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Short communication

Phylogenetic study of indigenous grapevine leaf rust fungi in North America and biological identity of an invasive grapevine leaf rust fungus in Brazil

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ABSTRACT

Two American grapevine leaf rust (GLR) fungi, *Phakopsora muscadiniae* and *P. uva*, were found to be phylogenetically different from Asian-Australasian GLR fungi. In the phylogram based on rDNA region sequences, a clade of the American fungi was sister to an inclusive clade of three autoecious species on *Meliosma* and a heteroecious species host-alternating between *Meliosma* and *Parthenocissus*. An invasive Brazilian GLR fungus (as *P. meliosmae-myrianthae*) was included in the Asian-Australasian GLR fungi clade, or the clade of the Thai population of *Phakopsora* sp. It could be assumed that the Brazilian GLR fungus originated from an inadvertently introduced Thai population.

Grapevine leaf rust (GLR) diseases are common in eastern Asia (Ono, 2000; Ono, Chatasiri, Pota, & Yamaoka, 2012) and have become emerging diseases in newly established vineyards in subtropical and tropical Asia (Pota et al., 2014) and South America (Bayer & Costa, 2006; Halefeld-Vieira, Nechet, & Barbosa, 2009; Ono, 2015; Primiano, Loehrer, Amorim, & Schaffrath, 2017; Ribeiro & Lima, 2007; Sonego, Garrido, & Gava, 2005; Tessmann, Dianese, Genta, Vida, & May-de Mio, 2004; Xavier, Dariva, Ribeiro, & Mizobutsi, 2012; de Souza, 2004). A GLR fungus was once detected in Northern Territory, Australia in 2001. However, intensive eradication programs succeeded to eliminate the fungus from Northern Territory in 2007 (International Plant Protection Convention, 2008). By contrast no GLR has been recorded in major grape producing regions in the wine belt in Europe and North America.

The name *Phakopsora ampelopsidis* Dietel & P. Syd. had been applied to a causative agent of GLR (Hiratsuka, 1935) until Ono (2000) clarified the biological and taxonomic relationships within the *P. ampelopsidis* species complex. *Phakopsora euvitis* Y. Ono [≡ *P. meliosmae-myrianthae* (Henn. & Shirai) Y. Ono] is now accepted as the real cause of the GLR in Asia. An additional rust fungus, *P. montana* Y. Ono & Chatasiri, host-alternating between *Meliosma tenuis* Maxim. and *V. coignetiae* Planch., was found to be pathogenic to grape cultivars in Japan (Ono et al., 2012). Grapevine leaf rust, widespread in Southeast Asia and Australasia, was once attributed to *P. euvitis* (Weinert, Shivas, Pitkethley, & Daly, 2003). However, Pota et al. (2014) showed that tropical Asian and Australasian GLR fungi were composed of three populations, which were distinct from the two temperate Asian GLR fungi.

In the Americas, Buriticá (1994) proposed a new species, *P. uva* Buriticá & J. F. Hennen (= *Uredo viala* Lagerh.), as a causal fungus of leaf rust of American bunch grapes in the genus *Vitis* subgenus *Vitis*. In addition, another American species, *P. muscadiniae* Buriticá (= *Uredo vitis* Thüm.), was described as a causal fungus of leaf rust of Muscadine grapes in the subgenus *Muscadinia* (Buriticá, 1999). Although the American GLR fungi were once identified as *P. euvitis* by Ono (2000), *P. uva* and *P. muscadiniae* are now accepted as distinct species.

In Brazil, a GLR fungus identified as *P. euvitis* (≡ *P. meliosmae-myrianthae*) was first recorded at a commercial vineyard of table grapes (*V. vinifera* L.) in Jandaia do Sul, northwestern Paraná State in March 2001 (Tessmann et al., 2004). Subsequent field surveys showed that the rust was also present in other counties of Paraná State. In 2003, the fungus was found also in Indaiatuba, Itupeva,

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Louveira and other areas of São Paulo State (Tessmann et al., 2004). According to Primiano et al. (2017), most cultivars of *V. labrusca* and *V. vinifera* in Brazil are susceptible to this fungus, and in highly susceptible cultivars the disease can affect 70% of the leaf area, resulting in early leaf drop (Angelotti, Regina, Buffara, Vieira, & Vida, 2014). Nogueira, Willocquet, and Amorim (2016) assumed that the early defoliation would result in a decline in vine vigor and reduce the fruit yield in subsequent years as shown for the GLR of a cultivar Delaware (*V. labrusca*) in Japan by Ozoe and Kadowaki (1971). Primiano et al. (2017) pointed out that the most important consequence of the invasion of the Asian GLR fungus into Brazil was an increase in frequency of fungicide treatments, particularly in cultivars of *V. labrusca*, stressing economic impacts by increasing control costs with the intensive fungicide spraying in the short term. The GLR is now spreading in large areas in Brazil and posing a serious threat to Brazilian viticulture. It is unlikely that the GLR fungus established in Brazil could be localized and controlled to prevent further spread in the Americas.

Despite their scientific and economic importance, biological and taxonomic identity of the fungi causing GLR in the Americas has undetermined and further clarification, with additional information on their genetic, morphological, ecological, and pathological properties and geographic distribution is needed. In this study, the GLR species in North America and Brazil were analyzed and compared to those distributed in Asia and Australasia, in order to clarify their phylogenetic relationships and taxonomic identities.

1. Molecular phylogenetic analysis

Dried specimens of rust-infected leaves of grapevine were used for molecular phylogenetic analyses (Table 1; Supplementary Table S1). DNA was extracted from uredinia and urediniospores produced on the leaves. Uredinia and urediniospores were suspended in 50 μL of DNA extraction buffer (10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% sodium dodecyl sulfate, 0.01% Proteinase K). The mixture was incubated at 37 °C for 60 min, then at 95 °C for 10 min. After being centrifuged at 15,000 rpm for 2 min, the aqueous phase was pipetted out and transferred to a new tube to collect total DNA.

The internal transcribed spacer (ITS) 2 rDNA and the large-subunit (LSU) rRNA gene (rDNA) D1/D2 regions were amplified by polymerase chain reaction (PCR) using primers Rust2inv (Aime, 2006) and NL4 (O'Donnell, 1993). PCR reaction was performed using 25 μL reaction volumes each containing: 1 μL genomic DNA, 12.5 μL of EmeraldAmp® PCR Master Mix (TaKaRa, Tokyo, Japan), 2.5 μL (0.2 μM) of each primer, and adding 6.5 μL distilled water to get 25 μL reaction volumes. PCR was carried out using a TaKaRa PCR Thermal Cycler Dice® Touch (TaKaRa, Tokyo) under the following protocol; 5 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 53 °C and 1 min at 72 °C, and a final step of 8 min at 72 °C. PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light. PCR products were purified using illustra ExoProStar (GE Healthcare UK, Little Chalfont, England) following the manufacturer's instructions. Sequencing was performed using the BigDye Terminator V3.1 Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions using the same primers used for PCR amplification and analyzed on an ABI 3500xL Genetic Analyzer (Applied Biosystems). Sequences were assembled and edited with BioEdit ver. 7.1.9 (Hall, 1999).

The DNA sequences were aligned using the MAFFT 7 with the G-INS-I option (Katoh & Standly, 2013). Sequences were manually edited when necessary using BioEdit ver. 7.1.9. All the sequences analyzed in this study have been deposited in the DNA Data Bank of Japan (DDBJ) as LC218323–LC218365 (Table 1; Supplementary Table S1).

Table 1
List of specimens of the Americas GLR fungi examined and accession numbers of ITS2-LSU rDNA D1D2 regions analyzed in this study.

Specimens	Species	Host plants	Collection sites	ITS2-D1D2 accession no
IBAR 10516	*Phakopsora muscadiniae*	*Vitis rotundifolia*	South Carolina, USA	LC218334
IBAR 10519	*P. muscadiniae*	*V. rotundifolia*	South Carolina, USA	LC218335
IBAR 10522	*P. muscadiniae*	*V. rotundifolia*	South Carolina, USA	LC218336
IBAR 10525	*P. muscadiniae*	*V. rotundifolia*	South Carolina, USA	LC218337
IBAR 10526	*P. muscadiniae*	*V. rotundifolia*	South Carolina, USA	LC218338
IBAR 10527	*P. muscadiniae*	*V. rotundifolia*	Georgia, USA	LC218339
IBAR 10533	*P. muscadiniae*	*V. rotundifolia*	Florida, USA	LC218340
IBAR 10546	*P. muscadiniae*	*V. rotundifolia*	Louisiana, USA	LC218341
IBAR 10547	*P. muscadiniae*	*V. rotundifolia*	Louisiana, USA	LC218342
IBAR 10548	*P. muscadiniae*	*V. rotundifolia*	Louisiana, USA	LC218343
IBAR 10551	*P. muscadiniae*	*V. rotundifolia*	Louisiana, USA	LC218344
IBAR 10611	*P. muscadiniae*	*V. rotundifolia*	Florida, USA	LC218345
IBAR 10628	*P. muscadiniae*	*V. rotundifolia*	Florida, USA	LC218346
IBAR 10631	*P. muscadiniae*	*V. rotundifolia*	Georgia, USA	LC218347
IBAR 10746	*P. muscadiniae*	*V. rotundifolia*	Florida, USA	LC218348
IBAR 10755	*P. muscadiniae*	*V. rotundifolia*	South Carolina, USA	LC218349
IBAR 10757	*P. muscadiniae*	*V. rotundifolia*	South Carolina, USA	LC218350
IBAR 10758	*P. muscadiniae*	*V. rotundifolia*	South Carolina, USA	LC218351
IBAR 10530	*P. uva*	*V. aestivalis*	Florida, USA	LC218352
IBAR 10534	*P. uva*	*V. aestivalis*	Florida, USA	LC218353
IBAR 10538	*P. uva*	*V. aestivalis*	Florida, USA	LC218354
IBAR 10540	*P. uva*	*V. aestivalis*	Florida, USA	LC218355
IBAR 10542	*P. uva*	*V. aestivalis*	Florida, USA	LC218356
IBAR 10544	*P. uva*	*V. aestivalis*	Florida, USA	LC218357
IBAR 10612	*P. uva*	*V. aestivalis*	Florida, USA	LC218358
IBAR 10613	*P. uva*	*V. aestivalis*	Florida, USA	LC218359
IBAR 10615	*P. uva*	*V. aestivalis*	Florida, USA	LC218360
IBAR 10617	*P. uva*	*V. aestivalis*	Florida, USA	LC218361
IBAR 10618	*P. uva*	*V. labrusca*	Florida, USA	LC218362
IBAR 10627	*P. uva*	*V. palmate*	Florida, USA	LC218363
IBAR 9676	*Phakopsora* sp.	*Vitis* sp.	Sao Paulo, Brasil	LC218364
IBAR 10578*	*Phakopsora* sp.	*Vitis* sp.	Sao Paulo, Brasil	LC218365

*IBAR 10578 is part of UB18453 that is the first GLR fungal specimen collected at Maringá, Paraná in Brazil in 2001.

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