Puccinia psidii infecting cultivated *Eucalyptus* and native Myrtaceae in Uruguay

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Abstract

Eucalyptus or guava rust caused by *Puccinia psidii* is a serious disease of *Eucalyptus* and other Myrtaceae. In Uruguay, it has been previously found on *Eucalyptus globulus* and *Psidium brasiliensis*. Almost nothing is known regarding the occurrence of this pathogen on other *Eucalyptus* species or native Myrtaceae in that country. In this study, we determined the presence of *P. psidii* on *Eucalyptus* species and native Myrtaceae trees in Uruguay and evaluated the pathogenicity of specimens from native myrtaceous hosts on *E. globulus* and *E. grandis*. Phylogenetic analyses based on the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA operon were used to confirm pathogen identity. Comparisons of ITS sequences confirmed the identity of *P. psidii* on *Eucalyptus globulus*, *E. grandis*, *Myrcianthes pungens*, and *Myrrhinium atropurpureum* var. *octandrum*. This is the first report of *P. psidii* on *M. atropurpureum* var. *octandrum*. Pathogenicity tests showed that isolates from native Myrtaceae could infect both *Eucalyptus* species tested, indicating a strong biological relationship between both introduced and native Myrtaceae. This study supplies relevant field data, morphological information, molecular phylogenetic analyses and infection studies that contribute to a better understanding of an important and little studied pathogen.

Introduction

The guava or *Eucalyptus* rust, *Puccinia psidii* Winter, was first found in 1884 on *Psidium guajava* L. (syn. *Psidium pomiferum* L.) in Brazil (Winter 1884) and was discovered on non-native eucalypts (*Corymbia citriodora* (Hook) Hill & Johnson syn: *Eucalyptus citriodora* Hook), in the same country in 1944 (Joffily 1944). This was the first record of the rust having undergone a host jump from a native to a non-native tree (De Castro *et al*. 1983). Subsequent to its first discovery, *P. psidii* has been recorded on many species in the Myrtaceae from the Americas, Hawaii and more recently in Japan (Acuña and Garran 2004; Alfenas *et al*. 2004; Dianese *et al*. 1984; Ferreira 1981, 1983; Kawanishi *et al*. 2009; MacLachlan 1938; Marlatt and Kimbrough 1979; Rayachhetry *et al*. 2001; Uchida *et al*. 2006; Walker 1983).

Puccinia psidii is considered a devastating pathogen of *Eucalyptus* in Brazil, causing severe damage on *Eucalyptus* trees younger than 2 years old (Alfenas *et al*. 2004; Coutinho *et al*. 1998). This rust is unique because of its exceedingly wide host range, for which Simpson *et al*. (2006) cite 71 host species. Its wide host range and aggressiveness on certain hosts make this rust a major threat to *Eucalyptus* and other Myrtaceae throughout the world (Coutinho *et al*. 1998; Glen *et al*. 2007; Grgurinovic *et al*. 2006; Langrell *et al*. 2008).

In Uruguay, *P. psidii* was first found on *Psidium brasiliensis* L. (Koch de Brotos *et al*. 1981). The rust has been more recently reported on plantation-grown *Eucalyptus globulus* Labill. subsp. *globulus* (hereafter *E. globulus*) where it caused severe damage to 1-year-old trees (Telechea *et al*. 2003). This was the first record of *P. psidii* on *E. globulus* and it raised concerns that the rust could threaten the important *Eucalyptus* forestry industry in Uruguay.

Little is known of the occurrence of *P. psidii* on cultivated *Eucalyptus* spp. or native Myrtaceae trees in Uruguay. For this reason, information on its host range on these taxa represents a fundamental requirement to develop an effective disease management program. Therefore, the aim of this study was to determine the occurrence of *P. psidii* on *Eucalyptus* species and native Myrtaceae trees in Uruguay. In addition, the pathogenicity of isolates obtained from native myrtaceous hosts on the two most important *Eucalyptus* spp. planted in Uruguay, *E. globulus* and *E. grandis* (Hill) Maiden, was evaluated.

Materials and methods

Rust collections

During 2005 to 2007, *Eucalyptus* plantations and natural forest were examined throughout Uruguay for rust pustules. Surveys included sites randomly selected in the provinces of Canelones, Durazno, Florida, Lavalleja, Maldonado, Paysandú, Río Negro, Rivera, Tacuarembó and Treinta y Tres. Each sampling site represented a location where Eucalyptus plantations and native trees were close to each other (<500 m). Each site was visited at least twice during this study and each visit was conducted during a different season to avoid season-associated variation as well as to insure the greatest diversity of fungi where obtained. A total of 22 Myrtaceae species native to Uruguay and 12 species of *Eucalyptus* were examined (Table 1). Only 22 out of 35 native Myrtaceae species were found on the sampled sites, the others were either not geographically located in the regions where *Eucalyptus* was planted or if present, they were in very low frequency and not found during the surveys.

Samples of infected leaves were collected in plastic bags, and transported in a cooler at 8°C to the laboratory. Each rust sample was divided in the laboratory, where a small amount of leaf tissue bearing pustules was dried in small paper envelopes for later analysis. Urediniospores were collected from fresh pustules and stored at −80°C in glass capsules until they could be used in pathogenicity tests.

Table 1. List of native and non-native Myrtaceae that were sampled and provinces where each host species was examined.

Myrtaceae species native to Uruguay	Provinces where each host was examined	Cultivated Myrtaceae	Provinces where each host was examined
Acca sellowiana	Rivera, Tacuarembó, Lavalleja, Treinta y Tres	*Eucalyptus bicostata*	Río Negro
Agariota eucalyptides	Rivera	*Eucalyptus camaldulensis*	Durazno, Paysandú, Río Negro, Treinta y Tres
Blepharocalyx salicifolius	Durazno, Florida, Lavalleja, Maldonado, Paysandú, Río Negro, Rivera, Tacuarembó, Treinta y Tres	*Eucalyptus cinerea*	Paysandú, Tacuarembó
Calyptranthes concinna	Rivera, Treinta y Tres	*Eucalyptus dunnii*	Durazno, Florida, Paysandú, Río Negro, Tacuarembó
Eugenia involucrata	Tacuarembó	*Eucalyptus ficifolia*	Paysandú
Eugenia mansonii	Durazno, Rivera, Tacuarembó	*Eucalyptus globulus*	Canelones, Durazno, Florida, Lavalleja, Maldonado, Paysandú, Río Negro, Tacuarembó
Eugenia repanda	Lavalleja, Río Negro, Treinta y Tres	*Eucalyptus grandis*	Durazno, Paysandú, Río Negro, Rivera, Tacuarembó
Eugenia uniflora	Durazno, Florida, Tacuarembó, Treinta y Tres, Rivera	*Eucalyptus maidenii*	Durazno, Lavalleja, Paysandú, Río Negro
Eugenia uruguayensis	Durazno, Paysandú, Río Negro, Rivera, Tacuarembó	*Eucalyptus robusta*	Tacuarembó
Gomidesia palustris	Rivera, Trienta y Tres	*Eucalyptus saligna*	Paysandú
Hexachlamis edulis	Paysandú, Río Negro	*Eucalyptus tereticornis*	Durazno, Florida, Lavalleja, Paysandú, Río Negro, Rivera
Myrceugenia euosma	Rivera, Tacuarembó	*Eucalyptus viminalis*	Lavalleja
Myrceugenia glaucescens	Durazno, Lavalleja, Maldonado, Paysandú, Río Negro, Rivera, Tacuarembó, Treinta y Tres	*Syzygium jambos*	Canelones
Myrcianthes cisplatensis	Durazno, Maldonado, Paysandú, Río Negro, Rivera, Tacuarembó, Treinta y Tres		
Myrcianthes gigantea	Treinta y Tres		
Myrcianthes pungens	Paysandú, Rivera, Tacuarembó, Treinta y Tres		
Myrciaria tenella	Lavalleja, Maldonado, Rivera		
Myrrhinium atropurpureum var. octandrum	Durazno, Lavalleja, Maldonado, Paysandú, Rivera, Tacuarembó		
Psidium cattleianum	Treinta y Tres		
Psidium luridum	Rivera		
Psidium incanum	Rivera		
Psidium pubifolium	Paysandú, Rivera		

Those species on which rust infections were observed are in bold

Rust morphology

Teliospores and urediniospores were compared using standard light microscope techniques. Teliospores were germinated on a slide with free water for 180 min and observed under the microscope to examine promycelia and cell number. In addition, urediniospore morphology was observed using a Hitachi S-3500 N Variable Pressure Scanning Electron Microscope (SEM) at the Imaging Center, College of Biological Science, University of Minnesota. For each sample, spores were attached to stubs with a thin layer of adhesive. Stubs were coated with gold and placed in the low-vacuum, variable pressure Environmental SEM and photographed with a digital camera at approximately ×2,000 magnification.

DNA extraction, PCR, sequencing and phylogenetic analysis

DNA was extracted from dried infected host leaf tissue (~20 mg) containing uredinial pustules. Dried host tissue with spores was shaken in tubes with sterile 1-mm glass beads (Lysing matrix C; Bio 101, Carlsbad, CA, USA) and 25 mg of sterile diatomaceous earth (Sigma-Aldrich, St. Louis, MO, USA) in a Savant FastPrep shaker (FP120; Holbrook, NY, USA) for 20 s at a speed setting of 5 (Zambino 2002). DNA extraction was performed using OmniPrepTM DNA Extraction Kit (Biosciences, Saint Louis, MO) following the manufacturer's instructions.

The internal transcribed spacer region of the ribosomal DNA (ITS) was amplified using primers ITS-1F (5' CTT GGT CAT TTA GAG GAA GTA A 3') and ITS-RUST1 (5' GCT TAC TGC CTT CCT CAA TC 3') (Kroop et al. 1995). Primers PR1 (5' AAA TCG TAA CAA GGT TTC CG 3') and PR2 (5' TAA GTT CAG CAG GTA GTC CC 3') (Langrell *et al*. 2008) were used for those samples for which no PCR product was obtained with the former pair of primers. Polymerase Chain Reaction (PCR) was performed in a 50-µl reaction mixture of 5.0 µl of 0.05% casein, 5.0 µl of 10X PCR Buffer, 1.5 µl of 50 mM $MgCl_2$, 1.0 µl of 10 mM dNTPs, 1.0 µl of 20 mM ITS-1F, 1.0 µl of 20 mM ITS-RUST1, 0.2 µl of Platinum Taq Polymerase, 30.3 µl of ddH_2O, 5.0 µl of DNA template. PCR amplifications were performed in a MJ Research PTC 200 DNA Engine Thermal Cycler PCR (MJ Research, Reno, NV) with the following parameters: an initial denaturation step of 2 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 44°C, 2 min at 72°C and final extension of 10 min at 72°C; hold at 4°C.

PCR products were visualized by 1.5% agarose gel electrophoresis, purified and prepared for sequencing using EXO-SAP-IT PCR clean-up kit (USB, Cleveland, OH, USA) following the manufacturer's instructions. Sequencing reactions were performed using the same primers as those for the PCR and the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Forest City, CA, USA) on an ABI Prism 377 automated DNA sequencer. Sequences were obtained in both directions and assembled using ChromasPro software (Technelysium, Eden Prairie, MN, USA). Assembled sequences were subject to BLAST searches in the NCBI GenBank. Phylogenetic analysis was performed to confirm species identification. Thus, *Puccinia psidii* sequences available in GenBank were downloaded along with sequences of the rust species that showed the closest match with *P. psidii*. Following a preliminary phylogenetic analysis, the alignment was trimmed leaving only representative species of closest related taxa (Table 3). *Phakopsora pachyrhizi* was chosen as the outgroup taxon. Multiple sequence alignments were made online using the E-INS-i strategy in MAFFT version 6 (Katoh *et al*. 2005).

Phylogenetic analysis was performed using PAUP Version 4.0b10 (Swofford 2002) for maximum parsimony analysis and Mr. Bayes v3.1.2 (Ronquist and Huelsenbeck 2003) for Bayesian analysis. Maximum parsimony analysis was performed using the heuristic search option with simple taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. Support for the nodes of the shortest trees was determined by analysis of 1,000 bootstrap replicas (Hillis and Bull 1993). Tree length (TL), consistency index (CI), retention index (RI), and homoplasy index (HI) were calculated.

The best nucleotide substitution model for the Bayesian analysis was determined using MrModeltest v2.2 (Nylander 2004). The general time reversible substitution model including a proportion of invariant sites and gamma-distributed substitution rates of the remaining sites (GTR + I + G) was selected using AIC. Two independent runs each using four MCMC chains starting from a random tree topology were run over 10 million generations. Trees were sampled every 100th generation and the "burnin" was set at 6,000 generations after which the likelihood values were stationary. To obtain the estimates for the posterior probabilities, a 50% majority rule consensus of the remaining 99,941 trees was computed from a total of 199,882 sampled trees. Bayesian analysis was repeated three times, showing identical tree topology, indicating that topology was independent from priors. Results for one out of the three replicates were randomly selected for presentation.

Pathogenicity tests

To assess pathogenicity of the rust samples collected on native Myrtaceae trees to *Eucalyptus*, *E. globulus* and *E. grandis* seedlings were inoculated with a suspension of urediniospores from each of the two rust collections (UY 220 and UY221) under controlled conditions. Three clones of *E. globulus* (A, B and C) and three clones of *E. grandis* (D, E and F) were inoculated with each rust

sample, using the urediniospores that were collected from fresh pustules and that had been stored at −80°C. In addition to *Eucalyptus*, *Syzygium jambos* (L.) Alston plants were inoculated, since this tree species has been shown to be highly susceptible to *P. psidii* and it is frequently used for inoculum preservation and multiplication (Junghans *et al*. 2003).

One 4-month-old seedling of each host was inoculated with each rust sample. Inoculation was conducted using a pipette to apply five drops of suspension per leaf on five leaves per plant. Each drop was approx. 20 µl of a spore suspension with 5 × 104 urediniospores/ml. Inoculated plants were incubated 24 h in a mist chamber at 25°C in the dark and then transferred to a growth chamber at 22°C with 12-h photoperiod, at 40 µmol photons/s/m^2 of light intensity. Twelve days later, plants were evaluated for the presence of rust pustules. Those plants showing negative results (i.e., no pustules) were evaluated again 21 days post-inoculation to confirm the absence of pustules. DNA was extracted from urediniospores present on pustules from inoculated plants, sequenced as described above, and compared with the inoculated specimen-sequence to confirm its identity. Pathogenicity tests were replicated once.

Results

Samples collected

Rust on native trees was very rare and after examining several trees of native Myrtaceae species during 2 years of surveys, this rust was found only on *Myrrhinium atropurpureum* Schott var. *octandrum* Benth and *Myrcianthes pungens* (Berg) Legrand. Rust pustules were also observed on *Eucalyptus globulus* and *Eucalyptus grandis* plantations. All diseased *Eucalyptus* trees were 1 year old, whereas the native trees with disease symptoms were adult specimens of unknown age. Rust infections were also observed on *E. globulus* and *E. grandis* cuttings in two nurseries located in Paysandú and Canelones, respectively. One sample collected on *S. jambos* from a nursery in Canelones was also included in this study (Table 2).

Table 2. Host, location and date of collection of specimens analyzed in this study.

Rust ID	Host	Location	Age of the host	Date of collection
UY217	*Eucalyptus grandis*	Tacuarembó, 31°41'S, 55°57'W	Re-growth, 1 year old	11/18/05
UY220	*Myrrhinium atropurpureum var. octandrum*	Tacuarembó, 31°35'S, 55°47'W	Adult tree	11/18/05
UY221	*Myrcianthes pungens*	Tacuarembó, 31°33'S 55°43'W	Adult tree	11/18/05
UY894	*E. globulus*	Lavalleja, 34°20'S, 55°09'W	1 year old	05/11/06
UY895	*E. globulus*	Maldonado, 34°19'S, 54°44'W	1 year old	04/05/06
UY1371	*E. grandis*	Paysandú, 32° 15'S, 58°05'W	4 months old cutting in a nursery	01/16/07
UY1372	*E. grandis*	Río Negro, 32° 25'S, 57°22'W	2 years old	01/22/07
UY1374	*E. globulus*	Canelones, 34° 40'S, 56°20'W	4 months old cutting in a nursery	01/30/07
UY1375	*E. globulus*	Canelones, 34° 40'S, 56°20'W	4 months old cutting in a nursery	01/30/07
UY1731	*E. grandis*	Canelones, 34° 40'S, 56°20'W	4 months old cutting in a nursery	12/04/07
UY1732	*Syzygium jambos*	Canelones, 34° 40'S, 56°20'W	1 year old cutting in a nursery	12/04/07

Symptoms and morphology

Similar symptoms were observed on different hosts infected with *P. psidii*. Lesions were primarily observed on young tissues such as actively growing leaves and shoots (Fig. 1). Bright orange pustules with orange-yellow urediniospores were present on all evaluated hosts, but dark orange-brown teliospores were observed only on *E. globulus* and *E. grandis*. Gray discoloration of old lesions was observed on E. grandis, and shoot tips were dead on *E. grandis* and *M. atropurpureum* var. *octandrum* (Fig. 1). Teliospores were similar to those reported by Walker (1983), roughly ellipsoidal to cylindrical to broadly clavate, one-septate, constricted at the central septum, 26–42 ×

15–22 µm, with the upper cell generally slightly wider and shorter than the lower, wall pale golden yellow, pore apical in the upper cell and just below the septum in the lower cell, pedicels either deciduous or short (up to 15 µm long). However, in sample UY895 teliospores had pedicels of up to 25 µm long (Fig. 2a). Germinated teliospores produced a four-celled basidium with four basidiospores (Fig. 2b).

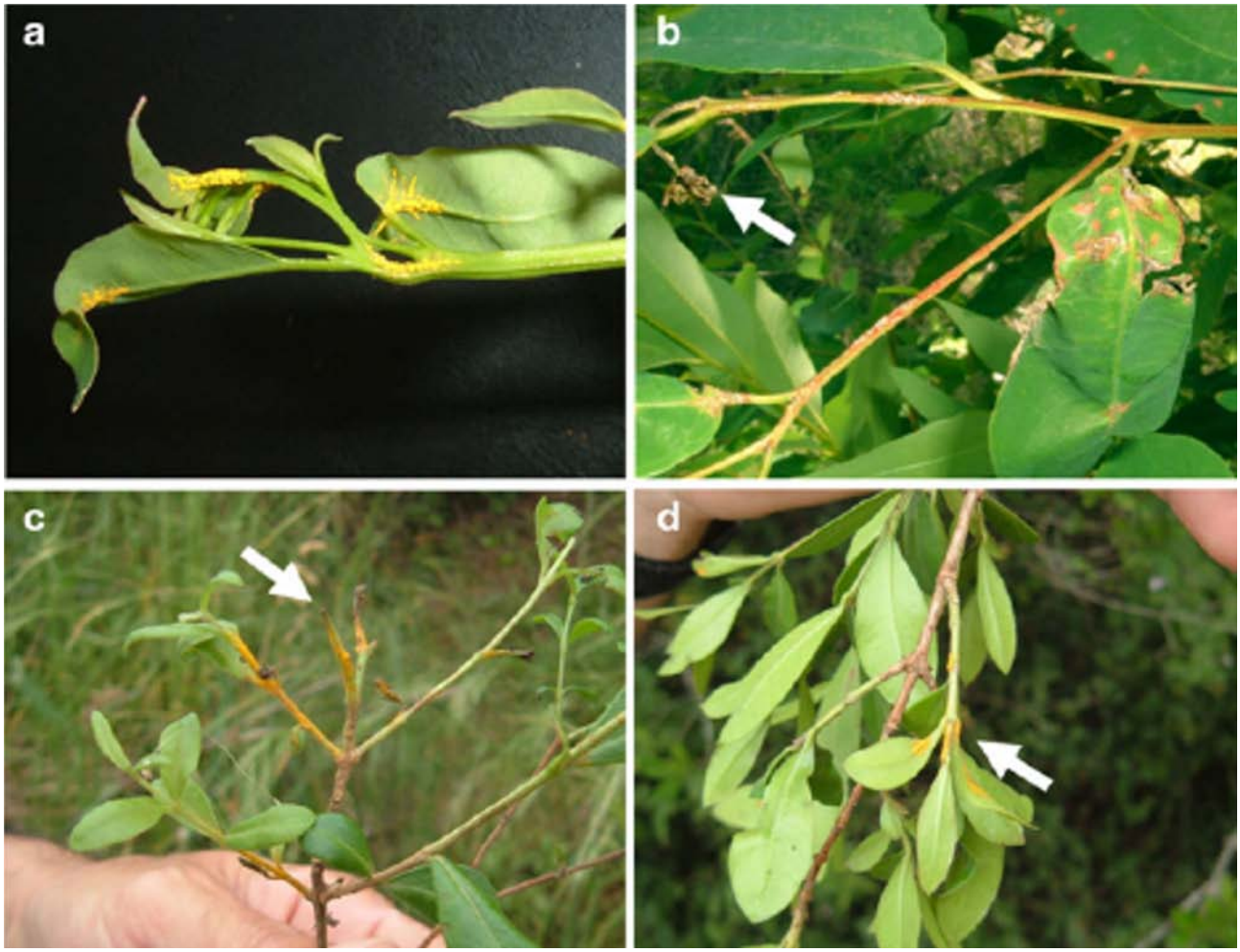


Figure 1. Symptoms of *Eucalyptus* rust on different hosts. **a** Young lesions on *E. grandis*, the pustules are bright orange on young tissue. **b** Old lesions on *E. grandis*, grey discoloration on leaves and twigs and dead shoot tip. **c**,**d** Pustules on twigs, leaves and petioles of *Myrrhinium atropurpureum* var. *octandrum* appear bright orange. Trees also have dead shoot tips. Arrows show areas of dying shoot tips and location of orange urediniospores on infected branches.

Urediniospores examined from each specimen showed a high level of similarity in spore size, spine density and spine distribution on the spore surface. Urediniospores were observed in all collected samples. They were of 19–26 x 15–22 µm, yellow, unicellular, spherical to elliptical, base truncate, finely and uniformly echinulate with spines up to 1 µm long, 0.5–1.5 µm apart. In some urediniospores, a bald patch without spines was observed (Fig. 2c–f).

Phylogenetic analysis

DNA fragments of approximately 640 bp were amplified for all specimens. Sequences were deposited in GenBank and accession numbers are shown in Table 3. The ITS dataset consisted of 34 ingroup sequences plus *Phakopsora pachirhizi* used as the outgroup taxon. Aligned DNA sequences of 596 total characters included the complete ITS region (ITS1, 5.8 S and ITS2 regions), of which 245 were constant, 53 variable characters were parsimony-uninformative and 298 were parsimony informative. Maximum parsimony and Bayesian analyses resulted in trees of identical topology.

The heuristic search analysis of the data resulted in 2 most parsimonious trees (TL=733 steps; CI= 0.748; RI=0.910; HI=0.252). The phylogram obtained from the Bayesian analysis is shown in Fig. 3. The aligned sequence data were deposited in TreeBASE (ID 10699).

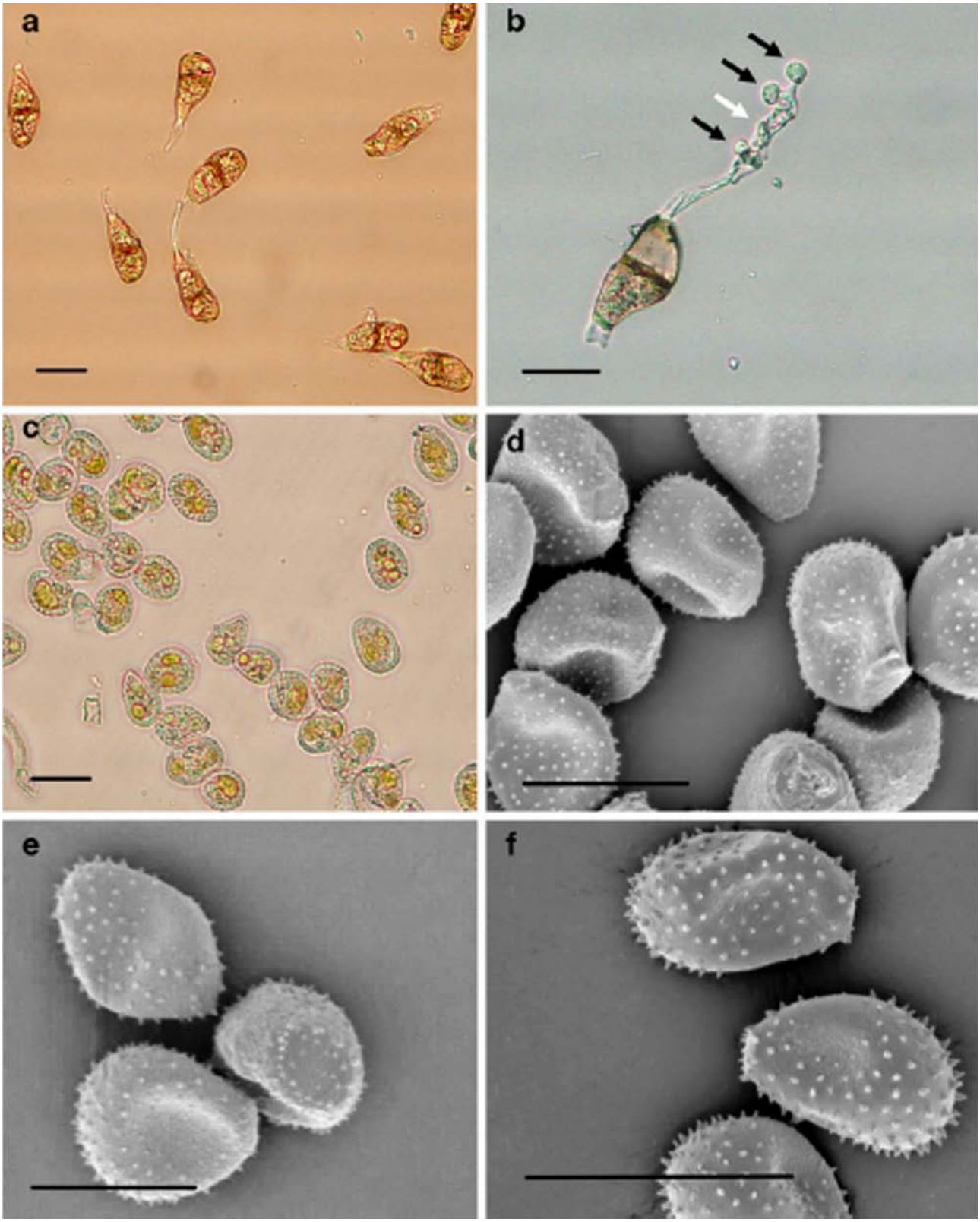


Figure 2. a–c Light micrographs of teliospores and urediniospores of *P. psidii*. **a** Teliospores observed in sample UY895 with most characteristics as previously described by Walker (1983). However, some spores display a pedicel up to 25 μm long. **b** Germinated teliospore. Black arrows indicate each basidiospore and the white arrow indicates the location where the fourth basidiospore had been ejected. **c** Urediniospores from sample UY217. **d–f** Scanning electron micrographs of

gold-coated urediniospores of P. psidii collected on **d** *E. grandis* (UY217), **e** *Myrrhinium atropurpureum* var. *octandrum* (UY220), and **f** *Myrcianthes pungens* (221). Bars 20 μm

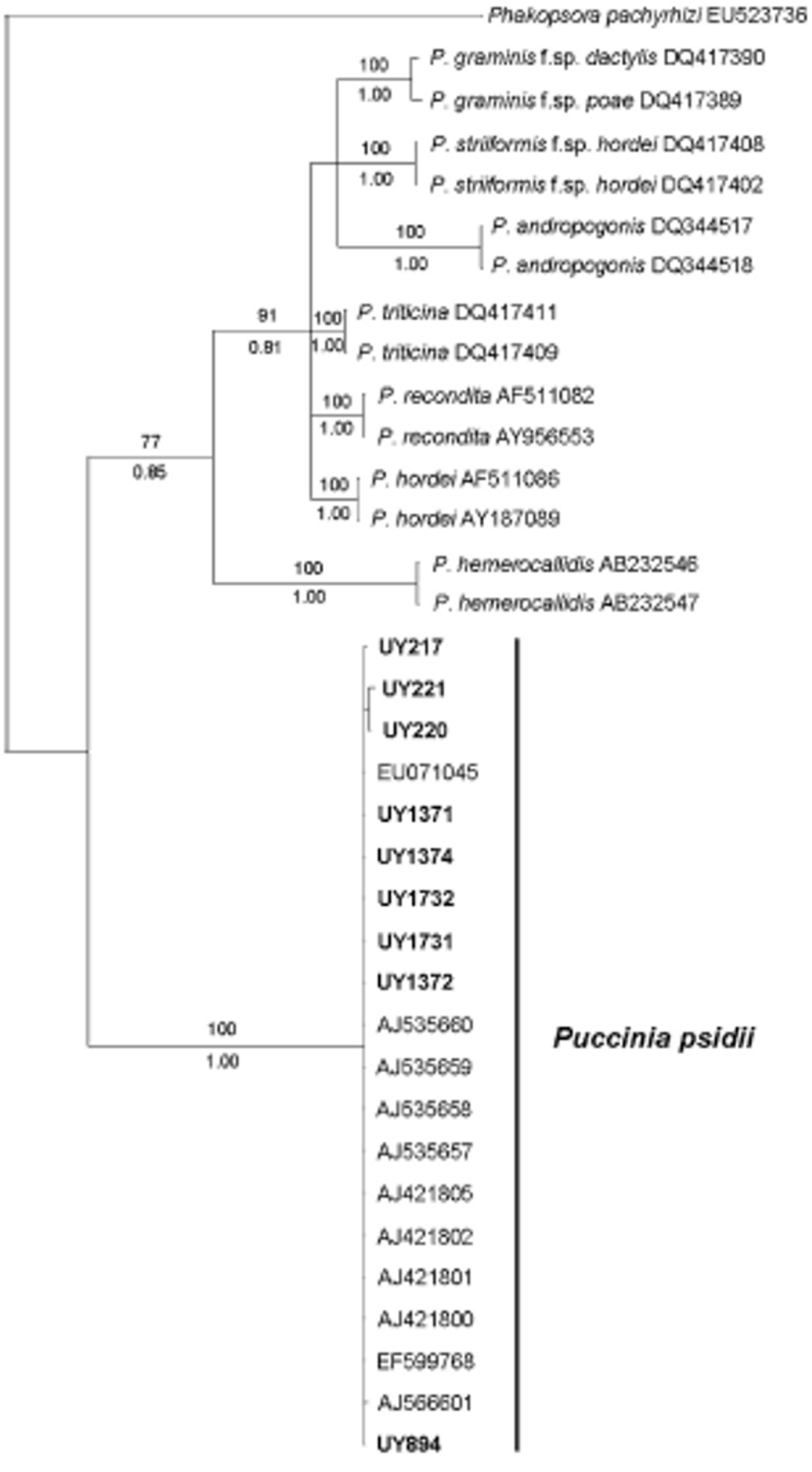
Table 3. List of sequences used in this study, including those for which sequences were obtained from GenBank.

Collection ID no.	Rust	Host species	Location[a]	GenBank accession no.	Reference
UY217[b]	***Puccinia psidii***	***Eucalyptus grandis***	**Tacuarembó, Uruguay**	**EU348742**	**This study**
UY220	***P. psidii***	***Myrrhinium atropurpureum* var. *octandrum***	**Tacuarembó, Uruguay**	**EU439920**	**This study**
UY221	***P. psidii***	***Myrcianthes pungens***	**Tacuarembó, Uruguay**	**EU439921**	**This study**
UY894	***P. psidii***	***Euc. globulus***	**Maldonado, Uruguay**	**EU348743**	**This study**
UY1371	***P. psidii***	***Euc. grandis***	**Paysandú, Uruguay**	**FJ710803**	**This study**
UY1372	***P. psidii***	***Euc. grandis***	**Río Negro, Uruguay**	**FJ710804**	**This study**
UY1374	***P. psidii***	***Euc. globulus***	**Canelones, Uruguay**	**FJ710805**	**This study**
UY1731	***P. psidii***	***Euc. grandis***	**Canelones, Uruguay**	**FJ710807**	**This study**
UY1732	***P. psidii***	***Syzygium jambos***	**Canelones, Uruguay**	**FJ710808**	**This study**
E-UFV8	*P. psidii*	*Euc. grandis*	Espírito Santo, Brazil	AJ535660	Langrell et al. 2008
MG27	*P. psidii*	*Eugenia uniflora*	Minas Gerais, Brazil	AJ421801	Langrell et al. 2008
MG32	*P. psidii*	*Melaleuca quinquinervia*	Minas Gerais, Brazil	AJ421802	Langrell et al. 2008
USA2	*P. psidii*	*Mel. quinquinerva*	Florida, USA	AJ535658	Langrell et al. 2008
SZ2.18	*P. psidii*	*Mel. quinquinervia*	Hawaii, USA	EU071045	Langrell et al. 2008
n/a	*P. psidii*	*Metrosideros polymorpha*	Hawaii, USA	EF599768	Uchida et al., 2006
MG63	*P. psidii*	*Myrcia jaboticaba*	Minas Gerais, Brazil	AJ421805	Langrell et al. 2008
USA3	*P. psidii*	*Pimenta dioca*	Florida, USA	AJ535659	Langrell et al. 2008
SC1	*P. psidii*	*Psidium guajava*	Santa Catarina, Brazil	AJ536601	Langrell et al. 2008
UFV18	*P. psidii*	*S. jambos*	Minas Gerais, Brazil	AJ421800	Langrell et al. 2008
USA1	*P. psidii*	*S. jambos*	Florida, USA	AJ535657	Langrell et al. 2008
HSZ0219	*P. andropogonis*	n/a	-	DQ344517	Szabo 2006
HSZ0027	*P. andropogonis*	n/a	-	DQ344518	Szabo 2006
HSZ0928	*P. graminis* f.sp. *dactylis*	*Dactylis glomerata*	-	DQ417390	Barnes and Szabo 2007
HSZ0929	*P. graminis* f.sp. *poae*	*Poa pratensis*	-	DQ417389	Barnes and Szabo 2007
IBA8759	*P. hemerocallidis*	n/a	-	AB232547	Chatasiri et al. 2006
IBA8749	*P. hemerocallidis*	n/a	-	AB232546	Chatasiri et al. 2006
CDL22/81	*P. hordei*	n/a	-	AY511086	Anikster et al. 2004
CDL64-2B	*P. hordei*	n/a	-	AY187089	Anikster et al. 2004
11506 F	*P. recondita*	n/a	-	AY956553	Abbasi et al. 2004
ANK77081	*P. recondita*	*Triticum turgidum*	-	AF511082	Barnes and Szabo 2007
HSZ0711	*P. striiformis* f. sp. *hordei*	*Hordeum vulgare*	-	DQ417402	Barnes and Szabo 2007
PSH13	*P. striiformis* f. sp. *hordei*	*Hor. vulgare*	-	DQ417408	Barnes and Szabo 2007
HSZ0741	*P. triticina*	*T. aestivum*	-	DQ417409	Barnes and Szabo 2007
HSZ0741	*P. triticina*	*T. aestivum*		DQ417411	Barnes and Szabo 2007
Brazil-1	*Phakopsora pachyrhizi*	*Glycine max*	-	EU523736	Silva et al. 2008

[a] Location only indicated for *P. psidii* collections

[b] Specimens sequenced in this study are shown in bold

Phylogenetic analysis showed a high level of similarity among the nine samples and they grouped together with ITS sequences of *P. psidii* obtained from GenBank while also clearly separated from the most closely related species for which sequences have been published. Minor variation in the analyzed ITS regions was observed among the sequences of the six samples collected on *Eucalyptus* spp. and the one collected on *S. jambos*. The only change observed was in the sequence UY1372, which showed ambiguity at the position 396 of the alignment with a double peak of guanine and adenosine. In contrast, the two samples collected on the native myrtaceous trees displayed most variation (5 changes) in the ITS2 region. The sequence of UY220 collected from *Myrrhinium atropurpureum* var. *octandrum* showed an insertion of a guanine in the position 311 of the alignment, and substitutions of guanine instead of adenosine in two different positions


Phakopsora pachyrhizi EU523736
100
1.00
P. graminis f.sp. dactylis DQ417390
P. graminis f.sp. poae DQ417389
100
1.00
P. striiformis f.sp. hordei DQ417408
P. striiformis f.sp. hordei DQ417402
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P. andropogonis DQ344517
P. andropogonis DQ344518
91
0.91
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P. triticina DQ417411
P. triticina DQ417409
100
1.00
P. recondita AF511082
P. recondita AY956553
77
0.85
100
1.00
P. hordei AF511086
P. hordei AY187089
100
1.00
P. hemerocallidis AB232546
P. hemerocallidis AB232547
UY217
UY221
UY220
EU071045
UY1371
UY1374
UY1732
UY1731
UY1372
100
1.00
AJ535660
AJ535659
AJ535658
AJ535657
AJ421805
AJ421802
AJ421801
AJ421800
EF599768
AJ566601
UY894
Puccinia psidii
0.05

Figure 3. Phylogram obtained from the Bayesian analysis based on the ITS region indicates the phylogenetic relationship among rust sequences obtained from rust on *Eucalyptus* and native Myrtaceae trees in Uruguay (labeled UY and in bold), *P. psidii* and other related rusts. Bootstrap values of 1,000 replicates of maximum parsimony (>75%) and posteriori probabilities are shown below and above branches, respectively. The tree was rooted to *Phakopsora pachyrhizi*.

(i.e. position 396 and 453, respectively). On the other hand, DNA sequence of UY221 from *Myrcianthes pungens* showed the same substitution in the position 396 plus insertions of one adenosine and one thiamine at the positions 503 and 530, respectively.

Pathogenicity tests

The two rust samples collected from native Myrtaceae (UY220 and UY 221) that were used in the pathogenicity tests were able to infect and produce new uredinial pustules on the different clones of *E. globulus*. However, UY220 was able to sporulate only on *E. grandis* clones D and F, and no infection was observed on *E. grandis* clone E. *Syzygium jambos* showed no signs of infection by either rust isolate used in the inoculations (Table 4).

Table 4. Results of pathogenicity tests performed on three clones of E. globulus and E. grandis as well as S. jambos inoculated with the two rust samples collected from native Myrtaceae trees (UY220 and UY221).

	Eucalyptus globulus			*Eucalyptus grandis*			*Syzygium jambos*
Rust ID	A[a]	B	C	D	E	F	NN[b]
UY220	+	+	+	+	-	+	-
UY221	+	+	+	-	-	-	-

+ and - indicate presence/absence of pustules 12 days post-inoculation

[a] code of the clone

[b] *NN* No name

Although severity of infection was not specifically assessed, clear differences in number and size of pustules were observed between rust samples on different clones of *E. globulus*, clone A was just slightly infected by both rust samples, clone C was more severely infected by UY220 but slightly infected by UY221 and clone B was slightly infected by UY220 and moderately infected by UY221.

Discussion

This study has led to the discovery of two previously unknown native Myrtaceae hosts of *P. psidii* in Uruguay. They further provide conclusive evidence based on DNA sequence comparisons that the rust fungus occurs on native Myrtaceae in the country and that it is the same fungus that is found on non-native *Eucalyptus* spp. in plantations. DNA-based evidence for these findings is supported by morphological characteristics of the fungus. The results have also shown, for the first

time, that rust isolates from native trees can infect *Eucalyptus* spp. in Uruguay.

Previously, *P. psidii* in Uruguay has been reported on *Psidium brasiliensis* (Koch de Brotos *et al*. 1981) and *Eucalyptus globulus* (Balmelli et al. 2004; Telechea *et al*. 2003). In this study, we found the fungus on *Eucalyptus globulus*, *E. grandis*, *Myrcianthes pungens*, and *Myrrhinium atropurpureum* var. *octandrum* although infections were not abundant. Finding *P. psidii* on native trees and the scarcity of infections observed support the view that the fungus is native in Uruguay. If so, it would be under strong ecological homeostasis. Uruguay has 35 native species of Myrtaceae (Brussa and Grela 2007) and we expect that many of these trees could be hosts to this rust with its unusually broad host range (Simpson *et al*. 2006). It has undergone significant host shifts to non-native trees such as *Eucalyptus* (Coutinho *et al*. 1998; Slippers *et al*. 2005) and *Metrosideros* (Uchida *et al*. 2006). Confirmed by morphological characteristics and phylogenetic comparisons, this is the first report of *P. psidii* on *M. atropurpureum* var. *octandrum* and the first report of *P. psidii* on these two native hosts in Uruguay, although it has been previously reported on *M. pungens* in Brazil (Hennen *et al*. 2005).

Symptoms on both *Eucalyptus* spp. and native Myrtaceae trees were consistent with those described previously (Alfenas *et al*. 2004; Old *et al*. 2003). The profuse production of teliospores observed in this study under field conditions suggests that *P. psidii* is a heteroecious macrocyclic rust for which the alternate aecial host is unknown. This view was also proposed by Simpson *et al*. (2006). However, it is possible that *P. psidii* is apomictic, since aecia and aeciospores have been observed after inoculations with basidiospores on *Eucalyptus* and *Syzygium jambos* (Ferreira 1989; Figueiredo *et al*. 1984).

Even though we did not examine the number of nuclei present in each basidiospore produced from germinated teliospores, four basidiospores were produced from each teliospore and we expect that these would give rise to monokaryotic basidiospores. Alfenas *et al*. (2004) made a similar observation, and it raises a question about when it undergoes dikaryotization. Pycnia have never been observed in *P. psidii* and the stage where dikaryon formation takes place has yet to be discovered.

Results of this study provide strong preliminary evidence that *P. psidii* is genetically diverse in Uruguay. Although the sample size was relatively small, DNA sequence data showed that isolates are genetically different. Furthermore, pathogenicity tests with different *P. psidii* isolates also suggested differences in the susceptibility of *Eucalyptus* hosts. Further studies will be needed to determine whether this represents intraspecific variation in the ITS region or whether *P psidii*, comprises several cryptic species. Genetic variation based on much larger collections of isolates should be undertaken to better understand the population genetics of this pathogen and thus differences in resistance and susceptibility that were observed in *Eucalyptus* clones.

An interesting observation in this study was that *Syzygium jambos* was not infected in pathogenicity tests. This tree is one of the hosts most susceptible to *P. psidii* elsewhere in the world (Junghans *et al*. 2003). Physiological variability is known in *P. psidii* and characterization of different physiological groups (or biotypes) based on cross-inoculations have been described previously (Aparecido *et al*. 2003; Coelho *et al*. 2001; De Castro *et al*. 1983). Lack of susceptibility to isolates of *P. psidii* in *S. jambos* emphasizes the fact that the pathogen is physiologically variable in Uruguay and that it is most likely native to the area in which it was discovered in this study. This is likely to complicate *Eucalyptus* forestry in Uruguay and it will mean that screening of clones will need to include the breadth of variability of the rust. It will also be important to understand the population structure of the rust to allow the development of effective breeding programs that will minimize the economic impact of *P. psidii* in Uruguay.

Acknowledgements

We acknowledge the financial and logistic support of Forestal Oriental, Rivermol, Stora Enso and Weyerhaeuser Uruguay, as well as Eufores and Forestal Oriental for supplying clones for inoculation. We also thank Andrés Berrutti, Brett Arenz, Charles Barnes, Gustavo Balmelli, Jason Smith, Kim Nguyen and Les Szabo for their assistance and discussions on various aspects of these investigations.

References

Abbasi M, Hedjaroude G, Scholler M, Goodwin SB (2004) Taxonomy of *Puccinia striiformis* s.l. in Iran. Rustaniha 5:199–224

Acuña M, Garran SM (2004) Detección de *Kirramyces epicoccoides*, *Puccinia psidii* y *Coniothyrium zuluense*, agentes causales de enfermedades en *Eucalyptus* spp. en la zona de Concordia, Entre Ríos, Argentina. Revista INTA Argentina 33:135–148

Alfenas AC, Zauza EA, Mafia RG, Assis TF (2004) Clonagem e Doenças do Eucalipto. Universidade Federal do Viçosa, Brazil

Anikster Y, Szabo LJ, Eilam T, Manisterski J, Koike ST, Bushnell WR (2004) Morphology, life cycle biology, and DNA sequence analysis of rust fungi on garlic and chives from California. Phytopathology 94:569–577

Aparecido CC, Figueiredo MB, Furtado EL (2003) Grupos de variabilidade fisiológica em populações de *Puccinia psidii*. Summa Phytopathol 29:234–238

Balmelli G, Marroni V, Altier N, Garcia R (2004) Potencial del mejoramiento genético para el manejo de enfermedades en *Eucalyptus globulus*. INIA, Serie Técnica 143

Barnes CW, Szabo LJ (2007) Detection and identification of four common rust pathogens of cereals and grasses using real-time polymerase chain reaction. Phytopathology 97:717–727

Brussa CA, Grela IA (2007) Flora arbórea del Uruguay con énfasis en las especies de Rivera y Tacuarembo. COFUSA, Mosca, Uruguay

Chatasiri S, Kitade O, Ono Y (2006) Phylogenetic relationships among *Puccinia hemerocallidis*, *P. funkiae*, and *P. patriniae* (Uredinales) inferred from ITS sequence data. Mycoscience 47:123–129

Coelho L, Alfenas AC, Ferreira FA (2001) Physiologic variability of *Puccinia psidii* – the rust of *Eucalyptus*. Summa Phytopathol 27:295–300

Coutinho TA, Wingfield MJ, Alfenas AC, Crous PW (1998) *Eucalyptus* rust: a disease with the potential for serious international implications. Plant Dis 82:819–825

De Castro HA, Krugner TL, Ideriha CF, Cappello MS, Marchi AB (1983) Inoculação cruzada de *Eucalyptus*, goiaba (*Psidium guajava*) e jamboeiro (*Syzygium jambos*) com *Puccinia psidii*. Fitopatol Bras 8:491–497

Dianese JC, Moraes TS, Silva AR (1984) Response of *Eucalyptus* species to field infection by *Puccinia psidii*. Plant Dis 68:314–316

Ferreira FA (1981) Ferrugem do eucalipto-ocurrência, temperatura para germinaçâo de uredosporos, produçâo de teliosporos, hospedeiro alternativo e resistencia. Fitopatol Bras 6:603–604

Ferreira FA (1983) Ferrugem do eucalipto. Revista Árvore 7:91–109

Ferreira FA (1989) Patología Forestal. Principais doenças florestais no Brazil. Sociedade de Investigaçòes Florestais, Brazil

Figueiredo MB, Coutinho LN, Hennen JF (1984) Estudos para determinação do ciclo vital de *Puccinia psidii* Winter. Summa Phytopathol 10:53–54

Glen M, Alfenas AC, Zauza EAV, Wingfield MJ, Mohammed C (2007) *Puccinia psidii*: a threat to the Australian environment and economy – a review. Australas Plant Pathol 36:1–16

Grgurinovic CA, Walsh D, Macbeth F (2006) *Eucalyptus* rust caused by *Puccinia psidii* and the threat it poses to Australia. EPPO Bulletin 36:486–489

Hennen JF, Figueiredo MB, de Carvalho AA, Hennen PG (2005) Catalogue of the species of plant rust fungi (Uredinales) of Brazil. Instituto de Pesquisas, Jardim Botânico do Rio de Janeiro, Brazil

Hillis DM, Bull JJ (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst Biol 42:142–152

Joffily J (1944) Ferrugem do eucalipto. Bragantia 4:475–487

Junghans DT, Alfenas AC, Maffia LA (2003) Escala de notas para quantificação da ferrugem em *Eucalyptus*. Fitopatol Bras 28:184–188

Katoh K, Kuma K, Toh H, Miyata T (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res 33:511–518

Kawanishi T, Uematsu S, Kakishima M, Kagiwada S, Hamamoto H, Horie H, Namba S (2009) First report of rust disease on ohia and the causal fungus, *Puccinia psidii*, in Japan. J Gen Plant Pathol 75:428–431

Koch de Brotos L, Boasso O, Riccio de Machado C, Gandolfo Antunez C (1981) Enfermedades de las plantas, hongos superiores y saprofitas en Uruguay. Departamento de Comunicaciones, Dirección de Sanidad Vegetal, Ministerio de Agricultura y Pesca, Uruguay

Kroop BR, Albee S, Flint KM, Zambino P, Szabo LJ, Thomson SV (1995) Early detection of systematic rust infections of Dyers Woad (*Isatis tinctori* L.) using polymerase chain reaction. Weed Sci 43:467–472

Langrell SRH, Glen M, Alfenas AC (2008) Molecular diagnosis of *Puccinia psidii* (guava rust) – a quarantine threat to Australian eucalypt and Myrtaceae biodiversity. Plant Pathol 57:687–701

MacLachlan JD (1938) A rust of the pimento tree in Jamaica. Phytopathology 28:157–170

Marlatt RB, Kimbrough JW (1979) *Puccinia psidii* on pimento dioica in South Florida. Plant Dis 63:510–512

Nylander JA (2004) MrModeltest v2.2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden Old KM, Wingfield MJ, Yuan ZQ (2003) A manual of diseases of Eucalyptus in South-East Asia. Center for International Forestry Research, Jakarta, Indonesia

Rayachhetry MB, Van TK, Center TD, Elliott ML (2001) Host range of *Puccinia psidii*, a potential biological control agent of *Melaleuca quinquenervia* in Florida. Biol Control 22:38–45

Ronquist F, Huelsenbeck JP (2003) MrBayes3: Bayesian phylogenetic inference under mixed models. Biometrics 19:1572–1574

Silva DCG, Yamanaka N, Brogin RL, Arias CAA, Nepomuceno AL, Di Mauro AO, Pereira SS, Nogueira LM, Passianotto ALL, Abdelnoor RV (2008) Molecular mapping of two loci that confer resistance to Asian rust in soybean. Theor Appl Genet 117:53–63

Simpson JA, Thomas K, Grgurinovic CA (2006) Uredinales species pathogenic on species of Myrtaceae. Australas Plant Pathol 35:549–562

Slippers B, Stenlid J, Wingfield MJ (2005) Emerging pathogens: fungal host jumps following anthropogenic introduction. Trends Ecol Evol 20:420–421

Swofford DL (2002) PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4.0b10a. Sinauer Associates, Sunderland, Mass.

Szabo LJ (2006) Deciphering species complexes: *Puccinia andropogonis* and *Puccinia coronata*, examples of differing modes of speciation. Mycoscience 47:130–136

Telechea N, Rolfo M, Coutinho TA, Wingfield MJ (2003) *Puccinia psidii* on *Eucalyptus globulus* in Uruguay. Plant Pathol 52:427

Uchida J, Zhong S, Killgore E (2006) First report of a rust disease on ‘Ohi'a caused by *Puccinia psidii* in Hawaii. Plant Dis 90:524

Walker J (1983) Pacific Mycogeography: deficiencies and irregularities in the distribution of plant parasitic fungi. Aust J Bot Suppl Ser 10:89–136

Winter G (1884) Repertorium. Rabenhorstii fungi europaei et extraeuraopaei. Cent. XXXI et XXXII. Hedwigia 23:164–172

Zambino EJ (2002) Dry grinding at near-ambient temperatures for extracting DNA from rust and other fungal spores. Biotechniques 33:48–51