

Original Article

PHYLOGENETIC ANALYSIS OF *FUSARIUM OXYSPORUM* STRAINS ISOLATED FROM STRAWBERRY CROPS *FRAGARIA ANANASSA* DUCH IN THE PROVINCE OF PICHINCHA (ECUADOR)

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ABSTRACT

Objective: Strawberry cultivation has acquired great importance for consumption, promoting the increase of its production in Ecuador. However, the process of importing plant material from producing countries in order to improve domestic production has contributed to the dissemination of the fungus *Fusarium oxysporum f. sp. fragariae*.

To identify the presence of the pathogen, by applying molecular techniques to the *Fusarium* strains isolated from strawberry crops.

Methods: Nine two diseased strawberry plants and 92 asymptomatic plants were analyzed. From these samples, 13 fungi with the characteristics of the *Fusarium* genus were identified. The isolates were analyzed at the molecular level, by PCR (Polymerase chain reaction) amplifying the ITS regions of the rDNA and the EF-1 α region.

Results: The PCR product was sequenced to elucidate the phylogenetic relationships between the isolates, identifying 12 strains as *F. oxysporum f. sp. fragariae*. These results confirmed the presence of the fungus in the strawberry crops analyzed, representing a contribution to the search for control alternatives to avoid the spread of the pathogen.

Conclusion: The PCR product was sequenced to elucidate the phylogenetic relationships between the isolates, identifying 12 strains as *F. oxysporum f. sp. fragariae*.

Keywords: Identification, Phylogenetic analysis, *Fusarium*, Strawberry

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INTRODUCTION

Strawberry (*Fragaria ananassa*) has become a very important industrial crop worldwide, in fact, the demand for strawberries in the world is increasing. In Ecuador, 12% of strawberry producers are exported and the rest of producers satisfy domestic demand [1].

The specific pathogenicity of *Fusarium oxysporum f. sp. fragariae* has a negative impact on agriculture worldwide given that it is the cause of vascular wilt and basal rot of a large variety of plants, in Ecuador this fungus affects fruit crops such as babaco [2], strawberry, tomato kidney among others [3].

Nowadays, the use of new biotechnological technologies based on the Polymerase Chain Reaction (PCR) technique allows the rapid detection of pathogens present in plants and in the soil [4].

Restriction fragment length polymorphism, or *RFLP*. Random Amplification of Polymorphic DNA (RAPDs), among others, are used in the analysis of DNA genetic polymorphism, which with the development of bioinformatics software designed for sequence analysis, allows to obtain efficient and timely information applicable

to multiple samples [5]. In this work it was proposed, identify, the fungus *Fusarium oxysporum f. sp. fragariae*, to establish its presence in strawberry crops in Ecuador, for which, by amplifying and sequencing the region of the intergenic spaces of the ribosomal DNA (ITS) and the Elongation Factor 1 alpha (EF-1 α).

MATERIALS AND METHODS

In this work 92 plants with symptoms such as: and 92 asymptomatic to the disease (soil/plant, root and leaves) were analyzed; these plants were in the phenological phases of vegetative development and fructification, according to the following procedure: Planting of the plant material (strawberry seedlings) was carried out on the previously prepared substrate of PDA (Potato dextrose agar, Oxoid, England), after a reseeded in selective medium MEA (Malt extract agar, Oxoid, England) the pathogen was isolated for conservation it

Molecular evaluation

It was evaluated at the molecular level (phylogenetic analysis) for which the amplification of the gene of interest was carried out by PCR using primers EF-1, EF-2, ITS-1, ITS-4 (table 1).

Table 1: Nucleotide sequences used for amplification of regions ITS (s) and TEF-1 α

Primer	Sequence	Authors
ITS-1	5'-TCCGTAGGTGAACCTGCGG-3'	Martin and rygiewicz [6]
ITS-4	5'-TCCTCCGTTATTGATATGC-3'	
EF-1	3'-ATGGGTAAGGARGACAAGAC-5'	O'Donnell <i>et al.</i> [7]
EF-2	3'-GGARGTACCAGTSATCATGTT-5'	

The amplification reaction was performed in a final volume of 25 μ L, with 5 μ L of template DNA. Sterile ultrapure water was included as a negative control in each reaction. The components concentrations of

the mixture were: 1.5 mmol MgCl₂, 0.2 mmol de cada dNTP, 0.5 μ M of each primer, 1.25 U/25 μ L de Taq polymerase (FLEXI), PCR buffer (Green FLEX 1X). The amplification was carried out according to the

following conditions: initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 seconds, primer binding at 53 °C for 60 seconds, initial extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR product was analyzed on a 0.5% agarose gel, in each electrophoretic analysis a molecular weight marker of 100 bp was used, the run of the gel was at 100 volts for 30 min. The gel was visualized in a UV transilluminator (The Enduro™ GDS, USA).

The PCR products with nonspecific bands were purified. All the amplified ones were sent to sequence at MACROGEN in Seoul, South Korea (https://dna.macrogen.com), the results were compared with the GenBank database (NCBI, 2015). From the sequences of the EF-1 α and the RTS ITS regions, probabilistic models were applied; for multiple sequence alignment, the MUSCLE algorithm was used and the Maximum Likelihood Algorithm software was used for the phylogenetic trees. The sequences were contrasted with probable homologous genes available in

the NCBI database (National Center for Biotechnology Information) using the BLAST local alignment application (Basic Local Alignment Search Tool) (www.ncbi.nlm.nih.gov/BLAST). The sequences obtained were edited and registered in the SeaView Version 4.5.4 program, for which the quality of the chromatograms was inspected, eliminating the bases that were not well defined.

RESULTS AND DISCUSSION

After isolation in PDA medium and confirmed its morphology by microscopic observation, all isolates were analyzed at the molecular level.

Molecular evaluation

The DNA concentration of the isolates obtained from the strawberry seedlings is shown in the table 2.

Table 2: DNA concentration of the isolates obtained from strawberry plants *Fragaria ananassa* duch

Treatments	Nucleic acids concentration (ng/ μ l)	260/280
AB ₁	4196.25	2.14
AB ₂	1542.90	2.19
AB ₃	1867.25	2.15
AB ₄	2475.70	2.08
AB ₅	555.85	2.11
AB ₆	4695.40	2.14
AB ₇	6546.45	2.05
AB ₈	696.05	2.08
AB ₉	84.40	1.87
AB ₁₀	1693.70	2.06
AB ₁₁	4598.75	2.09
AB ₁₂	119.95	1.97
AB ₁₃	2573.45	2.07
<i>Pestalotia</i> sp.*	1536.55	2.00
<i>Rhizoctonia</i> sp.*	3255.40	2.19

*Possible genres

The DNA of each isolate was quantified by spectrophotometry in a nano Drop One (Thermo Fisher scientific 8000 microvolume UV-Vis, USA), obtaining, as a result, a concentration range between 84.4 and 65.45 ng/ μ l and an average absorbance ratio of 2.0(260/280) This average is framed in the relation between 1.7 and 2.0 (260/280) [8] to define that DNA is pure.

Phylogenetic analysis based on the ITS fragment

It was carried out by PCR with the initiators ITS-1 and ITS-4, DNA of the 13 treatment and two possible pathogens previously characterized by optical microscopy such as *Pestalotia* sp. and *Rhizoctonia* sp. Where the 570 bp band was observed in the gel for the 13 treatments, including *Pestalotia* sp. In the case of *Rhizoctonia* sp. the band had a molecular weight slightly higher to 650 bp. The AB8 treatment and negative control (-) showed no visible band.

Multiple alignments with the MUSCLE algorithm based on the codons of the study sequences

The topology of the condensed tree obtained based on the ITS region started with the formation of a single group and a single divergent (polytomy), in the group 25 taxa are included, some of them grouped with good support and separated by small branches internal, also showing condensed branches that turned out to be polytomous. The rooting of the tree was achieved by including as sequences external groups of *Fusarium oxysporum* f. sp. *fragariae* (90436908), *Fusarium oxysporum* (827206934, 514825685 and 514825683). Sequences available in the NCBI nucleotide database (National Center for Biotechnology Information) found in the BLAST database. Relationships received moderate-high bootstrap support (0.83-0.92) in the maximum verisimilitude analysis (fig. 1).

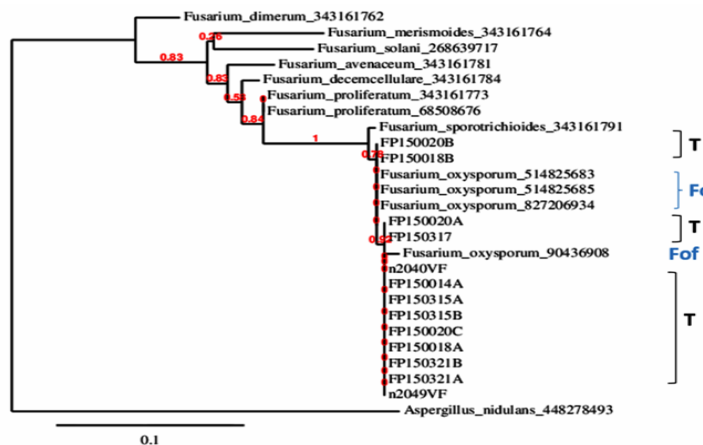


Fig. 1: Consensus tree constructed with the maximum verisimilitude method, based on the distances of the sequences of the regions of the internal transcribed spacer (ITS) in the ribosomal DNA; (T) treatments, (Fo) *Fusarium oxysporum*, (Fof) *Fusarium oxysporum* f. sp. *Fragariae*

Also, can be observed that the 9 reference sequences were grouped into a single clade (branch) with *Fusarium oxysporum* f. sp. *fragariae* (90436908). The rooting shows that *Fusarium oxysporum* f. sp. *Fragariae* and the strains are monophyletic based on the sequences to the ITS regions.

Phylogenetic analysis based on the EF-1 α region

The amplification and sequencing of the EF-1 α region were carried out with the EF1-EF2 primers plus the DNA of the 13 treatments. After PCR amplification, bands of 570 bp were visualized for the 13 treatments and the presence of nonspecific bands was observed in each of the isolates, so the Band-stab PCR technique was carried out in all the isolates.

The amplification of the products extracted with the Band-stab PCR technique allowed the visualization a band of 680 bp in the 13 treatments (AB₁-AB₁₃), with the follow concentrations of DNA: 80

ng/ μ l (AB₂, AB₃, AB₄, AB₅, y AB₈), 100 ng/ μ l (AB₁, AB₆, AB₇, AB₉, AB₁₀, AB₁₁, AB₁₂ y AB₁₃).

After sequencing in MACROGEN, the results were edited and registered in the Sea View program. The quality of the chromatograms was examined by eliminating bases not well defined and the result obtained was of 631 bp of acceptable quality. Multiple alignments were performed, applying the MUSCLE algorithm, under the default configuration in the Sea View program they showed similarity in the nucleotides, except for small differences in the treatments AB₂, AB₃, and AB₁₂, but a very significant difference in the alignment of the nucleotides of the AB₄ treatment (fig. 2). The alignment between the treatments establishes that the AB₄ treatment presents different nucleotide zones with the other treatments, determining that there is no degree of similarity between the amino acids.

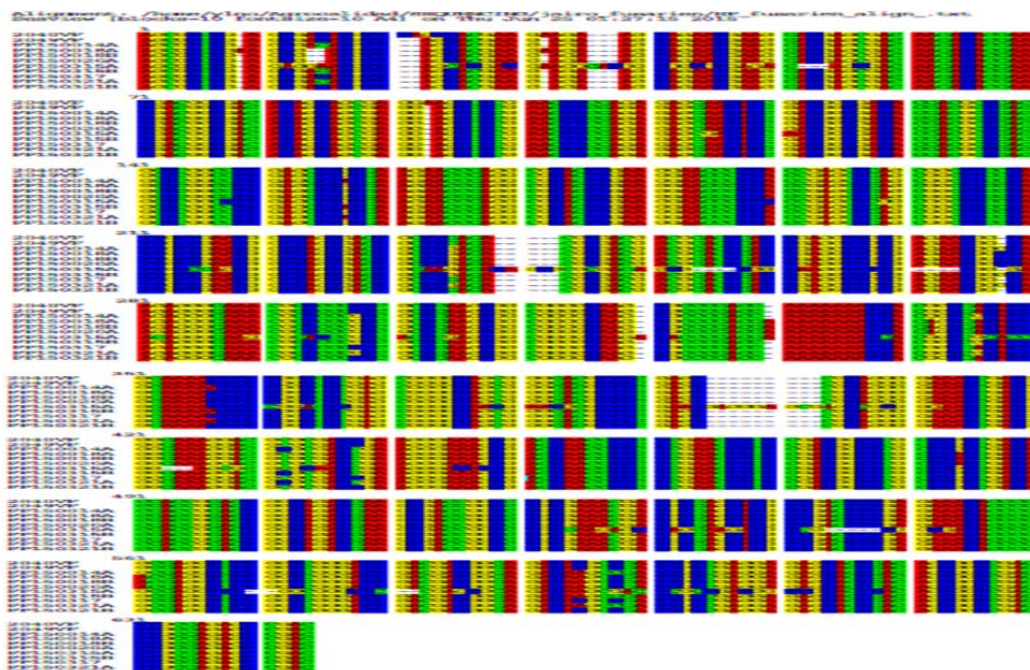


Fig. 2: Multiple alignments with the MUSCLE algorithm based on codons of the study sequences

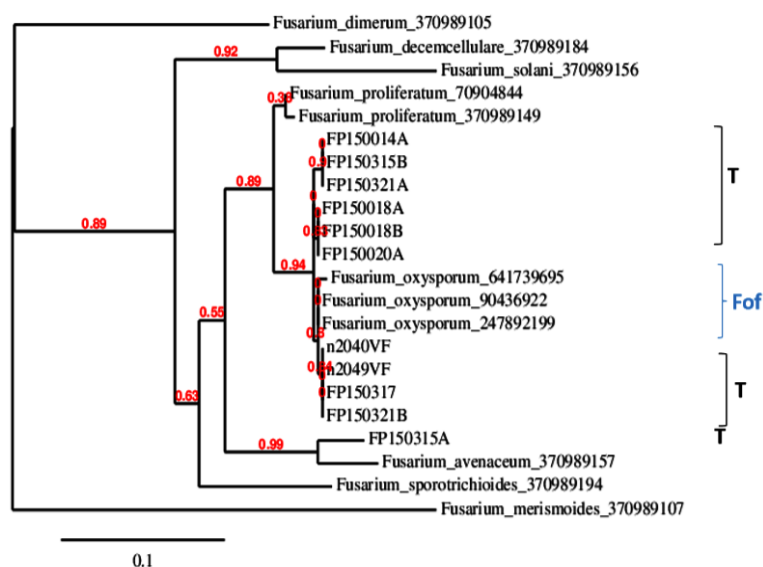


Fig. 3: Consensus tree constructed with the maximum verisimilitude method, based on the distances of the sequences of the EF 1 α region of the DNA: (T) treatments, (Fof) *Fusarium oxysporum* f. sp. *Fragariae*

The cladogram was constructed based on the sequences of the EF-1 α regions, with the maximum verisimilitude method visualizing 2 initial clades, in the formation of a single initial group and a single divergent (polytomy), in the group 25 taxa are included, some of them grouped with good support and separated by small internal branches, also protruding condensed branches that turned out to be polytomous. In the formation of the clades, sequences of other genera and sequences of *Fusarium oxysporum* f. sp. *fragariae* (90436922, 641739695, 2478921999), sequences available in the NCBI database (2015) (fig. 3).

In this cladogram, the sequences of treatments AB₅ and AB₆ were excluded because their sequences decay rapidly with few base pairs. Relationships received moderate-high bootstrap support (0.89-0.94) in the maximum verisimilitude analysis

Other authors have previously studied the ITS and EF-1 α regions, demonstrating their effectiveness in resolving some generated ambiguities and taxonomically locating new species of the *Fusarium* genus [9]. Although the use of universal primers allows us to study the inter-specific variability and establish identifications when comparing the obtained sequences with others previously deposited in the GenBank database, it should be noted that the best tool to identify quickly, unequivocally and less expensively the different species of fungi associated with vascular wilt is the use of specific primers [10], but everything will depend on the purity of the isolates and the PCR products, so several procedures were carried out until the best result.

Evaluation of the special form of *Fusarium oxysporum* with specific initiators

The specific oligonucleotides designed for *Fusarium oxysporum* f. sp. *fragariae* Fofra-1 and Fofra-2 [11], used in the PCR reaction, showed no amplification in the 13 DNA samples. Therefore, it should be taken into account that the isolates used in this investigation did not amplify because there are differences in evolution between species from one locality to another [12].

On the other hand, no significant statistical differences were detected between the treatments for the distribution, determining that there are no differences between the variables. The coefficient of variation is 19.46%, acceptable for field evaluation.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declare none

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