Fungal Ecology 33 (2018) 134–142

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Novel microsatellite markers reveal multiple origins of *Botryosphaeria dothidea* causing the Chinese grapevine trunk disease

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ARTICLE INFO

Article history:
Received 28 April 2017
Received in revised form
29 January 2018
Accepted 10 February 2018

Corresponding Editor: Luke Barrett

Keywords:
Botryosphaeriaceae
Fungal pathogens
Genetic diversity
Microsatellite genotyping
Population structure
SSR
Vitis

ABSTRACT

Botryosphaeria dothidea causes diseases in many different hosts worldwide. This species has become the most prominent fungal taxon causing grapevine trunk disease in China, with a recent disease outbreak. To reveal the genetic diversity and explore the origin of *B. dothidea*, six novel microsatellite markers were developed and used for the genotyping of 151 isolates obtained from China and overseas. The results demonstrated the high genetic diversity of the *B. dothidea* populations. Bayesian cluster analysis separated the total *B. dothidea* isolates into five genetic populations. *B. dothidea* isolates from Chinese grapevines were observed to share alleles with isolates from different hosts within China and from grapevines growing overseas, indicating both endemic host shifts and exotic introduction. In addition, unique pathogen genotypes were identified in Chinese grapevine isolates. Hence, we infer that *B. dothidea* isolates from multiple origins are contributing to the dieback and canker outbreak currently occurring in China.

1. Introduction

Pathogen invasion and emergence have become active, interesting research fields in ecology, evolutionary biology and plant pathology (Sakai et al., 2001; Gladieux et al., 2015). Identifying the source and pathways of a biological invasion is essential for understanding the origin of the pathogen (Robert et al., 2012). Disease emergence via multiple introductions is a well-known extrinsic factor in infectious disease outbreaks (Rajkumar et al., 2011; Khankhet et al., 2014; Biasi et al., 2015). The severity of introduced diseases is frequently determined by the combined effect of the aggressiveness of the pathogen and the susceptibility of the host (Brasier, 2001; McDonald, 2004; Milgroom, 2015). Soon after an introduction, the new ecological niche may enable the pathogen to expand rapidly. However, both abiotic and biotic factors, such as a new host, new competitors, and new climatic conditions, can impose selective pressure and influence the rate of adaptation and evolution of the pathogen (McDonald, 2004). Alternatively, an emerging disease could be due to a shift from one host to another (McDonald, 2004; Couch et al., 2005; Stukenbrock et al., 2007; Stukenbrock and McDonald, 2008; Gladieux et al., 2010). These alternative routes for the emergence of new infectious diseases lead to different outcomes. For example, if the disease results from

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https://doi.org/10.1016/j.funeco.2018.02.004

a recent single introduction, then the pathogen population will be genetically homogeneous, exhibiting genetic variation similar or identical to a subset of the source population (Mayr, 1982). In contrast, if the pathogen population is derived from admixture (an endemic source population recombined with an introduced population), then it will show high genetic diversity; consisting of alleles from both endemic and introduced populations (Facon et al., 2008).

Organisms with broad ecological niches likely exhibit greater potential to colonise and be re-distributed in new regions (Lee and Gelembiuk, 2008; Pavlic-Zupanc et al., 2015). Species of the fungal family Botryosphaeriaceae are widely distributed in both natural and human-associated areas and disturbed environments. Hence, the species in this family are assumed to exhibit particularly broad ecological niches (Pavlic-Zupanc et al., 2015). *Botryosphaeria dothidea* belonging to the fungal family Botryosphaeriaceae, is endophytic and pathogenic in a wide range of woody and horticultural plants (Liu et al., 2012; Phillips et al., 2013). Environmental changes often act as triggers for the pathogenicity of *B. dothidea* (Manawasinghe et al., 2016). Therefore, this fungus has been classified as an opportunistic pathogen (Chethana et al., 2016). *B. dothidea* seriously affects grapevines (*Vitis vinifera* and *Vitis* spp.), causing grapevine dieback and cankers (Úrbez-Torres, 2011). Li et al. (2010) first demonstrated that *B. dothidea* causes shoot dieback in grapevines in China. Since then, this species has been reported to be the most abundant botryosphaeriaceous taxon from Chinese grapevines (Yan et al., 2013). The symptoms caused by *B. dothidea* infection include canker, lack of or limited bud burst, stunted growth with shortened internodes, chlorosis, and fruit rot (Yan et al., 2012, 2013). In addition to causing diseases in grapevines, *B. dothidea* has been reported to cause diseases in apples (*Malus* spp.) (Tang et al., 2012; Xu et al., 2014), peaches (*Prunus* spp.) (Li et al., 1995), pears (*Pyrus* spp.) (Xu et al., 2014) and tea plants (*Camellia* spp.) (Jayawardena et al., 2016). Several phylogenetic studies have been conducted with the aim of understanding the distribution and taxonomical status of *B. dothidea* as a plant pathogen (Hyde et al., 2014). However, the patterns of population genetic variation in *B. dothidea* and its putative origin remain largely unknown.

The selection of molecular markers is one of the main steps for conducting population genetic studies (Faria and Miyaki, 2006). Among the numerous marker types available, microsatellite markers have become most widely used, particularly for analysing recently expanding populations. Microsatellite markers exhibit several desirable characteristics, such as codominance, high polymorphism, high accuracy, and simplicity with respect to inferring population genetic parameters (Breuillin et al., 2006; Zheng et al., 2008; Biasi et al., 2015).

A population of *B. dothidea* from Californian pistachio plants has been studied via microsatellite-primed polymerase chain reaction (MP-PCR), partial sequencing of the RNA polymerase 2 (RPB2) gene, random amplification of polymorphic DNA (RAPD), and analysis of vegetative compatibility (VC) types (Ma et al., 2001, 2003). Ma et al. (2001) demonstrated genetic stability and low genetic diversity of *B. dothidea* populations isolated from pistachios in California. In addition, higher genetic similarity was found within the *B. dothidea* populations from California than between Californian and non-Californian populations (Ma et al., 2003).

The aim of the present study was to develop microsatellite markers based on whole-genome sequence data, explore the genetic diversity and elucidate the potential introduction pathways of *B. dothidea* into Chinese vineyards using developed markers. To achieve these goals, we obtained *B. dothidea* isolates from grapevines as well as from apple, peach, pear, and tea plants from various regions in China. For comparison, representative isolates of *B. dothidea* from grapevines in Australia, Canada, Italy, and Thailand were also used. The main objectives of this study were to: (i) determine the genetic diversity within both individual subpopulations and the total *B. dothidea* samples; (ii) understand the relationships among *B. dothidea* isolates obtained from different hosts in China; and (iii) investigate the genetic relationships between the Chinese isolates and isolates from other countries. We hypothesised that the *B. dothidea* population is subdivided according to the climatological origin. We expected multiple origins of *B. dothidea* in Chinese vineyards, including host shifts in endemic genotypes and exotic introductions from other countries.

2. Materials and methods

2.1. Botryosphaeria dothidea isolates

B. dothidea isolates were collected from 18 grape-growing regions in China (Fig. 1A, Table S1). Samples were collected from symptomatic grapevine woods, exhibiting wedge-shaped cankers and shrivelled and rotten fruit clusters, as described by Yan et al. (2013). The isolates from apple, peach, pear, and tea plants were obtained from the culture collection of Institute of Plant and Environment Protection of Beijing Academy of Agriculture and Forestry Sciences (JZB culture collection). Additionally, *B. dothidea* isolates were obtained from different laboratories in Australia, Canada, Italy, and Thailand (Fig. 1B, Table S1).

According to the previous literature, *B. dothidea* shows variation in pathogenicity associated with different geographical origins (Manawasinghe et al., 2016). Therefore, we hypothesised that the *B. dothidea* population in China has differentiated into subpopulations according to the geographical origin of the fungi. Thus, the isolates obtained from Anhui (AH), Guangxi (GX), Hubei (HUB), Hunan (HUN), Jiangsu (JS), Sichuan (SC), Shanghai (SH) and Zhejiang (ZJ) provinces were grouped into the subtropical population (Fig. 1A green provinces). The isolates obtained from Beijing (BJ), Jilin (JL), Gansu (GS), Hebei (HEB), Henan (HEN). Liaoning (LN), Ningxia (NIX), Shandong (SD), Shanxi (SHX) and Tianjin (TJ) provinces were grouped into the temperate population (Fig. 1A blue provinces). Since the exact geographical origins of the isolates from different hosts and other countries were unknown, these isolates were included in the third population, referred to as the “other” population.

2.2. DNA extraction and PCR confirmation

The isolates were grown on potato dextrose agar (PDA) medium at 28 °C for 7–10 d. Initial species confirmation was performed by comparing basic morphological characters with the type specimen described by Phillips et al. (2013). Approximately 100 mg of mycelium was collected to extract DNA using the DNeasy Plant Mini Kit (QIAGEN GmbH, QIAGEN Strasse 1, 40742 Hilden, Germany). For species confirmation, the ITS (internal transcribed spacer) region was amplified using the ITS4 and ITS5 primers (White et al., 1990). PCR was carried out in a final volume of 25 μl. The PCR mixture consisted of 1 μl of genomic DNA, 0.3 μl TaKaRa Ex Taq DNA polymerase, 2.5 μl of 10 × Ex Taq buffer, 3.0 μl of dNTPs, 1 μl of each primer and 16.5 μl of ddH_20. The thermal cycler conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. The PCR products were visualised in 1% agarose gel stained with ethidium bromide, and sequencing was carried out by the Sun-biotech Company in Beijing, China. The sequencing results were subjected to searches in the NCBI database using the MegaBLAST tool for species confirmation. All the sequences were compared with the *B. dothidea* type (CBS115476) sequence. Isolates showing 99–100% query cover and more than 98–100% identity were used for genotyping.