Moko Disease-Causing Strains of *Ralstonia solanacearum* from Brazil Extend Known Diversity in Paraphyletic Phylotype II

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ABSTRACT

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The epidemic situation of Moko disease-causing strains in Latin America and Brazil is unclear. Thirty-seven *Ralstonia solanacearum* strains from Brazil that cause the Moko disease on banana and heliconia plants were sampled and phylogenetically typed using the endoglucanase (*egl*) and DNA repair (*mutS*) genes according to the phylotype and sequevar classification. All of the strains belonged to phylotype II and a portion of the strains was typed as the Moko disease-related sequevars IIA-6 and IIA-24. Nevertheless, two unsuspected sequevars also harbored the Moko disease-causing strains IIA-41 and IIB-25, and a new sequevar was described and named IIA-53. All of the strains were pathogenic to banana and some of the strains of sequevars IIA-6, IIA-24, and IIA-41 were also pathogenic to tomato. The Moko disease-causing strains from sequevar IIB-25 were pathogenic to potato but not to tomato. These results highlight the high diversity of strains of Moko in Brazil, reinforce the efficiency of the *egl* gene to reveal relationships among these strains, and contribute to a better understanding of the diversity of paraphyletic Moko disease-causing strains of the *R. solanacearum* species complex, where the following seven distinct genetic clusters have been described: IIA-6, IIA-24, IIA-41, IIA-53, IIB-3, IIB-4, and IIB-25.

Different *Ralstonia solanacearum* strains can infect triploid banana, heliconia (*Heliconia* sp.), and other ornamental Musaceae plants (11) and cause Moko disease. These strains are historically known as *R. solanacearum* race 2, biovar 1, which is a quarantine pest (EPPO A2 list) found in Brazil that only occurs in the Amazonas, Pará, Rondônia, Roraima, Pernambuco, and Sergipe states (18). *R. solanacearum* encompasses thousands of different strains with a broad host range and unusual genetic diversity, including the two closely related strains *R. syzygii* (the causal agent of the Sumatra disease in the clove) and banana blood disease bacterium (3). Such complexity justifies the use of the term "species complex" for *R. solanacearum* (3,4). However, the last two species have not been reported in Brazil. Recent studies suggest that the *R. solanacearum* species complex originated in Oceania or Indonesia, migrated to Africa, and subsequently migrated to South America and Asia, possibly before the fragmentation of the ancestral continent Gondwana (23,31).

Because of this unusually large strain diversity, *R. solanacearum* was historically classified according to phenotypic and genetic properties as follow: five races based on the host range (11) and six biovars based on biochemical characteristics (12,19). However, race and biovar classifications cannot mirror the broad genetic diversity of the different strains that compose the *R. solanacearum* species complex based on the development of "omics" technologies over the past decades. This issue was addressed in the 2000s and resulted in a hierarchical classification scheme by Fegan and Prior (3). This classification consisted of four phylotypes that were based on the geographical origins of the strains and 52 sequevars, which are groups of strains with highly conserved regions in a partial endoglucanase (*egl*) nucleotidic sequence (26) and clones. The phylotypes were correlated with their geographical origin: phylotype I included strains from Asia, phylotype II included strains from America, phylotype III included strains from Africa and the Indian Ocean, and phylotype IV included strains from Indonesia, Japan, and Australia (22).

The classifications as phylotypes and sequevars are working definitions (22) that are presently accepted by the scientific community as best reflecting the genetic diversity of the species complex. The phylotypes can be determined from a phylogenetic analysis of the *egl* gene or DNA repair (*mut*S) gene and routinely by multiplex polymerase chain reaction (PCR) with Nmult series oligonucleotides (internal transcribed spacer region of the 16S-23S ribosomal RNA), which was proposed by Fegan and Prior (3). All of the Moko disease-causing strains are phylogenetically distributed within phylotype II (American origin) and further subdivided into lineages IIA and IIB (1,4). More specifically, the literature has indicated the paraphyletic nature of the Moko pathogen that is distributed into four distinct sequevars: IIA-6, IIA-24, IIB-3, and IIB-4 (1,3,4). The sequevars are also a working definition and subdivision of the phylotypes and they are based on a <1% divergence of the nucleotidic sequence of the *egl* gene (3,21). The routine detection of the Moko ecotype can be achieved by multiplex (Mmx)-PCR, which discriminates every sequevar of the Moko disease strains: IIA-6, IIA-24, IIB-3, and IIB-4 (3).

In Brazil, most of the *R. solanacearum* diversity studies were conducted on race 1 (2,25,27,29). Unfortunately, the strains that are assigned to the obsolete race 1 classification are distributed across all four of the phylotypes. To our knowledge and based on

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the important role of the banana industry in Brazil, it is surprising that only one study has phylogenetically typed 19 Moko disease-causing strains by Mmx-PCR (20). Typical Moko disease symptoms in the wild include yellowing and wilting of the inner leaves caused by the infection, which initiates in the rhizomes and moves toward the pseudostem; fruit are then deformed, turn black, and shrivel up. Banana plants near maturity may show no apparent symptoms but the inner pulp can still present dry rot and the plants may die. In the Philippines, Bugtok disease shows atypical symptoms confined to the inflorescence of 'Saba' and 'Cardaba' and vascular discoloration that rarely extends into the lower part of the pseudostem. These atypical symptoms are a result of the interaction of these particular cultivars and strains of *R. solanacearum* sequevar IIB-3 (4). In the state of Sergipe, which is in northeastern Brazil, the symptoms are also atypical and differ from the typical symptoms, which are found exclusively in the Amazonia region. In Sergipe, the symptoms initiate in inflorescences and cause uneven and premature ripening of fruit that internally show dry rot. The bacteria can move toward the pseudostem and cause darkening of the vascular bundles; wilting of the banana mats rarely develop, such as in Bugtok disease, which occurs in the Philippines. This Sergipe syndrome of Moko will be investigated to determine whether it results from a particular Moko strain within the dispersed genetic clusters of Moko disease-causing strain members in the disputed phylotype II.

Diversity studies are essential for the adaptation of control strategies or development of new strategies based on resistance germplasms that have yet to be developed. Determining the genetic and phenotypic variability of the Moko disease-causing strains is of the upmost importance; however, this variability remains to be described in South America (4), especially in Brazil (20). The aim of this study is to explore the pathogenic and phylogenetic diversity of *R. solanacearum* strains that originates in Brazil where recent wilting has appeared on banana (*Musa* sp.) and heliconia (*Heliconia* sp.) plants in different production areas.

MATERIALS AND METHODS

Bacterial strains and DNA typing. In total, 37 strains were obtained from symptomatic banana (*Musa* sp.) and heliconia (*Heliconia* sp.) plants (Table 1). From this total, 7 strains were obtained from the Phytobacteria Culture Collection of the Biological Institute, São Paulo, Brazil; 27 were obtained from the National Institute of Research in Amazonia, Manaus, Brazil; and 2 were obtained from the Culture Collection of the Phytobacteriology Laboratory of the Federal Rural University of Pernambuco, Recife, Brazil; and 1 from Embrapa Tabuleiros Costeiros, Aracaju, Brazil. The standard of comparison in all of the analyses was the *R. solanacearum* type strain K60^T, which is the same as ATCC11696 (IBSBF292, Phytobacteria Culture Collection of the

TABLE 1. Brazilian *Ralstonia solanacearum* strains used in this study

Strain[a]	Host	Origin[b]	Phyl-seq (*egl* gene)[c]	Pathogenicity (banana/tomato)	GenBank[d]	
					egl	*mutS*
B1	*Musa* sp.	Anamã/AM	IIA-24	+/–	KF889439	KF896791
B11	Banana (AAB)	Anamã/AM	IIA-24	+/–	KF875412	KF896783
B13	Banana (AAB)	Anamã/AM	IIA-24	+/–	KF889438	KF896793
B133	*Musa* sp.	Manacapuru/AM	IIA-24	+/–	KF875407	KF896789
B14	Banana (AAB)	Anamã/AM	IIA-24	+/–	KF875408	KF896788
B15	Banana (AAB)	Anamã/AM	IIA-24	+/–	KF875409	KF896787
B17	Banana (AAB)	Anamã/AM	IIA-24	+/+	KF875406	KF896792
B3	Banana (AAB)	Iranduba/AM	IIA-24	+/–	KF875413	KF896782
B35	Banana (AAB)	Coari/AM	IIA-24	+/–	KF889437	KF896794
B5	Banana (AAB)	Anamã/AM	IIA-24	+/+	KF875419	KF896784
B6	Banana (AAB)	Anamã/AM	IIA-24	+/–	KF875411	KF896785
B67	Banana (ABB)	Parintins/AM	IIA-24	+/–	KF875405	KF896796
B9	Banana (AAB)	Anamã/AM	IIA-24	+/–	KF875418	KF896790
IBSBF1544	*Musa* sp.	Amazonas	IIA-24	+/–	KF875429	KF896769
IBSBF187	*Musa* sp.	Humaitá/AM	IIA-24	+/+	KF875431	KF896768
IBSBF188	*Musa* sp.	Humaitá/AM	IIA-24	+/+	KF875430	KF896770
IBSBF2571	*Musa* sp.	Tabatinga/AM	IIA-24	+/–	KF875428	KF896803
IBSBF615	*Musa* sp.	PA	IIA-24	+/+	KF875417	KF896771
B105	Banana (AAB)	Alto Solimões/AM	IIA-41	+/+	KF875410	KF896786
B54	Banana (AAB)	Manacapuru/AM	IIA-41	+/+	KF875424	KF896795
B64	Banana (AAB)	Parintins/AM	IIA-41	+/–	KF875422	KF896776
B66	Banana (AAA)	Parintins/AM	IIA-41	+/+	KF875403	KF896775
B73	Banana (AAB)	Rio Preto da Eva/AM	IIA–41	+/+	KF875423	KF896774
B74	Plantain	Tefé/AM	IIA-41	+/+	KF875404	KF896773
B75	Plantain	Tefé/AM	IIA-41	+/–	KF875421	KF896777
B95	*Musa* sp.	Alto Solimões/AM	IIA-41	+/+	KF875402	KF896780
B96	*Musa* sp.	Alto Solimões/AM	IIA-41	+/–	KF875415	KF896778
B106	Banana (AAB)	Alto Solimões/AM	IIA-41	+/–	KF875420	KF896779
BV136	*Musa* sp.	Paraná do Supia/AM	IIA-41	+/–	KF875414	KF896781
Cotpin2	*Musa* sp.	Propriá/SE	IIA-53	+/–	KF875416	KF896797
F2	Banana (*Musa* sp.)	Propriá/SE	IIA-53	+/–	KF875426	KF896801
F3	Banana (*Musa* sp.)	Propriá/SE	IIA-53	+/–	KF875425	KF896802
IBSBF2572	*Musa* sp.	Japoatã/SE	IIA-53	+/–	KF875427	KF896772
IBSBF2661	Heliconia	Abreu e Lima/PE	IIA-6	+/+	KF875432	NA
B4	Banana (AAB)	Anamã/AM	IIB-25	+/–	KF889435	KF896800
B7	Banana (AAB)	Anamã/AM	IIB-25	+/–	KF889436	KF896799
B10	Banana (AAB)	Anamã/AM	IIB-25	+/–	KF889434	KF896798

[a] Source of strains: IBSBF, strains from the Phytobacteria Culture Collection of the Biological Institute, São Paulo, Brazil; B, strains from the National Institute of Research in Amazonia, Manaus, Brazil; F, strains from the Culture Collection of Phytobacteriology Laboratory of the Federal Rural University of Pernambuco, Recife, Brazil; Cotpin 2, strain from the Embrapa Tabuleiros Costeiros, Aracaju, Brazil.
[b] AM = Amazonas State; PA = Pará State; SE = Sergipe State; PE = Pernambuco State.
[c] Phylotype-sequevar.
[d] NA = sequence not available.

Biological Institute); the standard was isolated from tomato plants and typed as phylotype IIA sequevar 7 (IIA-7) (Table 2).

The DNA extraction of strains for the phylotype analysis was performed from the bacterial cultures grown in Kelman medium (14) at 29°C for 48 h. The bacterial genomic DNA extraction was performed with the AxyPrep Bacterial Genomic DNA MiniPrep Kit (Axygen Biosciences, Union City) according to the manufacturer's recommendations. Strain typing was performed by a

TABLE 2. GenBank reference strains of *Ralstonia solanacearum* complex used for phylogenetic analysis[a]

Strain	Host	Origin	Alternative name	Phyl-seq (*egl* gene)[b]	GenBank	
					egl	*mutS*
A3909	Heliconia	Hawaii	RUN9	IIA-6	EF371812	AY756753
ACH732	Tomato	Australia	UW433	IV-11	GQ907150	AY756743
ACH92	Ginger	Australia	…	I-16	AF295254	AY756764
CFBP2957	Tomato	Martinique	RUN27	IIA-36	AF295265	EF371845
CFBP2958	Tomato	Guadeloupe (FWI)	RUN28	IIA-39	AF295266	AY756806
CFBP2972	Potato	Martinique (FWI)	RUN30	IIA-35	AF295264	AY756807
CFBP3059	Eggplant	Burkina Faso	JCG.AU28	III-23	AF295270	AY756766
CFBP6779	Canna Russian Red	Martinique (FWI)	ANT174-1, RUN288	IIA-38	EF371835.1	EF371872
CFBP6783	Heliconia	Martinique	RUN17	IIB-4NPB	EF371852	EF371852
CFBP7014	Anthurium	Trinidad	RUN297	IIB-51	AF371831	EF371875
CFBP7032	Tomato	Cameroon	CMR39, RUN150	IIA-41	EF439726	EF439803
CFBP7054	Tomato	Cameroon	CMR121, RUN203	IIA-52	EF439725	EF439800
CFBP7058	Huckleberry	Cameroon	CMR134	I-13	EF439740	EF439794
CIP10	Potato	Peru	UW477, RUN110	IIB-25	AF295260	AY756821
CIP240	Potato	Brazil	RUN482	IIB-26	EF647739	JF702714
CIP365	Potato	Philippines	RUN47	I-45	GQ907151	AY756787
CIP418	Peanut	Indonesia	MOH6	IIB-3	GU295005	AY756809
CMR15	Tomato	Cameroon	CFBP6941, RUN133	III-29	EF439743	JF702729
CMR32	Huckleberry	Cameroon	CFBP6942, RUN145	III-29	EF439749	EF439773
CMR34	Tomato	Cameroon	CFBP147	IIB-1	EF439750	EF439810
CMR66	Huckleberry	Cameroon	RUN166	III-49	EF439729	EF439783
DGBBC1125	Potato	Guinea	RUN369	III-43	GU295008	NA
DGBBC1138	Potato	Guinea	RUN362	III-44	GU295009	NA
DGBBC1227	Potato	Guinea	RUN364	III-42	GU295011	NA
GMI1000	Tomato	French Guyana	JS753, RUN54	I-18	AF295251	AY756804
GMI8044	Banana	Grenada	RUN585	IIA-6	GU295013	JF702718
GMI8254	Tomato	Indonesia	RUN597	I-47	GU295014	JF702719
IBSBF1900	Banana	Brazil	RUN301	IIA-24	EF371839	EF371871
IBSBF2001	Tomato	Brazil	RUN981	IIB-25	GU295017	NA
ICMP7963	Potato	Kenya	RUN55	IIA-7	AF295263	AY766776
ISBSF1712	Geranium	Brazil	RUN299	IIB-27	EF371833	EF371869
IPO1609	Potato	Netherlands	RUN1	IIB-1	EF371814	EF371849
J25	Tomato	Kenya	…	III-20	AF295279	AY756810
JT516	Potato	Reunion	RUN160	IIB-1	AF295258	AY756783
JT519	Geranium	Reunion Is.	RUN471	I-31	GU295032	JF702713
JT525	Geranium	Reunion	RUN60	III-19	AF295272	AY756786
K60[Tc]	Tomato	EUA	ATCC11696, IBSBF292	IIA-7	EF192970.1	AY756799.1
M2	Mulberry	China	RUN343	I-48	FJ561067	NA
MAD17	Pepper	Madagascar	RUN320	I-46	GU295040	NA
MAFF301558	Potato	Japan	RUN71	IV-8	DQ011558	AY756812
Molk2	Banana	Philippines	…	IIB-3	EF371841	EF371848
NCPPB1018	Potato	Angola	RUN479	III-21	AF295271	AY756772
NCPPB332	Potato	Zimbabwe	RUN75	III-22	AF295276	AY756760
NCPPB3987	Potato	Brazil	RUN81	IIB-28	AF295261	AY756785
O3	Olive tree	China	…	I-44	FJ561069	JF702706
P11	Peanut	China	…	I-17	FJ561068	JF702705
PSi7	Tomato	Indonesia	RUN83	IV-10	EF371804	AY756752
PSS175	Perilla	Taiwan	…	I-32	KF913847	NA
PSS219	Tomato	Taiwan	…	I-34	FJ561167	JF702700
PSS358	Tomato	Taiwan	…	I-15	EU407298	JF702699
PSS81	Tomato	Taiwan	…	I-14	FJ561066	JF702701
R229 (BDB)	Banana	Indonesia	RUN62	IV-10	GU295045	AY756811
R24 (RSY)	Clove	Indonesia	…	IV-9	JF702321	JF702735
R292	White mulberry	China	RUN91	I-12	AF295255	AY756801
RUN549	Tomato	Trinidad	RF38	IIA-37	JF702309	JF702716
T1-UY	Tomato	Uruguay	RUN448	IIA-50	GU295049	JF702712
UQRS555	Ginger	Mauritius	…	I-33	KF913848	NA
UQRS565	Ginger	Thailand	…	I-30	KF913846	NA
UW163	Plantain	Peru	RUN586	IIB-4	GU295052	AY756779
UW170	Heliconia	Colombia	RUN262	IIB-4	DQ011550	JF702702
UW181	Plantain	Venezuela	RUN454	IIA-6	GU295053	AY756754
UW469	Potato	Brazil	JT652	IIA-40	DQ657612	AY756808

[a] Phylotype, sequevar, host, origin, and alternative name information for the were obtained from previously published literature (1,15,21,22,28,30,31); NA = sequence not available.

[b] Phylotype-sequevar.

[c] *Ralstonia solanacearum* type strain, maintained at the Culture Collection of Phytobacteriology Laboratory of the Federal Rural University of Pernambuco, Recife, Brazil.

phylotype-specific multiplex (Pmx)-PCR, which characterized the appurtenance to the *R. solanacearum* species complex and phylotypes (3); strains belonging to phylotype II were further characterized by the specific Mmx-PCR for the Moko disease-causing strains (3,28).

PCR amplification and partial DNA sequencing of the *egl* and *mutS* genes. The PCR amplification of a 750-bp region of the *egl* gene was performed using the primer pair Endo-F (5′-ATGCATGCCGCTGGTCGCCGC-3′) and Endo-R (5′-GCGTTG CCCGGCACGAACACC-3′) (21). The reaction mixture (50 µl total volume) contained 2× PCR Master Mix (Thermo Scientific, San Jose, CA), 0.5 µM primers, and 100 ng of DNA. The reaction mixtures were heated for 9 min at 96°C and followed by 30 cycles (95°C for 1 min, 55°C for 40 s, and 72°C for 2 min), with a final extension step for 10 min at 72°C. The amplification of the 758-bp fragment of the *mutS* gene was performed in a total volume of 50 µl that contained 2× PCR Master Mix, 2.5% dimethyl sulfoxide, 100 ng of DNA, and 1 µM primers mutS-RsF1570 (5′-ACAGCGCCTTGAGCCGTACA-3′) and mutS-RsR1926 (5′-GCTGATCACCGGCCCGAACAT-3′) (28). The reaction mixtures were heated for 5 min at 96°C and followed by 35 cycles (94°C for 1 min, 66°C for 1 min, and 72°C for 90 s), with a final extension step for 5 min at 72°C. The PCR products were submitted to electrophoresis on a 1.5% agarose gel in 0.5× Tris-borate-EDTA buffer along with the GeneRuler 100-bp DNA ladder. The purification of the PCR products was performed with the PCR Clean-up Kit (Axygen Biosciences) and double strands were sequenced by Macrogen (Seoul, South Korea).

Sequence analysis and construction of phylogenetic trees. The alignment of partial sequences of the *egl* and *mutS* genes were performed with ARB Software Environment (15,17) (http://www.arb-home.de/). The phylogenetic reconstruction of strains was achieved with PhyML software (9) using the F81 nucleotide substitution model, which allows base frequencies to vary from 0.25 and aLRT branch support statistics while using the BEST topology search (best of NNI and SPR search). The *egl* and *mutS* sequences identified in this study were deposited in the GenBank database (Table 1).

Pathogenicity tests. The Moko strains and type strain K60^T (tomato, EUA) were tested for pathogenicity on 'Williams' banana plantlets (*Musa* sp.; Cavendish Group, AAA) obtained by micropropagation. The plantlets were grown in 500-ml plastic pots that contained Carolina Padrão substrate (sphagnum moss cake, expanded vermiculite, dolomitic limestone, gypsum, and traces of NPK) (Carolina Soil, Vera Cruz, Brazil) until six leaves were fully developed. The plantlets were then inoculated by injecting 1 ml of an *R. solanacearum* strain suspension (10^8 CFU/ml) into the pseudostem. The plants were kept in a greenhouse with a mean temperature of 36 ± 2°C and mean relative humidity of 85% and checked for 40 days to observe the occurrence of Moko disease symptoms.

The ability of the Brazilian *R. solanacearum* Moko disease-causing strains to infect the tomato plants was studied with the plantlets of 'Santa Clara', which is widely grown in Brazil and susceptible to bacterial wilt. Plants that were grown in 500-ml plastic pots that contained the Carolina Padrão substrate for 25 days were inoculated by the root wound method (5), then kept in a greenhouse with mean temperature and relative humidity values of 38 ± 2°C and 88%, respectively, and assessed for 30 days. The strain pathogenicity was recorded using a qualitative criterion (i.e., – for symptom-free plants and + for wilting or dead plants).

According to the phylogenetic analysis of the *egl* gene sequence, three Moko strains (B4, B7, and B10) were assigned to sequevar IIB-25 (reference strain CIP10, potato, Peru). This sequevar is not known to host the Moko strains; therefore the strains were further investigated for their ability to cause wilt in the 'Aracy' potato (*Solanum tuberosum*) plant within 21 days after budding. The plants were inoculated following the same methodology as the tomato pathogenicity tests. Once symptoms appeared, the bacterial streaming tests and reisolation in triphenil tetrazolium chloride (TZC) agar medium were performed. The strain IBSBF455 (potato, Brazil) was used as the standard strain for comparison.

Based on the phylogenetic analyses, 11 Brazilian *R. solanacearum* Moko disease-causing strains were assigned to sequevar IIA-41 (reference strain CFBP7032, tomato, Cameroon) and three Brazilian strains were closely related to sequevar IIB-25. To investigate the relationship between this unsuspected phylogenetic position and pathogenicity, representative strains of sequevar IIA-41 (CFPB7032, tomato, Cameroon; RUN434, irrigation water, French Guiana; RUN117, bell pepper, Brazil; and RUN118, chili, Brazil) and sequevar IIB-25 (CIP10, potato, Peru; RUN981, tomato, Brazil; and RUN1340 and RUN1341, potato, Iran) were inoculated on 'Grand Naine' banana (Cavendish group) according

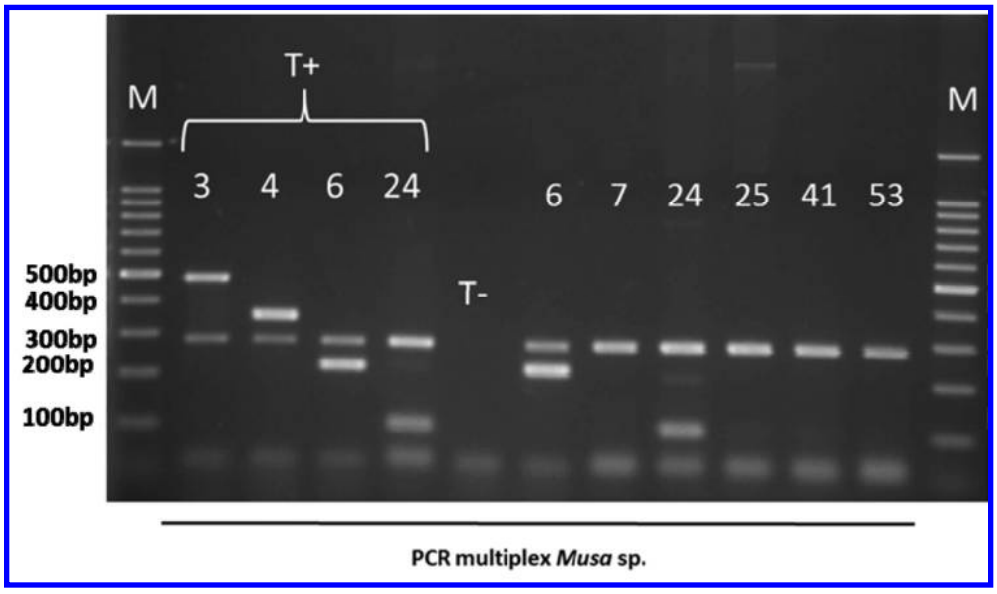

Fig. 1. Moko multiplex polymerase chain reaction (Mmx-PCR) from the Brazilian *Ralstonia solanacearum* Moko disease-causing strains. Lane M, 1-kb ladder; lanes 1 to 4, amplification of the reference strains for sequevars 3, 4, 6, and 24 by Mmx-PCR; lane 5, negative control (water); lanes 6 to 11, amplification of the Brazilian strains by Mmx-PCR, with sequevar 6 (IBSBF2661, sequevar IIA-6), sequevar 7 (K60^T, sequevar IIA-7), sequevar 24 (IBSBF1900, sequevar IIA-24), sequevar 25 (B4, sequevar IIB-25), sequevar 41 (B73, sequevar IIA-41), and sequevar 53 (IBSBF2572, sequevar IIA-53).

to Cellier and Prior (1). The pathogenicity test was performed in a high-security quarantine facility (NS3-Rotoplan, Cirad, Réunion Island, France). Banana plants with five fully developed leaves were inoculated by injecting the pathogen into the pseudostem and depositing the inoculum manually on wounded roots. Symptom development was visually assessed every 3 days for 30 days.

RESULTS

DNA typing. The Pmx-PCRs revealed two amplicons: the 282-bp amplicon specific to the *R. solanacearum* species complex (internal marker) and the 372-bp amplicon, indicating that all of the Brazilian Moko disease-causing strains belonged to phylotype II. The Mmx-PCR identified the sequevars IIA-6 (220 bp) and IIA-24 (100 bp) by using the VC46F/VC46E and SI28F/SI28R primer pairs, respectively. Other strains only amplified the 282-bp amplicon that was specific to *R. solanacearum* (759/760 primer pair); however, a specific amplicon was not amplified for any known Moko sequevars (Fig. 1).

Phylogenetic analysis. The phylotype and sequevar strain typing was based on a phylogenetic reconstruction from a partial sequence of the *egl* sequences along with reference *egl* sequences from the GenBank database that covered the know sequevar diversity within the *R. solanacearum* species complex. Trees constructed with the *egl* and *mutS* sets of sequences were generally consistent with previously reported topological differences (22) (Figs. 2 and 3), in which phylotype II was more closely related to phylotype III in the phylogenetic *egl* tree and closer to phylotype I in the phylogenetic *mutS* tree.

Among the Brazilian strains, the phylogenetic *egl* tree revealed the presence of the Moko disease-related sequevars IIA-6 (IBSBF2661) and IIA-24 (n = 18 strains) (Fig. 2). Furthermore, three new clusters of strains did not group with any previously described Moko disease clusters. The first group, which is reported here as a new sequevar IIA-53, consisted of strains F2, F3, Cotpin2, and IBSBF2572, which were all from Sergipe State. The second group consisted of 11 strains related to strain CFBP7032 (tomato, Cameroon) and assigned to the well-established sequevar IIA-41. The third group included clustered strains B4, B7, and B10 and was closely related to the potato strain CIP10 (Peru) assigned to the known sequevar IIB-25 (Fig. 1). The phylogenetic *mutS* tree (Fig. 3) also unraveled the Moko strains in the sequevars IIA-24 (n = 17 strains), IIA-41 (n = 12 strains), and IIA-53 (F2, F3, and IBSBF2571). An evaluation of

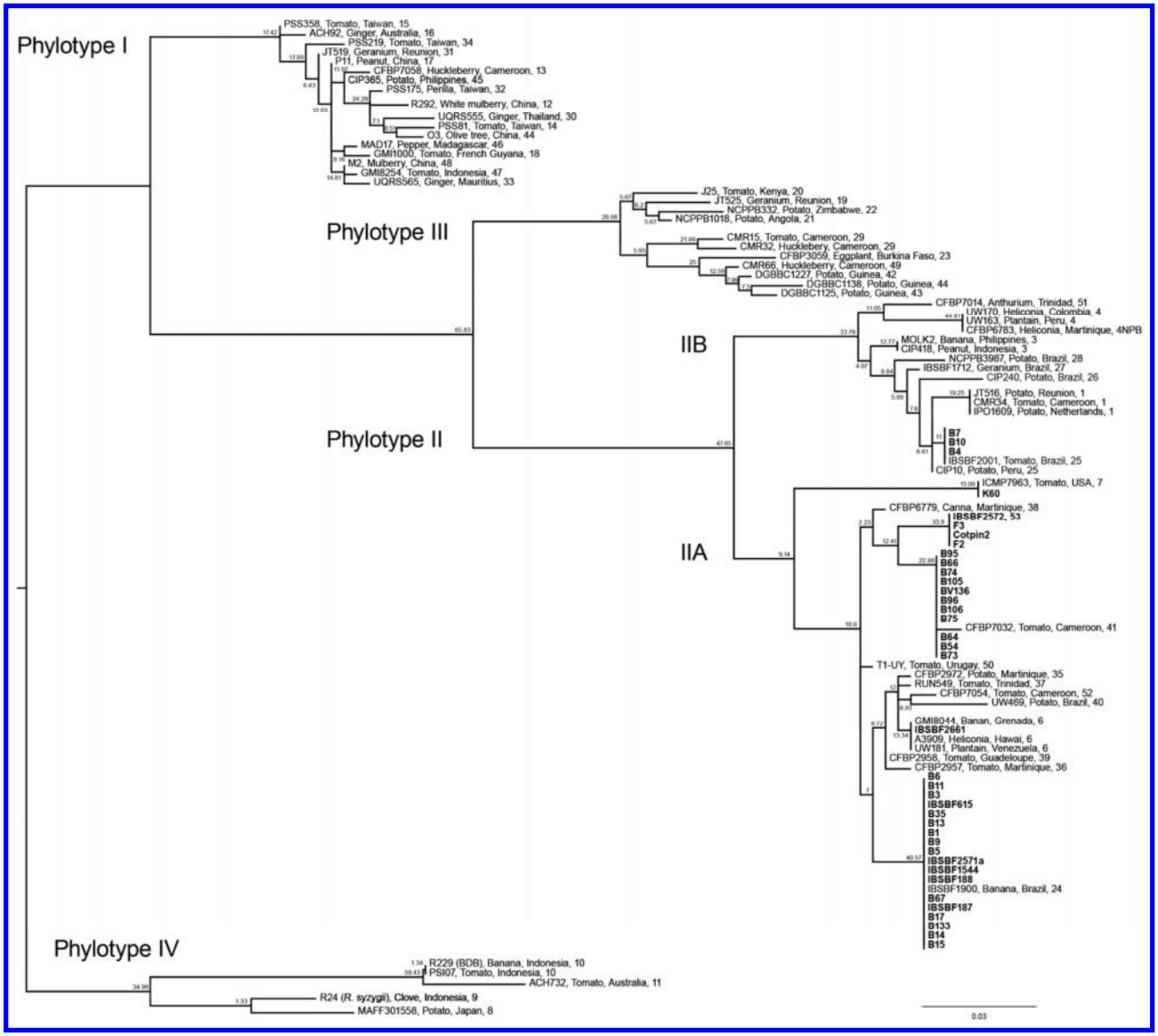

Fig. 2. Phylogenetic reconstruction based on the partial endoglucanase (*egl*) gene sequences from Brazilian Moko disease-causing *Ralstonia solanacearum* strains using the PhyML software and F81 nucleotide substitution model along with aLRT branch support statistics while using the BEST topology search (best of NNI and SPR search).

the Moko strains B4, B7, and B10 indicated that they were incongruent in their phylogenetic assignment between the phylogenetic *egl* and *mutS* trees; in the *mutS* tree, they were grouped in a distinct cluster within phylotype IIB and more closely related to sequevar IIB-3 strains MOLK2 (*Musa* sp., Indonesia), CFBP1409 (*Musa* sp., Honduras), UW9 (*Heliconia* sp., Costa Rica), and CFBP1183 (*Heliconia* sp., Costa Rica) (Fig. 3). There was also incongruence between the *egl* and *mutS* trees for strain IBSBF2571, which was grouped in the IIA-24 sequevar in the *egl* tree and IIA-53 in the *mutS* tree.

Pathogenicity testing. All of the Brazilian strains isolated from the banana and heliconia specimens induced characteristic Moko disease symptoms in the banana plants 15 to 30 days post-inoculation (dpi). The Santa Clara tomato plant also presented wilt symptoms following the inoculation of 12 of 37 strains (Table 1), including the type strain K60^T at 15 to 30 dpi. Among the 12 strains that were found to be pathogenic to tomato, 1 belonged to sequevar IIA-6, 5 belonged to IIA-24, and 6 belonged to IIA-41. Strains from sequevars IIB-25 (n = 3), IIA-53 (n = 4), IIA-41 (n = 5), and IIA-24 (n = 14) were not found to be pathogenic to tomato (Table 1).

The Moko strains B4, B7, and B10 and reference strain CIP10 (potato, Peru) were phylogenetically assigned to sequevar IIB-25 and found to be pathogenic to potato under greenhouse conditions; this result is consistent with the phenotype of the type strain IBSBF455 (potato, Brazil). The incubation period was 15 days. In all of the cases, the bacterial streaming test was positive and strains were reisolated in TZC medium. The reference strains belonging to sequevar IIA-41 obtained from other hosts (CFPB7032, RUN434, RUN117, and RUN118), IIB-25 (CIP10, RUN981, RUN1340, and RUN1341), and IIA-7 (K60^T) were characterized as nonpathogenic to banana because the plants did not develop any symptoms 4 weeks after inoculation.

DISCUSSION

The Moko disease caused by *R. solanacearum* in banana plants is a quarantine pest in Europe (EPPO A2 list) and Brazil, where it has been observed in the northern and northeastern regions and has severely limited crop production in some areas (27). This study investigates the genetic and pathogenic diversity and phylogenetic positions of 37 *R. solanacearum* Moko disease-causing strains from Brazil. It was not surprising that this relatively low sampling (n = 37) mirrored such a broad level of genetic diversity because limited studies have been conducted in Brazil on the genetic diversity of the *R. solanacearum* Moko disease-causing strains. Only scarce information by Pinheiro et al. (20) showed the existence of potential unsuspected genetic groups among the Moko disease agents within phylotype II.

The sequevar is a working definition based on the partial sequence of the *egl* gene. The phylogenetic assignments based on *egl* typing are congruent with the organismal phylogeny based on

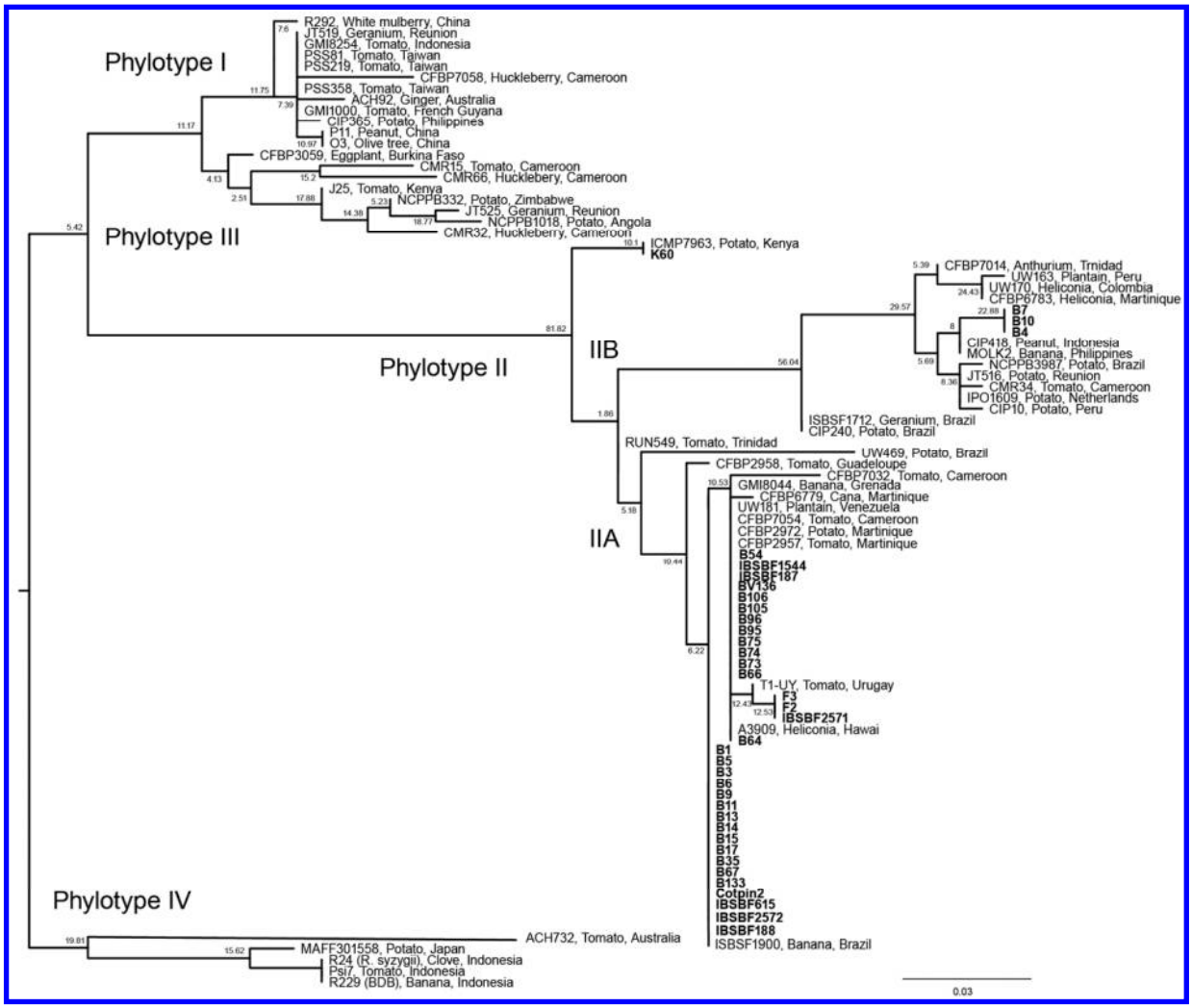

Fig. 3. Phylogenetic reconstruction based on the partial DNA repair (*mutS*) gene sequences from Brazilian Moko disease-causing *Ralstonia solanacearum* strains using the PhyML software and F81 nucleotide substitution model along with aLRT branch support statistics while using the BEST topology search (best of NNI and SPR search).

pangenomic DNA microarray hybridization (8), and this study confirmed that the *egl* gene is a reliable tool for determining the phylogenetic relationships of a particular strain within the *R. solanacearum* species complex (3,4,28).

All of the *R. solanacearum* Moko strains are historically known to infect species of both Musaceae and Heliconiaceae but only some of them infect members of the Solanaceae family under greenhouse conditions (1). Based on the hierarchical classification proposed by Fegan and Prior (4), these strains are typed into four sequevars in the phylotype II: IIA-6, IIA-24, IIB-3, and IIB-4. In this study, some of the Brazilian Moko disease-causing strains belonged to sequevars IIA-6 and IIA-24, whereas other strains were surprisingly assigned to sequevars IIA-41 and IIB-25. The Moko strains B4, B7, and B10 in sequevar IIB-25 were characterized as pathogenic to banana and potato and phylogenetically related to strain CIP10 (potato, Peru) in the phylogenetic *egl* tree; however, they showed a distinct phylogenetic cluster in the phylogenetic *mutS* tree. Such an unexpected assignment of Moko and non-Moko strains sharing the same phylogenetic position was also observed by Wicker et al. (30) in Martinique. This work reported on the emerging strains within the Moko sequevar IIB-4 that were shown to be new pathological variants. These emerging strains were nonpathogenic to banana (IIB4-NPB) but highly aggressive to eggplant, pepper, and tomato, and they even broke down the resistance in tomato 'Hawaii 7996' (30). A new emergent strain of *R. solanacearum* (RS37) obtained from geranium (*Pelargonium* sp.) in Florida colonized triploid banana and reduced the growth of the plant without causing wilting. Strain RS37 was phylogenetically related (*egl* gene) to the Martinique strain CFBP6801 (from *Heliconia caribaea*) and belonged to phylotype II; however, its sequevar was not defined (13). The phylogenetic position of sequevar IIB-25 was framed by the banana Moko IIB-3 strains and potato brown-rot IIB-1 strains; therefore, it may constitute an evolutionary link between the two well-known ecotypes and highlight their potential importance in evolutionary studies for understanding host adaptation.

Furthermore, the Moko disease-causing strains F2, F3, Cotpin2, and IBSBF2572 did not cluster with any previously referenced sequevars but formed the new sequevar IIA-53. The strains IBSBF2572 and Cotpin2 clustered with the strain-typed sequevar IIA-24 in the *mutS* phylogenetic tree; however, the sequevars were based on the *egl* phylogenetic tree and these two strains will remain in sequevar IIA-53 until further investigation. Therefore, it was confirmed that the *mutS*-tree resolution was insufficient to support the sequevar typing of the Moko strains. The new sequevar IIA-53 extended the known phylogenetic diversity of the paraphyletic Moko strains to seven different sequevars that are distributed as phylotypes IIA (IIA-6, IIA-24, IIA-41, and IIA-53) and IIB (IIB-3, IIB-4, and IIB-25). In addition to the newly proposed sequevar IIA-53, the existence of an even larger number of unreported sequevars in Brazil and South America remains possible. This idea is supported by the fact that the last Moko-disease-related sequevar was detected in Brazil (sequevar IIA-24) (4). Brazilian strains IIA-6 and IIA-24 amplified the expected amplicons in the specific Mmx-PCR; however, the Moko strains from sequevars IIA-41, IIA-53, and IIB-25 only amplified the internal marker of the *R. solanacearum* species, which indicated the necessity to develop additional molecular tools to produce reliable diagnostic protocols for all of the Moko sequevars.

All of the Brazilian *R. solanacearum* Moko disease-causing strains tested in this study were pathogenic to banana plants and showed characteristic symptoms of the disease, such as browning of the xylem vessels and wilting until death. These strains displayed variable pathogenic levels on the tomato plants, and no correlation with the sequevar classification was observed among strain IBSBF2661 (IIA-6) and 11 others pathogenic strains, which is consistent with Cellier and Prior (1), who reported that some strains are pathogenic to tomato plants. To our knowledge, the Moko disease-causing strains have never been witnessed in field-grown infected tomato or potato plants, although these strains could be virulent on susceptible potato and tomato plants. This raises a difficult question regarding the role of host adaptation as a bottleneck evolutionary process and its link with the development of strains that are likely to cause epidemics, such as Moko (1).

The correlation between the new syndrome in the state of Sergipe, known here as the "Sergipe facies", and new sequevar IIA-53 confirmed our hypothesis that the syndrome is caused by particular Moko strains in phylotype II. The group reported here as sequevar IIA-53 included strains from the state of Sergipe in northeastern Brazil that were obtained from the peduncle of banana plants showing atypical symptoms, which were similar to the Bugtok disease in Philippines and different from the typical symptoms found in the northern region of Brazil. These strains caused infection that was initiated in the inflorescence and followed by uneven and premature ripening of fruit that internally showed dry rot, and the disease occurred under an aggregated spatial pattern. The bacteria can move toward the pseudostem and cause darkening of the vascular system but it will never cause wilting or yellowing of the plants.

Sequevar IIB-25 included strains from Amazonas State that were found to be pathogenic to potato under experimental conditions. This ability to wilt members of the families Musaceae, Heliconiaceae, and Solanaceae from the Moko ecotype is important and should be considered from both an epidemiological and evolutionary viewpoint.

Central America and northern South America are reported as the centers of origin (10,24) and diversification of the Moko disease-causing strains, which might be related to the large geographical size of the banana production area. Because the phylogenetic analysis of the *egl* gene represents an organismal phylogeny, we can predict three possible hypotheses to explain this paraphyly: (i) the Moko disease-causing ability developed from closely related but genetically different strains (convergent evolution), (ii) all of the clusters in the phylogeny of *R. solanacearum* species complex except the Moko strains lost the ability to cause the disease in banana, and (iii) horizontal gene transfer (HGT) between pathogenic and nonpathogenic strains in the banana occurred and resulted in new pathotypes (4). Relative to this last hypothesis, HGT is known as a major driving force in bacterial evolution (6). By analyzing the comparative genomic hybridization on microarrays, Guidot et al. (7) demonstrated that DNA blocks of 30 kb and 33 genes could be integrated during a single transformation event. Lefeuvre et al. (16) reported that most of the putative HGT events were detected between strains clustered with the Molk2 strain (IIB-3) and in recombinogenic strains of phylotypes I and IIA. Moreover, a limited number of HGT events was found among strains of phylotypes I, III, and IV. From the multilocus sequence analysis data, Wicker et al. (31) reported that the recombination between strains, particularly within phylotype IIB, had a different shift pattern, with phylotype IIB being nearly clonal and phylotype IIA being recombinogenic, highly diverse, and expanding.

This study highlights that, in Brazil, Moko disease is caused by *R. solanacearum* strains from a broad genetic diversity. In addition to the well-characterized Moko sequevars, two sequevars (IIA-41 and IIB-25) previously unrelated to Moko disease were typed as pathogenic to banana, with similar symptoms. Additional environmental strains must be investigated to determine if the Sergipe facies of the Moko disease in the field can be attributed to the new sequevar IIA-53 only. This new phylogenetic aspect of the Moko ecotype must be further investigated and characterized to provide (i) clues for epidemiological studies, (ii) guidance for disease control programs, and (iii) new molecular diagnostic tools for Moko disease-causing strains.

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