Isolation and Primary Identification of Leaf Rust on Black Cottonwood (Populus deltoides) at the South of China

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Molecular Pathogens, 2020, Vol.11, No.2  doi: 10.5376/mp.2020.11.0002

Received: 13 Aug., 2020
Accepted: 18 Aug., 2020
Published: 28 Aug., 2020

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Preferred citation for this article:
Zhou Y., Dai M.L., Dai X.G., Li S.X., Yin T.M., and Li X.P., 2020, Isolation and primary identification of leaf rust on black cottonwood (Populus deltoides) at the South of China, Molecular Pathogens, 11(2): 1-8 (doi: 10.5376/mp.2020.11.0002)

Abstract  To explore the species of pathogen of leaf rust on black cottonwood (Populus deltoides) at the south of China, in this study, leaf rust spores were isolated and identified on P.deltoides in JiangSu Province(Nanjing, SiHong, SiYang), AnHui Province (HeFei) and HuBei Province (ShiShou). Totally eleven isolates (three in NanJing, four in SiYang, two in HeFei, one in SiHong, one in ShiShou) were obtained by leaf single conidia isolation method. Through scan electron microscope (SEM), the spores of the length, width, thorn distance and diameter of bald surface of each segregation line were found to be largely different between them (p<0.05). By ITS and LSU sequencing, the urediniospores of SY92, SY39, SY40, SY16, NJ10, NJ30, NJ65, HF58 and HF59 were similar with M.larici-populina, the urediniospores of SH48 and SS101 were similar with M.medusae. According to molecular clustering, the urediniospores from NanJing, SiYang and HeFei were categorized into M.larici-populina and those from SiHong and ShiShou were classified into M.medusae. Intriguingly, M.medusae found is at first recorded at the south of China. Our study provides an important reference for further research on the pathogen of leaf rust on P. deltoides at the south of China.

Keywords  Populus deltoides; Poplar leaf rust; Melampsora medusa; Melampsora larici-populina

Poplar (Populus spp.) is an important fast-growing wood species. Among them, Populus spp. is widely planted worldwide due to its cold tolerance, fast growth, and easy cultivation, occupying more than 90% of poplar plantations (Liu et al., 2016). There are three main species of black cottonwood: one is Populus deltoides (Segelquist et al., 1993), which is mainly distributed along the Mississippi River in America. It is the fastest growing tree species among poplars and the largest planting area in China. The second species is the European Black Cottonwood (P. nigra L.), which is mainly distributed in southern Europe and western Asia (Arens et al., 1998); the third species, P. Euramericana, is a hybrid of American and European black cottonwood, often called Canadian poplars or Italian poplars, mainly distributed in America. Poplar rust is one of the most serious diseases affecting the development of the poplar industry. The fungus was introduced into Australia in the 1970s, which had a devastating impact on the local poplar production, with the volume loss reaching more than 80% (Brown, 1984). In China, leaf rust frequently breaks out and spreads due to the large planting area and single stand of Populus deltoides (Liang et al., 2006). In May and June, the first onset of leaf rust occurs, and injured leaves showed flaky orange-yellow spots the affected leaves show flaky-orange spots. In August and September entered the second peak of onset, the leaves fell off early.

The main pathogen of poplar leaf rust is Melampsora, and 32 species have been found (Vankraay et al., 1974), most of which are allogeneic (Bagyanarana et al., 1984; Shang et al., 1990a; Liang et al., 2006), most of which need further separation and identification. In the existing reports, the color, shape and size of the spores of rusts, the number of bud holes germinated, the arrangement of bud holes and the presence or absence of bald spots were mostly preliminarily identified (Arthur, 1934; Wang et al., 1983; Cao et al., 2000), leading to inaccurate identification of some rust fungi. The presence or absence of vulgaris bald spots divides rust fungi into two categories: (1) There are no bald spots, including, burrs, including M. laricis, M. ciliata, M. occidentalis, M. populnea, M. pruinosa, M. pulcherrima, M. nuiangensis and M. cumminisii; (2) There were bald spots, including...
M. larici-Populina and M. Allii-populinata at the apex of bald spots, and M. Abilitis-Canadensis and M. Medusae at the equator. According to the morphology (length, width, spines and wall thickness) of the urediniospores of rust, Tian et al. (2009) divided 196 samples into I-V class, among which the size of the IV urediniospores was 17.7~27.9 μm × 9.2~23.4 μm, which was classified as M. larici-populina. However, the length of the urediniospores of M. larici-populina, which was first discovered in North America in 2005, was 32~48 μm, with a typical smooth apical region (Grondin, 2005). The investigation and research of (Xiao et al., 2009) poplar leaf rust mainly focuses on northern of China (Xiao et al., 2009), and there are few systematic studies on black poplar leaf rust in south of China. In order to further understand the species and distribution patterns of leaf rust of P. deltoides from South China, we investigated the incidence of rust in the main planting areas of P. deltoides and collected a large number of samples. Preliminary separation and morphological observation of rust samples were carried out by single spore separation method, and with the help of ITS and LSU sequencing, the molecular identification and clustering of isolates were further carried out. The analysis found that the M. larici-populina rusts were found in Nanjing, Sihong and Hefei, while the M. Medusae were found in Sihong and Shishou. Among which the M. Medusae rusts were reported for the first time on the P. deltoides in South China. This study will provide an important reference for the prevention and control of poplar rust and the mechanism of poplar rust resistance.

1 Results and Analysis

1.1. Morphological observation of P. deltoides leaf rust disease symptoms and pathogen isolation lines

The P. deltoides leaves infected with rust fungus showed orange-yellow spots (Figure 1). When the disease was severe, the yellow lesions were distributed in one piece. Eleven isolates were isolated from Nanjing, Sihong, Siyang, Hefei and Shishou by the leaf monospore separation method (Table 1). Environmental scanning electron microscopy (SEM) showed that the urediniospores of the isolated strain were oval (Figure 2A), ovoid (Figure 2B) and obovate (Figure 2C); from the position of the bald spots, the isolates can be classified as type I (SY92, SY39, SY40, SY16, NJ10, NJ30, NJ65, HF58 and HF59), urediniospores with smooth apex (Figure 2A), inferred to be M. larici-populina and M. allii-populinata and class II (SH48 and SS101), urediniospores with smooth central patch (Figure 2D) and are inferred to be M. abietis-canadensis or M. medusae.

1.2 Statistical analysis of the length, width, spines and smooth region diameter of urediniospores in five sites

The morphological characteristics of the urediniospores of the 11 isolates were statistically analyzed by location showed that the length, width, spines and smooth region diameter of urediniospores at the five sites were different to different degrees (p<0.05, Figure 3). The length of urediniospores of rusts in Sihong (SH) and Hefei (HF) was significantly different from that in the other three regions, and the variation range was 18.57~28.06 μm (Figure 3A). Siyang (SY) and Sihong (SH) rust fungi had a significant difference in the width of urediniospores from the other three regions (Nanjing, Hefei, and Shishou), and the variation range was 10.87-16.52 μm (Figure 3B). However, there is no significant difference between Sihong (SH) and Shishou (SS) in the urediniospores distance of spines (Figure 3C). Shishou (SS) is significantly different from the other four regions, and the variation range is 1.37~2.12 μm. Siyang (SY), Hefei (HF) and Shishou (SS) have no difference in the smooth region diameter of urediniospores (Figure 3D), while Nanjing (NJ) and Sihong (SH) have significant differences, with a variation range of 3.81~9.07 μm.

The correlation analysis of the length, width, spores distance between spines and spores diameter of smooth region of urediniospores in five sites (Table 2). The urediniospores of width and spores distance between spines, spores distance between spines and spores diameter of smooth are all significantly positively correlated (p<0.01 ); There was no significant correlation between the length and width of urediniospores, the length and spores diameter of smooth region of urediniospores; the width and spores diameter of smooth region of urediniospores.
Figure 1 Symptoms of leaf rust on *P. deltoides*
Note: A: The normal leaf; B: leaf rust on *P. deltoides*

Table 1 Information on leaf rust collected from different hosts

| Isolations | The host (Populus clones) | The collection position |
|------------|---------------------------|-------------------------|
| NJ10       | *Conviction yourself deltoides' 2-2'* | Nanjing, JiangSu Nanjing Forestry University |
| NJ30       | *P. x euramericana CV. 'Nanlin895'* |                      |
| NJ65       | *P. deltoides Bartr.cv. 'Haryard EX I-63/51* |                      |
| SY16       | *P. x euramericana CV. 'Nanlin895'* | SiYang, JiangSu SiYang Farm |
| SY39       | *Conviction yourself deltoides' T120'* |                      |
| SY40       | *Conviction yourself deltoides' T120'* |                      |
| SY92       | *Conviction yourself deltoides' T20'* |                      |
| SH48       | *Conviction yourself deltoides' 3412'* | SiHong, JiangSu ChenWei Forest Farm |
| HF58       | *Conviction yourself deltoides x P. nigra* | HeFei, AnHui AnHui Agricultural University |
| HF59       | *Conviction yourself deltoides x P. nigra* |                      |
| SS101      | *P. deltoides CV. 'Huazhi 1'* | ShiShou, HuBei Populur Research Institute |

Figure 2 Morphological feature of rust urediniospores
Note: Spores Oval (A), Oval (B, D) and Obovate (C); Urediniospores with smooth apex (A); Urediniospores with smooth central patch (D)
Figure 3 Comparison of five company sites on length, width, short between spines and smooth region diameter of urediniospores

Note: A: Comparison of five company sites on length of urediniospores; B: Comparison of five company sites on width of urediniospores; C: Comparison of five company sites on short between spines of urediniospores; D: Comparison of five company sites on smooth region diameter of urediniospores

Table 2 Correlation analysis between spores length, spores width, spores distance between spines and spores diameter of smooth region

| Trait                        | Length | Width  | Distance between spines | Diameter of smooth region |
|------------------------------|--------|--------|-------------------------|----------------------------|
| Length                       | 1      |        |                         |                            |
| Width                        | 0.242  | 1      |                         |                            |
| Distance between spines      | 0.343  | 0.745 **| 1                       |                            |
| Diameter of smooth region    | 0.316  | 0.219  | 0.600 **                | 1                          |

Note: **: The significance level of correlation analysis was 0.01

1.3 Identification and isolation of pathogens by ITS and LSU sequences

The DNA of rust bacteria isolated from five sites in Nanjing (NJ), Sihong (SH), Siyang (SY), Shishou (SS), and Hefei (HF) from P. deltoides were used as templates, and the universal primers NL1 and NL4, ITS1-F and ITS4-B were amplified by PCR, and LSU (large nuclear subunit: D1/D2 region) of 470 bp to 651 bp and ITS of 846 bp to 860 bp (internal transcribed spacer: ITS 1-5.8 were obtainedS-ITS 2) fragments were cloned and sequenced to determine its base sequences.

ITS multiple alignment found that the ITS sequence of class I (NJ10, NJ30, NJ65, SY92, SY39, SY40, SY16, HF58, and H59) had the highest consistency with M. larici-populina (99.34%); and class II (SH48 and SS101)) and M. medusae have the highest consistency (99.92%). LSU multiple comparison found that SY92, SY39, SY40, SY16, NJ10, NJ30, NJ65, HF58 and HF59 had the highest agreement with M. larici-populina (99.85%), and SH48
and SS101 had the highest agreement with M. medusae (99.75%). ITS and LSU sequencing results and database (GenBnak) of 11 rust fungi in Nanjing (NJ), Sihong (SH), Siyang (SY), Shishou (SS) and Hefei (HF) using Mega7.0 software Sequence alignment of the ITS and LSU region sequences of the 4 genus Prussia (M. epitea; M. allii-populina; M. caprearum; M. pruinosa) of the same genus downloaded in the download to construct a phylogenetic tree (Figure 4). The results showed that the above-mentioned SY92, SY39, SY40, SY16, NJ10, NJ30, NJ65, HF58 and HF59 isolates clustered in the same branch with M. larici-populina, and SH48 and SS101 clustered in the same branch with M. medusae. This indicates that there is no difference in the isolates of rust in leaf samples collected from three locations in Nanjing (NJ), Siyang (SY) and Hefei (HF) are not different, and there is a large degree of similarity. They are from the same species M. larici-populina. There is no difference between the rust fungi isolated from Sihong and Shishou which are M. medusae. Combining with morphological and molecular techniques, we obtained the identification results of the isolated lines at various locations (Table 3).

![Figure 4: Phylogenetic tree based on sequences of ITS and LSU between isolates and their relatives in Melampsora spp](image)

Note: A: Phylogenetic tree of isolations ITS sequence (JQ912668: M.larici-populina; GQ479861: M.medusae; GQ479290: M. allii-populina; GQ479208: M.caprearum; GQ479898: M.pruinosa); B: phylogenetic tree of isolations LSU sequence (JQ042251: M. larici-populina; AB116800: M. allii-populina; AB116792: M.pruinosa; KU550033: M.caprearum; JQ42246: M.medusae)

### Table 3 Identification of Melampsora spp. isolations in five sites

| Species          | Site   | Isolations | Size(μm)                     |
|------------------|--------|------------|------------------------------|
| M. larici-populina | NanJing | NJ10       | 13.97~28.60×10.80~16.59      |
|                  |        | NJ30       | (23.32±0.50×13.33±0.47)      |
|                  |        | NJ65       |                              |
|                  | SiYang | SY16       | 16.79~26.29×10.87~16.69      |
|                  |        | SY39       | (20.73±0.48×13.70±0.68)      |
|                  |        | SY40       |                              |
|                  |        | SY92       |                              |
| M. medusae       | SiHong | SH48       | 16.79~26.29×10.87~16.69      |
|                  |        | SS101      | (20.73±0.48×13.70±0.68)      |

### 2 Discussion

*Puccinia spp.* rust is the most important pathogenic bacterium that causes poplar leaf rust. It generally occurs in early summer, usually from the end of August to the beginning of September, it can cause poplar leaves to wither and fall in advance. In serious cases, it can cause premature bud to fall early, or make trees vulnerable to infection
by other pathogens, thus causing harm to its growth. It was found that there were 9 species of phyllostachys parasitizing on Chinese poplar (Shang and Pei, 1984; Yuan, 1984; Liang et al., 2006), among which 5 species are the most common: *M. medusae*, *M. abietis-Canadensis*, *M. aecidioides*, *M. laricisi-Populina* and *M. Allii-populina*. However, their morphological characteristics are similar without obvious differences, and they are only judged to have certain risks according to their morphological characteristics.

In this study, the identification of rusts from the main planting areas of *P. deltoides* (Nanjing (NJ), Sihong (SH), Siyang (SY), Hefei (HF), Shishou (SS)) were carried out by using the method of morphological combination molecule. Morphological and molecular identification results showed that (Table 3): I class (SY92, SY39, SY40, SY16, NJ10, NJ30, NJ65, HF58 and HF59) are closely related to *M. larici-populina*, so the rust pathogens of Nanjing (NJ), Siyang (SY) and Hefei (HF) as *M. larici-populina*; II class (SH48 and SS101) are closely related to *M. medusae*, so the rust pathogens of Sihong and Shishou as *M. medusae*, this conclusion is consistent with the predecessors' research results part (Wang et al., 2017). Through SPSS’s significance T test, it was found that the value of *M. larici-populina* urediniospores is consistent with the results of domestic research by Liu et al. (2006) and Dai et al. (2019); but there is a big difference between the researches of Phillips and Xue (1991) (*p*=0.05), and the measured values are all smaller than those in foreign studies. It is speculated that the size of urediniospores may be related to the geographical environment. So far, there are few reports on the factors affecting the size of urediniospores, so a larger sample size and more specific analysis of samples from more regions are needed. The measurements of urediniospores of *M. Medusae* were smaller than those in New South Wales, Australia (Walker et al., 2010) and Florida, USA (Loyd and Smith, 2018). Zheng et al. (2019) identified *M. Medusa* on *P. deltoides* cv. ‘Zhonghua hongye’, *P. szechuanica*, *P. simonii* and *P. yunnanensi* in Shaanxi and Sichuan, China, in February 2019, which was similar to the survey time of this study, indicating that the transmission speed was fast and there was a trend of continuous transmission. *M. Medusae* was first discovered on *P. deltoides* in Shishou (SS) and Sihong (SH), which has important guiding significance for the quarantine of *M. Medusae* in China. In this study, *M. larici-populina* and *M. medusae* were separated, identified by morphology and molecular biology, and morphological characteristics of urediniospores were statistically analyzed, the specific distribution of the disease is not yet known, and its biological characteristics and pathogenic mechanism need to be further studied, so as to promote the scientific prevention and control of leaf rust of *P. deltoides*.

3 Materials and Methods
3.1 Collection of pathogenic bacteria

In the Poplar Research Institute of Shishou City, Jingmen, Hubei Province: 112°42′E, 29°72′N; Nanjing Forestry University, Nanjing City, Jiangsu Province: 118°81′E, 32°08′N; Siyang City, Jiangsu Province County Siyang Farm: 118°70′E, 33°72′N; Malang Lake, Sihong County, Suqian City: 118°31′E, 33°32′N; Hefei City, Anhui Province, Anhui Agricultural University: 117°26′E, 31°86′N five sites diseased leaves of *P. deltoides* were collected.

3.2 Collection of isolated strains

The collected leaves with poplar leaf rust in each area were classified and labeled according to the region, and then the monospore pile separation was carried out in the laboratory using the leaf disk method (Barrès et al., 2008). First samples taken sick leaves disinfection treatment, and out of the leaves remove the circular blade (semal) perforator, we get semal placed in the size of 90 mm in a petri dish, eppendorf company produces the pipetting gun disperse monospore pile in the 4μm microns Agar in water, and then to inoculation experiment, mixing has been good with urediospore liquid to infection of NL895 leaves, place the inoculation of good young leaves at a temperature of 19℃, light cycle for 16 h / 24 h in greenhouse cultivation, further breeding in rust, infection after the blade. The collected spores are stored in a refrigerator at 4℃ at room temperature for further use.
3.3 Morphological characteristics of pathogenic bacteria

The morphological characteristics were mainly observed by scanning electron microscopy (QUANTA 200) (Tian et al., 2004). The materials were prepared by scanning electron microscopy: double-sided adhesive tape was affixed to glass slides, a small amount of summer spores powder was dipped into the injection needle and slowly and evenly applied to the double-sided adhesive tapes, and the glass slides were placed in a critical point dryer for drying, and then placed in a vacuum coating machine for coating. The coated samples were observed in the scanning electron microscope, and 30 citricaria were randomly selected for photographing in each separation system (XT Microscope Server software). Use Photoshop software to measure the length, width, thorn distance and diameter of bald spots in the photos. Use SPSS for statistical analysis. Use Excel to draw comparison histograms of measured values.

3.4 Sequence identification of ITS and LSU of pathogenic bacteria

The rust fungus DNA was extracted using the glass bead method column fungal DNAout kit of Beijing Tianenze Gene Technology Co., Ltd. The primers ITS1-F and ITS4-B were used to amplify the ITS sequence; the primers NL1 and NL were used to amplify the ribosomal large subunit (LSU) gene sequence and the PCR amplification program was started (Table 4). The specific PCR program is: pre-denaturation at 94°C for 3 min; (denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 3 min). After a total of 35 cycles of reaction, extension at 72°C for 10 min, and finally cooling at 4°C. Keep warm. The amplified fragments were submitted to Sangong Biotech for sequencing.

Phylogenetic trees were constructed by using ITS and LSU sequences of 11 isolates and sequences of four other grid rust genera mentioned above. using MEGA7.0 software (Kumar et al., 2016) to build the tree, using the neighbor joining method (Neighbor joining), and proceeding 1,000 Bootstrap statistical tests (Zhang et al., 2019).

Table 4 Primer sequence

| Primer name | Primer sequence (5'–3') Primer Sequence (5'–3') |
|-------------|--------------------------------------------------|
| ITS1-f      | CTTGGTCATTTAGAGGAAGTAA                           |
| ITS4-B      | CAGGAGACTTGTACACGGTGCCAG                        |
| NL1         | GCATATCAATAAGCGGAGGAAAAG                        |
| NL4         | GGTCCGTGTTTCAAGACGG                             |

Authors’ contributions

Zhou Y, Dai ML, Dai XG, Li SX, Yin TM, and Li XP are the executers of the experimental design and research of this study. Zhou Y, Dai ML, Dai XG, Li SX, Yin TM, and Li XP completed the writing of data analysis and the first draft of the paper; Li XP is the corresponding author of the project. All authors read and approved the final manuscript.

Acknowledgments

This study was co-funded by the Nature Foundation-General Project of Jiangsu Province (BK20161525) and the National Natural Science Foundation of China (31570662, 31561123001).

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