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SHORT COMMUNICATION

No alternative hosts of the sugarcane pathogen *Leifsonia xyli* subsp. *xyli* were identified among grass and non-grass species using novel PCR primers

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Abstract *Leifsonia xyli* subsp. *xyli* (Lxx) is an endophytic bacterium that colonizes the xylem vessels of sugarcane and causes substantial losses in biomass. It is generally accepted that sugarcane is the only known natural host of Lxx despite the fact that there is just one systematic study to support this claim. Lxx-specific primers were developed and tested against a collection of cultivable bacterial endophytes of sugarcane to ensure their specificity. They were used to screen plants from twelve grass species collected in sugarcane fields and fifteen diverse species grown from seeds in the greenhouse. PCR did not detect Lxx in any case, confirming that the host range of this bacterium is most likely restricted to *Saccharum* spp.

Keywords Ratoon stunting disease · *Saccharum* · PCR detection · Host range

The genus *Leifsonia* comprises fourteen species and two subspecies isolated from very diverse environments such as Antarctic pond (Reddy et al. 2003), Himalayan soil (Reddy et al. 2008), lichens (An et al. 2009), teak rhizosphere soil (Madhaiyan et al. 2010) and nematode galls (Evtushenko et al. 2000). *Leifsonia xyli* is the only plant pathogenic species of the genus described so far, and comprises two subspecies: *Leifsonia xyli* subsp. *cynodontis*, a pathogen of bermudagrass (*Cynodon dactylis*) and *Leifsonia xyli* subsp. *xyli* (Lxx), which causes ratoon stunting disease (RSD) in sugarcane (Davis et al. 1984). Although Lxx can experimentally colonize various different species (Gillaspie and Teakle 1989), it is generally accepted that its only natural host is sugarcane. Evidences support the possibility that Lxx is an endophyte that was transmitted to commercial sugarcane varieties after artificial interspecific hybridization with a wild progenitor, *Saccharum spontaneum* (Young 2016) and was disseminated during the breeding of modern sugarcane. Today, Lxx can readily be found worldwide where sugarcane is commercially grown. In Brazil, recent surveys detected the pathogen in 23–67 % of the fields sampled (Ponte et al. 2010; Urashima and Marchetti 2013). Surprisingly, however, there is only one systematic survey of natural alternative hosts of Lxx carried out in Australia to support this claim (Mills et al. 2001). Thus, additional investigations of alternative hosts of Lxx are needed as these may bring new insights on the biology of this pathogen that are relevant for RSD management.

This study surveyed possible hosts of Lxx by PCR using primers developed in previous studies based on the nucleotide sequence of the ITS region CxFor/CxxREV/CxcREV (Fegan et al. 1998) and Cxx1/Cxx2 (Pan et al. 2001) and a novel primer pair, FL12650F2 (GCGTGGAGAAGTTCATCGTT)/FL12650R2 (AGCGGCTGAAGGGAGTAGTT) which was tested against a collection of 20 endophytic bacterial strains isolated from sugarcane. These primers were designed based on the sequence of the gene Lxx12650 (GenBank: AE016822.1, locus_tag = "Lxx12650") using the softwares Primer 3 V.4 (Rozen and Skalestsky 2000), NetPrimer (PREMIER Biosoft 2013) and GeneRunner (GENERUNNER 2013). Up to this date, this gene is considered unique to Lxx as there is no other gene sequence deposited in GenBank with a similar sequence. PCR for the novel primer pair was performed in a final volume of 25 μL containing 2 μL of total DNA at 50 ng/μL; 12.5 μL of amplification buffer GoTaq Green Master Mix (Promega®); 0.5 μL of a 10 μM

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solution of each primer and 9.5 μL of nuclease-free water (Ambion®) to complete the reaction. The amplification program consisted of an initial cycle at 95 °C for 5 min followed by 30 cycles of 95 °C for 10 s, 62 °C for 30 s, 72 °C for 30 s, and a final cycle at 72 °C for 5 min. The PCR for the primers CxFor/CxxREV/CxcREV and Cxx1/Cxx2 were performed according to Fegan et al. (1998) and Pan et al. (2001), respectively. DNA was extracted from cultures of the Lxx strain CTCB07, *Leifsonia xyli* subsp. *cynodontis* (Lxc) strain DSMZ46306 and of sugarcane plants from the variety SP80-3280 previously known to be infected by Lxx, according to Carvalho et al. (2016). DNA concentrations were determined by spectrophotometry using a Nanodrop 1000 (Thermo Scientific). To test the sensibility of the new primers the DNA extracted from Lxx strain CTCB07 was diluted (1:10) from 100 ng/μL to 0.01 pg/μL. PCR products were resolved by electrophoresis at 80 W in 1.5 % agarose gels in 0.5X TBE buffer and visualized after staining with Sybr Safe (Invitrogen).

In order to evaluate the specificity of the primers FL12650F2/FL12650R2, cultivable endophytic bacteria were isolated from Lxx-infected plants of the varieties CB49-260 and SP80-3280 which are maintained as a source of inoculum for experimental purposes and were previously screened for the presence of Lxx by PCR using the Cxx1/Cxx2 primers. Five basal internodes of each variety were surface sterilized by sequential washing in 70 % ethanol for 1 min and in 2 % sodium hypochlorite for 3 min followed by rinsing two times with deionized and sterilized water. The internode cuttings were centrifuged for 15 min at 3,000 rpm twice and 50 μL aliquots of the collected vascular fluid were plated on M-SC medium (Teakle and Ryan 1992) modified by the addition of 5 mg mL^{-1} methionine (Monteiro-Vitorello et al. 2004). Leaves from the same plants were also superficially sterilized by the process described above, cut with a sterile scalpel, incubated in 4 ml of NaCl 100 mM sterile solution for 40 min and 50 μL aliquots were plated on the same medium. Aliquots of the second rinsing were also plated to confirm the absence of epiphytic contamination. The plates were incubated at 28 °C for 7 days and the isolates were transferred to new plates. Colonies of these isolates were scraped with a sterile toothpick, suspended in 100 μL of sterile ultrapure water and used for DNA extraction (Carvalho et al. 2016) which was used in PCR with the primers FL12650F2/FL12650R2 as described above. In order to identify the endophytic bacteria, the 16S rDNA region of each isolate was amplified using the primers 16F27 and 16R1542 (Gürtler and Stanisich 1996) and the resulting PCR products were sequenced. A consensus sequence was obtained for each isolate using the Bioedit software (Hall 1999) and deposited in Genbank (Table 1). Sequences were compared with those deposited in the same database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/genbank/).

In the search for alternative hosts of Lxx two adult plants of 12 species of Poaceae family were collected in different sugarcane fields in the County of Piracicaba (latitude 22°42′30″S and longitude 47°38′00″ W; Table 2). The twelve species of the family Poacea were collected randomly in areas where sugarcane is grown in order to verify if they may act as sources of inoculum,

Table 1 Identification of endophytic bacteria isolated from sugarcane plants based on the similarity sequence of the 16S region

Isolate	Species	Cover	e-value	Ident	GenBank accession number
CTC3	*Nocardioides hankookensis*	97 %	0.0	97 %	KX262683
CTC5	*Microbacterium laevaniformans*	97 %	0.0	99 %	KX262684
CTC8	*Microbacterium testaceum*	97 %	0.0	99 %	KX262685
CTC9	*Microbacterium testaceum*	98 %	0.0	98 %	KX262686
CTC11	*Curtobacterium* sp.	98 %	0.0	98 %	KX262687
CTC13	*Burkholderia jiangsuensis*	98 %	0.0	98 %	KX262688
CTC15	*Microbacterium* sp.	97 %	0.0	98 %	KX262689
CTC16	*Curtobacterium luteum*	98 %	0.0	99 %	KX262690
CTC17	*Curtobacterium* sp.	97 %	0.0	99 %	KX262691
CTC31	*Xanthomonas albilineans*	97 %	0.0	99 %	KX262692
CTC34	*Aeromicrobium* sp.	97 %	0.0	97 %	KX262693
CTC35	*Leifsonia* sp.	97 %	0.0	99 %	KX262694
CTC36	*Methylobacterium* sp.	98 %	0.0	99 %	KX262695
CTC37	*Enterobacter asburiae*	95 %	0.0	99 %	KX262696
CTC38	*Rhizobium* sp.	98 %	0.0	98 %	KX262697
CTC40	*Sphingomonas* sp.	97 %	0.0	98 %	KX262698
CTC42	*Curtobacterium* sp.	94 %	0.0	84 %	KX262699
CTC44	*Marmoricola korecus*	95 %	0.0	98 %	KX262700
CTC45	*Aeromicrobium* sp.	96 %	0.0	99 %	KX262701
CTC46	*Sphingomonas mali*	97 %	0.0	95 %	KX262702

Table 2 Results of PCR reactions using Lxx specific primers from plants collected in the field or raised from seeds in the greenhouse

Host species	Common name	Family	Field	seeds
Pennisetum purpureum	elephant grass	Poaceae	–[1]	-
Brachiaria spp.	signal grass	Poaceae	-	-
Panicum maximum	guinea grass	Poaceae	-	-
Setaria italica	foxtail millet	Poaceae	-	-
Cynodon dactylon	bermuda grass	Poaceae	-	-
Rhynchelytrum repens	natal grass	Poaceae	-	nt[2]
Digitaria spp.	crabgrass	Poaceae	-	nt
Cenchrus echinatus	southern sandbur	Poaceae	-	nt
Eragrostis pilosa	Indian lovegrass	Poaceae	-	nt
Digitaria insularis	sourgrass	Poaceae	-	nt
Sorghum halepense	Johnson grass	Poaceae	-	nt
Eleusine indica	Indian goosegrass	Poaceae	-	nt
Sorghum bicolor	grain sorghum	Poaceae	nt	-
Sorghum bicolor	sweet sorghum	Poaceae	nt	-
Sorghum bicolor	Sudan grass	Poaceae	nt	-
Oryza sativa	rice	Poaceae	nt	-
Solanum lycopersicum	tomato	Solanaceae	nt	-
Cucumis melo	melon	Cucurbitaceae	nt	-
Arabidopsis thaliana	arabidopsis	Brassicaceae	nt	-
Crotalaria juncea	sunn hemp	Fabaceae	nt	-
Cyperus rotundus	nut grass	Cyperaceae	nt	-
Ipomoea trifoliata	morning glory	Convolvulaceae	nt	-

[1] (–) no PCR amplification

[2] not tested

thus representing a new route of infection. In addition to field plants, we also investigated the possibility of seed transmission of Lxx by diverse potential hosts (Table 2). These species were chosen considering their roles in sugarcane fields: *Setaria italica*, *Cynodon dactylon*, *Pennisetum purpureum*, *Brachiaria* spp., *Panicum maximum*, *Cyperus rotundus* and *Ipomoea trifoliate* are weeds; *Crotalaria juncea* is used as a green cover; *Solanum lycopersicum* and *Sorghum* spp. can be used in crop rotations. *Oryza sativa* and *Cucumis melo* were chosen as outgroup species, since they are not related to sugarcane cropping in Brazil. Five plants of each species were grown from seeds in a greenhouse in 15 L pots containing PlantMax substrate. Leaves of these plants were washed three times in sterile water for 1 min and surface sterilization was confirmed by plating aliquots of 100 μL of the final wash water into M-SC medium amended with methionine followed by incubation at 28 °C. The leaves were macerated in liquid nitrogen and 100 mg were used for DNA extraction according to Carvalho et al. (2016). PCR was performed as described above using the three sets of primers.

The FL12650F2/FL12650R2 pair generated one fragment with the expected size (394 bp) when DNA from a pure culture of Lxx or from sugarcane leaves of the Lxx-infected plants of the variety SP80-3280 were used as templates and it resulted in amplification products when the DNA of Lxx was diluted down to 1 pg/μL of DNA/reaction (Fig. 1). The specificity of the primers was demonstrated as there were no amplifications in reactions using DNA of the 20 bacterial isolates and of the Lxc strain as template (data not shown). As inferred from the sequence of the 16S region, the endophytic isolates comprised twelve different genera, six Actinobacteria, including *Leifsonia* sp., and six Proteobacteria (Table 1). Thus, primers FL12650F2/FL12650R2 can be used in

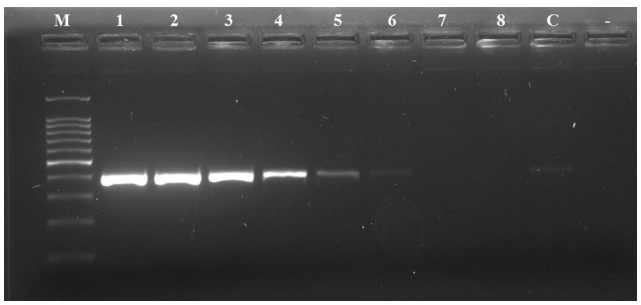

Fig. 1 Electrophoresis of fragments amplified with primers FL12650F2/FL12650R2. (M) Marker 100 bp; (1) to (8) serial dilution (1:10) of DNA extracted from Lxx strain CTCB07 ranging from 100 ng/μL (lane 1) to 0.01 pg/μL (lane 8); (C) sugarcane variety SP80-3280 infected with Lxx; (–) negative control

diagnostic tests in conjunction or in substitution to existing ones designed based on the ITS.

PCR with all primer pairs resulted in no amplifications both in plants collected in sugarcane fields and in plants grown from seeds in the greenhouse (data not shown) indicating that they are no natural hosts of Lxx. This is in agreement with a report from Australia, where plants from 53 species, including 9 genera also used in our study, were surveyed and none was found to be infected with Lxx, although a bacterium similar to Lxx, probably *Leifsonia xyli* subsp. *cynodontis*, was found in almost half of the tested plants comprising 18 grass species (Mills et al. 2001). Thus, considering the results of both surveys and also the fact that RSD occurs in high frequency in sugarcane fields in Brazil (Ponte et al. 2010; Urashima and Marchetti 2013), presently the most likely conclusion is that the only host of Lxx is indeed sugarcane or that, if existent, infected alternative hosts are not ubiquitous. Thus, for practical purposes, this conclusion is important since it minimizes the possibility of any of the species, which occur as weeds in sugarcane fields or are used in crop rotations, being sources of inoculum and thus transmitting Lxx to sugarcane during harvest. On the other hand, the fact that sugarcane is the only natural host of Lxx is intriguing. A possible explanation for this is that this restricted ecological niche of Lxx relates to the surprisingly high number of non-functional genes found in its genome (Monteiro-Vitorello et al. 2004). The driving forces that guide the accumulation of these so-called pseudogenes might result from a process of genome decay in which the function of genes that no longer play adaptive roles are lost to mutations. A typical example of this phenomenon among the Actinobacteria is represented by *Mycobacterium leprae* (Ventura et al. 2007). In the case of Lxx, it is presently unknown if gene decay is the cause or the consequence of its restricted host range, but comparative genomic studies within Lxx and among other species of *Leifsonia* should bring original insights into the evolution of this pathogen.

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