COMPARATION OF SYMPTOM TYPE DIFFERENCES IN ENVIRONMENTAL PRICKLYASH RUST AND POPULATION GENETIC STRUCTURE OF COLEOSPORIUM

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Abstract. Pricklyash has important economic, medicinal and ecological value. Pricklyash rust is one of the main diseases of the plant, it often causes discoloration, necrosis and abscission of Pricklyash leaves. When the disease is serious, it can cause entire leaves of pricklyash to fall off and infect repeatedly, which can lead to the weakening of pricklyash, even death, and seriously affects its yield and quality. The spore morphology of pricklyash rust with different symptoms was observed and ITS sequence was analyzed and compared, the results showed that 60 samples of pricklyash rust collected from wild pricklyash, Qujing pricklyash, red pricklyash and green pricklyash belong to Basidio-mycotina, Teliomycetes, Uredinales, Coleosporaceae, *Coleosporium*, *Coleosporium zanthoxyli*. By comparing and analyzing the results of previous studies on *Dendrobium* rust with of pricklyash rust, it was found that *Dendrobium* rust and pricklyash rust were clustered in two populations and the genetic similarity coefficient of ISSR was 0.67. The genetic diversity of *Dendrobium* rust was richer than of pricklyash rust.
Keywords: *pricklyash rust, coleosporium, population genetic structure, molecular marker*

Introduction

Pricklyash (*Zanthoxylum* spp.) belongs to deciduous small trees or shrubs of Rutaceae, *Zanthoxylum* spp. There are about 250 species of pricklyash in the world, widely distributed in tropical and subtropical regions of Asia, Africa, America and Oceania (Huang, 1997). China is the first country to produce pricklyash and also one of it main cultivating countries in Asia (Li, 2014). There are about 39 species and 14 varieties of pricklyash in China, ranging from Liaodong Peninsula in the Hainan Island in the south, Taiwan in the southeast, and Southeast Tibet in the west (Huang, 1997). Artificially cultivated varieties have green (green pricklyash) and red (red pricklyash) fruits after ripening (Li, 2014).

Pricklyash is not only an important seasoning, but also a key traditional Chinese medicine (Gu, 2015). Pricklyash has a strong aromatic odor, which can mask other smells, disperse cold in the warm, cool down body temperature and relieve pain, thus

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can be used to treat many diseases. With the continuous understanding and analysis of chemical constituents of pricklyash, it has been found that pricklyash contains a variety of chemical constituents which are beneficial to human health and disease treatment. Its rich α-linolenic acid, palmitic acid, linoleic acid and polyunsaturated fatty acid have anti-cancer, anti-inflammatory, anti-oxidation, bacteriostasis, insecticidal and antiseptic effects (Chen, 2016; Chang, 1997; Lee, 2012; Li, 2009; Liu, 2007; Mi, 2014; Paik, 2005). In addition, pricklyash has a strong root system which grows rapidly and has many advantages, such as water and soil conservation, good growth in arid and barren areas, and thus can be used to improve the ecological environment (Gu, 2015). With important economic, medicinal and ecological values, pricklyash has attracted the attention of governments at all levels and enterprises. By 2014, the planting area of pricklyash in China was 1.67×10^6 hm^2 while that in Yunnan Province was 3.03×10^5 hm^2, which accounts for more than 10% of the total planting area of pricklyash in China. Yunnan Province is one of the important production bases of pricklyash in China (Gu, 2015).

Pricklyash rust (*Coleosporum zanthoxyli*) is a common leaf disease in the cultivation area of pricklyash which is less harmful to fruits and infects both seedlings and grown plants of pricklyash (Cao, 1989, 1994; Zhang, 2006). Tt often causes discoloration, necrosis and abscission of pricklyash leaves. When the disease is serious, it can cause the whole leaves of pricklyash to fall off and infect repeatedly, which can lead to the weakening of pricklyash, even death, and seriously affects the yield and quality of pricklyash. In recent years, pricklyash rust has occurred in large areas in Shandong, Shaanxi and other major pricklyash production areas, causing great losses to pricklyash production (Cao, 1989, 1994; Chen, 2007).

Coleosporium zanthoxyli which causes pricklyash rust, belongs to Basidiomycetes, Uredinales, Melampsoraceae, *Coleosporium* fungus. The process of the study of Chinese prickly ash rust, *Coleosporium* fungus can be observed from rust spores of summer and winter spore on the pricklyash, the other rust spore type has not been observed, and its life cycle was unclear. At present, there are few studies on the morphology of *Coleosporium zanthoxyli* and the population genetic structure of *Coleosporium zanthoxyli* on different types of pricklyash. Therefore, on the basis of previous investigation, rust samples from red, green and wild pricklyash in Qujing, Yunnan Province were collected in 2018 and were observed carefully. The population genetic structure of *Coleosporium* on different *Coleosporium zanthoxyli* was analyzed by ISSR molecular markers, and was compared with those on Dendrobium rust. The comparative analysis was carried out in order to provide theoretical basis for the prevention and control of pricklyash rust and the related research fields of *Coleosporium.*

Materials and methods

Materials

Collection of pricklyash rust samples

In this study, rust samples from red, green, wild and local pricklyash were collected from Qujing (N26°53’49”, E103°34’42”), Yunnan Province. When collecting rust samples, the collected rust samples were packed independently with absorbent paper, recorded and then air-dried or stored in a refrigerator at – 80 °C for reserve. Details of the samples are shown in *Table 1*.

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Table 1. *Collection information of pricklyash rust samples*

Pricklyash type	No. of samples	Sampling time	Sample number
Red pricklyash	15	2018.10	HHJ
Wild pricklyash	15	2018.10	YHJ
Qujing pricklyash	15	2018.10	QJHJ
Green pricklyash	15	2018.10	LHJ
Total	60		

Primer ISSR

The molecular markers used in this study were fungal ribosomal rDNA-ITS primers (forward primers: TCCGTAGGTGAACCTGCGG; reverse primers: TCCTCCGCTTATTGATATGC) (Chen, 2007), and six ISSR primers designed and developed by our team. All the primers were synthesized by Shanghai Invitrogen Company. Detailed information is shown in *Table 2*.

Table 2. *The primers used in genetic analysis*

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

Instruments used

The main instruments used in this experiment are: ultra clean workbench, autoclave, drying box, electronic balance, HH-4 digital constant temperature water bath pot, Microfuge18 centrifuge, Blue Shield 522 visible light gel electrophoresis transmission meter, ABI-9700 PCR, 78HW-1 constant temperature heating magnetic stirrer, GeneQuant Pro protein nucleic acid analyzer, Imagequant-300 gel imaging system, ABI-3730XL genetic analytical system, Lycra fluorescence microscope and stereomicroscope.

Methods

Morphology identification of Coleosporium

The symptoms of rust samples collected were carefully observed, and both sides of the susceptible parts of rust plants' leaves were recorded by camera. The morphology of pathogens in the susceptible parts was observed by stereomicroscope, the symptoms were recorded, the spores of rust were picked up for observation, and the infected tissues were cut into water slides to observe the structure of sporulation tissues. The spore morphology, color and size were recorded.

Extraction of Coleosporium DNA

The genomic DNA of plant rust was extracted by Chelex-100 method (Liu, 2015). The details are as follows: 150 uL Chelex-100 (concentration percentage is 20%, 1/3

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chelex should be absorbed) solution is absorbed by a pipette into a 1.5 mL centrifugal tube, 5 uL supernatant of Chelex-100 is absorbed by a 10 uL pipette to the lesion with rust spores, and the rust spores on the lesion are washed repeatedly so as to elute the spores off the lesion as far as possible, and the washed spores are sucked into a 1.5 mL centrifugal tube containing 150 uL Chelex-100 solution by a 10 uL pipette. The centrifugal tube was placed in boiling water for 2 min, oscillated on vortex oscillator for 20 s, centrifuged for 10 s, then water bath for 5 min, oscillated for 20 s and centrifuged for 30 s. The obtained supernatant is the extracted DNA, which is stored in the refrigerator at -20 °C for reserve.

Molecular identification of Coleosporium

The total volume of PCR reaction was 50 uL according to ITS sequence analysis of common primers of fungi ITS sequence, among which: 2×phanta max ×Buffer 25 uL (the final concentration of Mg^{2+} is 2 mM), dNTP Mix 0.2 mM, Phanta Max Super-Fidelity DNA Polymerase 1U, primers ITS1 and ITS4 0.5 mM, template DNA 25 ng/uL. A negative control group of ddH_2O was established for each reaction. Amplification was performed by ABI-PCR. The reaction procedures were as follows: pre-denaturation for 3 min at 95 °C, denaturation for 15 s at 95 °C, annealing for 15 s at 57 °C, extension for 30 s at 72 °C, running for 35 cycles; extension of 5 min at 72 °C, preservation at 4 °C; after the end of PCR, 5 uL PCR fidelity product was mixed with 2 uL loading-buffer which contains anthocyanin (1 mL Loading buffer + 10 uL Anthocyanin), conducted electrophoresis with 1.5% agarose gel for 45 min under 120 V, and then photographed with gel imaging system so as to analyze PCR product initially. The PCR product with identical fragment size was recycled with gel, and then cloned to test its sequences; sequence comparative analysis of the tested ITS sequences was conducted on NCBI website, and the ITS sequences of different plant Coleosporium were clustered by the neighbor-joining method using the software of molecular evolutionary genetics analysis (MAGE 5.1) (Saitou, 1987).

Population genetic structure analysis

The total volume of ISSR-PCR reaction for population genetic structure analysis of various plant rust was 30 uL, including: 10×Easy Buffer (containing Mg^{2+}) 3.0 uL, 2.5 mM dNTPs 2.4 uL, ISSR primer 1.2 uL, TaqE 0.3 uL, template DNA (25 ng/uL) 1 uL, ddH_2O 22.1uL. Coleosporium was used as positive control group and ddH_2O as negative control group each time. The reaction procedures were as follows: pre-denaturation for 2 min 30 s at 95 °C; denaturation for 30 s at 94 °C, annealing for 1 min at 50 °C (the specific annealing temperature of each primer was shown in *Table 2*), extension for 1 min at 72 °C, running for 35 cycles; extension for 10 min at 72 °C, preservation at 4 °C; after the end of PCR, 5 uL PCR product was mixed with 2 uL Loading-buffer which contains anthocyanin (10 uL/mL anthocyanin), conducted electrophoresis with 1.5% agarose gel for 45 min under 120 V, and then photographed with gel imaging system so as to analyze PCR product initially. Samples with PCR products were sent to Kunming Shuo Qing Biological Co., Ltd. for further analysis with ABI-3730XL genetic analysis system.

ISSR-PCR amplified fragments were analyzed by genetic analysis system. The size of ISSR-PCR amplified fragments ranged from 100 bp to 700 bp (internal standard ABI-LIZ1200). The fragments of PCR products obtained by genetic analysis system

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were transformed into 01 matrix. The PCR products were packed in a box with a width of 10 bp. The population structure was analyzed by unweighted pair group method of with arithmetical averages (UGPMA) cluster analysis NTSY spc 2.1 soft (Rohlf, 2000).

Results and analysis

Oberservation of pricklyash rust symptoms

By observing the rust samples collected from wild pricklyash, Qujing pricklyash, green pricklyash and red pricklyash, it was found that there are various types of symptoms of rust infection (*Fig. 1*). In the early stage of pricklyash rust, pricklyash leaves produced one or more spots of varying sizes. With the deepening of infection, yellow to orange naked summer spore piles appeared on the back of pricklyash leaves. The size of summer spore piles was 0.1-0.7 mm, and the size of winter spore piles was 0.2-1.0 mm at the corresponding parts of late summer spore piles. Some of the lesions of pricklyash rust are irregularly distributed, forming a lesion (wild pricklyash rust) by a single summer spore pile; some are centered on one point with others forming a circle (Qujing pricklyash rust and green pricklyash rust); others are centered on one point, surrounded by two circles, and at the later stage of infection, the outer circle turns red-brown (red pricklyash rust).

Figure 1. *Symptoms of different types of pricklyash rust*

The pathogenic morphology of pricklyash rust was observed under a microscope. It was found that the color of summer spores of pricklyash rust varied from light yellow to yellow. Most of the summer spores were oval, elliptical, rough and warty. The size of summer spores was 30-60 um × 18-25 um (*Fig. 2*). There are 3-4 cells in the winter spores, the top 3 cells are yellow and the base cell is colorless, and the spore size is 55-91 um × 18-29 um (*Fig. 3*). The winter spore often germinates from the top cells and grows germ tubes.

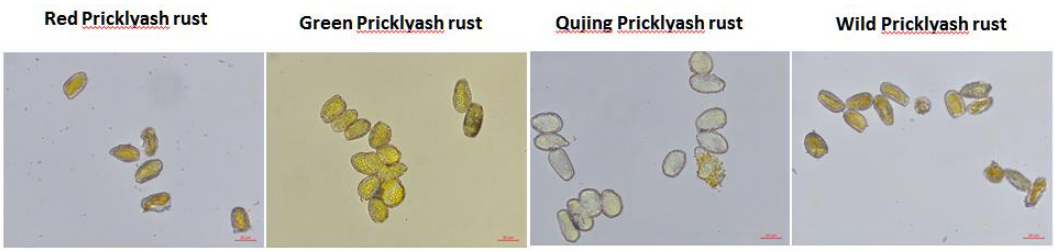

Figure 2. *Urediospore morphology of pricklyash rust fungus with different symptoms*

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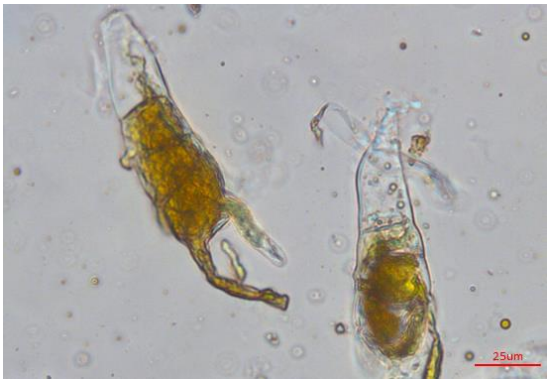

Figure 3. *Teleutospore morphology of pricklyash rust fungus (germinated teleutospore)*

ITS sequence analysis of pricklyash rust

In this paper, the ITS sequences amplified by fungal ITS universal primers were compared and analyzed on NCBI website. It was found that the rust collected from red pricklyash, green pricklyash, Qujing pricklyash and wild pricklyash belongs to Basidiomycotina, Teliomycetes, Uredinales, Coleosporaceae, *Coleosporium*, *Coleosporium zanthoxyli* (*Table 3*). The genetic relationship of the rust samples collected from different types of pricklyash was analyzed by MAGE 5.1. The results showed that except for YHJ-2 having 96% coverage rate and 97.83% similarity rate with *Coleosporium zanthoxyli* of registration number MH465095.1 published on NCBI website, ITS sequences of other samples have 99% or above coverage rate and 99% and above similarity rate with *Coleosporium zanthoxyli* of registration number MH465095.1, especially the 7 samples of QJHJ-1, QJHJ-2, YHJ-3, HHJ-1, HHJ-3, LHJ-1, HHJ-2, which have 99% coverage rate and 100% similarity rate.

Table 3. *Comparison of ITS sequences of pricklyash rust fungus with different symptom types*

Sample name	Query cover%	E-value	Ident %	Homologous species	Accession	Host
QJHJ-1	99	0.0	100.00	Coleosporium zanthoxyli	MH465095.1	Qujing pricklyash
QJHJ-2	99	0.0	100.00	*Coleosporium zanthoxyli*	MH465095.1	Qujing pricklyash
QJ-HJ-3	99	0.0	99.87	*Coleosporium zanthoxyli*	MH465095.1	Qujing pricklyash
YHJ-1	100	0.0	99.85	*Coleosporium zanthoxyli*	MH465095.1	Wild pricklyash
YHJ-2	96	0.0	97.83	*Coleosporium zanthoxyli*	MH465095.1	Wild pricklyash
YHJ-3	99	0.0	100.00	*Coleosporium zanthoxyli*	MH465095.1	Wild pricklyash
HHJ-1	99	0.0	100.00	*Coleosporium zanthoxyli*	MH465095.1	Red pricklyash
HHJ-2	99	0.0	99.73	*Coleosporium zanthoxyli*	MH465095.1	Red pricklyash
HHJ-3	99	0.0	100.00	*Coleosporium zanthoxyli*	MH465095.1	Red pricklyash
LHJ-1	99	0.0	100.00	*Coleosporium zanthoxyli*	MH465095.1	Green pricklyash
LHJ-2	99	0.0	100.00	*Coleosporium zanthoxyli*	MH465095.1	Green pricklyash

In this paper, the genetic relationships between the obtained ITS sequences of pricklyash rust, the ITS sequences of Dendrobium Coleosporium in our previous studies and the ITS sequences of *Coleosporium zanthoxyli* of registration number MH465095.1

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published on NCBI website were analyzed by MEGA5.10 software. It was found that Dendrobium Coleosporium could be clustered into categories I, II and III, and all of *Coleosporium zanthoxyli* were clustered in category I except from TP-SH-3 and TP-SH-4, which were clustered in category III. The rust collected from other Dendrobium species were clustered in category II.

Genetic diversity analysis of different pricklyash rust

Popgene 1.32 software was used to analyze the genetic diversity of four different types of pricklyash rust. The results (*Table 4*) showed that the population level, Nei's gene diversity index (H), Shannon's information index and percentage of polymorphic loci of wild pricklyash rust were the largest, indicating that the genetic diversity of wild pricklyash rust was the richest, followed by Qujing pricklyash rust, while red pricklyash and green pricklyash have similar genetic diversity. Generally speaking, the genetic diversity of the four types of pricklyash rust is not much different. At the same species level, the percentage of polymorphic loci was 82.72%, the number of observed alleles (Na) was 1.8272, the number of effective alleles (Ne) was 1.2383, the genetic diversity index (H) and information index (I) were 0.1551 and 0.2560, respectively. The number of polymorphic loci was 268, and the percentage of polymorphic loci was 82.72%, indicating the rich genetic diversity of pricklyash rust population (*Fig. 4*).

Table 4. *Genetic diversity of rust fungi populations on different pricklyash*

Population code	Alleles (Na)	Effective allele (Ne)	Nei gene diversity index (H)	Shannon's (I) Shannon information index	Number of polymorphic loci	Percentage of polymorphic loci
HHJ	1.5556	1.1976	0.1277	0.2068	180	55.56
LHJ	1.537	1.2041	0.1292	0.2069	174	53.70
YHJ	1.6296	1.2465	0.1577	0.2519	204	62.96
QJHJ	1.5031	1.2368	0.1424	0.2199	163	50.31
Average	1.5563	1.2213	0.1393	0.2214	180	55.63
Total of species	1.8272	1.2383	0.1551	0.2560	268	82.72

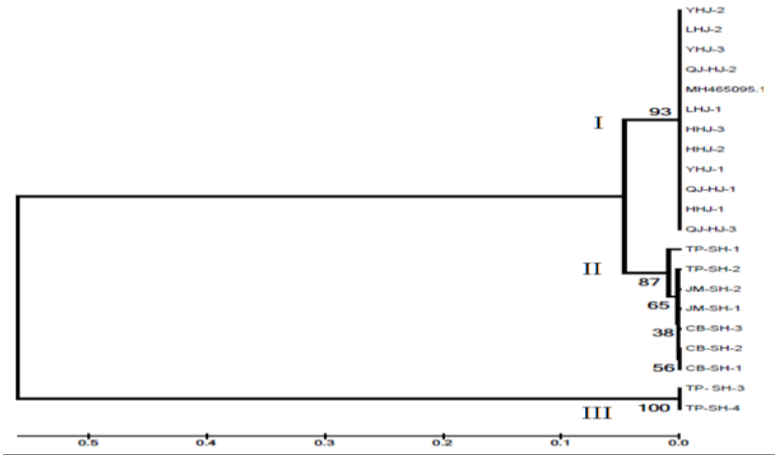

Figure 4. *ITS sequence comparison dendrogram of different pricklyash and Dendrobium rust. Note: MH465095.1 is: ITS sequence of Zanthoxylum bungeanum with login number MH465095.1 published by NCBI website; ITS sequences of TP-SH-1, TP-SH-2, TP-SH-3, TP-SH-4, JM-SH-1, JM-SH-2, CB-SH-2, CB-SH-3 are the experimental data of this group (Pu, 2019)*

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NTsis software was used to analyze the population genetic structure of different types of pricklyash rust. The results (*Fig. 5*) showed that the similarity of four pricklyash rust populations was above 0.74, which was relatively high, and the genetic structure was more complex, and the classification was not very obvious. Especially for wild pricklyash rust and Qujing pricklyash rust, the clustering was dispersed.

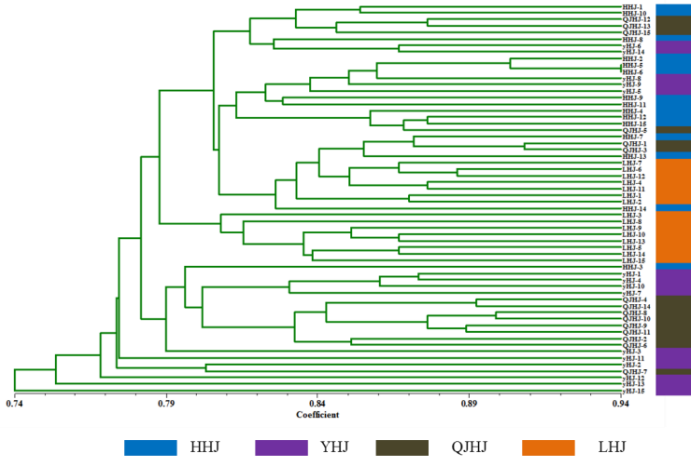

Figure 5. *Population genetic structure of rust on different pricklyash*

Population structural genetic analysis of Coleosporium

The ISSR molecular markers designed by the team were used to analyze the population genetic structure of rusts collected from different varieties of pricklyash and Dendrobium. NTsis software analysis results (*Fig. 6*) showed that rusts on pricklyash and on Dendrobium were clustered in two groups, namely, rusts on Dendrobium clustered in category I while rusts on pricklyash clustered in category II with a similarity coefficient of 0.67, which means that the population differentiation between the rusts on pricklyash and the rusts on Dendrobium was obvious.

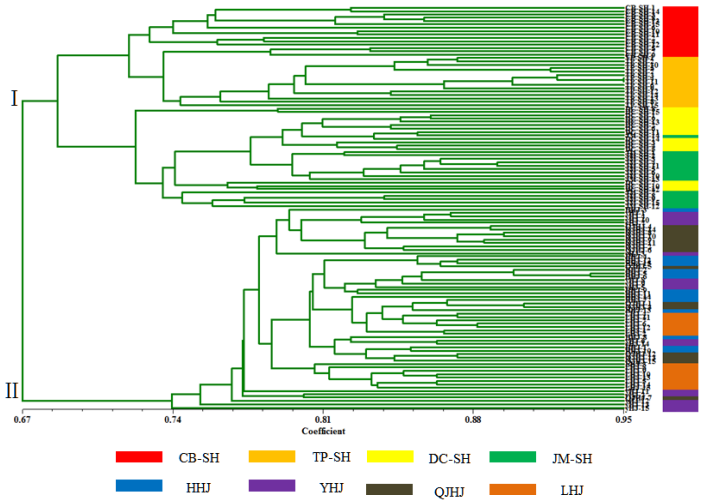

Figure 6. *Population genetic structure of Coleosporium on different pricklyash and Dendrobium. Note: CB-SH, TP-SH, DC-SH and JM-SH are the experimental data of our group (Pu, 2019)*

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Discussion

Pricklyash (*Zanthoxylum* spp.) has many species. There are about 250 species of *Zanthoxylum* spp. plants in the world. There are about 50 species, 13 varieties and 2 variants of *Zanthoxylum* in China. Artificially cultivated varieties have green (green pricklyash) and red (red pricklyash) fruits after ripening. pricklyash rust is one of the common diseases on pricklyash, it often causes discoloration, necrosis and abscission of pricklyash leaves, which seriously affects the yield and quality of pricklyash.

The symptoms and spore morphology of 60 samples of rust collected from red pricklyash, green pricklyash, Qujing pricklyash and wild pricklyash were observed in this study. It was found that the symptoms of rust were varied and the size of summer spores of rust varied greatly. ITS sequence analysis showed that these pricklyash rusts with different symptoms and collected from different types of pricklyash were caused by *Coleosporium*. Scholars have reported that *Coleosporium zanthoxyli* is hosted by 9 species of *Zanthoxylum* plants, such as pricklyash and wild pricklyash, as well as *Dendrobium candidum*, *Dendrobium globosa*, etc. (Xi, 2018). Moreover, the ITS sequences of pricklyash and Dendrobium rusts were compared and analyzed to find that most of them had a high similarity of 0.96 (*Fig. 4*), but the population genetic structure of *Coleosporium* derived from pricklyash and Dendrobium was quite different. Therefore, for the highly host-specific *Coleosporium rust*, whether pricklyash rust and Dendrobium rust can be infected by each other requires further research and study.

After analysis, it was found that the population genetic structure of rust on pricklyash was rich. From the ISSR population genetic structure map, we can see that rust on wild pricklyash are distributed in all large populations, which indicates that some rust on wild pricklyash may have close genetic relationship with rust samples on Qujing pricklyash, red pricklyash and green pricklyash. It is speculated that rust on wild pricklyash may be transmitted to each other. *Figure 5* shows that the population differentiation of rust on Dendrobium and rust on pricklyash is obvious. The population genetic similarity coefficient of ISSR is 0.67. The rust on Dendrobium and rust on pricklyash are clustered in two populations respectively. Moreover, Dendrobium rust is closely related to the host varieties of *Dendrobium*, but pricklyash does not show this pattern. This may be mainly due to the fact that the collected samples of *Dendrobium* rust come from greenhouse cultivation, and the possibility of its transmission in the air is relatively small, while pricklyash rust is collected from the field, and its rust spores can be transmitted with the airflow. It can be seen from this paper that while preventing and treating pricklyash rust on different varieties of pricklyash, control of rust on wild pricklyash needs to be further strengthened.

Conclusions

(1) The spore morphology of pricklyash with different symptoms of pricklyash rust was observed, and ITS sequence was analyzed and compared to show that the symptoms of rust on wild pricklyash, Qujing pricklyash, red pricklyash and green pricklyash were various. The pathogens causing different symptoms of pricklyash rust belong to Basidio-mycotina, Teliomycetes, Uredinales, Coleosporaceae, *Coleosporium*, *Coleosporium zanthoxyli.*

(2) By comparing and analyzing the results with previous studies on Dendrobium rust, we found that rust on Dendrobium and rust on pricklyash were clustered in two populations, and the genetic similarity coefficient of ISSR was 0.67.

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(3) The genetic structure of *Coleosporium* on wild pricklyash was rich. The *Coleosporium* on wild pricklyash can be clustered into *Coleosporium* on other varieties of pricklyash, which means that *Coleosporium* on wild pricklyash may be the source of infection of other types of pricklyash rust.

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