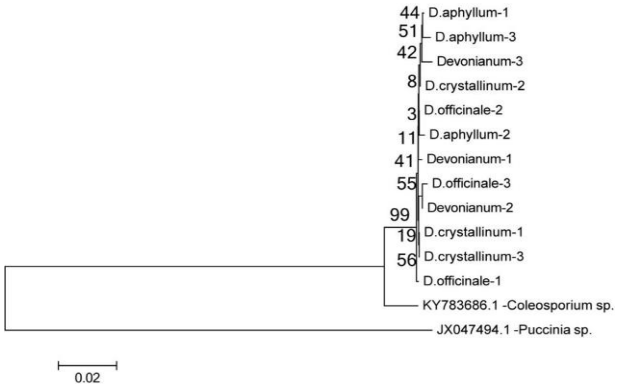

Fig. 4: Comparative dendrogram of rust fungi of different *Dendrobium* species
Note: KY783686.1: The ITS1 sequence of *Coleosporium* spp. from NCBI; JX047494.1: The ITS1 sequence of *Puccinia* spp. from NCBI

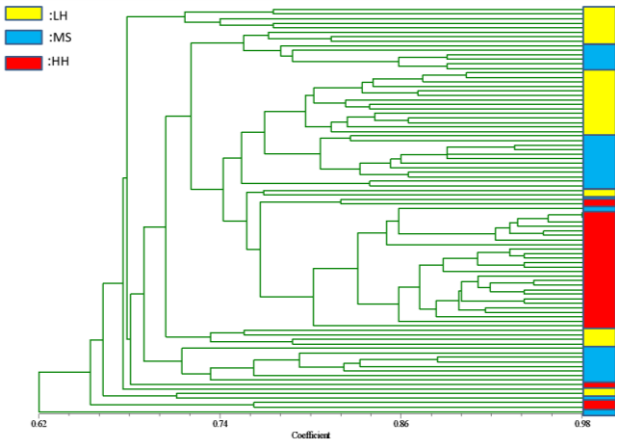

Fig. 5: Cluster diagram of rust fungi of *Dendrobium* at different places
Note: Yellow is the rust population of *Dendrobium* in Lianghe County; Blue is the rust population of *Dendrobium* in Mangshi City; Red is the rust population of *Dendrobium* in Honghe County

subpopulation (Fig. 5).

Cluster Analysis of the Rust Fungi of Different Species of *Dendrobium* in Different Places

The conclusion of the cluster analysis of the rust fungi of different species of *Dendrobium* in the same place is shown in Fig. 6. There was a genetic similarity coefficient between 0.59 and 0.92 for different species of *Dendrobium* in the same place. Except JM-SH-14 and *D. candidum* all the other bacterial strains in the same species were clustered into one group, except JM-SH-14 bacterial strain and bacterial strain of *D. aphyllum*, with an indication that there was a higher similarity on the rust fungi of the same species of *Dendrobium*. Based on similarity coefficient of 0.59, the rust fungus of *D. devonianum* and *D. candidum* infected were classified into a population, while for similarity coefficient of 0.61, the rust fungus of *D. crystallinum* and *D. aphyllum* infected were divided into B population. Based on

Table 3: Comparison and analysis of ITS sequence of different species of *Dendrobium*

Sample name	query cover%	E-value	Ident%	Homologous species	Accession	Host
*D. devonianum*1	100	0	96	*Coleosporium* spp. AM-2013	KY783677.1	*D. devonianum*
*D. devonianum*2	100	0	96	*Coleosporium* spp. AM-2013	KY783677.1	
*D. devonianum*3	100	0	96	*Coleosporium* spp. AM-2013	KY783677.1	
*D. crystallinum*1	100	0	96	*Coleosporium* spp. AM-2013	KY783677.1	*D. crystallinum*
*D. crystallinum*2	100	0	96	*Coleosporium* spp. AM-2013	KY783677.1	
*D. crystallinum*3	100	0	96	*Coleosporium* spp. AM-2013	KY783677.1	
*D. aphyllum*1	100	0	96	*Coleosporium* spp. AM-2013	KY783677.1	*D. aphyllum*
*D. aphyllum*2	100	0	95	*Coleosporium* spp. AM-2013	KY783677.1	
*D. aphyllum*3	100	0	96	*Coleosporium* spp. AM-2013	KY783677.1	
*D. officinale*1	100	0	96	*Coleosporium* spp. AM-2013	KY783677.1	*D. candidum*
D. officinale 2	100	0	96	*Coleosporium* spp. AM-2013	KY783677.1	
D. officinale 3	100	0	96	*Coleosporium* spp.	KY783677.1	

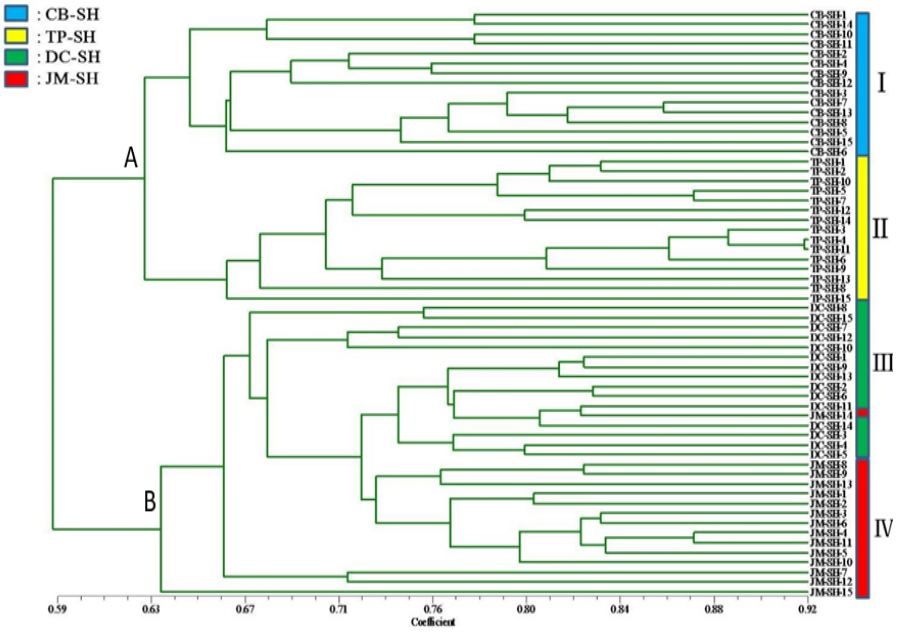

Fig. 6: Cluster diagram of rust fungi of *Dendrobium* of different species in the same place
Note, Blue is the rust population of *D. devonianum*; Yellow is the rust population of *D. candidum*; Green is the rust population of *D. aphyllum*; Red is the rust population of *D. crystallinum*

genetic similarity coefficient of 0.61, the rust fungus of *D. devonianum* and *D. candidum* were classified as the two populations (&II) according to the species of *Dendrobium*. While for genetic similarity coefficient of 0.72, most of the rust fungus of *D. crystallinum* and *D. aphyllum* were also classified as the two populations III & IV according to the species of *Dendrobium*, there were a few bacterial strains separately divided into different populations for the genetic similarity coefficient of 0.61–0.71 (Fig. 6) (JM-SH-7, 12, 15 and DC-SH-7, 8, 10, 12, 15. There was a high similarity of 0.82 between JM-SH-14 and *D. aphyllum*. By comparison, the rust fungus of *D. devonianum* and *D. candidum* showed a higher similarity, and between the rust fungus of *D. aphyllum* and *D. crystallinum*, it is a relatively high similarity.

Discussion

Dendrobium, with a great variety, is one of the maingenus of Orchidaceae. At present, there are more than 1000 species of Dendrobium in the world, 74 species in China, among which more than 50 species have medicinal value, such as *D. candidum*, *D. nobile*, etc. *D. candidum*is considered as the one with the highest medication value among all *Dendrobiums* (Xi and Zhao, 2012). Yunnan, having abundant resources of *Dendrobium*, has 58 species of *Dendrobium* plants and 2 variants of *Dendrobium plant* (Yao, 2004; Bai *et al*., 2006). With the increase in planting area of *Dendrobium*, its diseases are increased (Li *et al*., 2008).

In this study, 150 samples the rust fungus, from four varieties of *D. candidum*, *D. aphyllum*, *D. devonianum* and

D. crystallinum, were gathered in Honghe and Dehong of Yunnan Province, and their disease symptoms and spore morphology were observed. It was found that the disease symptoms and spore morphology were complicated. Based on the results of ITS molecular marker, cloning sequencing and ISSR analysis, the rusty diseases of *D. candidum*, *D. aphyllum*, *D. devonianum* and *D. crystallinum* were caused by the *Coleosporium* fungi, which was consistent with that of purple *D. candidum* identified (Zhao *et al.*, 2016). Hu *et al*. (2013) found that the rusty diseases of *D. devonianum* were caused by *Puccinia* fungi, and whether it was caused by the Puccinia fungi and *Coleosporium* fungi, *Puccinia* or another fungus should be further studied. It was also the first time for the research to identify the pathogen of the rust diseases of *D. aphyllum* and *D. crystallinum* by molecular means, and whether the rusty diseases of other *D.* were also caused by the *Coleosporium* fungi or many kinds of rusty fungus should also be further studied and determined.

The analysis of the genetic structure of the rust fungi populations of Dendrobium has indicated that the rust fungi of the same species of *Dendrobium* aggregated together if it was involved in different species in the same places, with the exception of individual bacterial strains, and there was a higher similarity on the rust fungi of *D. devonianum* and *D. candidum*, a higher similarity on that of *D. aphyllum* and *D. crystallinum*. However, according to the cluster analysis of the rust fungi of *Dendrobium* in different places, it is not completely divided into different subpopulations according to the local differences, and the rust fungus of several places is mixed in different subpopulations. By comparison with the geographical factors, the species of *Dendrobium* had a bigger effect on the genetic structure of the rust fungi populations of *Dendrobium* and the relationships among rust fungi populations of *Dendrobium* may be related to the ones between species of *Dendrobium*. By collecting the samples of the rust fungus from more places and many species of *Dendrobium*, understand the relationships between the rust fungus of different species of *Dendrobium i*n different places or the same place, which plays an important role in the study of the mechanism of rust resistance of *Dendrobium* and in the breeding of rust resistance of *Dendrobium.*

Conclusion

On comparing the results from ITS molecular marker and clone sequencing with NCBI website, it was shown that the rusty diseases of *D. candidum*, *D. aphyllum*, *D. crystallinum* and *D. devonianum* were caused by the *Coleosporium* fungi. Its species will be confirmed in the future. The *Coleosporium* causing the *Dendrobium* rust disease in Honghe and Dehong was mainly aggregated regionally, and a small amount of rust in Honghe was found to be aggregated with that in Dehong, and the *Coleosporium* fungus causing the Dendrobium disease for different species of *Dendrobium* were aggregated alone together, which indicated that the rust fungi of *Dendrobium* had a stronger specificity.

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