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Identification and genetic diversity of *Rosellinia* spp. associated with root rot of coffee in Colombia

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Abstract The genus *Rosellinia* includes species that cause root rot on a wide range of herbaceous and woody hosts. In Colombia, these fungi cause serious diseases of potato, forest and fruit trees, as well as coffee plants. The aim of this study was to identify isolates of *Rosellinia* collected from coffee and other hosts using DNA sequence comparisons of the internal transcribed spacer (ITS) region. Pathogenicity tests were conducted on coffee seedlings to confirm the role of the collected species in coffee root disease. Twenty six isolates were obtained and these were grouped into two clades representing *R. bunodes* and *R. pepo*. Isolates from *Coffea arabica*, *Hevea brasiliensis, Macadamia integrifolia, Psidium guajava* and *Theobroma cacao* were identified as *R. pepo,* while *R. bunodes* was obtained only from coffee plants. Low levels of genetic variability were observed among isolates of the two species. Pathogenicity tests on coffee with *R. bunodes* resulted in 98 % seedling death in an average of 10 days, while *R. pepo* killed 54 % of inoculated seedlings in an average of 16 days confirming the compatibility of both species with this host. Pathogen characterization will be useful for further research in disease diagnosis, soil recovery and breeding for resistance.

Keywords *Coffea arabica* · ITS · Phylogeny · *Rosellinia bunodes* · *Rosellinia pepo* · Soil-borne pathogens

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Introduction

Based on symptoms and morphological characteristics, two species of *Rosellinia* are known in Colombian coffee growing areas, *Rosellinia bunodes* (Berk & Brome) Sacc., which causes a disease known as black root rot, and *R. pepo* Pat. causing stellate root rot (Fernández and López 1964; Castro and Esquivel 1991). Other than in Colombia, these pathogens are known to affect coffee in Africa (Saccas 1956), Brazil (Ponte 1996), Costa Rica (Bautista and Salazar 2000), Cuba (Herrera 1989), El Salvador (Procafé 1996), Guatemala (Hernández 1967) and Puerto Rico (Garcia 1945), while *R. arcuata* Petch, and/or *R. bunodes* are mentioned infecting coffee in India (Sivanesan and Holliday 1972; Muthappa 1977; Kannan 1995).

Many *Rosellinia* spp. are saprophytes, some live endophytically and occasionally become pathogenic, and some species are well-known root pathogens on commercially grown plants such as potato (Guerrero 1990) and woody perennial trees in tropical and sub-tropical areas globally (Petrini and Petrini 2005; Ten Hoopen and Krauss 2006). Among the best known root pathogens are *R. necatrix* Berl.: Prill and *R. desmazieresii* (Berk. & Br.) Sacc. (= *R. quercina*

Hart), mostly known from temperate climates causing diseases on pear, apple and grape in Japan (Eguchi et al. 2009; Takemoto et al. 2009a, b), on avocado in Spain (López-Herrera 1998; Pliego et al. 2012) and in Argentina on peach, plum, apple, pear, grapevine and other hosts (Sarasola and de Sarasola 1975). *Rosellinia bunodes, R. pepo* and *R. arcuata*, are known only from the tropics (Kannan 1995; Ten Hoopen and Krauss 2006). Various other *Rosellinia* spp. that infect coffee were mentioned by Saccas (1956), e.g. *R. coffeae* Sacc., *R. didolotii* Sacc, *R. echinocarpa* Sacc, *R. lobayensis* Sacc., *R. mastoidiformis* Sacc. and *R. megalospora* Sacc., but very little is known about these species.

Rosellinia bunodes and *R. pepo* occur in the soil as saprophytes (Aranzazu 1996). After infection of suitable living hosts, patches of dying plants extend in a circular pattern due to the pathogen's spread through root contact or via mycelial aggregations (Fernández and López 1964; Merchán 1988; Aranzazu 1996; Bautista and Salazar 2000). Many shade and fruit trees grown in association with coffee (e.g. *Inga* sp., *Leucaena* sp., *Erytrina* sp., *Cordia alliodora* (Ruiz & Pav.) Oken, *Tabebuia rosea* DC, *Cedrela odorata* L., *Alnus acuminata* Kunth) are susceptible to infection by *Rosellinia* spp. and are thought to provide initial sources of inoculum for coffee tree infection (Bermúdez and Carranza 1990; Aranzazu 1996; Castro and Serna 2009). In addition, debris of cassava (*Manihot esculenta* Crantz) left in the soil after co-cultivation with coffee has been mentioned as increasing the survival of the pathogen and thus damage due to subsequent *Rosellinia* infection in Colombian coffee growing areas (Castro and Serna 2009).

Infection of coffee plants by *Rosellinia* spp. results in chlorosis, wilt, die-back and death of plants. This may occur within a few weeks in the case of seedlings or young plants in the field or take up to three or four years after infection in the case of adult plants (Fernández and López 1964; Castro and Esquivel 1991; Ibarra et al. 1999). Important economic losses have been recorded by Castro and Serna (2009) in Colombian coffee growing areas. The diseases caused by *Rosellinia* spp. are also known to be difficult to control and numerous integrated measures have been investigated, with variable results (Merchán 1988; Ten Hoopen and Krauss 2006; Gutiérrez et al. 2006). Barceló-Muñoz et al. (2007), implemented a program aimed at selecting avocado rootstocks tolerant to white rot caused by *R. necatrix* in Spain. However, little research on resistance to tropical *Rosellinia* spp. has been published (Ten Hoopen and Krauss 2006).

The genus *Rosellinia* belongs to the family Xylariaceae (Class Euascomycetes, subclass Pyrenomycetes, order Sphaeriales, syn. Xylariales) and includes more than one hundred species (Pliego et al. 2012). Teleomorphic structures, such as ascospore morphology, are considered valuable taxonomic characters for the identification of *Rosellinia* spp. (Pérez-Jiménez et al. 2003; Petrini and Petrini 2005; Takemoto et al. 2009a, b; Pliego et al. 2012). However, in tropical areas, stromata bearing fruiting bodies are rarely found in nature, making the identification of *Rosellinia* spp. reliant on characteristics of the anamorph (*Dematophora*) (Fernández and López 1964; Bermúdez and Carranza 1992; Ibarra et al. 1999; Realpe et al. 2006). A major diagnostic character at the generic level has been the presence of pear-shaped swellings at the septa of the hyphae (Saccas 1956; Sarasola and de Sarasola 1975; Pérez-Jiménez 2006; Pliego et al. 2012). At the species level, *R. pepo* and *R. bunodes* have been distinguished based on the mycelial aggregates formed on the roots. *R. pepo* produces grayish cobweb-like strands, which become black and coalesce into a woolly mass. Beneath the bark, white, star-like fans can be observed macroscopically. *Rosellinia bunodes* shows black branching strands firmly attached to the roots, forming black dots and lines embedded in the tissues (Waterston 1941; Fernández and López 1964; Ibarra et al. 1999; Realpe et al. 2006).

Rosellinia species have mostly been characterized based only on morphology (Petrini and Petrini 2005), with only limited DNA sequence data available for species in the genus. However, in the last decade, molecular tools have provided important means to elucidate genetic variation and phylogenetic relationships among global members of the Family Xylariaceae (Bahl et al. 2005; Peláez et al. 2008; Hsieh et al. 2010; Pliego et al. 2012). Sequencing of the internal transcribed spacer regions (ITS), fragments of the β-tubulin (BT), adenosine triphosphatase (ATP) and translation elongation factor 1α (TEF) gene regions and random amplified polymorphic DNA (RAPD) amplifications have mostly been used for identification of *R. necatrix* (López et al. 2008; Takemoto et al. 2009a, b). ITS Scorpion primer pairs have been successfully developed for large-scale detection of *R. necatrix* by real-time Scorpion- polymerase chain reaction (PCR) in soils and in plant materials (Schena and Ippolito 2003; Ruano-Rosa et al. 2007), and recently Takemoto et al. (2011) developed a species-specific PCR diagnostic for *R. necatrix* and *R. compacta* Takemoto in Japan.

At the population level, inter-simple sequence repeat (ISSR) markers have been used to study *R. necatrix* diversity in *Cyperus esculentus* L. (Armengol et al. 2010). However, there is still a lack of information on tropical *Rosellinia* spp. that cause damage to commercially propagated plants such as coffee. López (2004), made a first attempt to study the genetic variability of *R. bunodes* and *R. pepo* from coffee, cocoa and potato in Colombia, using ITS and RAPD sequences and mentions high variability in these species.

The primary aim of this study was to identify the species of *Rosellinia* damaging coffee and other associated plants in the Central Colombian coffee growing area. A pathogenicity test through artificial inoculation was carried out to confirm compatibility of the species with coffee.

Materials and methods

Sample collection and fungal isolation

During 2008 and 2009, samples were collected from plants showing macroscopic signs of root rot caused by *Rosellinia* spp. Plant hosts sampled included coffee (*Coffea arabica* L.), macadamia (*Macadamia integrifolia* Maiden & Betche), rubber (*Hevea brasiliensis* Müll. Arg.), cocoa (*Theobroma cacao* L.) and guava (*Psidium guajava* L.). The areas sampled were located in the central coffee growing area of Colombia and included the Caldas, Risaralda and Quindío Provinces. Samples were selected and preliminary identifications were made based on in situ macroscopic observation of symptoms and signs as described by Fernández and López (1964) and Realpe et al. (2006).

Plants thought to have root rot were identified based on external symptoms, including wilting, yellowing or dead trees. For fungal isolations, small segments (4–5 cm) were removed from fresh roots of symptomatic plants and placed in 2 % NaClO for 15 min, rinsed in sterile water and dried as described by López (2004) and Realpe et al. (2006). Small pieces of tissue, including fungal mycelium, were removed from the root sections and transferred to 2 % Malt Extract Agar (MEA), (Oxoid), pH 5.7, containing thiamine (100 μg/l) and antibiotic (100 mg/l rifampicin). Six to seven pieces were transferred to each Petri dish and the plates incubated at 24 °C for 3 to 4 days in the dark. Resultant colonies were transferred to fresh medium to obtain pure isolates, which were distinguished by the pear-shaped swellings at the septa, characteristic of *Rosellinia* spp. (Saccas 1956; Realpe et al. 2006; Pérez-Jiménez 2006; Pliego et al. 2012). Isolates were stored in liquid nitrogen (−196 °C) using the technique described by Ten Hoopen et al. (2004).

DNA extraction, amplification and sequencing

Twenty six isolates, identified as possible *Rosellinia* spp. based on morphology, and representing each of the hosts and areas sampled, were selected for characterization using DNA sequence comparisons. For each isolate, small pieces of mycelium from 15-day-old cultures were transferred to 100 ml Erlenmeyer flasks containing 100 ml Sabouraud medium (peptone-glucose-yeast extract). Flasks were incubated at 27 °C, for 8 days in darkness, with continuous shaking at 150 rpm. The resultant mycelium was harvested by filtration through Whatman No.1 filter paper and DNA was extracted using the method of Lee and Taylor (1990). Resultant DNA was diluted 20-fold with distilled water and stored at −20 °C until further use.

Amplification of the ITS1, 5.8S and ITS2 nuclear gene regions of the ribosomal RNA operon was performed for 26 isolates as described by Hillis et al. (1996) using the primers ITS1 (5′TCC GTA GGT GAA CCT GCG G3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) (White et al. 1990). PCR reactions consisted of 1.25 U of Taq polymerase (Promega, Southhampton, UK), 2 mM MgCl2; 0.2 mM dNTPs; 1X PCR Buffer; 0.2 μM of each Primer (ITS1, ITS4) and 100 ng of template DNA. Reactions were conducted with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C. A final elongation step was at 72 °C for 5 min. PCR products were separated on a 1.5 % agarose gel and stained with 1 μl ethidium bromide. Amplified products were visualized under UV light and their molecular mass estimated by comparison with Lambda DNA/HindIII Marker.

PCR products were purified using PCR purification kit (QIAGEN). Sequencing reactions were conducted using BigDye terminator cycling conditions on an Applied Biosystems Automatic Sequencer 3730XL (Macrogen Inc, Seoul, Korea). Sequences were aligned with MAFFT 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) and a tree was generated using PAUP 4b10 (Swofford 2002). Analyses were done using the heuristic search option with 100 random addition sequence replications (Efron 1986). Sequences of known *Rosellinia* spp. were retrieved from Genbank National Center for Biotechnology Information (NCBI) and incorporated into the analyses (Table 1). *Hypoxylon intermedium* (Achwein.) Y.M. Ju & J.D. Rogers (Sánchez-Ballesteros et al. 2000) and *Amphisphaeria umbrina* (Fr.) de Not., both members of Xylariales, were used as outgroup taxa.

Pathogenicity tests

In order to preliminary evaluate the ability of the species of *Rosellinia* collected in the surveyed area to infect coffee, two pathogenicity tests (one in November and another in December 2009) were conducted under greenhouse conditions at Planalto-Cenicafé, in Chinchiná, Colombia, using seedlings of *C. arabica* variety Caturra. Isolates encoded as RCQ 60 (CBS134099), obtained from coffee with black root rot in a farm of Quimbaya (Quindio), and RCACC 67 (CBS 134106) obtained from cocoa roots with star rot, in Palestina (Caldas), were grown on twice-autoclaved parboiled rice (Doña Pepa ®) placed in plastic bags. One mycelial disc (6 mm diameter) taken from cultures of each isolate growing on MEA plates was added to each bag, then incubated at 25 °C in darkness for twenty five days, to allow the mycelium to completely colonize the rice.

Coffee seedlings (65-days-old) previously grown in sterilized sand, were planted in plastic pots (one plant/pot) containing 150 g of sterilized soil (sandy loam, pH=4.9, organic matter=10 %). Eight days after planting, the seedlings were inoculated with 0.18 g of rice/plant, placing the inoculum in contact with the roots. For the controls,

Table 1 Sequences of isolates retrieved from GenBank included in this study

Taxon	Host and geographic origin	Culture number	Gen bank accession number	Reference
Amphisphaeria umbrina (Fr.) de Not.	Unknown	–	AF009805	
Hypoxylon intermedium (Achwein.) Y.M. Ju & J.D. Rogers	Unknown	H4A	AJ390396	Sánchez-Ballesteros et al. (2000)
Rosellinia bambusae Henn.	*Dendrocalamus latiflorus* Munro (Taiwan)	ATCC 66430	AY908998	Peláez et al. (2008)
Rosellinia bambusae Henn	*Calamus* sp. (Australia)	–	AY862573	Bahl et al. (2005)
Rosellinia buxi Fabre	Unknown (England)	ATCC 32869	AY909000	Peláez et al. (2008)
Rosellinia capetribulensis J. Bahl, Jeewon & K.D. Hyde	*Calamus* sp. (Australia)	–	AY862570	Bahl et al. (2005)
Rosellinia corticium (Achwein.) Sacc.	Unknown	F-160.845	AY908999	Peláez et al. (2008)
Rosellinia compacta Takemoto	Unknown (Japan)	–	AB430457	Takemoto et al. (2009b)
Rosellinia compacta Takemoto	Unknown (Japan)	89112602	AB430456	Takemoto et al. (2009b)
Rosellinia mirabilis (Berk & Broome) Y.M. Ju & J.D. Rogers	*Calamus* sp. (Australia)	–	AY862572	Bahl et al. (2005)
Rosellinia necatrix Berl. Ex Prill	*Ehretia microphylla* Lam (Taiwan)	R210	EF592569	Sun et al. (2008)
Rosellinia necatrix Berl. Ex Prill	*Camellia sinensis* (L.) Kuntze (Taiwan)	R301	EF592564	Sun et al. (2008)
Rosellinia necatrix Berl. Ex Prill	*Serissa japonica* (Thunb.) Thunb (Taiwan)	R203	EF592563	Sun et al. (2008)
Rosellinia necatrix Berl. Ex Prill	Unknown (Japan)	W536	AB430450	Takemoto et al. (2009b)
Rosellinia necatrix Berl. Ex Prill	*Acer morrisonense* Pax (Taiwan)	R101	EF592568	Sun et al. (2008)
Rosellinia pepo Pat.	Unknown	CBS350.36	AB017659	
Rosellinia quercina R. Hartig	Unknown	ATCC36702	AB017661	
Rosellinia subiculata (Achwein.)Sacc.	Wood (Illinois, USA)	ATCC 58850	AY909002	Peláez et al. (2008)
Rosellinia bunodes (Berk & Broome) Sacc.	*Hibiscus mutabilis* L. (Bahamas)	CBS. 347.36	AB609598.1	

uninoculated soil was used. The experimental unit was made up of ten seedlings placed individually in a row of plastic pots; the treatments corresponded to the isolates and five replicates were used per treatment as well as for the controls. Inoculated and control experimental units were placed in a fully randomized design in a greenhouse at an approximately 28 °C day and 20 °C night temperature regime.

In both trials, plants were checked daily for symptoms for 35 days post-inoculation. Symptomatic and dead plants were inspected for the presence of mycelium on their stems or roots to confirm infection by *Rosellinia* spp. Experimental data, including the number of dead plants and days to mortality, were statistically analyzed using ANOVA and Tukey's mean test (p=0.05) (SAS Statistical Software 2010).

Results

Sample collection and fungal isolations

Twenty coffee farms located at an altitude of between 1,200 and 1,500 (m) where patches with symptoms of root rot were present were sampled. In these plantations, areas including infected coffee plants ranged from three to 3,000 trees. Cocoa plantations (two) and macadamia (three) had smaller numbers of infected trees ranging from three to 50 plants affected. Other hosts growing on coffee farms had fewer than 10 plants with symptoms per stand. Trees from which samples were collected showed typical symptoms of root rot caused by *Rosellinia* spp. including foliage yellowing or wilting as well as dead plants. In the root collar area, the bark was cracked and small black lines and dots could be seen macroscopically, embedded in the wood (Fig. 1a). These symptoms were most common on coffee and are similar to those recorded for *R. bunodes* (Fernández and López 1964; Ibarra et al. 1999; López 2004). Other plants, including cocoa, had white fan-shaped or stellate mycelial growth under the bark of roots (Fig. 1b), which is typical for *R. pepo* (Waterston 1941; Sivanesan and Holliday 1972; Merchán 1988; Realpe et al. 2006).

Rosellinia spp. were successfully isolated from 90 % of the root samples collected, resulting in a total of 26 putative isolates of the fungus (Table 2). Cultures were grouped based on the signs of the pathogen observed on the roots

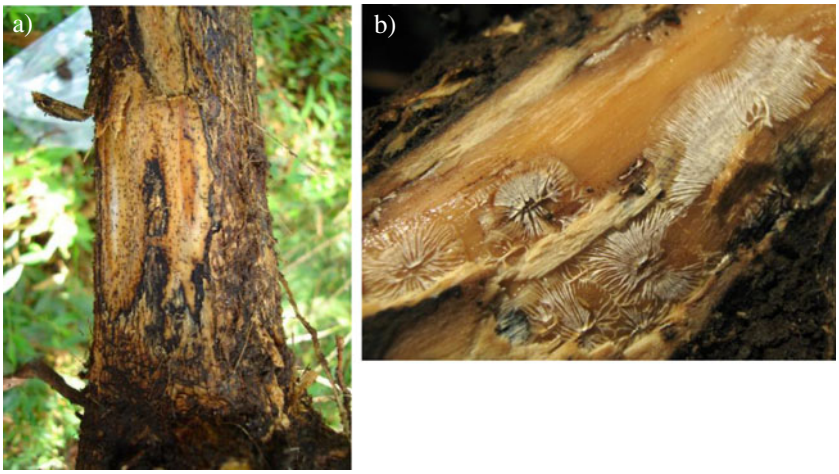

Fig. 1 Signs of *Rosellinia* infection observed on coffee trees. **a** Black streaks and spots caused by *Rosellinia bunodes,* **b** white mycelial stars under the bark caused by *Rosellinia pepo*

at the time of sampling. Colonies of morphological group A, representing 18 isolates obtained from roots with star rot, were initially white and then turned dark gray and olive green or dark brown. Colonies of group B isolates, consisting of eight cultures originated from black root rot, produced a fluffy mycelium that was also initially white and later turned gray, brown or black, or with some areas white and other black. However there was no consistency with regard to the color of the colonies in each group after ten days of cultivation. No other structures were observed in addition to the mycelium, either on infected roots or in culture after 25 days of cultivation. The most important morphological characteristic observed using light microscopy for these isolates was the pyriform-swellings at the junctions of the septa in the hyphae (Fig. 2), as has been previously described for these fungi (Fernández and López 1964; Ibarra et al. 1999; López 2004; Realpe et al. 2006). The size range of the swellings was between 6.0 and 12.5 μm wide for both species, increasing as the mycelium aged. Strains of three (3) isolates of *R. bunodes* and eight (8) of *R. pepo* were deposited in the Centraalbureau voor Schimmelcultures (CBS, Netherlands). The deposit numbers are to be found in Table 2.

ITS sequence comparisons

PCR amplification of the ITS regions for 26 isolates putatively representing *Rosellinia* spp. yielded fragments of ~633 bp in length. All sequences generated for the phylogenetic analyses in this study were deposited in Genbank (Table 2). Parsimony analysis produced a data set with 363 constant characters, 101 parsimony-uninformative and 257 parsimony informative characters. Four hundred and sixty four uninformative characters were excluded and 30 trees were obtained, from which one was chosen for presentation (Fig. 3). This tree consisted of two major clades, the largest of these included 18 isolates from coffee and other hosts (colony type A), exhibiting little diversity, and strongly supported by a 100 % bootstrap value. The reported sequence in this clade corresponds to *R. pepo* (AB 017659) from the CBS (Netherlands). The second clade, including 8 isolates from Colombia (colony type B), all obtained from coffee plants, was strongly supported by a 100 % bootstrap value and was related to an *R. bunodes* sequence (AB 609598). The tree had a consistency index (CI) of 0.45, homoplasy index (HI) of 0.54, retention index (RI) of 0.76, and rescaled consistency index (RC) of 0.34 (TreeBase number TB2: S12799).

Pathogenicity tests

Isolates of both *R. bunodes* (RCQ 60) and *R. pepo* (RCACC 67) were pathogenic to *C. arabica* seedlings. In both trials, wilting symptoms caused by *R. bunodes* (RCQ 60) were seen as early as 9 days post-inoculation on most of the seedlings. All seedlings were killed in the first trial and 98 % in the second trial, within 10 to 11 days post-inoculation. Wet rot, as well as brown, sunken discoloration were noticed in the tissues at the bases of the seedlings, with fine white mycelium invading the roots infected by *R. bunodes.*

Wilt symptoms caused by *R. pepo* (RCACC 67) were evident 14 days post-inoculation on most of the seedlings in both trials. For the first trial, 62 % of the seedlings were killed and 46 % died in the second trial after 16 and 24 days post-inoculation respectively. Dry rot and brown tissue discoloration, but no sunken tissue, were observed at the bases of the seedlings, with gray mycelium invading the roots. Differences in mortality and days to death were statistically significant ($P<0.0001$) in both tests. No mortality was found in any of the control plants for either of the tests.

Table 2 Isolates of *Rosellinia* spp. from coffee and other hosts in Colombia used in this study and for which internal transcribed spacer (ITS) regions sequence data were generated

Taxon	Culture number	Host	Origin	Gen bank accession number
Rosellinia bunodes	RCQ 48.2 (CBS 134097)[b]	*Coffea arabica* L.	Circasia (Quindío)	JF263537
"	RCQ 68 (CBS 134098)[b]	"	"	JF263538
"	RCQ 67.2	"	"	JF263539
"	RCQ 67	"	"	JF263540
"	RCQ 66	"	"	JF263541
"	RCQ 65	"	"	JF263542
"	RCQ 60[a] (CBS 134099)[b]	"	Quimbaya (Quindío)	JF263543
"	RCQ 48	"	Circasia (Quindío)	JF263544
Rosellinia pepo	RCACC 65 (CBS 134100)[b]	*Theobroma cacao* L.	Palestina (Caldas)	JF263545
"	RMACC 45 (CBS 134101)[b]	*Macadamia integrifolia* Maiden & Betche	Chinchiná (Caldas)	JF263546
"	RGUC 46 (CBS 134102)[b]	*Psidium guajava* L.	"	JF263547
"	RGUC 45	"	"	JF263548
"	RGUC 28	"	"	JF263549
"	RCR 14.2 (CBS 134103)[b]	*Coffea arabica* L.	Pereira (Risaralda)	JF263550
"	RCC 67 (CBS 134104)[b]	"	Chinchiná (Caldas)	JF263551
"	RCC 64.2	"	Palestina (Caldas)	JF263552
"	RCC 64	"	"	JF263553
"	RCC 60	"	Chinchiná (Caldas)	JF263554
"	RCAUR 18 (CBS 134105)[b]	*Hevea brasiliensis* Müll. Arg.	Pereira (Risaralda)	JF263555
"	RCAUR 17	"	"	JF263556
"	RCACC 67[a] (CBS 134106) [b]	*Theobroma cacao* L.	Palestina (Caldas)	JF263557
"	RCACC 66	"	"	JF263558
"	RCACC 36	"	"	JF263559
"	RMACQ 46 (CBS 134107)[b]	*Macadamia integrifolia* Maiden & Betche	Buena Vista (Quindío)	JF263560
"	RCR 24	*Coffea arabica* L.	Pereira (Risaralda)	JF263561
"	RCR14	"	"	JF263562

Codes: *RCC Rosellinia*-Coffee-Caldas, *RCQ Rosellinia*-Coffee-Quindío, *RCR Rosellinia*-Coffee-Risaralda, *RCACC Rosellinia*- Cocoa-Caldas, *RMACC Rosellinia*-Macadamia-Caldas, *RGUC Rosellinia*-Guava -Caldas, *RCAUR Rosellinia*-Caucho (Hevea)-Risaralda, *RMACQ Rosellinia*-Macadamia-Quindío

[a] Isolates included in pathogenicity tests. [b] CBS deposit number

Discussion

This study provides the first detailed identification of *Rosellinia* species in Colombian coffee growing areas using DNA sequence data. Previous studies in the country relied on identification based only on morphology. We identified *R. bunodes* and *R. pepo* affecting several hosts, including coffee plants in three provinces of Colombia. Symptoms, signs and molecular characterization of *R. bunodes* and *R. pepo* in this study are consistent with the etiology of the diseases known as black root rot (*R. bunodes*) and stellate root rot (*R. pepo*).

In this study, ITS sequence data confirmed that both *R. bunodes* and *R. pepo* are present in Colombia, confirming previous reports based on disease symptoms. Isolates obtained grouped into two distinct clades with 100 % bootstrap support and separate from any other *Rosellinia* sp. for which sequence data are available in GenBank. Currently, there are more than 100 reported *Rosellinia* species known (Kirk et al. 2001), but molecular data for these species is minimal or non-existent for most. Most of the sequence data available for the genus in GenBank are for *R. necatrix*. Our data for *R. bunodes* broadens the single sequence report, previously available for this species.

The sequence data emerging from this study supports the reliability of observed differences in macroscopic characters on infected plant roots as diagnostic for discriminating between *R. pepo* and *R. bunodes*. Neither culture morphology nor microscopic features were sufficient to differentiate between species. Differences in mycelium color in culture were not consistent among isolates of the same species, or between species. Pyriform hyphal swellings at the junctions

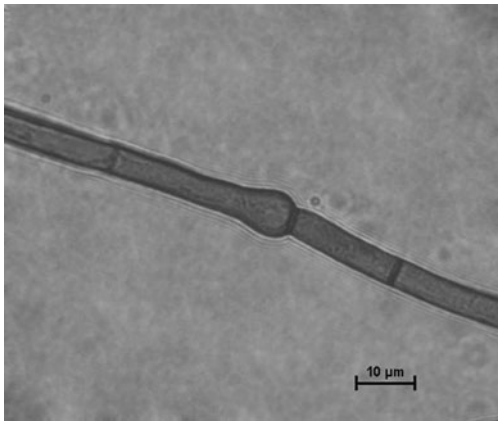

Fig. 2 Morphological characteristics of *Rosellinia bunodes* and *Rosellinia pepo* growing in culture. Typical pear-shaped swelling in the septa union of mycelia

of septa were also observed for both species, as previously reported by Fernández and López (1964), López (2004) and Realpe et al. (2006). These swellings have also been reported for *R. necatrix* (Saccas 1956; Pérez-Jiménez 2006; Pliego et al. 2012). Our observations showed that these swellings develop in mature hyphae (more than 8-days-old) rather that in young mycelium. Eventually, synnemata and conidia were observed in some old cultures (more than 30 days), but these structures had dimensions very similar in *R. pepo* and *R. bunodes*, as reported by Saccas (1956) and Petrini and Petrini (2005). Thus, they did not add any taxonomic information useful for diagnostics. Fruiting bodies, such as stromata bearing perithecia were not observed, as frequently reported in other studies (Bermúdez and Carranza 1992; Ibarra et al. 1999). This might be related to the condition of the samples and the

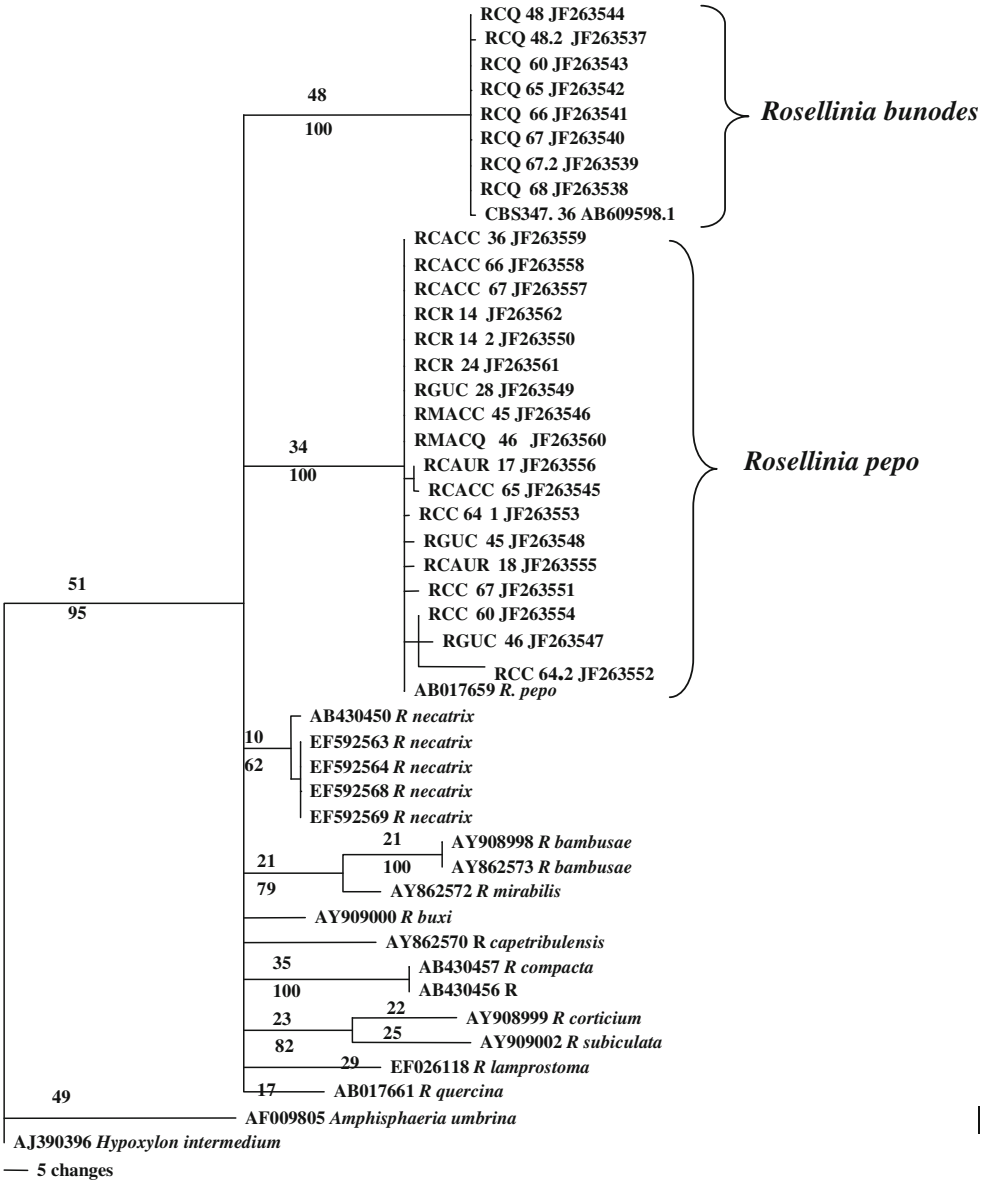

Fig. 3 The most parsimonious tree generated from DNA sequence data of the ITS regions for isolates of *Rosellinia* spp. Branch lengths are shown above and boostrap values below the branches. CI=0.4535; RI=0.7659; HI=0.5465; RC=0.3474 Tree Length=452

time required for these structures to appear after infection (Sarasola and de Sarasola 1975; de Texeira et al. 1995; Nakamura et al. 2000; Pliego et al. 2012). It could also mean that the pathogen does not require those parts of its life cycle under Colombian conditions where the temperature and humidity is high all year round. An effort should be made, nonetheless, to locate such structures and thus complement the molecular taxonomic understanding of *Rosellinia* spp. in Colombia.

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