Mycologia, 99(6), 2007, pp. 877–883.
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Genetic variability of *Phytophthora sojae* isolates from Argentina

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Abstract: *Phytophthora sojae* causes root and stem rot, one of the most important diseases of soybean worldwide. Genetic diversity of 32 *Phytophthora sojae* isolates of different geographic origin from Argentina was evaluated with RAPD markers. The isolates were collected from diseased soybean plants and soil samples from Santa Fe, Buenos Aires, Córdoba and Entre Ríos provinces, in the Pampeana Region. DNA was amplified with 20 decanucleotides primers. Seven primers amplified 49 fragments, of which 35 were polymorphic, indicating high variability. RAPD analysis detected intraspecific variability even among isolates of the same geographic origin.

Key words: Argentina, Phytophthora root rot, RAPD, soybean

INTRODUCTION

Argentina is the primary exporting country of soybean meal and oil, and third exporter of grain, after USA and Brazil. Soybean production rose to 38 300 000 metric tons from 144 000 000 hectares in 2004/2005 growing season. Yield losses for the 1997/1998 growing season were estimated at 8% (Wrather et al 2001) and more recently at 8–10% (Vallone 2002). During the past decade the situation has worsened due to the widespread use of no-till practices, soybean monocropping and genetic uniformity of cultivars. Late season diseases and several root rot diseases presently are among the most damaging pathologies. Phytophthora root rot (PRR) of soybean (*Glycine max* L. Merr.) caused by *Phytophthora sojae* Kaufmann and Gerdemann, first was noted in Argentina in southern Santa Fe and northern Buenos Aires provinces in Pampeana Region (PR) in the 1970s (Martínez and Ivancovich 1979). In 1989 the pathogen was isolated and identified by Barreto et al (1991). From 1991 to 1992 isolated outbreaks were recorded (Barreto et al 1995). Since then prevalence of the disease has continued to increase and it has become epidemic (Barreto et al 1998, Vallone et al 1999). Incidence registers for PRR rose to 60% in Pergamino, Buenos Aires, and 70% in Marcos Juárez, Córdoba (Vallone et al 1999).

P. sojae is a homothallic Oomycota with more than 50 races described worldwide. In Argentina race 1 was identified in 1989 (Barreto et al 1991) and prevailed for several years, but increasing variability in pathogenicity has been reported (Barreto et al 1997, 1998; Gally et al 1999). The knowledge of pathogenic composition of populations is essential for addressing local breeding programs but demands significant amounts of time and space.

Molecular technologies have been applied recently to study genetic variation in plant pathogen populations. Direct analysis of DNA polymorphisms is a reliable approach to detect genetic variation, and random amplified polymorphic DNA (RAPD) (Williams et al 1990) is a powerful tool for such detection. This method uses primers of arbitrary sequence, which allows an extensive random sampling of the genome. For this reason RAPD has been used to detect genetic variation at the species level (McDermont et al 1994, Gosselin et al 1996, Frazzon et al 2002, Doherty et al 2003, Silva et al 2003, Gouveia et al 2005, Zhou et al 2005).

RAPD has been used to estimate genetic diversity of other *Phytophthora* species (Chang et al 1996, Peters et al 2005). Whisson et al (1994) used RAPD and other molecular techniques to establish the genetic relation among species of the *P. megasperma* complex. RAPD technology also has been used to confirm sexual recombination of *P. sojae* in vitro and to study the segregation of avirulence genes (Whisson et al 1994). Meng et al (1999) used RAPD markers to study populations of *P. sojae* from Indiana, Iowa and Minnesota and found no correlation of populations with geographic origin. Wang et al (2003) analyzed genetic diversity of isolates from China and the United States through RAPD and distinguished 12 genetic groups. RAPD markers also were used to detect interspecific hybrids between *P. sojae* and *P. vignae* (Drenth et al 2003).

The aim of this study was to estimate genetic variability of isolates of *P. sojae* from Argentinean provinces and to estimate phenetic relationships among 32 Argentinean isolates.

Accepted for publication 11 August 2007.
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TABLE I. Origin of Argentine isolates of *P. sojae* in this study

Number of isolate	Locality	Province	Isolated from plant (P) or soil (S)
15, 21, 34, 36, 126	Ocampo	Buenos Aires	P
62, 64	Ocampo	Buenos Aires	S
89, 123, 153, 282	Pergamino	Buenos Aires	P
174, 175	Pergamino	Buenos Aires	S
165, 178	Rafael Obligado	Buenos Aires	S
501, 502	Hughes	Santa Fe	P
500, 503, 504, 505	Hughes	Santa Fe	S
149, 180	Godeken	Santa Fe	P
177	Godeken	Santa Fe	S
49	Bigand	Santa Fe	P
370	Los Molinos	Santa Fe	S
52, 55, 75	Marcos Juárez	Córdoba	P
168, 211	Marcos Juárez	Córdoba	S
600	Gualeguaychú	Entre Ríos	P

MATERIALS AND METHODS

Thirty-two *P. sojae* isolates from diseased plants and infested soils from Buenos Aires, Córdoba, Santa Fe and Entre Ríos provinces, supplied by Dora Barreto from the Instituto Nacional de Investigaciones Agropecuarias (Argentina), were used in the study (TABLE I). Two isolates from Canada, one from Essex (isolate CA1A4) and one from Ottawa (CA9826), supplied by Terry Anderson from the Agriculture and Agrifood Research Centre, were included as controls.

Growth in liquid media.—Erlenmeyer flasks containing 25 mL of liquid V8 medium (40 mL V8 juice, 1 g sucrose, 0.6 g $CaCO_3$, 0.2 g yeast extract and 0.01 g cholesterol/L of bidistilled water) were inoculated by transferring a 5 mm disk cut from the margin of a 5 d old colony growing on VA medium (V8 medium with 2% agar). Cultures were grown at 24 C in a New Brunswick G-27 incubator chamber at 23 C. The mycelium was harvested on the fifth d (Gally et al 2003), vacuum filtered in a Buchner funnel through filter paper, rinsed three times with bidistilled water, fractionated and kept at −70 C until used.

DNA extraction.—Genomic DNA was extracted from mycelia (Dellaporta et al 1983). DNA concentration was estimated by comparing electrophoretic patterns on 0.8% agarose (in 1× TAE buffer) gels with standard DNA marker sets (phage λ double digested with EcoRI and HindIII).

Polymerase chain reaction.—PCR was carried out in 50 µL final volume with 40–60 ng genomic DNA, 5 µL L dNTP mix (100 mM), 6 µL L $MgCl_2$ (25 mM), 10 µL L primer (3 ng/mL), 5 µL L 10× Taq DNA polymerase buffer (10 mM tris-HCl, pH 9.0 at 25 C, 50 mM KCl, 0.1% Triton X-100) and 0.5 µL L Taq DNA polymerase (Promega) (0.5 units/µL). The mixture was amplified in a thermal cycler (Techne Gene E). The thermal cycler was programmed for one cycle of 94 C for 6 min; 45 cycles of 94 C for 1 min, 36 C for 1 min and 72 C for 2 min, followed by a final extension step of 72 C for 6 min.

Two PCR amplifications per isolate were carried out to ensure reproducibility of banding patterns. Measures were taken to prevent contamination of PCR experiments with previously amplified fragments; in particular pre- and postamplification procedures were separated and fresh aliquots of reagents were used for each experiment wherever possible. To test the reliability of PCR products several controls were used routinely, one without primer, a second without Taq DNA polymerase and the third with no genomic DNA.

Primers.—Twenty arbitrary primers (Promega, series A and B) were screened for suitability in a small number of individuals. Only those primers that produced clear and reproducible RAPD bands for all fungal isolates were chosen.

DNA fragment analysis.—Amplification products were resolved on 1.4% agarose gels stained with EtBr (0.5 µg/mL). Fragments were observed and photographed on a UV transilluminator (312 nm).

Statistical methods.—RAPD bands were scored as present (1) or absent (0) across all genotypes to create a binary matrix, which was analyzed with the program NTSYS-PC version 1.8 (Rohlf 1993). The unweighted pair-group arithmetic mean method (UPGMA) cluster analysis was performed based on the simple matching (SM), Jaccard (J), and Dice (D) association coefficients (Sneath and Sokal 1973). Phenograms showing similarity relations were generated by the same program, with the tree display option (TREE). Correlation cophenetic coefficients (r) were calculated for each association coefficient (Sokal and Rohlf 1962). A cophenetic correlation of $r > 0.8$ was considered a good fit. A three-dimensional graphic was obtained with the principal coordinates ordination method (Gower 1966).

The ability of the primers to differentiate among isolates was assessed by calculating their resolving power (Rp) (Prevost and Wilkinson 1999). Genetic variability among isolates from Buenos Aires, Córdoba and Santa Fe provinces was quantified by the percentage of polymorphic loci (P = number of polymorphic loci/number of loci analyzed) and the mean heterozygosity (He) (Nei 1978). The percentage of polymorphic loci and the mean heterozygosity (He) were estimated from allelic frequencies by Biosys 1.7 (Swofford and Selander 1981).

RESULTS

Characterization of RAPD markers.—Of the 20 primers screened seven produced clear and reproducible bands by RAPD amplification for all isolates. The seven primers A01 (5′CCC AAG GTC C3′), A02 (5′GGT GCG GGA A3′), A03 (5′AAG ACC CCT C3′), A5 (5′CAC CAG GTG A3′), A6 (5′GAG TCT CAG G3′), A10 (5′ACG GCG TAT G3′), and B06 (5′GTG ACA TGC C3′) were used for the analysis. They

TABLE II. Data pertinent to six primers. B = number of total bands, PB = number of polymorphic bands, Rp = resolving power and P% = percentage of polymorphic loci

Primer	B	PB	Rp	P%
A01	5	3	9,1	60
A02	6	5	7,11	83
A03	10	9	13,6	90
A06	8	6	13,56	75
A10	6	4	7,72	67
B06	9	9	8,22	100

allowed the scoring of 49 bands that appeared consistently in all experiments among the amplified fragments of the 34 isolates. The Rp of the primers varied from 7.1 for A02 primer to 13.6 for A03 primer (TABLE II). Primers A03 and B06 were more effective in detection of polymorphisms. All bands produced with primer B06 were polymorphic across all isolates. Primer A05 was monomorphic and was not included in the table. Thirty-five (71%) of the total bands were polymorphic, suggesting high genetic variability among the isolates.

Isolates from Buenos Aires Province presented the highest genetic variability and isolates from Córdoba the lowest. Results of isolates from Santa Fe were intermediate (TABLE III).

Cluster analysis.—Three phenograms were obtained based on Jaccard, simple matching and Dice coefficients. High levels of correlation cophenetic coefficients were achieved by the three association coefficients used, indicating low distortion, 0.82 by Jaccard and 0.84 by simple matching and Dice. Dice was chosen for the analysis. The phenogram showed that the relatedness of the 34 isolates of *P. sojae* was 0.76–1.0 (FIG. 1). Grouping analysis revealed that 30 isolates clustered in one group (FIG. 1, group G1) with an origin of ramification in 0.87. A second group (FIG. 1, group G2) included only three isolates, 126, 503 and 178. Isolate 15 from Manuel Ocampo, Buenos Aires, was genetically unique as it was sister of the other 33 isolates, according to analysis (FIG. 1). It presented the lowest values of Dice coefficient, even with respect to isolates of the same province and the same locality (data not shown). The main group (FIG. 1, group G1) comprised two subgroups (FIG. 1, groups SG1 and SG2). Group SG1 with 21 isolates included two conjuncts (C1 and C2), a nucleus of two isolates (89 and 52) and two isolates separated from the rest, one of which was CA9826, from Ottawa, Canada. Group SG2 comprised two conjuncts (FIG. 1, groups C3 and C4). Control isolates from Canada were included in SG1 with 19 Argentinean isolates, which indicated a high degree of similarity among them.

TABLE III. Genetic variability of isolates of *P. sojae* from each province. P% = percentage of polymorphic loci, He = mean heterozygocity (standard errors in parentheses)

Province	Mean sample size per locus	*P	**He
Buenos Aires	15.0	77.8	0.315 (0.03)
Santa Fe	11.0	58.3	0.237 (0.04)
Córdoba	5.0	33.3	0.162 (0.04)

* P: percentage of polymorphic loci.
** He: mean heterozygocity.

Only two pairs of isolates presented 100% of similarity (association coefficient 1.0), and they belonged to different conjuncts of group SG1; isolates174 and 153, both from Pergamino, Buenos Aires, were in group C2; isolates 211 and 505 from Córdoba and Santa Fe respectively were in group C1.

The ordination of isolates through the principal coordinates method let us distinguish five groups (FIG. 2, groups GI to GV) in three-dimensional dispersion and showed nearly the same relations between isolates as the phenogram (FIG. 1). Group GI included isolates of C1 of the phenogram, group GII comprised the remainder of isolates of SG1, group GIII comprised all isolates of C3 (in SGII of the phenogram), group GIV comprised the isolates of C4 and the isolate 15 and Group GV included isolates 178, 126 and 503, which were clustered in a separated group in the phenogram (G2).

DISCUSSION

The main factors that contribute to genetic changes within fungi populations are mutation, mating system, gene flow, migration, population size and selection. However in agricultural ecosystems pathogen populations evolve adapting to constant changes in environment conditions, such as the use of resistant varieties, applications of fungicides and fertilizers, irrigation and crop rotation. These changes impose a strong directional selection, which may be the main force acting in these systems (McDonald 1997).

The evolution of populations of *P. sojae* in USA and Canada has responded mainly to the resistance genes included in the commercial genotypes of soybean cultivated (Hobe 1981, Buzzell and Anderson 1982, Schmitthenner 1994).

Although populations of *P. sojae* in soil usually present high variability, the origin of that variability has not been elucidated fully (Forster et al 1994). A high degree of homozigosis of populations of *P. sojae* could be presumed due to its homothallic character (Tyler et al 1995). However a high level of heterozygosity was

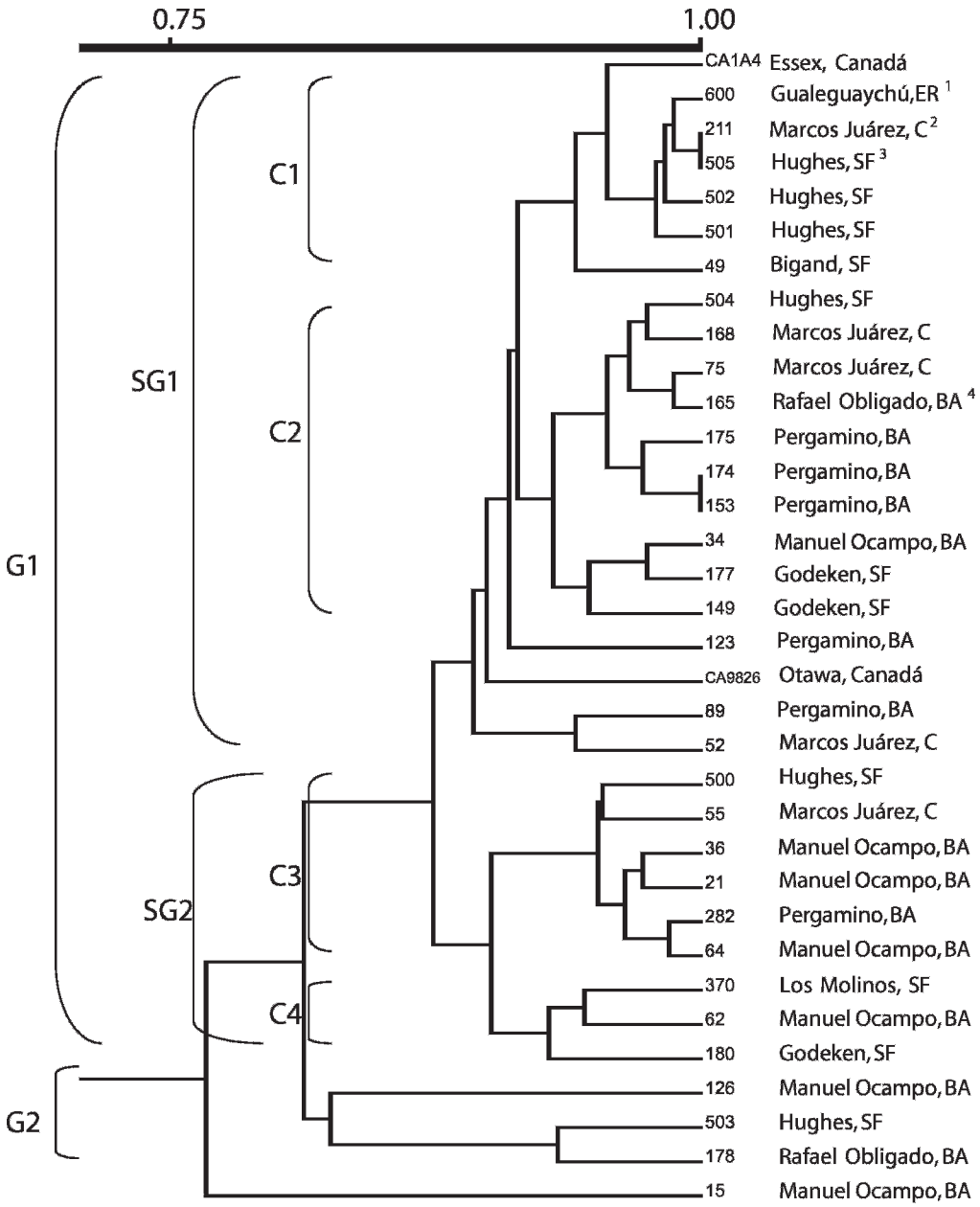

FIG. 1. Phenogram of the relationships between *P. sojae* isolates based on RAPD analysis (Dice coefficient). [1]Entre Ríos, [2]Córdoba, [3]Santa Fe, [4]Buenos Aires.

reported for virulence genes (Bhat and Schmitthenner 1993). Sexual recombination between pathotypes has been demonstrated in vitro and is presumed to play an important roll in the generation and maintainance of genetic diversity in the field (Bhat and Schmitthenner 1993, Tyler et al 1995, Whisson et al 1994). Parasexual recombination, also demonstrated in vitro (Long and Keen 1977, Layton and Khun 1989), may be a less important factor because of its low frequency in field conditions and the adaptative advantage of sexual populations, considering the longer viability of oospores compared to zoospores (Goodwin 1997). In this study RAPD markers were useful for detecting high genetic variability among Argentinean isolates of *P. sojae*. Genetic structure of a population reflects its evolutionary history and its potential to evolve (McDonald 1997). Considerable diversity within Buenos Aires and Santa Fe isolates suggests rapid evolution. The higher variability found in Buenos Aires probably is related to a longer evolution compared to the other sites. *P. sojae* first was isolated in 1989 from diseased plants from Pergamino, north of Buenos Aires (Barreto et al 1991). From 1989 to 1992 the disease was detected only in the same area, and as growers

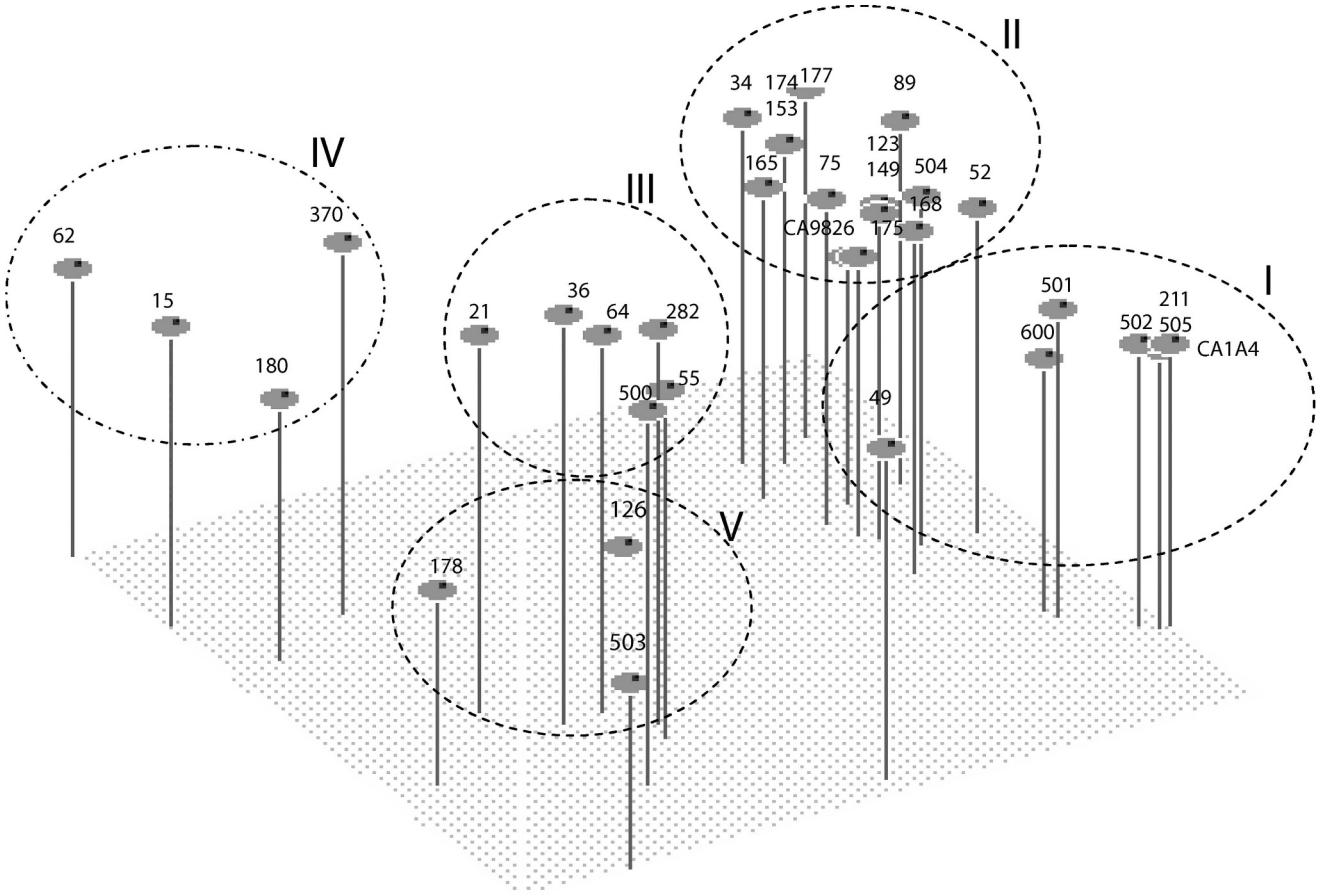

FIG. 2. Grouping of isolates through the principal coordinates method.

adopted conservation tillage systems and continued monocropping the disease began to spread and was detected in other provinces (Ploper 2004). The lower variability obtained from Córdoba isolates probably is related to the fact that they were collected from the same locality.

The range of amplification products obtained with the seven primers in this study clearly demonstrated high intraspecific variation among *P. sojae* isolates even from the same location. Molecular variation yielded up to 71% of polymorphic bands, which represents high degree of polymorphism. The isolates from Canada, used as controls, were clustered in the same subgroup with 19 Argentinean isolates, which indicated a high degree of similarity among them. This arrangement confirmed that with the primers used in this study we did not find any relation between the grouping of isolates and their geographic origin. These results can be compared with those of Wang et al (2003) who used RAPD markers to analyze 75 isolates from China and 11 from USA. They found 12 genetic groups but most of the isolates (44 Chinese and 10 American) were included in one group and no relationship between clustering and geographic origin was found. That study indicated a higher level of polymorphism than in the Argentinean isolates. Meng et al (1999) also found genetic diversity among isolates of *P. sojae*, with four distinct groups obtained by RAPD, with no relationships detected with geographic origin of isolates, the same finding of Peters et al (2005) with *P. erythroseptica*.

Our study indicates that *P. sojae* is a highly variable pathogen in Argentina because great variability was found among isolates from the same locality, and thus RAPD markers are useful to detect intraspecific genetic variation. Its rapid evolution and high genetic diversity indicates increasing fitness of the population, which might improve the pathogen's abilities to overcome single gene resistance, widely used to manage Phytophthora root rot of soybean. This paper constitutes the first report of genetic variability in *P. sojae* populations from Argentina.

ACKNOWLEDGMENTS

We thank Dr Laura Ferreyra for her critical revew of the manuscript and Dr Terry Anderson and Dora Barreto for their kindly collaboration with some strains.

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