**Fulvia fulva** [syn. *Cladosporium fulvum, Passalora fulva*] races in Argentina are evolving through genetic changes and carry polymorphic *avr* and *ecp* gene sequences

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**Abstract** The aim of this work was to study further the population of *Fulvia fulva* [syn. *Cladosporium fulvum, Passalora fulva*] in Argentina particularly in terms of diversity at the genetic level by means of ISSR. Also, we studied polymorphisms within *avr* and *ecp* sequences, what incidentally might lead to the development of new races. Argentinian tomato cultivars only are affected by two races of *F. fulva* and the data we provide here indicates that the populations of these two races are under an evolutionary process. Representatives of them had several polymorphisms within the coding sequences of effectors, but they are not constrained to *avr* genes, probably because, based on field data, resistant cultivars are not available. Most polymorphisms observed in *avr* and *ecp* genes were deletions or insertions (INDELs) or single nucleotide polymorphisms (SNPs). Therefore, probably the environment and management practices are the most important factors driving evolution of these races in Argentina and this might explain why the pathogen population is not driven at the pathogenic or race level.
Keywords *Cladosporium fulvum* · Plant-pathogen interaction · Polymorphism · Extracellular proteins · Avirulence genes · Tomato

Introduction

*Fulvia fulva* (Cooke) Cif. [syn. *Cladosporium fulvum* Cooke, the name traditionally quoted in the literature for this fungus and because of this used throughout this paper], and *Passalora fulva* (Cooke) U. Braun & Crous] (Mycosphaerellaceae, Ascomycota) is a hemibiotrophic fungal pathogen that causes leaf mould of tomato (*Solanum lycopersicum* L.; Thomma et al. 2005). This is a disease that occurs mainly in greenhouse-grown tomatoes, where high relative humidity and temperatures around 20 °C prevail (Thomma et al. 2005). Conidia on the adaxial leaf surface germinate and runner hyphae penetrate through stomata (De Wit 1977; Lazarovits and Higgins 1976). Within a week, diffuse light green to yellowish spots develop on the abaxial leaf surface that gradually increase in size. Within two weeks the fungus starts to sporulate on the abaxial leaf surface and the lesions gradually become necrotic (Blancard and Iglesias 1988; Thomma et al. 2005).

The *C. fulvum*-tomato interaction complies with the gene-for-gene relationship, in which each pathogen avirulence (*avr*) gene product is recognized by a receptor protein encoded by a corresponding dominant host Cf resistance gene (Stergiopoulos and De Wit 2009). AVR proteins serve dual functions; in the absence of a matching Cf receptor protein in the plant, they contribute to virulence and serve as effector proteins facilitating apoplastic leaf colonization (compatible interaction). However, in tomato plants carrying Cf receptors, matching effector proteins are recognized and trigger a Cf-mediated resistance response (incompatible interaction). This is usually associated with a hypersensitive response (HR) (Heath 2000) and by the accumulation of callose, phytoalexins and pathogenesis-related proteins (De Wit 1982; De Wit 1992), which are effective tools according to their virulence, which depends on the presence/absence of Avr proteins (Gabriëls 2006). In addition to these five *avr* genes, seventy genes coding for extracellular proteins (*ecp*) from *C. fulvum* have now been cloned recently (Mesarich et al. 2018). A total of fourteen Ecps have been shown to trigger HR in wild species of tomato (*Solanum* spp.). These were Ecp1, Ecp2–1, Ecp4, Ecp5, Ecp6, Ecp8, Ecp9–1, Ecp10–1, Ecp11–1, Ecp12, Ecp13, Ecp14–1, Ecp15 and Ecp16 that carry the cognate Cf-ecp genes (Laugé et al. 1996; Laugé et al. 2000; Van der Hoorn et al. 2001; Krujtt et al. 2005; Thomma et al. 2011; Mesarich et al. 2018).

Plant-pathogenic fungi can be characterized by their lifestyle, which is the result of complex interactions with their hosts with whom they coevolved. Plant immune receptors impose a high selection pressure on pathogen effectors that mostly evolved in such a way that they avoid or escape recognition though retaining their function, which has been found in many pathosystems including the *C. fulvum*-tomato interaction (Allen et al. 2004; Dodds et al. 2006; Ma and Guttmann 2008; Soanes and Talbot 2008; Stavrinides et al. 2008; Van Den Hooven et al. 1999, 2001). Also, horizontal gene transfer might increase pathogens aggressiveness, expanding in this way their host range (Stergiopoulos and De Wit 2009). Since the 1930s, different Cf resistance genes against *C. fulvum* have been introduced in tomato cultivars (Lindhout et al. 1989; Rivas and Thomas 2005), which raised the selection pressure on the fungal population, leading to various types of adaptations in effector proteins. Regarding this, gene loss or pseudogenization might contribute to the adaptation of fungal pathogens to a hemibiotrophic lifestyle like that revealed by *C. fulvum*. An example of this might be the limited number of genome encoded proteases that are expressed during *C. fulvum* infection (Jashni et al. 2019).

In *C. fulvum*, five *avr* genes (*avr2*, *avr4*, *avr4E*, *avr5* and *avr9*) have been cloned (Joosten et al. 1994; Luderer et al. 2002b; Van den Ackerveken et al. 1992; Van Kan et al. 1991; Westerink et al. 2004a, b; Mesarich et al. 2014), all encode proteins that trigger HR in tomato plants carrying the cognate Cf-2, Cf-4, Hcr9-4E, Cf-5 and Cf-9 genes, respectively (Dixon et al. 1996; Jones et al. 1994; Takken et al. 1999; Thomas et al. 1997; Dixon et al. 1998). *Cladosporium fulvum* Avr proteins are small, secreted, cysteine-rich, and the nomenclature of the races of the strains of *C. fulvum* is according to their virulence, which depends on the presence/absence of Avr proteins (Gabriëls 2006). In addition to these five *avr* genes, seventy genes coding for extracellular proteins (*ecp*) from *C. fulvum* have now been cloned recently (Mesarich et al. 2018). A total of fourteen Ecps have been shown to trigger HR in wild species of tomato (*Solanum* spp.). These were Ecp1, Ecp2–1, Ecp4, Ecp5, Ecp6, Ecp8, Ecp9–1, Ecp10–1, Ecp11–1, Ecp12, Ecp13, Ecp14–1, Ecp15 and Ecp16 that carry the cognate Cf-ecp genes (Laugé et al. 1998; Laugé et al. 2000; Van der Hoorn et al. 2001; Krujtt et al. 2005; Thomma et al. 2011; Mesarich et al. 2018; Stergiopoulos et al. 2012; Ökmen et al. 2013; Mesarich et al. 2014; Mesarich et al. 2018).
Iakovidis et al. (2020; Kahlone et al. 2020). Recently, Iakovidis et al. (2020) described the genes encoding recognition of the C. fulvum Ecp5 that are encoded by several loci within the genome of tomato.

Various types of mutations in AVR genes of C. fulvum have been reported, including deletions and changes that can either lead to their loss or provide stability/instability to effector proteins that might be no longer recognized by their cognate Cf receptors (Westerink et al. 2004b; Van Kan et al. 1991, Van Den Burg et al. 2006). In this respect, Stergiopoulos et al. (2007) found that nucleotide variation in ECP genes occurred at a lower frequency than in AVR ones and also that they are mostly silent substitutions or modifications in non–protein-coding sequences (Stergiopoulos et al. 2007), which might be explained by the fact that Cf-ecp genes have not been introduced in commercial tomato varieties regularly (Laugé et al. 1998).

Hybrid cultivars of tomato grown in Argentina are susceptible to C. fulvum and this fungus causes significant economic losses in greenhouse-grown tomatoes in the provinces Corrientes and Buenos Aires (Rollan et al. 2013). Medina et al. (2015) isolated, characterized and analysed several isolates of this fungus collected from diseased tomatoes in these provinces. We monitored the disease in recent years and collected additional isolates, all belonged either to race 0 or 2.

The aim of the present work was to increase our knowledge regarding the population of C. fulvum in Argentina particularly in terms of its diversity. We analysed diversity of C. fulvum isolates by means of ISSR and then we studied polymorphisms within AVR and ECP sequences that code for virulence factors, which might lead to the appearance of new races.

Materials and methods

Isolates of Cladosporium fulvum

Eight new isolates of C. fulvum were obtained from tomato cultivars with typical leaf mould disease symptoms. They were collected in different locations of the two main tomato production areas of Argentina (Table 1). Monosporic cultures of isolates were maintained on potato dextrose agar (PDA) at 4 °C (Rollan et al. 2013), and deposited in the culture collection of the Centro de Investigaciones de Fitopatología (CIDEFI), Universidad Nacional de La Plata (UNLP). In addition, 12 previously described isolates were also included in this study (Medina et al. 2015).

Genomic DNA extraction

Total genomic DNA was extracted from monosporic cultures using the Wizard® Genomic DNA Purification kit. The quality and quantity of genomic DNA was evaluated by electrophoresis at 90 V for 30 min in a 0.7% (w/v) agarose gel that was stained with ethidium bromide (10% v/v). Gels were visualized using UV light and images were captured with the GeneSnap software. Genomic DNA was quantified by comparing DNA bands with those of a molecular marker lambda genome digested with HindIII (Invitrogen) through the GeneTools in an image analyser (SynGene, Cambridge, UK), according to the methodology described by Franco et al. (2017).

Genetic diversity of C. fulvum isolates

Diversity among the isolates was done by analysis of Inter Simple Sequence Repeat (ISSR) fingerprints. As outgroup S. lycopersici CIDEFI 216 was included in the analysis (Franco et al. 2017). Seven ISSR primers were used (AA5, AN, GA5, IA5, BA3, FA5, and LA5, Additional Table 1) to perform PCR amplifications as described by Franco et al. (2017). PCRAs were run in a PTC-0150 MiniCycler (MJ. Research) thermocycler programmed as follows: an initial denaturation step at 94 °C for 7 min, followed by 33 cycles of 94 °C for 1 min, 48 °C (primers AA5, AN, GA5, IA5 and BA3) or 53 °C (primers FA5 and LA5) for 75 s and 72 °C for 4 min, and then a final extension of 72 °C for 7 min. ISSR-PCR products were resolved in 1.5% agarose gels stained with ethidium bromide. Gels were run at 70 V, and then were exposed to UV illumination and images were captured with GeneSnap software (SynGene). The ISSR-PCR banding patterns obtained from stable amplified bands were analysed according to statistical methods used by Franco et al. (2017).

PCR of internal transcribed spacer (ITS)

The ITS region was amplified by ITS-4 and ITS-5 primers (Additional Table 2, White et al. 1990), using fungal genomic DNA as template. Reaction mixtures contained 1× amplification buffer (Inbio Highway), 1.5 mM MgCl2 (Inbio Highway), 40 ng of each primer,
200 mM each deoxynucleoside triphosphate (Inbio Highway), 25 ng of DNA template, and 1 U of Taq DNA polymerase (Inbio Highway) in a 15-μl volume.

Reactions were performed in a PTC-1152 Mini Cycler (MJ Research) programmed as follows: an initial step at 94 °C for 4 min; followed by 33 cycles of a denaturing step at 94 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min; with a final extension at 72 °C for 5 min.

PCR products were resolved by electrophoresis in 1% (w/v) agarose gels stained with 10% ethidium bromide (v/v). A 100 to 1000 bp DNA ladder marker (Inbio Highway) was used to estimate the size of the amplicons. Gels were observed and documented in a GeneGenius analyser (Syngene) and the bands were processed by means of GeneSnaps, GeneTools, and GeneDirectory software (Syngene). Amplicons were purified as described by Sambrook et al. (1989), precipitated, and sequenced at MACROGEN Inc. (Seoul, South Korea). ITS sequences were deposited at the National Centre for Biotechnology Information (NCBI) GenBank (Additional Table 3).

PCR amplification of \textit{avr} genes and race identification

The primers used in the amplification reactions were those described by Stergiopoulos et al. (2007), which were modified since the universal M13 sequence was removed (Additional Table 2). PCRs were performed in a 15-μl volume with a thermocycler (Model Multigene gradient; Labnet International, In) programmed as follows: an initial denaturing step at 94 °C for 5 min; then, 40 cycles of a denaturing step at 94 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min; with a final extension at 72 °C for 5 min.

PCR products were resolved by electrophoresis in 1% (w/v) agarose gels stained with 10% ethidium bromide (v/v). A 100 to 1000 bp DNA ladder marker (Inbio Highway) was used to estimate the size of the amplicons. Gels were observed and documented in a GeneGenius analyser (Syngene) and the bands were processed by means of GeneSnaps, GeneTools, and GeneDirectory software (Syngene). Amplicons were purified as described by Sambrook et al. (1989), precipitated, and sequenced at MACROGEN Inc. (Seoul, South Korea). ITS sequences were deposited at the National Centre for Biotechnology Information (NCBI) GenBank (Additional Table 3).

| Isolate CIDEFI | Race | Tomato cultivar | Year of sampling | Site of sampling | Reference |
|----------------|------|-----------------|------------------|-----------------|-----------|
| 300            | 2    | Elpida          | 2011             | Los Hornos, La Plata | Medina et al. 2015 |
| 301            | 2    | Elpida          | 2011             | Abasto, La Plata  | Medina et al. 2015 |
| 303            | 0    | Elpida          | 2012             | Los Hornos, La Plata | Medina et al. 2015 |
| 305            | 2    | Colibri         | 2012             | Arana, La Plata  | Medina et al. 2015 |
| 307            | 2    | Cherry          | 2012             | Arana, La Plata  | Medina et al. 2015 |
| 309            | 0    | Elpida          | 2012             | Arana, La Plata  | Medina et al. 2015 |
| 310            | 0    | Compak          | 2012             | Arana, La Plata  | Medina et al. 2015 |
| 311            | 2    | Colibri         | 2012             | Arana, La Plata  | Medina et al. 2015 |
| 312            | 2    | Cherry-Colly    | 2012             | Arana, La Plata  | Medina et al. 2015 |
| 315            | 0    | Elpida          | 2012             | Olmos, La Plata  | Medina et al. 2015 |
| 318            | 0    | Elpida          | 2012             | Etcheverry, La Plata | Medina et al. 2015 |
| 320            | 2    | Keitor          | 2013             | Corrientes       | Medina et al. 2015 |
| 322            | 2    | Yigido          | 2014             | Abasto, La Plata  | This study |
| 323            | 2    | Verde Cebra     | 2014             | Abasto, La Plata  | This study |
| 325            | 2    | Elpida          | 2015             | Gorina, La Plata  | This study |
| 326            | 0    | Pantano         | 2015             | Abasto, La Plata  | This study |
| 327            | 2    | Banano          | 2015             | Abasto, La Plata  | This study |
| 329            | 2    | Banano          | 2012             | Abasto, La Plata  | This study |
| 330            | 0    | Elpida          | 2012             | Los Hornos, La Plata | This study |
| 332            | 0    | Elpida          | 2015             | Abasto, La Plata  | This study |
Table 2 Allelic variation in the avirulence (Avr) and extracellular protein (Ecp) effector genes of *Cladosporium fulvum*. In black font are listed the variations found in the isolates of *C. fulvum* presented in this study.

| Gene | Polymorphism at DNA level | Isolates | Gene region | Predicted mutation in protein |
|------|--------------------------|----------|-------------|-------------------------------|
| **Avr 2** | 15 del. G | 318 | Exon 1 | – |
| | 22 del. C | 309 | Exon 1 | – |
| | 26 A > C | 330 | Exon 1 | – |
| | 55 T > G | 309 | Exon 1 | Trp > Gly Signal peptide |
| | 56 G > T | 315 | Exon 1 | Trp > Leu Signal peptide |
| | 191 A > G | 309 | Intron 1 | – |
| | 387 del. G | 309 | Exon 2 after stop | – |
| | 424 del. T | 309 | Exon 2 after stop | – |
| | 441 del. A | 309 | Non coding region | – |
| | 460 del. A | 309 | Non coding region | – |
| | 469 del. A | 309 | Non coding region | – |
| **Avr 4** | 1 C > A | 310 | Non coding region | – |
| | 23 ins. C | 301 | Non coding region | – |
| | 48 G > T | 309 | Non coding region | – |
| | 63 del. C | 325 | Non coding region | – |
| | 75 A > C | 309 | Non coding region | – |
| | 77 A > T | 309 | Non coding region | – |
| | 80 G > A | 300, 301, 305, 307, 311 | Non coding region | – |
| | 80 G > A | 322, 323, 327, 329, 332 | Non coding region | – |
| | 82 A > C | 309 | Non coding region | – |
| | 83 C > T | 309 | Non coding region | – |
| | 89 A > C | 309 | Non coding region | – |
| | 90 A > G | 309 | Non coding region | – |
| | 103 C > T | 309 | Non coding region | – |
| | 114 A > C | 309 | Non coding region | – |
| | 220 C > A | 309 | Exon 1 | Synonymous (Signal peptide) |
| | 493 G > A | 325 | Exon 1 | Synonymous |
| | 678 A > T | 305 | Non coding region | – |
| **Avr 4E** | 364 C > T | All | Exon 1 | Phe > Leu |
| | 364 C > T | All | Exon 1 | Phe > Leu |
| | 398 C > T | All | Exon 1 | Met > Thr |
| | 398 C > T | All | Exon 1 | Met > Thr |
| **Avr 9** | 18 G > T | 326 | Non coding region | – |
| | 137 T > G | 311 | Exon 1 | – |
| | 225 A > T | 325 | Intron 1 | – |
| | 363 del. C | 312 | Exon 2 | – |
| | 412 del. G | 311 | Exon 2 after stop | – |
| | 426 del. T | 311 | Exon 2 after stop | – |
| | 501 G > C | 332 | Exon 2 after stop | – |
| | 505 ins. T | 332 | Exon 2 after stop | – |
| | 516 T > C | 332 | Exon 2 after stop | – |
| | 518 ins. G | 332 | Exon 2 after stop | – |
| | 535 ins. A | 332 | Exon 2 after stop | – |
| **Ecp 1** | 93 A > T | 307 | Non coding region | – |
| Gene     | Polymorphism at DNA level | Isolates | Gene region   | Predicted mutation in protein |
|----------|--------------------------|----------|---------------|------------------------------|
| 97 A > G | 307                      | Non coding region | –                  |
| 120 ins. C | 300, 305, 307, 318, 320 | Non coding region | –                  |
| 120 ins. C | 323, 325, 326, 327, 329, 330, 332 | Non coding region | –                  |
| 217 del. G | 307                      | Non coding region | –                  |
| 487 ins. G | All                      | Non coding region | –                  |
| 487 ins. G | All                      | Non coding region | –                  |
| 522 C > A | 323                      | Non coding region | –                  |
| 801 ins. T | 322                      | Intron 1 | –                  |
| 826 C > T | 307                      | Intron 1 | –                  |
| 915 C > T | All (except 305)         | Intron 2 | –                  |
| 915 C > T | All (except 323)         | Intron 2 | –                  |
| 943 del. C | All (except 305)         | Intron 2 | –                  |
| 943 del. C | All (except 322)         | Intron 2 | –                  |
| 944 G > C | 329                      | Intron 2 | –                  |
| 949 C > A | 329                      | Intron 2 | –                  |
| 964 G > A | 329                      | Exon 3   | –                  |
| 969 T > G | 329                      | Exon 3   | Asp> Asn          |
| 970 A > T | 329                      | Exon 3   | –                  |
| 974 C > G | 329                      | Exon 3   | –                  |
| 979 ins. A | 329                      | Exon 3   | Frameshift        |
| 1003 ins. G | 329                      | Exon 3 after stop | –                  |
| 1025 ins. G | 318                      | Exon 3 after stop | –                  |
| 1076 ins. G; ins. TT | 326, 329 | Exon 3 after stop | –                  |
| 1078 G > A | 329                      | Exon 3 after stop | –                  |
| 1079 G > A | 326, 329                 | Exon 3 after stop | –                  |
| 1085 G > A | 329                      | Exon 3 after stop | –                  |
| 1086 C > G | 329                      | Exon 3 after stop | –                  |
| 1090 G > A | 329                      | Exon 3 after stop | –                  |
| 1092 G > C | 329                      | Exon 3 after stop | –                  |
| 1095 ins. A | 325, 326, 329 | Exon 3 after stop | –                  |
| 1107 A > T | 329                      | Exon 3 after stop | –                  |
| 1108 C > G | 329                      | Exon 3 after stop | –                  |
| 1122 ins. A | 326                      | Exon 3 after stop | –                  |
| 1123 G > T | 329                      | Exon 3 after stop | –                  |
| 1126 G > C | 329                      | Exon 3 after stop | –                  |
| 1129 G > T | 329                      | Exon 3 after stop | –                  |
| 1134 G > A | 326, 329                 | Exon 3 after stop | –                  |
| 1135 A > G | 326                      | Exon 3 after stop | –                  |
| 1137 T > A; T > C | 326, 329 | Exon 3 after stop | –                  |
| 1138 G > T; G > A | 326, 329 | Exon 3 after stop | –                  |
| 1139 T > G; T > A | 326, 329 | Exon 3 after stop | –                  |
| 1140 G > T | 326, 329                 | Exon 3 after stop | –                  |
| 1141 C > G | 326, 329                 | Exon 3 after stop | –                  |
| 1142 C > T | 329                      | Exon 3 after stop | –                  |
| Gene Polymorphism at DNA level | Isolates | Gene region | Predicted mutation in protein |
|-------------------------------|----------|-------------|-----------------------------|
| 1144 T > G; T > C            | 326, 329 | Exon 3 after stop | –                           |
| 1146 C > T                   | 326      | Exon 3 after stop | –                           |
| 1148-1153 ACGTCG > CTACGT    | 326      | Exon 3 after stop | –                           |
| 1154–1157 ins. TCAA; ins. TCGA| 326, 327 | Exon 3 after stop | –                           |
| 1158 A > G                   | 326, 327 | Exon 3 after stop | –                           |
| 1161 G > T                   | 329      | Exon 3 after stop | –                           |
| 1162 G > C; G > T            | 326, 329 | Exon 3 after stop | –                           |
| 1163 C > G; C > A; C > T     | 323, 326, 329 | Exon 3 after stop | –                           |
| 1165 G > A                   | 326      | Exon 3 after stop | –                           |
| 1167 G > A                   | 320      | Exon 3 after stop | –                           |
| 1167 G > C                   | 326      | Exon 3 after stop | –                           |
| 1169 G > T                   | 329      | Exon 3 after stop | –                           |
| 1170 A > C                   | 329      | Exon 3 after stop | –                           |
| 1177 G > C                   | 326      | Exon 3 after stop | –                           |
| 1178 C > C                   | 329      | Exon 3 after stop | –                           |
| 1179 C > A                   | 329      | Exon 3 after stop | –                           |
| 1182 ins. T                   | 326      | Exon 3 after stop | –                           |
| 1188 G > A                   | 326, 329 | Exon 3 after stop | –                           |
| 1190 C > A                   | 326      | Exon 3 after stop | –                           |
| 1192 A > C                   | 326      | Exon 3 after stop | –                           |
| 1193 C > T                   | 326, 329 | Exon 3 after stop | –                           |
| 1195 A > C                   | 326, 329 | Exon 3 after stop | –                           |
| 1197–1198 ins. AA            | 326      | Exon 3 after stop | –                           |
| 1201 A > T                   | 329      | Exon 3 after stop | –                           |
| 1202 A > C                   | 307      | Exon 3 after stop | –                           |
| 1206 ins. A                  | 326      | Exon 3 after stop | –                           |
| 1210 G > A                   | 326, 329 | Exon 3 after stop | –                           |
| 1212 C > A                   | 326      | Exon 3 after stop | –                           |
| 1213 Ins. C                  | 320      | Exon 3 after stop | –                           |
| 1213 ins. C; ins. G          | 326, 322, 332 | Exon 3 after stop | –                           |
| 1214 G > T                   | 322, 332 | Exon 3 after stop | –                           |
| 1216 del. T                  | 322      | Exon 3 after stop | –                           |
| 1218 A > C                   | 329      | Exon 3 after stop | –                           |
| 1219 G > A                   | 326      | Exon 3 after stop | –                           |
| 1223 T > C                   | 326      | Exon 3 after stop | –                           |
| 1224 G > T                   | 326, 329 | Exon 3 after stop | –                           |
| 1225 ins. G                  | 326      | Exon 3 after stop | –                           |
| 1229 T > C                   | 329      | Exon 3 after stop | –                           |
| 1231 G > A                   | 329      | Exon 3 after stop | –                           |
| 1238 T > A                   | 301      | Exon 3 after stop | –                           |
| 1238 T > A                   | 326, 329 | Exon 3 after stop | –                           |
| 1239 C > A                   | 301      | Exon 3 after stop | –                           |
| 1239 C > A                   | 322, 326, 332 | Exon 3 after stop | –                           |
| 1242 C > T                   | 329      | Exon 3 after stop | –                           |
| Gene     | Polymorphism at DNA level | Isolates | Gene region | Predicted mutation in protein |
|----------|--------------------------|----------|-------------|-----------------------------|
| 1249 T > C | 326                      | Exon 3 after stop |
| 1251 ins. C | 326                      | Exon 3 after stop |
| 1266 C > T | 326                      | Non coding region – |
| 1269 T > C | 326                      | Non coding region – |
| 1270 T > C | 326                      | Non coding region – |
| 1271–1273 ins. TTG | 326 | Non coding region – |
| 1284 A > G | 326                      | Non coding region – |
| 1287–1288 ins. AC | 326 | Non coding region – |
| 1293 C > G | 326                      | Non coding region – |
| 1294 G > C | 326                      | Non coding region – |
| 1296 A > C | 326                      | Non coding region – |
| 1302 del. T | 326                      | Non coding region |
| 1311 ins. A | 326                      | Non coding region – |
| 1312 C > T | 326                      | Non coding region – |
| 1319–1321 AGC > CCG | 326 | Non coding region – |
| 1323 C > T | 326                      | Non coding region – |
| 1326 C > G | 326                      | Non coding region – |
| 1328 T > C | 326                      | Non coding region – |
| 1329 del. G | 326                      | Non coding region – |
| 1331 A > G | 326                      | Non coding region – |
| 1332 G > A | 326                      | Non coding region – |
| 1335 G > T | 326                      | Non coding region – |
| 1336 A > G | 301                      | Non coding region – |
| 1336 A > G | 327                      | Non coding region – |
| 1337 C > A | 301                      | Non coding region – |
| 1337 C > A; C > T | 326, 327 | Non coding region – |
| 1338 C > T | 326                      | Non coding region – |
| Ecp2 22 del. T | 318 | Non coding region – |
| 22 del. T | 323                      | Non coding region – |
| 22–25 del. TGCA | 300 | Non coding region – |
| 28 del. C | 329                      | Non coding region – |
| 30–32 del. TAC | 320 | Non coding region – |
| 35 T > A | 320                      | Non coding region – |
| 41 C > T | 300                      | Non coding region – |
| 42 A > C | 320                      | Non coding region – |
| 75 del. G | 329                      | Non coding region – |
| 75–76 del. GA | 300 | Non coding region – |
| 79 G > C | 320                      | Non coding region – |
| 82 A > T | 320                      | Non coding region – |
| 89–90 GT > CC | 305 | Non coding region – |
| 123–124 TT > AC | 326 | Non coding region – |
| 193 ins. C | 326                      | Non coding region – |
| 205 C > A | 326                      | Non coding region – |
| 257 T > C | 326                      | Exon 1 Synonymous (Signal peptide) |
The molecular race identification was based on the presence/absence/sequence of \textit{avr} genes (Medina et al. 2015). \textit{Avr} gene sequences were deposited at National Centre for Biotechnology Information (NCBI) GenBank (Additional Table 3).

### Table 2 (continued)

| Gene | Polymorphism at DNA level | Isolates | Gene region | Predicted mutation in protein |
|------|---------------------------|----------|-------------|------------------------------|
| 305  | C > A                     | 326      | Exon 1      | Synonymous                   |
| 409  | G > T                     | 326      | Exon 1      | Arg > Ile                    |
| 414  | T > G                     | 326      | Exon 1      | Ser > Ala                    |
| 663  | ins. A                    | 300      | Exon 2      | Frameshift                   |
| 680  | ins. G                    | 301      | Exon 2      | Frameshift                   |
| 783–791 ins. GCCCGCGGC | 300      | Non coding region | – |
| \textit{Ecp4} | G > T                  | 300, 301, 305, 307, 320 | Exon 2 | Cys > Trp |
| 380  | G > T                     | All      | Exon 2      | Cys > Trp                    |
| 395  | ins. A                    | 326      | Exon 2      | Gly > Val                    |
| 408  | ins. A                    | 326      | Exon 2      | Frameshift                   |
| 423  | ins. T                    | 326      | Non coding region | – |
| \textit{Ecp5} | A > T                  | 300, 301 | Exon 1 | Premature stop codon |
| 516  | del. G                    | 322, 323, 327, 329 | Exon 2 | Frameshift |
| 541  | G > A                     | 318      | Exon 2      | Cys > Tyr                    |
| 541  | G > A                     | 325, 330, 332 | Exon 2 | Cys > Tyr |
| 691  | ins. G                    | 307      | Exon 2      | Frameshift +premature stop codon |
| 863  | del. A                    | 323      | Non coding region | – |
| 867  | del. A                    | 305      | Non coding region | – |
| 867  | del. A                    | 323, 327 | Non coding region | – |

### Table 3

| Genes | \( N_a \) | Nucleotide diversity | \( \pi \) | \( \theta_S \) | \( (\omega) \) |
|-------|----------|----------------------|----------|--------------|--------------|
| \textit{Avr2} | 8       | 0.005658 (0.003810)  | 2.7500   | 1.54270      | /            |
| \textit{Avr4} | 19      | 0.002459 (0.016880)  | 1.6670   | 4.00562      | 0            |
| \textit{Avr4E} | 19      | 0 (0)                | 0.0000   | 0.00000      | 0            |
| \textit{Avr9} | 19      | 0.002050 (0.001569)  | 1.1110   | 1.45368      | 1.5464       |
| \textit{Ecp1} | 14      | 0.018247 (0.009580)  | 24.7250  | 29.4405      | 0.7045       |
| \textit{Ecp2} | 14      | 0.006648 (0.003827)  | 5.3850   | 4.40233      | 0.3414       |
| \textit{Ecp4} | 14      | 0.001171 (0.001131)  | 0.5710   | 0.31445      | 21,952       |
| \textit{Ecp5} | 13      | 0.002178 (0.001486)  | 1.9230   | 0.64449      | 23,095       |

\( N_a \): Number of isolates used for the analysis. CIDEFI 326 was excluded from calculation of ECP5 due the large deletion.

\( \pi \): Nucleotide diversity based on the average number of nucleotide differences between two random sequences estimated for the entire length of the sequences analyzed for only non-synonymous substitutions, and synonymous ones.

\( \theta_S \): The statistic of the number of segregating sites, estimated for the entire length of the sequences analyzed, for non-synonymous and synonymous substitution.

\( (\omega) = Ka/Ks \): ratio of non-synonymous (Ka) to synonymous (Ks) nucleotide substitution rates, is an indicator of selective pressures on genes.
PCR amplification of ecp genes

Primers used in the reactions were those described by Stergiopoulos et al. (2007), except the ecp1 and ecp4 forward primers, which were modified to increase the specificity of the reaction (Additional Table 2). PCRs were performed in a 15-μl volume with a thermocycler (Model Multigene gradient; Labnet International, In) programmed as follows: an initial denaturing step at 94 °C for 5 min; then, 25 cycles of 30 s at 94 °C; 30 s of annealing at 55, 60, 64 and 64 °C for ecp1, ecp2, ecp4 and ecp5 amplification, respectively; and a 1 min extension step at 72 °C according to Stergiopoulos and co-workers (Stergiopoulos et al. 2007). All reactions included a final extension at 72 °C for 7 min.

Reactions were performed with 40 ng of genomic template DNA from monosporic cultures (Table 1), containing 1.5 mM MgCl₂ (Inbio Highway), 0.40 mM of each primer pair (corresponding to ecp1, ecp2, ecp4 and ecp5, respectively; Additional Table 2), 0.2 mM dNTPs (Inbio Highway), and 1 U of T-Plus DNA polymerase (Inbio Highway) in 1× reaction buffer (Inbio Highway). PCR amplifications were processed as previously described. Ecp sequences obtained were deposited in the NCBI GenBank (Additional Table 3).

Polymorphism within avr and ecp genes

Polymorphisms within avr and ecp genes of the 8 new isolates of C. fulvum as well as those of 12 isolates stored at the CIDEFI collection were identified by comparing nucleotide sequences as well as alignments of the PCR amplicons sequenced (Table 1). DNA sequences were aligned with ClustalW (Thompson et al. 1994) whereas multiple alignments of predicted proteins were made using Muscle (Edgar 2004) in Geneious v9.1.2 (Kearse et al. 2012). Nucleotide diversity were calculated using ARLEQUIN 3.5.2.2 (Excoffier and Lischer 2010). The ratio of nonsynonymous (Ka) to synonymous (Ks) nucleotide substitution rates is an indicator of selective pressures on genes and was calculated through the Ka/Ks service provided by Universitetet I Bergen (services.cbu.uib.no/tools/kaks).

Results

Genetic diversity within isolates of Cladosporium fulvum

The eight isolates described in this study were identified as Fulvia fulva (Syn. Cladosporium fulvum; Passalora fulva) based on the homology of the amplified ITS sequences. This was further supported by the growth of these isolates on PDA, where they developed colonies as well as conidia with morphological characteristics typical of C. fulvum (data not shown), and by the fact that we successfully amplified C. fulvum avr2, avr4, avr4E and avr9 genes, providing in this way evidence that they belonged either to race 0 or race 2 of C. fulvum (Table 1; Medina et al. 2015). While isolates CIDEFI 326, CIDEFI 330 and CIDEFI 332 were identified as representatives of race 0, CIDEFI 322, CIDEFI 323, CIDEFI 325, CIDEFI 327 and CIDEFI 329 were identified as representatives of race 2.

The seven ISSR primers selected amplified bands that ranged from 250 bp to 6500 bp and were used to assess genetic diversity. We found that the isolates were genetically diverse since 54 amplicons were polymorphic (30.86%). Based on the ISSR data we built a dendrogram using the UPGMA algorithm and Dice.
All the isolates were clustered in two groups at a similarity coefficient of 0.883 (Fig. 1). CIDEFI 305 and CIDEFI 325; CIDEFI 307, CIDEFI 329, CIDEFI 300, CIDEFI 320, CIDEFI 322, CIDEFI 318, CIDEFI 330, CIDEFI 332 and CIDEFI 327 isolates were clustered with higher homologies (>95%); however, no relationship was found between clusters and geographical origin, cultivar of pathogen isolation, or race of the isolates.

Polymorphism within avr and ecp genes

Polymorphisms within avr genes were analysed by aligning sequences, including those available in public databases (Fig. 2, Table 2, and See supporting electronic material). We analysed a 486 bp fragment of avr2, which was 100% homologous to the sequence of a European isolate (accession number: AJ421629). Only one variation was found within the avr2 sequence of the isolates presented in this study. This was a single nucleotide polymorphism (SNP) due to a transversion (26A > C) in the 5′ exon-1 sequence before the start codon of strain CIDEFI 330.

Also we analysed a 678 bp DNA fragment of avr4 gene and compared it with the sequence obtained by Joosten et al. (1997; accession number: Y08356). The CIDEFI strain sequences presented seven variations, which were found in non-coding sequences (over 98%); they included five SNPs of nucleotide transitions (80G > A) and one deletion (63delC). Furthermore, a nucleotide transition was detected in the avr4 coding sequence of isolate CIDEFI 325, which resulted in a synonymous modification, 493G > A.

We also amplified, analysed and compared the sequence of a 520 bp fragment of avr4E with the available sequence of a European isolate (AY546101). Within our isolates we found 16 polymorphisms, which were located within the coding region. Interestingly, all Argentinian isolates presented an SNP in one codon by a transition (364C > T; 398C > T), which resulted in a non-synonymous modification. One modification consisted of a substitution of Phe by Leu (F82L), while another consisted of a substitution of a Met by Thr (M93T).

The 545 bp partial sequence of the avr9 gene, was compared with that of a European (accession number: X60284). Interestingly, the isolates described in this study were polymorphic within the non-coding sequence, which has not been reported before in isolates from Argentina. One SNP was found in a non-coding region of avr9 of CIDEFI 326 (18G > T) and one within the intron sequence of CIDEFI 325 (225A > T), both due to transversions. Most mutations were found within the exon-2 sequence of avr9. Two SNPs occurred as transition and transversion of a nucleotide: 497G > C and 516 T > C and three indels (503-504insT; 516-517insG and 534-535insA) were observed in the sequence of CIDEFI 332 isolate. These changes did not provoke changes in the protein sequence since they occurred after the stop codon (382_384).

Based on nucleotide diversity analysis the avr2 gene presented the highest number of changes with the analyzed sequence (Table 3), but since they did not lead to amino acid substitutions, we cannot infer if they occur in response to evolutionary forces. In contrast with this, within the avr9 gene sequence the substitutions observed by Medina et al. (2015) most probably were due to the positive selective pressure since for the sequence of the Avr9 effector ω was greater than 1.

Polymorphisms within ecp genes were analysed by aligning our sequences and those available in public databases (Fig. 3, Table 2, and See supporting
The 1338 bp partial sequence of ecp1 was compared with the only sequence available at the NCBI (accession number: Z14023). The sequences of the Argentinian isolates presented one hundred seventy-five polymorphisms compared to the sequence of a European isolate, 111 SNPs and 64 indels. Interestingly, 166 variations occurred within non-coding sequences of CIDEFI isolates. All the isolates carried two nucleotide insertions (120-121insC and 487-488insG), except CIDEFI 322 and CIDEFI 301 that only carried the latter insertion. Strain CIDEFI 307, carried two SNPs (93A>T and 97A>G) and a deletion (217delG) in a non-coding region. CIDEFI 318 carried a SNP (522C>A). CIDEFI 307 and CIDEFI 322 carried one SNP and one insertion, respectively (826C>T and 801-802insT) in the intron-1 sequence. All isolates, but CIDEFI 305 and CIDEFI 323, carried a SNP causing a transition (915C>T) and a deletion (943delC), respectively, in the intron-2 sequence. Also, CIDEFI 329 showed two additional transversions (944G>C and 949C>A). Again, variations were found within the exon-3 sequence. The sequence of isolate CIDEFI 329 contained one transition (964G>A) and three transversions (969T>G, 970A>T and 974C>G). While the transition led to a synonymous modification, transversions resulted in non-synonymous modifications, where an Asp was replaced by Asn (D62N). Additionally, we found one insertion in CIDEFI 318 (1025-1026insG) and two (979-980insA and 1003-1004insG) in the sequence of CIDEFI 329 that caused frameshifts. While the isolates reported by Medina et al. (2015) presented, six SNPs and one indel after the stop codon, the new isolates described in this work contained twenty indels and eighty-one SNPs in the same region.

The 811 bp ecp2 gene fragment of CIDEFI isolates was analysed and compared to other available sequences of C. fulvum like as European isolate accession number: Z14024 and twenty-three variations were found. Seventeen of these polymorphisms were located within non-coding regions; among them, six occurred in sequences of the new isolates described in this study. Deletions were found in non-coding sequences of both previously reported and new isolates of C. fulvum (22delT; 24delG; 22_25delTGCA; 28delC; 30_32delTAC; 75delG; 75_76delGA). Furthermore, an insertion was found within the same DNA sequence (783-791insGCCGCCGCGC) of CIDEFI 300. Two insertions were found within the coding region of CIDEFI 300 and CIDEFI 301, respectively, (663_664insA and 680_681insG) and the analysis predicted a frameshift (>130 and >137). CIDEFI 326 was the isolate that presented the highest level of variation within the coding sequence. It presented two SNP that led to synonymous mutations, one in the signal peptide sequence (257T>C) and one within exon-1 (305C>A). Additionally, exon-1 had two SNPs by transversion of nucleotides that resulted in non-synonymous changes (409G>T and 414T>G). Arg was substituted by Ile (R64I) and Ser by Ala (S66A).

Within the 488 bp sequence of the ecp4 gene, 16 polymorphisms were detected within the coding region of all the isolates analysed that were compared to the only available sequence (EF104526). Except for the sequence of isolate CIDEFI 318, a transversion was detected, (380G>T), that resulted in a non-synonymous change, where Cys was replaced by Trp (C108W). Isolate CIDEFI 326 showed three single nucleotide insertions (395-396insA, 408-409insA and 423-424insT) that resulted in a non-synonymous change, where Gly was replaced by Val (G109V) and with a predicted frameshift (>113).

The 883 bp sequence of ecp5 showed 17 polymorphisms within both non-coding (over 36%) and coding (over 64%) regions compared to the available sequence of a European isolate (accession number: EF104527). The ecp5 sequences of CIDEFI 300 and CIDEFI 301 showed a codon change, where AAG (65A>T) was replaced by TAG leading to a premature stop codon (>21, as shown in Fig. 4), which might lead to a truncated protein. In the coding region, isolates CIDEFI 322, CIDEFI 323, CIDEFI 327 and CIDEFI 329 presented a deletion (516delG) with a frameshift, whereas the isolates CIDEFI 318, CIDEFI 325, CIDEFI 330 and CIDEFI 323 had a transition (541G>A) that led to a non-synonymous mutation, where Cys was replaced by Tyr (C48Y). Within the same region, CIDEFI 307 had an insertion (691insG), that causes a frameshift and premature stop. Besides, deletions were also detected after the stop codon, in isolates CIDEFI 305 (866delA), CIDEFI 332 (863delA and 866delA) and CIDEFI 327 (866delA). Finally, CIDEFI 326 carried a 358 bp sequence deletion (Fig. 5), that corresponded to the 3′ terminal intron and 5′ start of exon-2 of the ecp5 gene (213-572del).

Based on nucleotide diversity analysis, ecp1 was the extracellular protein where changes occurred the most within the analyzed sequences and also presented the sites segregating the most (Table 3). Interestingly, by the
Ka/Ks ratio analysis, it appears that \textit{ecp1} and \textit{ecp2} are under a negative pressure so that the protein sequence is conserved whereas \textit{ecp4} and \textit{ecp5} are under positive selective pressure (Table 3).

\textbf{Discussion}

\textit{Cladosporium fulvum} collected from production areas in Argentina belonged either to race 0 or 2 (Medina et al. 2015) and the new isolates described here also belonged to these two races. However, we found that genetic diversity was considerable and unrelated to the tomato cultivar and the area of collection. Identification of pathogens races is critical to develop a strategy to manage diseases through resistance genes. Identification of races of \textit{C. fulvum} can be done either by inoculating isolates on a set of tomato cv Money Maker differentials carrying known Cf genes (Cf-2, Cf-4, Cf-5, Cf-9; Rollan et al. 2013) as well as by the amplification of \textit{avr} genes in a PCR multiplex (Medina et al. 2015). Such amplicons can be sequenced and in this way we might identify mutations that eventually might overcome known Cf- genes. Until now the Argentinian population of \textit{C. fulvum} belong to race 0 or 2 (Medina et al. 2015), the fact that most cultivars are susceptible to the disease suggest that in Argentina tomato hybrids lack the necessary resistance genes to prevent the disease.

Despite the fact that many polymorphisms were found within the partial sequences of \textit{avr} genes, the \textit{avr4E} gene was the only one that presented a non-synonymous variation within the coding sequence, which was found in all the isolates from Argentina suggesting that the parental strains already carry the mutation and then additional genetic changes occurred in the strains of the CIDEFI collection. Two-point mutations led to two amino acid residue substitutions in AVR4E proteins, such changes have already been reported in isolates from other countries (Westerink et al. 2004b; Medina et al. 2015; Iida et al. 2015). Westerink et al. (2004b) found that the substitution of Phe by Leu within AVR4E protein was enough to avoid recognition by Cf-4E (Westerink et al. 2004b). Furthermore, Iida et al. (2015) found within Japanese isolates two substitutions within the AVR4E protein, the first one was similar to the non-synonymous change described in this work, whereas in the other one a Met was replaced by an aromatic amino acid, Tyr. In the isolates analysed here, Met was replaced by Thr. The fact that a non-synonymous substitution provoked the replacement within AVR4E protein sequence, where a hydrophobic amino acid (Met) was replaced by a hydrophilic one (Thr) should modify the peptide function, suggesting this that this is under positive selection (Stergiopoulou et al. 2014).

\textit{Ecp} genes encode proteins that have been considered virulence factors, although their roles remain to be isolated described by Medina et al. (2015) are indicated by black flags and by white flags for isolates described in this study. Of the non-synonymous DNA modifications, the changes in aminoacid sequence are indicated.
elucidated (Stergiopoulos et al. 2010). However, these proteins play an essential role in infection and colonization, since their deletion results in less virulent C. fulvum mutants (De Wit et al. 2009a; De Wit et al. 2012; Iakovidis et al. 2020). Stergiopoulos et al. (2007) stated that the frequency of loss of these genes within fungal populations is low, which might explain their presence in Argentinian isolates as well as their key role in pathogenesis. The biological role of ECP6 has been described; it codes a protein with three lysin motif domains that alter chitin-triggered immunity (Bolton et al. 2008; Bolton and Thomma 2008; De Jonge et al. 2010; Sánchez-Vallet et al. 2013). Luderer et al. (2002b) predicted that ECP1 and ECP5 might be rich in Cys residues and many of them form intramolecular disulphide bonds in protein, which is required for stability and function in the protease-rich leaf apoplast of tomato. Regarding the function of the other ECP proteins, fungal transformants where Ecp1 or Ecp2 were knocked out were impaired in aggressiveness in mature tomato plants (Laugé et al. 1997). Based on this and on the changes reported within the gene sequence of the predicted ECP1 protein, of CIDEFI 318 and CIDEFI 329 most probably these sequence changes impaired aggressiveness of the isolates. Furthermore, the change of Asp by Asn in the deduced sequence from CIDEFI 329; where an electrophilic amino acid was replaced by a hydrophilic one might alter the peptide’s function, which deserves to be studied further.

Stergiopoulos et al. (2014) found many polymorphisms within ecp2 genes from Mycosphaerella fijiensis. The authors found a change at position 66, where Ala was substituted by His or Gly. In our study, we found that Ser, a hydrophilic amino acid, was replaced by Ala, that is hydrophobic, which, at this position, might affect protein folding and probably its activity. Furthermore, the aggressiveness of CIDEFI 300 and CIDEFI 301, while interacting with tomato, should be affected due to the change in the C-terminal region of Ecp2. So, it appears that it is crucial to evaluate the role of such changes in virulence, which should be done by generating mutants.

Regarding the ECP4 protein, Mesarich et al. (2018) found that all six Cys residues are mostly conserved between Ecp4 and its homolog, Ecp7. Interestingly, in Argentinian isolates, one Cys residue at position 108 was replaced by Trp, a change that might affect their aggressiveness as well as fitness. Additionally, ECP4 of CIDEFI 326 most probably has no activity due to the loss of a stop codon; consequently, fitness of the isolate to interact with tomato might have been negatively affected.

Regarding the ECP5 protein, it has six cysteine residues and is the least polymorphic C. fulvum effector (Stergiopoulos et al. 2007; De Wit et al. 2009b). Iakovidis et al. (2020) found that genes encoding ECP5 that inducing an HR segregated as a monogenic trait, mapping to distinct loci in the tomato genome and identified at least three loci within chromosomes that might harbour distinct Cf-ecp5 genes. Based on an analysis of nucleotide and amino acid sequences, we can infer that the predicted change of Cys by Tyr as well as the premature stop codons that produce truncated ECP5 products and the frameshifts that change N-terminal or C-terminal regions might harm fitness of C. fulvum, to infect and colonize the apoplastic space.

It is possible that the large variation (SNP, indels) that occurred in non-coding sequences of ecp genes provide fungal isolates with an adaptive advantage in

![Fig. 5 Agarose gel (1% w/v) of the amplification product of the ecp5 gene (882 bp). Line 1: molecular weight marker. Line 2: negative control. Line 3: Product amplified from CIDEFI 305 strain. Line 4: Product amplified from CIDEFI 326 strain. Line 5: Product amplified from CIDEFI 322 strain.](image)
the environments assayed though this has to be studied in further detail.

Conclusions

Argentinian tomato cultivars are affected by only two races of *C. fulvum* whose populations are under an evolutionary process. Representatives of these two races present several polymorphisms in effectors, which are not constrained to *avr* genes, probably because, based on field data, resistant cultivars are not available. Variations found in the gene sequences of *avr2*, *avr4* and *avr9* within CIDEFI isolates were neutral for pathogenesis on tomato. This is the first report showing polymorphisms within *ecp* genes and the changes observed in Argentinian isolates suggest that they might not be such relevant factors of pathogenesis. There seems to be different evolutionary forces acting upon *ecp* genes, since, while *ecp1* and *ecp2* seemed to be under a negative pressure for changes, suggesting that they might probably play a key biological role, *ecp4* and *ecp5* are under a positive selective pressure, reflected by the amino acid changes and nonfunctional predicted peptides generated by frameshift and premature stop codons. Still additional studies regarding virulence and aggressiveness of the isolates described should be done in order to evaluate the effect of polymorphisms within *ecp* genes. Therefore, probably the environment and management practices are the most important factors driving evolution of these races in Argentina and this explains why the pathogen population is evolving but not at the pathogenic or race level.

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Compliance with ethical standards

Declaration of competing interest The authors declare that they have no competing interests.

This work does not include research involving human participants and/or animals.

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