J Gen Plant Pathol (2011) 77:178–181
DOI 10.1007/s10327-011-0303-4

FUNGAL DISEASES

Characterization of *Puccinia psidii* isolates in Hawaii using microsatellite DNA markers

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Received: 25 December 2010 / Accepted: 4 February 2011 / Published online: 8 March 2011

Abstract We studied genetic variation in *Puccinia psidii*, a newly introduced rust fungus in Hawaii, using microsatellite markers. All 36 Hawaiian *P. psidii* isolates analyzed had the same genotype at 11 microsatellite loci, while three genotypes were identified among four isolates from Florida and 11 genotypes among 18 isolates from Brazil. One of the isolates from Florida had 95% similarity to the Hawaiian isolates. We conclude that the genetically homogeneous *P. psidii* isolates in Hawaii might have derived from the same strain originally introduced into the Islands and are closely related to one of the strains found in Florida.

Keywords *Puccinia psidii* · Genetic diversity · Ohia plants · Rust · Microsatellite DNA marker · Simple sequence repeat

Puccinia psidii is a rust fungus that causes a disease commonly known as guava rust or eucalyptus rust. It was first observed on guava (*Psidium guajava* L.) in 1884 and later found on eucalypts (*Eucalyptus citriodora* Hook) in 1944 in Brazil (Coutinho et al. 1998). Since then, the fungus has spread to the Caribbean Islands and many other regions of the world, including South Africa (Coutinho et al. 1998), Florida (Marlatt and Kimbrough 1979; Rayachhetry et al. 1997), Hawaii (Uchida et al. 2006) and Japan (Kawanishi et al. 2009). At least 71 myrtaceous species have been documented as hosts of the fungus (Coutinho et al. 1998; Rayachhetry et al. 2001; Simpson et al. 2006). The wide host range of the rust fungus and its potential threats to many native or introduced species in Myrtaceae has become a major concern among many countries including New Zealand, Australia, South Africa and Brazil where Myrtaceae species are the major components in the forests and ecosystem (Coutinho et al. 1998; Glen et al. 2007; Tommerup et al. 2003).

In Hawaii, *P. psidii* was first found in 2005 when a young ohia plant covered with rust spores was sent to the University of Hawaii at Manoa for disease diagnosis (Killgore and Heu 2007). Based on host family associations, the rust pathogen was tentatively considered to be *P. psidii* (Killgore and Heu 2007). Further examination of the urediniospores and analysis of internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA) confirmed that the rust on the ohia plants was caused by *P. psidii* (Uchida et al. 2006). Since its first report on the Oahu Island, the rust disease has been observed on native ohia trees and other related species of Myrtaceae, including the endangered species *Eugenia koolauensis*, on several other major islands of Hawaii (Killgore and Heu 2007). Because native and non-native tree species of Myrtaceae have been planted widely for reforestation and commercial plantations in Hawaii, the economic and ecological effects of the disease to the region could be significant. However, lack of information about the origin and genetic variation of the pathogen population has hindered quanrantine measures in Hawaii. The main objective of this study was to characterize *Pucconia psidii* from Myrtaceae in Hawaii, USA, using the microsatellite (or simple sequence repeat, SSR) markers developed by Zhong et al. (2008).

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Table 1 Collection information for *Puccinia psidii* isolates used in the present study and their geographic and host origins

Isolate	Geographic origin	Host origin	Collector/provider	Year of collection
Hawaii01[a]	Oahu/HI/USA	*Metrosideros polymorpha*	Eloise Killgore	2005
Hawaii02	Oahu/HI/USA	*Metrosideros polymorpha*	Eloise Killgore	2006
Hawaii04	Oahu/HI/USA	*Metrosideros polymorpha*	Eloise Killgore	2006
Hawaii10	Oahu/HI/USA	*Syzygium jambos*	Eloise Killgore	2006
Hawaii12	Oahu/HI/USA	*Syzygium jambos*	Eloise Killgore	2006
Hawaii17	Oahu/HI/USA	*Myrtus communis*	Eloise Killgore	2006
Hawaii18	Oahu/HI/USA	*Melaleuca quinquenervia*	Eloise Killgore	2006
Hawaii19	Oahu/HI/USA	*Eugenia koolauensis*	Eloise Killgore	2006
Hawaii20	Oahu/HI/USA	*Metrosideros polymorpha*	Robert Hauff	2006
Hawaii22	Oahu/HI/USA	*Metrosideros polymorpha*	Robert Hauff	2006
Hawaii23	Oahu/HI/USA	*Metrosideros polymorpha*	Robert Hauff	2006
Hawaii24	Oahu/HI/USA	*Metrosideros polymorpha*	Janice Uchida	2006
Florida05	na/FL/USA	na	Min Rayachhetry	na
Florida06	na/FL/USA	na	Min Rayachhetry	na
Florida07	na/FL/USA	na	Min Rayachhetry	na
Florida08	na/FL/USA	na	Min Rayachhetry	na
Brazil13	na/na/Brazil	na	Acelino Alfenas	na
Brazil14	na/na/Brazil	na	Acelino Alfenas	na
Brazil15	na/na/Brazil	na	Acelino Alfenas	na
Brazil16	na/na/Brazil	na	Acelino Alfenas	na
UFV01	Itapetininga/SP/Brazil	*Eucalyptus*	Acelino Alfenas	2007
UFV03	Eunápolis/BA/Brazil	*Psidium guajava*	Acelino Alfenas	2007
UFV04	Vicosa/MG/Brazil	*Myrciaria cauliflora*	Acelino Alfenas	2007
UFV05	Tijuca/SC/Brazil	*Psidium guajava*	Acelino Alfenas	2007
UFV07	Valverde/MG/Brazil	*Eucalyptus*	Acelino Alfenas	2007
UFV08	Vicosa/MG/Brazil	*Psidium guajava*	Acelino Alfenas	2007
UFV09	Vicosa/MG/Brazil	*Syzygium jambos*	Acelino Alfenas	2007
UFV10	na	*Eucalyptus*	Acelino Alfenas	2007
UFV11	Aracruz/ES/Brazil	*Eucalyptus*	Acelino Alfenas	2007
UFV20	Cataguases/MG//Brazil	*Eucalyptus*	Acelino Alfenas	2007
UFV21	Vicosa/MG/Brazil	*Syzygium jambos*	Acelino Alfenas	2007
UFV22	Vicosa/MG/Brazil	*Psidium guajava*	Acelino Alfenas	2007
UFV23	Vicosa/MG/Brazil	*Psidium guajava*	Acelino Alfenas	2007
UFV24	Vicosa/MG/Brazil	*Syzygium jambos*	Acelino Alfenas	2007
Pp01	Kauai/HI/USA	*Syzygium jambos*	Rob Anderson	2006
Pp02	Kauai/HI/USA	*Myrtus*	Rob Anderson	2006
Pp03	Oahu/HI/USA	*Myrtus* sp.	Rob Anderson	2006
Pp04	Oahu/HI/USA	*Metrosideros polymorpha*	Rob Anderson	2006
Pp05	Oahu/HI/USA	*Metrosideros polymorpha*	Janice Uchida	2006
Pp06	Oahu/HI/USA	*Syzygium jambos*	Janice Uchida	2006
Pp07	Oahu/HI/USA	*Syzygium jambos*	Janice Uchida	2006
Pp08	Big Island/HI/USA	*Metrosideros polymorpha*	Anne Marie LaRosa	2007
Pp09	Big Island/HI/USA	*Metrosideros polymorpha*	Anne Marie LaRosa	2007
Pp10	Big Island/HI/USA	*Metrosideros polymorpha*	Anne Marie LaRosa	2007
Pp11	Big Island/HI/USA	*Metrosideros polymorpha*	Anne Marie LaRosa	2007
Pp12	Big Island/HI/USA	*Metrosideros polymorpha*	Anne Marie LaRosa	2007
Pp13	Big Island/HI/USA	*Syzygium jambos*	Anne Marie LaRosa	2007
Pp14	Big Island/HI/USA	*Syzygium jambos*	Anne Marie LaRosa	2007

Table 1 continued

Isolate	Geographic origin	Host origin	Collector/provider	Year of collection
Pp15	Big Island/HI/USA	*Syzygium jambos*	Anne Marie LaRosa	2007
Pp16	Big Island/HI/USA	*Syzygium jambos*	Anne Marie LaRosa	2007
Pp17	Big Island/HI/USA	*Syzygium jambos*	Anne Marie LaRosa	2007
Pp18	Big Island/HI/USA	*Syzygium jambos*	Anne Marie LaRosa	2007
Pp19	Big Island/HI/USA	*Syzygium jambos*	Anne Marie LaRosa	2007
Pp20	Big Island/HI/USA	*Syzygium jambos*	Anne Marie LaRosa	2007
Pp21	Big Island/HI/USA	*Syzygium jambos*	Anne Marie LaRosa	2007
Pp22	Big Island/HI/USA	*Syzygium jambos*	Anne Marie LaRosa	2007
Pp23	Big Island/HI/USA	*Melaleuca quinquenervia*	Anne Marie LaRosa	2007
Pp24	Big Island/HI/USA	*Melaleuca quinquenervia*	Anne Marie LaRosa	2007

na = data not available

[a] Hawaii01 was the isolate first recovered from an ohia plant infected by *P. psidii* in Hawaii in 2005

To obtain single-pustule-derived isolates, rust samples collected from three islands (Oahu, Kauai and Big Island) of Hawaii (Table 1) were used as sources of inoculum to inoculate paperbark plants (*Melaleuca quinquenervia*) using the method described by Rayachhetry et al. (2001). At 14 days after inoculation, single pustules were collected with a small cyclone collector (G-R Manufacturing, Manhattan, KS, USA) attached to a vacuum pump. The urediniospores from single pustules were suspended in Soltrol oil and used to reinoculate new plants to increase more urediniospores. Four isolates from Florida and 18 isolates from Brazil used in a previous study (Zhong et al. 2008) were also included in this study for comparison. Urediniospores of these *P. psidii* isolates were kindly provided by Dr. Acelino Alfenas (Department of Plant Pathology, Federal University of Viçosa, Viçosa, MG, Brazil) and Dr. Min Rayachhetry (USDA/ARS Aquatic Plant Control Laboratory at Fort Lauderdale, FL, USA), respectively.

We extracted DNA from the urediniospores (20–50 mg) or plant samples bearing uredinia (100–200 mg) using a FastDNA kit (MP Biomedicals, Solon, OH, USA) and a FastPrep Instrument (MP Biomedicals) according to the manufacturer's protocol. The DNA samples were subjected to microsatellite marker analysis using 11 (PpSSR12, 14, 18, 22, 78, 80, 136, 146, 161, 195, and 208) of the 15 primer pairs of Zhong et al. (2008), which consistently generated clear and specific amplicons in *P. psidii* during a preliminary test. For each primer pair, a M13 sequence (5′-CACGACGTTGTAAAACGAC) was added to the 5′ end of the forward primer during primer synthesis so that a fluorescent-labeled M13 primer could be included in PCR to generate PCR products for detection with a LI-COR 4300 DNA sequencer (LI-COR, Lincoln, NE, USA). PCR amplifications were performed in a thermocycler under the following conditions: 95°C for 5 min, 3 cycles at 95°C for 30 s, 50°C for 30 s, 72°C for 80 s; 33 cycles at 94°C for 15 s, 52°C for 15 s, 72°C for 45 s; followed by a 4°C holding step. Each PCR reaction contained 1× PCR buffer (10 mM Tris–HCl, 50 mM KCl), 200 μM of dCTP, dGTP, dTTP and dATP, 3.0 mM $MgCl_2$, 5 pmol M13 primer labeled by IRD700 or IRD800 at the 5′ end (LI-COR), 1 pMol 5′-tagged forward primer, 5 pMol reverse primer, 1 U *Taq* DNA polymerase (Bioline, Taunton, MA, USA) and 10 ng of genomic DNA in a total volume of 10 μL. The IRD700- or IRD800- labeled PCR products were diluted 10–20-fold, loaded onto a 7% polyacrylamide gel, and simultaneously detected with the LI-COR 4300 DNA system. The IRDye 700 or IRD800 size standard (50–700 bp, LI-COR) was also loaded on the gel to estimate the sizes of all alleles identified.

The alleles of each microsatellite locus amplified from individual isolates of *P. psidii* were scored based on their sizes in base pairs (bp) and designated as 0 or 1 according to the absence or presence of each allele in the isolates. Genetic similarity between isolates was measured as coefficients using the SimQual program of NTSYSpc version 2.021 (Exeter Software, Setauket, NY, USA). The unweighted pair group method of arithmetic averages (UPGMA) (SAHN program, NTSYSpc version 2.021) was used to construct a dendrogram representing genetic similarities based on the microsatellite data. To estimate the strength of the groupings generated by the cluster analysis, we conducted a bootstrap analysis with 500 replications using the WinBoot computer program (Nelson et al. 1994).

Our microsatellite analysis revealed a high level of genetic diversity in the population of *P. psidii*. A total of 45 polymorphic alleles were scored at the 11 microsatellite loci for the 58 *P. psidii* isolates analyzed. The number of alleles varied at different loci, ranging from 2 to 7 alleles. Cluster analysis indicated that the *P. psidii* isolates were clustered into three clades (I, II, and III) at 65% similarity

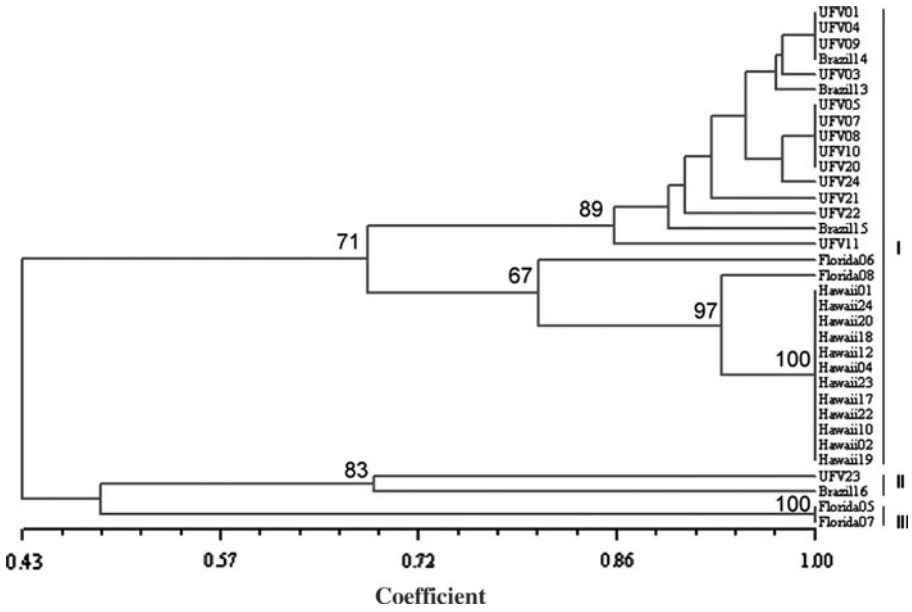

Fig. 1 A dendrogram of 34 isolates of *Puccinia psidii* generated by cluster analysis with the unweighted pair group method with arithmetic averages. A total of 45 alleles from 11 microsatellite loci were used in the analysis. Since the Hawaiian isolates have the same genotype at the 11 loci, only 12 of the 36 isolates are shown in the dendrogram. The numbers at the nodes of major clusters represent bootstrap values generated by 500 replications using the WinBoot program (Nelson et al. 1994)

(Fig. 1). The grouping was supported by the bootstrap analysis with bootstrap values ranging from 71 to 100. On the basis of the 11 microsatellite loci analyzed, we identified 15 genotypes among the 58 *P. psidii* isolates from Florida, Brazil and Hawaii. However, genetic diversity differs among the three regions. In Hawaii, no genetic variation was detected among the 36 isolates analyzed at the 11 microsateliite loci, suggesting that they may have originated from the same strain that was first introduced into Hawaii. The introduction probably has occurred very recently because the rust was not found before 2005 (Killgore and Heu 2007). In Brazil where the fungus is native, the genetic diversity was high; 11 genotypes were identified among the 18 samples analyzed. Although only four isolates from Florida were analyzed, three distinct genotypes were identified, indicating that at least three different strains occur in this region.

In this study, no isolates from Florida or Brazil were found to be exactly the same as those from Hawaii. However, one of the isolates from Florida (Florida08) is genetically close to the Hawaiian isolates with a similarity coefficient of 95% (Fig. 1). It is possible that the Hawaiian isolates were derived from a strain closely related to Florida08. Because only four isolates from Florida were analyzed and the collections from Brazil were not comprehensive, we still do not know whether the Hawaiian strain originated from Florida or some other place. Analysis of *P. psidii* isolates collected from all the regions where the rust commonly occurs using the microsatellite markers will provide more information on the origin, genetic diversity and population structure of the rust fungus.

Acknowledgments We thank Robert Anderson, Robert Hauff, Anne Marie LaRosa, Eloise Killgore, Janice Uchida, Min Rayachhetry, Les Szabo, and Acelino Alfenas for providing spores of *Puccinia psidii* or their DNA samples for this study.

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