Polymorphisms of the ITS region of *Inocutis jamaicensis* associated with *Eucalyptus globulus, Vitis vinifera* and native plants in Uruguay

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Eucalyptus globulus was introduced in Uruguay from Australia and planted on former prairie soils, sometimes nearby evergreen shrubs or small trees growing on low hills and riparian forests. *Inocutis jamaicensis* is associated with lesions of variable length and width on the stem of standing *E. globulus* trees and *Vitis vinifera* plants. The natural distribution of *I. jamaicensis* is restricted to North and South America where the fungus is colonising several native plants. Thus, it was hypothesised that the occurrence of this fungus on *E. globulus* and vines originated from a host jump from native plants. The aim of this work was to evaluate the genetic variation among *I. jamaicensis* isolates collected from *E. globulus, V. vinifera* and native plants in Uruguay and to detect possible host preferences of different isolates. The ITS1-5.8S-ITS2 rDNA region was amplified by PCR and then digested with four endonucleases which allow to differentiate eight RFLP patterns. There were no significant differences in allelic frequencies among isolates collected from different hosts. There was no indication of host specificity, but a support of the hypothesis that basidiospores originating from basidiomata on native plants can colonize *E. globulus, V. vinifera* and vice versa.

Keywords: white rotting fungi, stem cankers, genetic variation.

Uruguayan eucalypt plantations cover in total an area of approximately 474 000 hectares, 270 000 ha are planted with *Eucalyptus globulus* Labill. (Balmelli *et al.* 2004). This species was introduced to Uruguay from Australia in 1853 (Brussa 1994) and it was planted on former prairie soils, sometimes near evergreen shrubs or small trees on low hills, riparian and ravine forest (Bettucci *et al.* 2004). Currently, *E. globulus* is planted in Uruguay due to its fast growth rate and an advantageous wood quality for pulping proposes (Speranza 2003). It is supposed that forests will be expanded in the

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coming years in accordance with the favourable legislation and the installation of pulping factories in this country.

During the last years, lesions of variable length and width on the stem of standing *E. globulus* trees were observed mainly in plantations in the south-east of Uruguay. Using morphological characteristics Martínez (2005) has identified the basidiomycete fungus associated with these lesions as *Inocutis jamaicensis* (Murrill) Gottlieb, J.E. Wright and Moncalvo, previously named *Inonotus jamaicensis* Murrill (Gilbertson & Ryvarden 1987), Hymenochaetaceae (Hymenochaetales). The geographic distribution of this species is restricted to North America and South America from the south of Arizona to the south of Argentine, colonizing several native plants of different plant families (i.e. Asteraceae, Anacardiaceae, Fabaceae, Rhamnaceae, Timelaceae and Sapindaceae) (Martínez 2005). Moreover, *I. jamaicensis* can also be found on stems of old grapevines in Argentine (Lupo *et al.* 2006). In these plants an infestation with this fungus is associated with foliar symptoms known as "hoja de malvón" (Gatica *et al.* 2000; Lupo *et al.* 2006).

Polymorphisms in the ITS region within single individual strains have been reported from many basidiomycetes (eg. Aanen *et al.* 2001, Kauserud & Schumacher 2002). Secondary mycelium (isolated from fruit bodies) of basidiomycetes are characterized by the presence of complementary mating nuclei originated from primary mycelia. Since the rDNA region is composed of several tandem repeats, detected polymorphisms in the secondary mycelium can be due to polymorphism within one nucleus or due to different alleles in the two nuclei of dikaryotic mycelium (Aanen *et al.* 2001). In a previous work, it could be demonstrated that polymorphisms in ITS sequences of *I. jamaicensis* are caused by differences between the two nuclei in the secondary mycelium (Lupo *et al.* 2006). The mixed RFLP patterns observed in heterokaryotic isolates segregated in the homokaryotic progeny in Mendelian ratio (Lupo *et al.* 2006). Taking into consideration that *I. jamaicensis* is not present in Australia, where *Eucalyptus* trees are native, it was hypothesized that infections of *E. globulus* and grape vines originated from a host jump from American native plants. The aim of this work was to evaluate the genetic variation among *I. jamaicensis* isolates collected from native plants, *E. globulus* plantations and vineyards in Uruguay in order to understand if there are any host preferences of diferent isolates.

Materials and Methods

Fungal isolates

Fruiting bodies of *I. jamaicesis* (Fig. 1) were collected in autumn 2003 and 2004 from commercial *Eucalyptus globulus* plantations in

Fig. 1. Basidiomata of *Inocutis jamaicensis* on *Eucaluptus globulus* stem.

different regions of Uruguay (Table 1). In addition, fruiting bodies were collected from *Acacia longifolia* (a naturalized Fabaceae in Uruguay) and from the native species, *Baccharis dracunculifolia* (Asteraceae), *Eupatorium buniifolium* (Asteraceae), *Heterothalamus alienus* (Asteraceae); *Daphnosis racemosa* (Timelaceae), *Dodonaea viscosa* (Sapindaceae), *Lithraea brasiliensis* (Anacardiaceae), *Parkinsonia aculeata* (Fabaceae) and *Scutia buxifolia* (Rhamnaceae). All

Tab. 1. – Occurrence of alleles in isolates of *I. jamaicencis* from different hosts.

Host	**Isolate**	**Endonuclease**			
		Hae III	*Hha* I	*Taq* I	*Alu* I
Acacia longifolia	MVHC 11507	AA	BB	AB	AA
Acacia longifolia	MVHC 11505	AB	BB	AA	AA
Acacia longifolia	MVHC 11508	AB	AA	AB	AA
Baccharis dracunculifolia	MVHC 11729	AA	BB	AB	AA
Baccharis dracunculifolia	MVHC 11733	AA	AA	BB	AB
Baccharis dracunculifolia	MVHC 11730	AB	AB	AB	AB
Daphnosis racemosa	MVHC 11504	AB	AA	AB	AB
Dodonaea viscosa	MVHC 11404	AB	BB	AA	AA
Dodonaea viscosa	MVHC 11392	AB	AA	AB	AA
Eupatorium bunifolium	MVHC 11474	BB	BB	AA	AA
Eupatorium bunifolium	MVHC 11707	BB	BB	AA	AA
Eupatorium bunifolium	MVHC 11712	BB	BB	AA	AA
Eupatorium bunifolium	MVHC 11710	BB	AB	AB	AB
Eupatorium bunifolium	MVHC 11711	BB	AB	AB	AB
Eupatorium bunifolium	MVHC 11704	AB	AB	AB	AA
Eupatorium bunifolium	MVHC 11709	AB	AB	AB	AA
Eupatorium bunifolium	MVHC 11714	AB	AB	AB	AA
Heterothalamus alienus	MVHC 11395	AB	BB	AB	AA
Lithraea brasiliensis	MVHC 11506	AB	AA	AB	BB
Parkinsonia aculeata	MVHC 11501	AB	AA	AB	AB
Scutia buxifolia	MVHC 11502	BB	AA	AB	AB
Scutia buxifolia	MVHC 11503	AB	AB	AB	AB
Vitis vinifera	MVHC 11909	BB	BB	AA	AA
Vitis vinifera	MVHC 11919	AA	AB	AB	AA
Vitis vinifera	MVHC 11913	AA	AA	BB	AB
Vitis vinifera	MVHC 11914	AA	AB	AB	AB
Vitis vinifera	MVHC 11917	AB	BB	AB	AA
Vitis vinifera	MVHC 11918	AB	AA	AB	BB
Vitis vinifera	MVHC 11910	AB	AA	BB	AB
Vitis vinifera	MVHC 11915	AB	AB	AA	AA
Vitis vinifera	MVHC 11911	AB	AB	AA	AB
Vitis vinifera	MVHC 11912	AB	AB	AA	AB
Eucalyptus globulus	MVHC 11383	BB	AB	AA	AB
Eucalyptus globulus	MVHC 11469	AA	AA	BB	BB
Eucalyptus globulus	MVHC 11394	AA	AA	BB	AB
Eucalyptus globulus	MVHC 11415	AA	AA	BB	AB
Eucalyptus globulus	MVHC 11391	AB	BB	AA	AA
Eucalyptus globulus	MVHC 11297	AB	AB	BB	AB
Eucalyptus globulus	MVHC 11421	AB	AB	AA	AA
Eucalyptus globulus	MVHC 11386	AB	AB	AA	AB
Eucalyptus globulus	MVHC 11426	AB	AB	AA	AB
Eucalyptus globulus	MVHC 11390	AB	AB	AB	AB
Eucalyptus globulus	MVHC 11408	AB	AB	AB	AB

of these species occur within and around the *E. globulus* plantations. Finally, in autumn 2005, fruiting bodies were collected from a vineyard in the south of Uruguay. A total of 22 isolates from native plants, 11 isolates from *E. globulus* and 10 isolates from *V. vinifera* from different provinces of Uruguay were studied (Table 1).

Fungal isolates (tissue cultures) were obtained from basidiomata and cultivated on malt extract agar (MEA) at 25 °C. All cultures were deposited in the MVHC fungal collection (Laboratorio de Micología, Facultad de Ciencias – Ingeniería, Universidad de la República, Montevideo, Uruguay).

DNA extractions and PCR reactions

Total DNA was obtained from fresh aerial mycelium grown on MEA. DNA extractions were conducted as described previously by Lupo *et al.* (2006). The entire ITS1-5.8S-ITS2 rDNA region was amplified using the universal primer pair ITS1F – ITS4 (Gardes & Bruns 1993, White *et al.* 1990). PCR was performed in 25 µL volumes containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ (pH 8.3); 50 µM of each deoxiribonucleotide triphosphate; 1 µM of each primer; and 1 unit of Taq polymerase (ATGen) on a Gene-tech SPCR1 MKII – Termoblock. The following cycling parameters were used: 35 cycles of 70 s at 94 °C, 45 s at 52 °C and 90 s at 72 °C preceded by 5 min at 94 °C and an extension step of 72 ćC for 5 min.

RFLP analyses.

Aliquots of 10 µl of the amplified DNA were digested directly without further purification. Each sample was digested with 2 units of *Alu* I, *Hae* III, *Hha* I and *Taq* I (BioLabs) in single-enzyme essays. Each restriction digestion was conducted twice. The restriction fragments were separated by electrophoresis in 2 % (w/v) agarose gel (USB), stained with ethidium bromide (10 µg μL^{-1}), visualized and photographed under UV light.

Each RFLP pattern was considered as one allele and arbitrarily named using the name of the enzyme followed by a capital letter as subscripts. In this way homozygous isolates were coded as AA or BB and heterozygous as AB. Allelic frequencies were calculated using PopGene version 1.31 software (Yeh *et al.* 1997). Chi-square tests were performed to test significant differences in allelic frequencies across the isolates associated with different hosts.

Results

A fragment of approximately 850 bp was obtained from the amplification of the entire ITS1-5.8S-ITS2 rDNA region of all 43

isolates. When this fragment was digested with each of the four endonucleases, two banding patterns ('alleles') were obtained for each enzyme. Using the endonuclease *Hae* III a single fragment of 850 pb (Hae $_A$) was observed in some isolates which corresponded to the absence of any restriction site. The second allele, named Hae $_B$, consisted of two bands of 650 pb and 200 pb (Fig. 2, Table 1). Hha $_A$ corresponded to 3 bands of approximately 100, 350 and 400 bp and Hha $_B$ corresponded to 4 bands of 100, 150, 250 and 350 bp. Taq $_A$ corresponded to 3 bands of 75, 350 and 425 bp and Taq $_B$ corresponded to 4 bands of 75, 200, 225 and 350 bp. Alu $_A$ corresponded to 3 bands of 75, 175 and 600 bp and Alu $_B$ with 2 bands of 250 and 600 bp.

The two complementary mating nuclei in the secondary mycelium showed identical or different banding patterns. Every single allele was observed in double dose (homozygosis; Fig. 2 lanes 7 and 8, Table 1) single dose respectively (heterozygosis; Fig. 2 lane 9, Table 1). As expected, in heterozygotic isolates the sum of restriction fragments is longer than the original PCR product without digestion.

No specific alleles were associated with any host; moreover every single allele was present in isolates from every host (*E. globulus, V. vinifera* and native plants). In addition, no significant differences in allele frequencies ($p < 0.05$) were found either among the isolates collected from different hosts or from any host with respect to the entire population (Table 2). A total of 20 genotypes were observed in the entire population when combining the eight alleles. Seven genotypes were found only once, all others from twice to four times. Summarising, the same genotype was found in different hosts and no host-genotype relationship was observed.

Tab. 2. – Distribution of the allele frequencies across the *I. jamaicensis* population associated with different hosts.

Endonuclease	**Alleles**	**Allelic frequencies**				χ^2 *	***P***
		Native plants	*V. vinifera*	*E. globulus*	Entire population		
Hae III	A	0.43	0.60	0.59	0.51		
	B	0.57	0.40	0.41	0.49	2.30	0.32
Hha I	A	0.48	0.55	0.59	0.52		
	B	0.52	0.45	0.41	0.48	0.83	0.66
Taq I	A	0.59	0.60	0.55	0.58		
	B	0.41	0.40	0.45	0.42	0.16	0.92
Alu I	A	0.77	0.65	0.55	0.69		
	B	0.23	0.35	0.45	0.31	3.67	0.16
Number of isolates		22	10	11	43		

* Degrees of freedom = 2

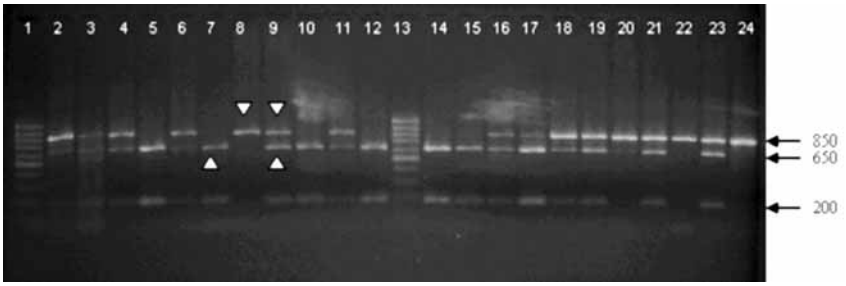

Fig. 2. ITS RFLP patterns of different *Inocutis jamaicensis* strains on Uruguayan native plants generated with the restriction enzyme *Hae* III.

1. 100bp Ladder	7. MVHC 11502	13. 100bp Ladder	19. MVHC 11505
2. MVHC 11392	8. MVHC 11507	14. MVHC 1711	20. MVHC 11506
3. MVHC 11395	9. MVHC 11704	15. MVHC 11712	21. MVHC 11508
4. MVHC 11404	10. MVHC 11707	16. MVHC 11714	22. MVHC 11729
5. MVHC 11474	11. MVHC 11709	17. MVHC 11503	23. MVHC 11730
6. MVHC 11501	12. MVHC 11710	18. MVHC 11504	24. MVHC 11733.

Single arrow heads indicate homozygotic isolates, two arrow heads indicate heterozygotic isolates.

Discussion

The four endonucleases evidenced a high variation of the ITS region of *I. jamaicensis*. This variability could be observed even within one heterokaryotic individual. Similar observations were made in studies of mycorrhizal and wood decaying basidiomycetes (Aanen *et al*. 2001, Glen *et al*. 2001, Kauserud & Schumacher 2003).

Inocutis jamaicensis was first reported in Uruguay in 2000 colonizing primarily *E. globulus* (Martinez *et al*. 2005). Considering that this fungus had not been reported in any other country where *Eucalyptus spp*. are grown, we hypothesized that this fungus must be present on plants of the native flora. Early surveys were conducted on native Myrtaceae expecting a host jump within the same plant family as it is known from *Puccinia*. The native host of this rust fungus is *Psidium guajava* but the rust nowadays also known as *Eucalyptus* pathogen (Alfenas *et al*. 2004). In contrast to this, *I. jamaicensis* has not been found on any native Myrtaceae in Uruguay but could be observed on host plants belonging to eight different families. Therefore, a broad host range of this fungus is evident.

No associations among genotypes and hosts were observed in the present study. In general, saprotrophic, wood rotting fungi tend to be less host specific than primary pathogens (Rayner & Boddy 1988; Schwarze *et al*. 2000). Inoculation trials on different provenances of *E. globulus* in Uruguay showed that *I. jamaicensis* is a non pathogenic fungus in despite of being observed on living trees (Bettucci *et al*. 2007). Balmelli et al. (2004) found that the colonization by

I. jamaicensis was correlated with the susceptibility of *E. globulus* to adverse environmental conditions. So, the absence of host preferences observed in this research, are in line with the assumption that *I. jamaicensis* is a non-pathogenic species.

Isolates obtained of fruit bodies collected from different hosts shared the restriction patterns and consequently the same alleles were observed. Wood decaying basidiomycetes colonise their hosts by (sexual) basidiospores (Schwarze *et al.* 2000). Mating experiments of monosporic isolates of *I. jamaicensis* originating from *E. globulus* in Uruguay and *V. vinifera* in Argentina demonstrated interfertility between the different isolates (Lupo *et al.* 2006). Together with the data presented within this study it can be concluded that basidiospores from basidiomata from on native plants intercompatible with basidiospores from basidiomata growing on eucalyptus or grape vine.

From a practical point of view, native plants are acting as reservoirs of basidiospores which have the potential to colonize *E. globulus* and *V. vinifera* plantations and vice versa.

Together with the expansion of *E. globulus* forests during the last years, the increasing colonization of *I. jamaicensis* was observed in nearly 30–50% of planted trees at the centre north of Uruguay (Bettucci 2003). This represents an important economical impact.

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