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**Caracterização de fungos associados à doença do Pé Negro da Videira (*Vitis vinifera* L.) no Peru**

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica do Doutor Artur Jorge da Costa Peixoto Alves, Investigador Principal do Departamento de Biologia da Universidade de Aveiro

"It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change."
— Leon C. Megginson

**o júri**

Presidente

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**palavras-chave**     Fungos, *Vitis vinifera*, *Fusarium*, *Clonostachys*, *Pleiocarpon*,
                       *Cylindrocladiella*, Peru, Doença do Pé Negro

**Resumo**     As doenças do lenho da videira (*Vitis vinifera* L.) são maioritariamente
               causadas por fungos e geram grandes perdas económicas a nível
               mundial.
               Uma das doenças é a Doença do Pé Negro. Os sintomas mais comuns
               são lesões radiculares necróticas, redução da biomassa da raíz,
               descoloração negra sobre a casca e necrose dos tecidos xilémicos na
               base dos porta enxertos. Ao longo das décadas, a incidência e
               severidade da doença tem vindo a aumentar, sendo a causa de perda
               de produtividade e morte de videiras jovens.
               Neste estudo caracterizou-se uma coleção de 138 fungos isolados de
               videira no Peru com sintomas de Doença de Pé Negro. Primariamente
               realizou-se tipagem dos isolados de forma a avaliar a diversidade
               genética da coleção de isolados. Foram escolhidos 38 isolados
               representativos desta diversidade para posterior identificação por
               sequenciação e análise da região ITS (Internal Transcribed Spacer).
               Esta permitiu a identificação inicial dos géneros Fusarium,
               Cylindrocladiella e Clonostachys. Posteriormente foi feita a análise de
               marcadores secundários de modo a permitir a correta identificação dos
               isolados ao nível da espécie
               Foram obtidas sequencias do Fator de Alongamento de Transcrição 1-
               alfa (tef1-α) para todos os isolados permitindo a identificação do género
               Pleiocarpon que não havia sido identificado usando a região ITS. Usou-
               se ainda a sequência parcial do gene que codifica para a β–Tubulina
               (tub2) para diferenciar as espécies de Clonostachys e Cylindrocladiella.
               A análise filogenética das sequências de ITS e tef1-α combinadas para
               os géneros Cylindrocladiella e Pleiocarpon indicam a presença das
               espécies Cylindrocladiella peruviana e Pleiocarpon strelitziae. A análise
               filogenética das sequências ITS, tef1-α e tub2 combinadas confirmou a
               identidade de C. peruviana. A espécie P. strelitziae é descrita pela
               primeira vez em associação com videiras sintomáticas.
               A análise filogenética do género Fusarium/Neocosmospora utilizando
               sequências de tef1-α permitiu agrupar 26 isolados em três complexos
               de espécies: 12 em Neocosmospora solani species complex, 1 em
               Fusarium incarnatum-equiseti species complex e 13 em Fusarium
               fujikuroi species complex. As espécies de Fusarium/Neocosmospora
               não são normalmente associadas à doença do Pé Negro da Videira. O
               seu potencial envolvimento nesta patologia necessita de ser estudado
               no futuro.
               A análise filogenética das sequências ITS e *tub2* combinadas e das
               sequências ITS, *tef1*-α e *tub2* combinadas do género *Clonostachys*
               indicam que os isolados deste género pertencem a duas possíveis
               novas espécies, *Clonostachys viticola* e *Clonostachys peruviana*. Estas

foram caracterizadas em termos morfológicos e taxas de crescimento a diferentes temperaturas, sendo apresentadas descrições taxonómicas para ambas.

**abstract**    Gravepine trunk diseaseas (*Vitis vinifera* L.) are mostly caused by fungi,
which can cause serious economic losses worldwide.
One of these diseases is Black Foot Disease. The most common
symptoms are necrotic root lesions, reduced root biomass, under-bark
black discoloration, and necrosis of xylem tissue at the base of the
rootstocks.
Over the last decade, the intensity and the incidence of the disease
increased, causing loss of productivity and death of young grapevines.
In this study were characterized a collection of 138 fungi isolated from
grapevine in Peru with Black foot disease symptoms. Primarily, a
genetic typing of the isolates was performed to assess the genetic
diversity of the isolate collection. Identification by sequencing and
analysis of the Internal Transcribed Spacer (ITS) was carried out with 38
representative isolates of the diversity. This allowed the initial
identification of the genera *Fusarium*, *Cylindrocladiella* and
*Clonostachys*. Afterwards, it was done a secondary marker analyses to
allow a correct identification of the isolates to the species level.
Sequences of the Translation elongation factor 1- alpha (*tef1-α*) were
obtained for all the isolates, allowing the identification of the genus
*Pleiocarpon,* previously unidentified when using the ITS region. The
partial sequence of the gene that codes for β–Tubulin (*tub2*) was used
to differentiate the species of *Clonostachys* and *Cylindrocladiella*.
The phylogenetic analyses with the combined sequences of ITS and
*tef1-α* for the genera *Cylindrocladiella* and *Pleiocarpon* indicated the
presence of the species *Cylindrocladiella peruviana* and *Pleiocarpon
strelitziae*. The phylogenetic analyses of the combined sequences of
ITS, *tef1-α* and *tub2* confirmed the identity of *C. peruviana*. The species
*P. strelitziae* was described for the first time in association with
symptomatic grapevine.
The phylogenetic analyses of genus *Fusarium*/*Neocosmospora* using
*tef1-α* sequences allowed to group 26 isolates in three different species
complexes: 12 in *Neocosmospora solani* species complex, 1 in
*Fusarium incarnatum-equiseti* species complex and 13 in *Fusarium
fujikuroi* species complex. The *Fusarium*/*Neocosmospora* species are
not normally associated with grapevine Black Foot Disease. Their
potential involvement in this pathology needs to be studied in the future.
The phylogenetic analyses of the combined sequences of ITS and *tub2*
and ITS, *tef1-α* and *tub2* of the genus *Clonostachys* indicated that the
isolates of these genus belong to two possible new species
*Clonostachys viticola* and *Clonostachys peruviana*. These species were

characterized in terms of morphology and growth rates at different temperatures, and taxonomic descriptions are given for both.

# Indice

# List of Abbreviations

- BFD: Black Foot Disease
- bp: base pairs
- CIA: Chloroform and Isoamyl Alcohol
- CTAB: Cetyltrimethylammonium bromide
- DNA: deoxyribonucleic acid
- dH$_2$O: Distilled water
- EDTA: Ethylenediaminetetraacetic acid
- EtBr: Ethidium Bromine
- FIESC: *Fusarium incarnatum-equiseti* species complex
- FFSC: *Fusarium fujikuroi* species complex
- FSSC: *Fusarium/Neocosmospora solani* species complex
- g: grams
- G: gravitational force
- GDP: Gross Domestic Product
- GTD: Grapevine Trunk Diseases
- ha: hectare
- *his3*: histone
- ITS: Internal Transcribed Spacer
- l: litre
- M: Molar
- MEA: Malt extract agar
- mg: milligrams
- min: minutes
- ML: Maximum Likelihood statistical method
- ml: millilitre
- MSP-PCR: Microsatellite primed PCR
- NaCl: Sodium chlorine
- NaOH: Sodium hydroxide
- OA: Oatmeal Agar
- PCR: Polymerase chain reaction
- PDA: Potato Dextrose Agar
- RNA: Ribonucleic acid
- RPB2: partial RNA Polimerase II gene
- SDS: Sodium dodecyl sulphate
- Sec: second
- TAE: Tris – Acetate EDTA
- TE: Tris – EDTA
- *tef1*-α: Translation Elongation Factor 1-alpha
- TES: Tris – EDTA SDS
- Tris: Tris(hydroxymethyl)aminomethane
- *tub2*: partial sequence of the gene that codifies β – Tubulin
- UPGMA: Unweighted Pair Group Method with Arithmetic Mean
- UV: ultraviolet
- µg: micrograms
- µl: microlitre
- °C: Degrees Celsius

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are presented (%) resulting of 1000 replicas. The samples used in the study are represented in bold with a black box.

- Figure 13 - Phylogenetic tree for *Fusarium fujikuroi* species complex, based on Maximum Likelihood (ML) analysis of *tef1*-α sequences alignment. The applied evolutionary model was the Kimura-2-parametre model with Gamma distribution. The bootstrap values are presented (%) resulting of 1000 replicas. The samples used in the study are represented in bold with a black box.

- Figure 14 - Phylogenetic tree based on Maximum Likelihood (ML) analysis of a combined data set of ITS and *tub2* sequence alignments. The applied evolutionary model was the Kimura-2-parameter with Gamma distribution permitting invariable sites. The bootstrap values are presented (%) resulting of 1000 replicas. The samples used in the study are represented in bold with a black box.

- Figure 15 - Phylogenetic tree based on Maximum Likelihood (ML) analysis of a combined data set of ITS, *tef1*-α and *tub2* sequence alignments. The applied evolutionary model was the Jukes-Cantor model with Gamma distribution. The bootstrap values are presented (%) resulting of 1000 replicas. The samples used in the study are represented in bold with a black box.

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- Figura 17 – Growth rate for *Clonostachys* sp. 2 (*peruviana*) for 10°C, 15°C, 20°C, 25°C, 30°C and 35°C

- Figure 18 – *Clonostachys viticola* – sample 47. Primary Conidiophores with adpressed branches and phialides (A); Secondary Conidiophores with adpressed branches and phialides (B); Conidia (C); Colonies of *Clonostachys viticola* (47) in OA and PDA respectively (d) and reverse colonies of *Clonostachys viticola* (47) in OA and PDA respectively (e)

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# 1. Introduction

## 1.1. Host Plant and Economic Importance

*Vitis vinifera* L. (Figure 1 and 2), common name grapevine, is a woody climbing plant belonging to the family Vitaceae, genus Vitis. It is present in temperate climatic regions and produces berries, known as grapes, that are used for many purposes, including wine production and grape consumption (Hardie, 2000; Terral *et al.*, 2010).

The wine industry is an important socioeconomic and cultural sector in many countries worldwide (Fraga et al., 2016). Between 2014 and 2016, the world wine GDP was 88 913 billion US$ (approximately 74 154 billion Euros) (Anderson *et al.*, 2017).

Figure 1 – *Vitis vinifera* (subsp. *Silvestris*) representation retrieved from http://www.floraiberica.es/floraiberica/texto/pdfs/09_110_01_Vitis.pdf

## 1.2. Grapevine Trunk Diseases

Grapevine trunk Diseases (GTDs) are a major problem for grapevine, mostly because of its economic impact on the wine industry and the quality impact on wine (Armengol & Gramaje, 2016; Carlucci *et al.*, 2017). The occurrence of these diseases can be increased by various stresses imposed to the young vines, either in nurseries or in vineyard. These can be environmental stresses, like high

Figure 2 - *Vitis vinifera* L. leaf

temperatures and/or pest effects, or it can be from vineyard management practices like poor drainage, soil compaction, inadequate planting, poor nutrition and heavy cropping of young vines (Halleen, Fourie, & Crous, 2007; Probst *et al.*, 2012; Stamp, 2001).

The GTDs are caused by fungal pathogens that can entry the plant through wounds and/or natural openings (Agustí-Brisach & Armengol, 2013; Carlucci *et al.*, 2017).

There are several GTDs, related with different pathogens as presented in table I.

Table I- Grapevine trunk diseases causing agents and relative age of affected vines (adapted from Maldonado-

| **Grapevine Trunk Diseases (GTD)** | Young vine (<8-10 years) | Black foot disease (BFD) | *Dactylonectri*a spp., *Thelonectri*a spp., *Campylocarpon* spp., *Neonectria* spp., *Ilyonectria* spp., *Cylindrocarpon* spp. |
| | | Petri disease | *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., *Cadophora luteo-olivacea* |
| | | Botryosphaeria dieback | *Botryosphaeriaceae* |
| | Adult vine (>8-10 years) | Esca disease | *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp. |
| | | Eutypa dieback | *Diatrypaceae* |
| | | Botryosphaeria dieback | *Botryosphaeriacea*e |

González *et al.*, 2018).

Characteristic symptoms of BFD on affected vines are sunken necrotic root lesions with root biomass reduction, under-bark black discoloration, and necrosis of xylem tissue at the base of the rootstock. The more external effects shown by affected plants are reduced vigour, shortened internodes and sparse and chlorotic foliage with necrotic margin (Agustí-Brisach & Gramaje, 2013; Carlucci *et al.*, 2017).

Originally, the causal agents of BFD were identified as *Cylindrocarpon* spp. The genus was meanwhile divided into different genera based on morphology complemented with phylogenetic analyses (Carlucci *et al.*, 2017; Chaverri *et al.*,2011).

Some fungal genera are commonly associated with Black foot disease (BFD): *Cylindrocarpon*, *Campylocarpon*, *Dactylonectria*, *Ilyonectria* (Armengol & Gramaje, 2016; Cabral *et al.*, 2012b;  Halleen *et al.*, 2003; Halleen *et al.*, 2004; Lombard *et al.*, 2014; Probst *et al.*, 2012), *Neonectria* (Armengol & Gramaje, 2016) and *Cylindrocladiella* (Agustí-Brisach *et al.*, 2012; Armengol & Gramaje, 2016; Van Coller et al., 2005 ). In a recent study, Carlucci *et al.* (2017) associated another genus, *Thelonectria,* with BFD.

In table II we can see the most common fungi associated with BFD.

Table II – List of most common species associated with BFD (retrieved and adapted from Carlucci et al., 2017)

| Species | Reference |
| --- | --- |
| *Campylocarpon fasciculare* | Carlucci *et al*., 2017; |
| *Campylocarpon peseudofasciculare* | Halleen *et al*., 2004 |
| *Cylindrocladiella parva* | Agustí-Brisach *et al*., |
| *Cylindrocladiella peruviana* | 2012; Carlucci *et al.,* 2017; Pham *et al*., 2018 |
| *Cylindrocladiela lagenifromis* | Koike *et al*., 2016; Pham *et al*., 2018; Van Coller *et al*., 2005 |
| *Dactylonectria alcacerensis* | |
| *Dactylonectria estremocensis* | |
| *Dactylonectria macrodidyma* | Carlucci *et al*., 2017; |
| *Dactylonectria novozelandica* | Lombard *et al*., 2014 |
| *Dactylonectria pauciseptata* | |
| *Dactylonectria pinicola* | |
| *Dactylonectria torresensis* | Carlucci *et al.,* 2017; |
| *Dactylonectria vitis* | Lombard *et al*., 2014 |
| *Ilyonectria europea* | Cabral *et al*., 2012a; |
| *Ilyonectria liriodendri* | Cabral *et al*., 2012b; Carlucci |
| *Ilyonectria lusitanica* | *et al*., 2017; Chaverri *et al*., |
| *Ilyonectria pseudodestructans* | 2011a |
| *Ilyonectria robusta* | |

These fungi associated with BFD can be isolated not only from grapevines but also from the soil around them, suggesting that the soil can be one of the sources of inoculum of these pathogens. These soilborne pathogens are challenging because of their capacity to survive for long periods in the soil, and because of the difficulty to detect, predict and diagnose future pathogenicity (Agustí-Brisach *et al*., 2014; Agustí-Brisach *et al*., 2013; Armengol & Gramaje, 2016).

## 1.3. Country, Geographic Area and Climate



Figure 3 - Map from Peru retrieved from http://legacy.lib.utexas.edu/maps/peru.html

Peru is a country located in the western and central part of South America. It has northern borders with Ecuador and Colombia, east borders with Brazil and Bolivia and south borders with Chile. Peruvian terrestrial territory reaches 1 285 215.60 km$^2$ and its maritime domain covers approximately 200 nautical miles (https://www.peru.travel/about-peru/location-geography-and-climate.aspx, http://embaixadaperu.pt/peru/).

Peru has 3 main regions according to the method of dividing the country by altitude: the coast, the mountains, and the jungle, all of them with distinct climate (https://www.peru.travel/about-peru/location-geography-and-climate.aspx, http://embaixadaperu.pt/peru/). The coast has a warm-temperate climate, with high humidity and dense fog that exuberates the cold in winter. In summer there is very little fog and temperatures reach 30 °C. The coast in the north is hot almost all year, with a short period of rain in November and December. The central and southern coast has two distinct seasons, winter from April to October and summer from November to March. The highland is the mountainous region of Peru and it is dominated by the Andes mountains. The northern Andes are lower and more humid than the rest. The highland has two seasons: summer from April to October with sunny days, cold nights and little rain, and winter from November to March when it rains heavily. The jungle is in the east of Peru, and it is characterized by its cloud forest and lowland jungle. It has two distinct seasons: From November to March it rains frequently, while from April to October it is dry. There is humidity all year round (https://www.peru.travel/about-peru/location-geography-and-climate.aspx).

In *Serie de Estadistica de Produccíon Agricola (SEPA)* of Ministerio de Agricultura y Riego of Peru (http://frenteweb.minagri.gob.pe/sisca/?mod=consulta_cult), we can check the values of production, the cultured area, and other variables from all the country and also for the regions separately of different types of crops, including grapes.

In the total Peru area, 689 957 tons of grapes were produced in 2016 within a total cultured area of 27 946 ha. The top 3 regions of grapes production in Peru are Piura, Ica, and Lima. In 2016, Piura region produced 278 366 tons of grapes within a cultivated area of 5 809

ha, followed by the Ica region with 224 666 ton within a cultivated area of 11 150 ha and finally Lima with 72 773 ton within a cultivated area of 3 995 ha.

## 1.4. Objectives

The aim of this study was to characterise a collection of fungal isolates obtained from young and adult grapevines showing symptoms of Black foot disease from various locations of Piura, Peru. These fungal isolates were obtained by Professor Edgar Rodríguez-Gálvez from Departamento de Sanidad Vegetal of Universidad Nacional de Piura.

# 2. Materials and Methods

## 2.1. Media and Reagents

- Potato Dextrose Agar (PDA) (1L)
    - 20g of Dextrose
    - 15g of Agar
    - 4g of Potato starch
- Half strength PDA (1L)
    - 19.5g of PDA
    - 7.5g of Agar
- Malt Extract Agar (MEA) (1L)
    - 30g of Malt extract
    - 3g of Peptone from soymeal
    - 15g of Agar
- MEA 3% (1L)
    - 1.44g of MEA
    - 14.45g of Agar
- Oatmeal Agar (1L)
    - 20g of Oat Flakes
    - 15g of Agar
- CIA (24:1)
    - 24ml of chloroform
    - 1ml of Isoamyl Alcohol

- CTAB 10%
    - 10g of CTAB
    - 90ml of $dH_2O$

- EDTA 0.5M pH = 8 (1L)
    - 186g OF EDTA
    - Adjust pH with NaOH

- Glycerol 15%
    - 15ml of Glycerol
    - 85ml of dH$_2$O
    - Autoclave

- NaCl 5M
    - 29.22g of Sodium chloride
    - 100ml of dH$_2$O
    - Autoclave

- Ammonium acetate (NH3AcO) 5M pH=5.2
    - 38.54g of NH$_3$AcO
    - 80ml of dH$_2$O
    - Autoclave

- SDS 10%
    - 10g of SDS
    - 100ml of dH$_2$O
    - Autoclave
- Streptomycin
    - 1 x 10$^5$ mg de streptomycin
    - 1 l of dH$_2$O
    - Filtrate
- TAE
    - 3 l of dH$_2$O
    - 60 ml of TAE 50x
- TE
    - 1ml of Tris-HCl 1M pH=8
    - 200µl of EDTA 0.5M pH=8
    - 98.8ml of dH$_2$O
    - Autoclave
- TES
    - 10ml of Tris-HCl 1M pH=8

- 2ml of EDTA 0.5M pH=8
- 20ml of SDS 10%
- 68ml of $dH_2O$
- Autoclave

- Tetracycline
  - $1 \times 10^4$ µg of tetracycline
  - 1 ml of $dH_2O$
  - Filtrate

- Tris-HCl 5M pH = 8
  - 6,055g of Tris base
  - 50ml of $dH_2O$
  - Use HCl to adjust the pH

## 2.2. Isolates

The fungal isolates studied were obtained from several regions of Piura, Peru. These were isolated from young and adult grapevine plants more precisely from pith of young grapevine, xylem from necrotic streaks from the base of rootstock and from necrotic tissue from roots of grapevine.

In attachment 1 can be seen a table with all the isolates used in the study.

Upon arrival from Peru, fungal cultures were transferred to half strength PDA plates and incubated at room temperature (between 21°C to 25°C). Cultures were routinely under these same conditions and also maintained at -80°C with 15% glycerol for long term storage.

## 2.3. Extraction of DNA

Total genomic DNA was extracted from fungi growing in half-strength PDA using a modified protocol of Möller *et al.*, 1992.

**Extraction of genomic DNA according to Mollër (modified)**

1. Grow fungus in PDA medium until it has an adequate development of mycelia
2. Transfer the mycelia to 2ml Eppendorf with 500µl of TES
3. Boil for 3 minutes at 100°C
4. Put the samples 10 min in ice
5. Add 10µl of proteinase K (20mg/ml) and mix

6. Incubate at 65°C for 30 min, swirl occasionally

7. Add 140µl of NaCl 5M

8. Add 65µl of 10% CTAB, mix by inversion

9. Incubate at 65°C for 30 min, swirl occasionally

10. Add 1ml of CIA (24:1) and mix by inversion

11. Incubate for 30 min in ice

12. Centrifuge during 10 min at 12 000 rpm at 4°C

13. Transfer supernatant (±800µl) to new 1.5ml Eppendorf

14. Add 225µl of $NH_4OAc$ and mix

15. Incubate for 30 min in ice

16. Centrifuge during 10 min at 12 000 rpm at 4°C

17. Transfer supernatant (±1000µl) to new 1.5ml Eppendorf

18. Add 500µl of ice-cold Isopropanol and mix

19. Incubate for 30 min in ice

20. Centrifuge during 10 min at 12 000 rpm at 4°C

21. Discard the supernatant

22. Wash with 1ml of ice-cold ethanol 76%

23. Centrifuge during 10 min at 12 000 rpm at 4°C

24. Discard supernatant

25. Repeat the last 3 steps again

26. Dry DNA pellet on bench

27. Dissolve the pellet in 50 µl TE buffer

28. Store the DNA at -20°C

## 2.4. PCR Typing (MSP–PCR)

The isolates were genetically typed using the MSP – PCR fingerprinting. The PCR reactions were performed in 25 µl volume using 15.75 µl of Water, 6.25 µl of NZYTaq 2x Green Master Mix, containing buffer, dNTP's, $MgCl_2$ and *Taq* DNA polymerase enzyme (NZYTech, Lisboa, Portugal) and 2 µl of primer $GTG_5$ (MWG-Biotech AG, New York City, USA) and 1 µl of DNA in a My Cycler™ Thermal Cycler (Bio-Rad, Hercules, California, USA).

The conditions used for the amplification are shown in Table III.

Table III - Primer and amplification conditions used for MSP- PCR

| Amplification Region | Primers | Conditions | | | | | |
|---|---|---|---|---|---|---|---|
| | | Initial denaturation | Denaturation | Annealing | Extension | no of cycles | Final Extension |
| **GTG5** | GTG5 | 95°C | 94°C | 53°C | 65°C | 30 | 65°C |
| | | 5 min | 1 min | 1 min | 8 min | | 16 min |

To visualize the amplified regions, an electrophoresis was carried out in a 1.5% agarose gel in TAE 1x using 5 µl of each PCR reaction, using the following conditions: 80V, during 165 min. The agarose gel was then stained with EtBr (0.5 µg/ml) during 15 min, washed in water for 1 hour and the image captures using the BIO-RAD Molecular Imager® Gel Doc™ XR+ With Image Lab Software (Bio-Rad, Hercules, California, USA).

The profiles generated by the PCR were analysed with the program GelCompar II version 5.2 (Applied Maths NV). Gels were normalised using the Molecular weight marker GeneRuler™ DNA Ladder Mix (Thermo Scientific) in both sides of the gel. The ''rolling disk'' background subtraction option was applied. DNA bands were detected by the software and were carefully verified by visual examination. Levels of similarity between the profiles were calculated using the band matching Pearson coefficient. Cluster analyses was performed by the UPGMA method to produce a dendrogram.

## 2.5. Amplification of ITS, *tef1*-α and *tub2* Regions

The Internal Transcribed Spacer (ITS) region and the Translated Elongation Factor 1-α (*tef1-α*) regions were amplified for all samples. For some specific samples, the β – Tubulin region (*tub2*) was also amplified.

The PCR reactions were performed in 25 µl volume using 15.75 µl of Water, 6.25 µl of NZYTaq 2x Green Master Mix, containing buffer, dNTP's, MgCl$_2$ and *Taq* DNA polymerase enzyme (NZYTech, Lisboa, Portugal) and 1 µl of each primer forward and reverse (10 pmol/µl) and 1 µl of DNA in a My Cycler™ Thermal Cycler (Bio-Rad, Hercules, California, USA). All the primers and conditions can be seen in Table IV and attachment 2.

Table IV - Primers and amplification conditions for ITS, *tef1-α* and *tub2*

| Amplification Region | Primers | Conditions | | | | no of cycles | Final Extension |
|---|---|---|---|---|---|---|---|
| | | Initial denaturation | Denaturation | Annealing | Extension | | |
| **ITS** | ITS5 | 95 °C | 95 °C | 50/55 °C | 72 °C | 30 | 72 °C |
| | NL4 | 3 min | 30 secs | 30 secs | 1 min 30 s | | 10 min |
| ***tef1-α*** | EF1 - 688F | 95 °C | 94 °C | 50/52 °C | 72 °C | 35 | 72 °C |
| | EF1 - 1251R | 3 min | 30 secs | 30 secs | 45 secs | | 10 min |
| ***tub2*** | T1 | 95 °C | 94 °C | 50 °C | 72 °C | 35 | 72 °C |
| | BT-2b | 3 min | 30 secs | 30 secs | 1 min | | 10 min |

The amplified regions were separated by electrophoresis in a 1% agarose gel in TAE 1x using 5 µl of each PCR reaction, in the following conditions: 80V, during 60 min. The agarose gel was stained with EtBr (0.5 µg/ml) during 15 min, washed in water for 30 min and the image captured using the BIO-RAD Molecular Imager® Gel Doc™ XR+ With Image Lab Software (Bio-Rad, Hercules, California, USA).

### 2.5.1. Purification of the amplicons

The PCR products were purified using the NZYGelpure Kit (NZYTech, Lisboa, Portugal) and DNA Clean & Concentrator™-5 (Zymo Research, Irvine, USA) before sequencing. The DNA Clean and Concentrator™-5 was used when the PCR product's DNA concentration was insufficient for sequencing.

**Purification of the samples using NZYGelpure Kit**

1.  Mark NZYTech Spin Columns + Collection tubes and 1.5 µl Eppendorf's equal to the number of samples to use
2.  Add 5 volumes of Binding buffer to the sample, mix by up and down and put it in the NZYTech Spin Columns
3.  Centrifuge at room temperature during 1 min at 12000 rpm
4.  Discard the liquid present in the collection tubes
5.  Add 600 µl of Wash buffer to the NZYTech Spin Columns
6.  Centrifuge at room temperature during 1 min at 12000 rpm
7.  Discard the liquid present in the Collection tubes
8.  Centrifuge at room temperature during 1 min at 12000 rpm

9. Discard the Collection tubes and put the NZYTech Spin Columns in the 1.5 µl marked Eppendorf's

10. Add 25 µl of distilled water in the centre of the filter. Rest for 1 min.

11. Centrifuge at room temperature during 1 min at 12000 rpm

12. Discard NZYTech Spin Columns.


**Purification of the samples using Zymo Research DNA Clean & Concentrator™-5**

1. Mark Columns + Collection tubes and 1.5 µl Eppendorf's equal to the number of samples to use

2. Add 5 volumes of Binding buffer to the sample, mix by up and down and put it in the Columns

3. Centrifuge at room temperature during 30s min at 13000 G

4. Discard the liquid present in the collection tubes

5. Centrifuge at room temperature during 30s min at 13000 G

6. Discard the liquid present in the collection tubes

7. Discard the Collection tubes and put the Zymo Columns in the 1.5 µl marked Eppendorf's

8. Add volume ≥ 6µl of dH$_2$O to the column and let rest for 1 min

9. Centrifuge at room temperature during 30s at 13000 G

10. Discard Zymo Spin Columns.

After the purification, add 5 µl of primer (2.5 µl of primer (10pmol/µl) and 2.5 µl of water) to 5 µl of purified DNA for sequencing.

## 2.6. Sequence Analysis

The sequencing of the amplicons was carried out at GATC Biotech (Konstanz, Germany). The chromatograms were visualised and edited using the program FinchTV (Geopiza, inc.). The identification of the isolates was done by comparing them with sequences deposited in a database, GenBank (NCBI - National Center for Biotechnology Information) using the bioinformatic tool BLAST (Basic Local Alignment Search Tool), accessible on-line in https://blast.ncbi.nlm.nih.gov/Blast.cgi.

The sequences, both from the samples and from sequences deposited in GenBank, were aligned using the program ClustalX2 version 2.1 (Larkin *et al*., 2007) with the following parameters: Pairwise alignment parameters (Gap opening = 0.1; Gap extend = 0.1) and multiple alignment parameters (Gap opening = 0.1; Gap extension = 0.2; Delay divergent sequences (%) = 25; DNA transition weight = 0.5) manually edited (Santos *et al*.,2017). The obtained alignment was then edited using the program BioEdit version 7.0.5.3 (Hall, 1999). The program used to perform

phylogenetic analyses for each alignment was MEGA: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (Kumar *et al.*, 2016) using the Maximum Likelihood (ML) method. MEGA7 was also used to determine the best evolution models for each alignment.

## 2.7. Morphologic Characterization

Morphological characterization of the isolates representing potential novel species was adapted from Moreira *et al.*, 2016.

To observe macroscopic characters, 5mm disk of mycelium from the isolates were inoculated on 90-mm petri dishes with PDA (PDA, VWR, Leuven, Belgium) and OA. They were incubated during 14 days at 25°C in the dark.

To observe micromorphological characters cultures on OA incubated during 14 days at 25°C in the dark were used.

The colony growth rate was evaluated using malt extract agar 3% (MEA, Merck KGaA, Darmstadt, Germany) and incubation at 10, 14, 20, 25, 30 and 35°C for 14 days in the dark. There were used 3 replicas of each isolate in each temperature. Plugs with 5mm of medium with mycelium were put in the border of the plate and the radius of the isolate was measured every day for 14 days with a ruler.

The microscopic slides were prepared with 100% lactic acid. The size and shape of conidia, metulae and phialides, the branching pattern and the size of conidiophores and the presence or absence of intercalary phialides were observed and recorded.

The structures were observed with a Nikon 80i microscope equipped with differential interference contrast. The pictures of the structures were captured with a Nikon DS-Ri1 camera and treated with NIS-Elements D program version 3.22.15 64bit.

# 3. Results and Discussion

## 3.1. Fungal identification

A total of 148 isolates were subjected to genetic typing. This was done using MSP–PCR which generates band profiles (Figure 4) that are capable of solving taxonomic inter- and intraspecific issues (Meyer & Mitchell, 1995; Weising *et al*., 1995).

A similarity analysis of the band profiles was performed (Figures 5 and 6) through which the representative profiles (or isolates) of each group or the most different ones within the group were chosen for further molecular identification through sequencing of the ITS marker. A total of 38 representative isolates were selected for ITS sequencing and analysis.

The results of the standard nucleotide BLAST search of the ITS sequences obtained are shown in table V. For each isolate it shows the maximum identity with the closest relative and corresponding accession numbers.
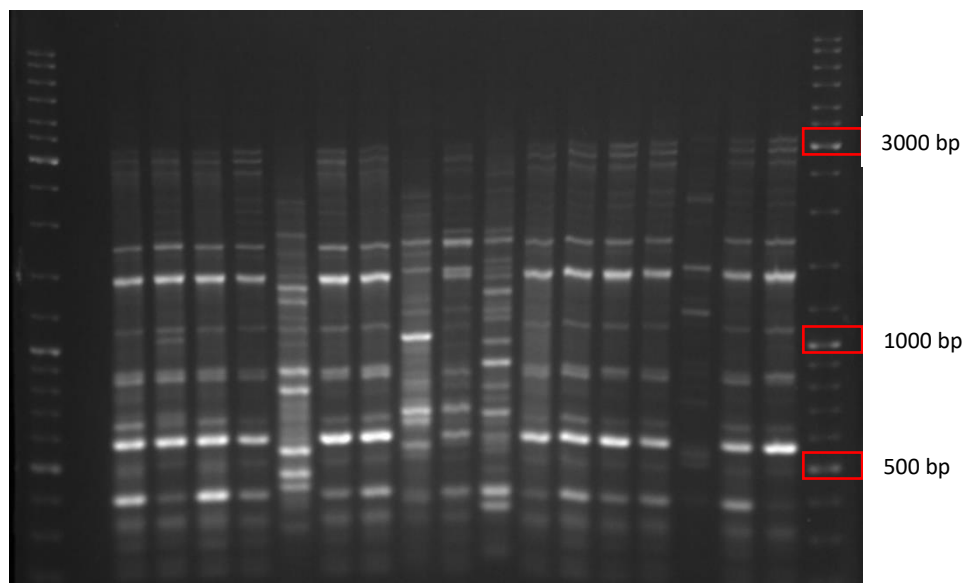


Figure 4 - Example of an agarose gel showing the band profile obtained by MSP-PCR revealed with BIO-RAD Molecular Imager® Gel Doc™ XR+ With Image Lab Software
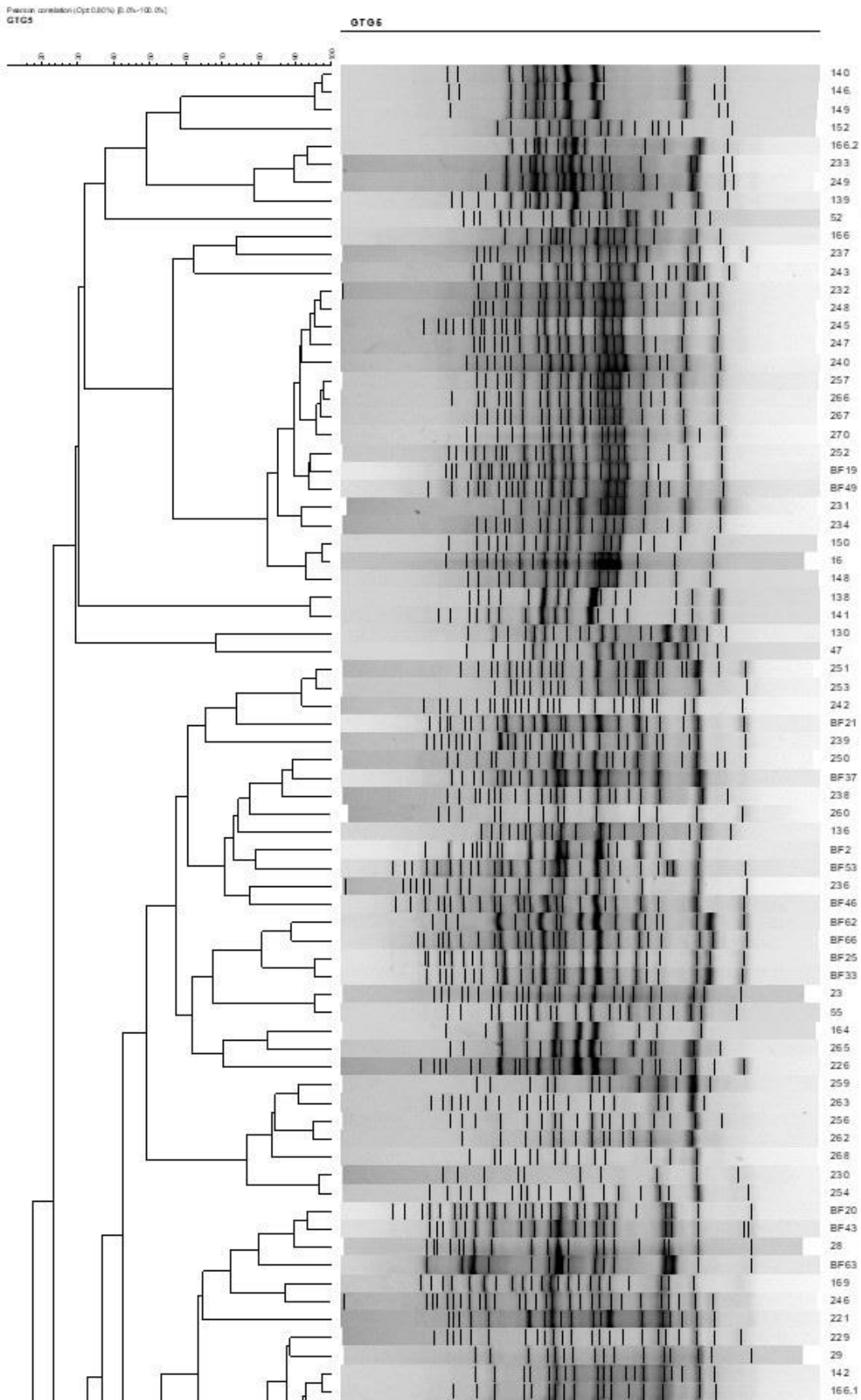
Figure 5 - Dendrogram resulting from the similarity analysis of the band profiles resultant from the MSP - PCR
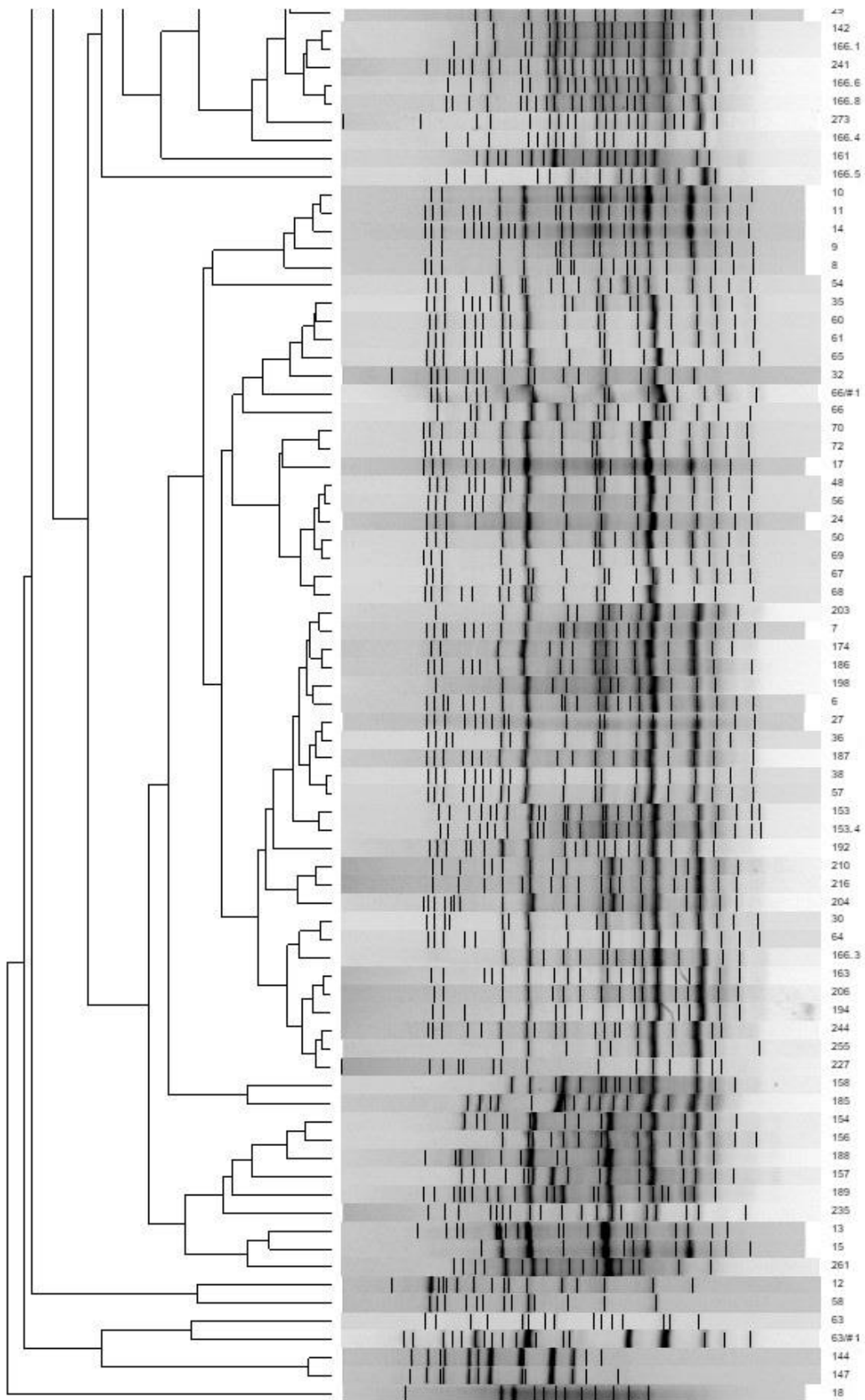
Figure 6 - Dendrogram resulting from the similarity analysis of the band profiles resultant from the MSP – PCR (continuation)

Table V - BLAST results of the ITS sequences of the chosen isolates, host information, maximum identity and GenBank accession numbers

| Isolates | ITS | Host | Maximum Identity | GenBank accession number |
|---|---|---|---|---|
| 12 | *Fusarium proliferatum* CanR-8 | healthy canola root | 100% | JF817300 |
| 13 | *Fusarium proliferatum* 10R-7 | cotton | 99% | MF614934 |
| 14 | *Fusarium proliferatum* CanR-8 | healthy canola root | 100% | JF817300 |
| 16 | *Nectriaceae* sp. Mdf-26 | *Paeonia ostii* root | 99% | MF574246 |
| 18 | *Fusarium proliferatum* 10R-7 | cotton | 100% | MF614934 |
|  | *Fusarium fujikuroi* H03S_C1 | grain | 100% | KX681574 |
|  | *Fusarium verticillioides* BPS180 | medicinal plant | 100% | KX262965 |
| 23 | *Fusarium solani* seqID3 | Biofilter treating methanol | 100% | MH348945 |
|  | *Fusarium keratoplasticum* ATS104 | toenail | 100% | MF411133 |
| 28 | *Fusarium solani* DET-33 | *Aspidosperma polyneuron* | 100% | KX385049 |
|  | *Fusarium lateritium* CBPPR0046 | cassava with root rot symptoms | 100% | KT211538 |
| 32 | *Fusarium proliferatum* 10R-7 | cotton | 100% | MF614934 |
|  | *Fusarium fujikuroi* H03S_C1 | grain | 100% | KX681574 |
|  | *Fusarium verticillioides* BPS180 | medicinal plant | 100% | KX262965 |
| 35 | *Fusarium proliferatum* 10R-7 | cotton | 100% | MF614934 |
|  | *Fusarium fujikuroi* H03S_C1 | grain | 100% | KX681574 |
|  | *Fusarium verticillioides* BPS180 | medicinal plant | 100% | KX262965 |
| 47 | *Trichoderma* sp. yi0319_1 | unknown | 99% | MH284309 |
|  | *Clonostachys rosea* genomic DNA sequence | unknown | 99% | LT576164 |
| 52 | *Fusarium solani* DET-33 | *Aspidosperma polyneuron* | 100% | KX385049 |
|  | *Fusarium lateritium* CBPPR0046 | cassava with root rot symptoms | 100% | KT211538 |
| 54 | *Fusarium proliferatum* 10R-7 | cotton | 100% | MF614934 |
|  | *Fusarium fujikuroi* H03S_C1 | grain | 100% | KX681574 |
|  | *Fusarium verticillioides* BPS180 | medicinal plant | 100% | KX262965 |
| 58 | *Fusarium proliferatum* 10R-7 | cotton | 100% | MF614934 |
|  | *Fusarium fujikuroi* H03S_C1 | grain | 100% | KX681574 |
|  | *Fusarium verticillioides* BPS180 | medicinal plant | 100% | KX262965 |
| 63 | *Phanerochaete sordida* CFR-V50 | forest soil | 99% | KF916581 |
| 72 | *Fusarium proliferatum* 10R-7 | cotton | 100% | MF614934 |
|  | *Fusarium fujikuroi* H03S_C1 | grain | 100% | KX681574 |
|  | *Fusarium verticillioides* BPS180 | medicinal plant | 100% | KX262965 |
| 139 | *Clonostachys rosea* 197WS | Open batch fermentation broth | 100% | MG396999 |

Table V - BLAST results of the ITS sequences of the chosen isolates, host information, maximum identity and GenBank accession numbers (Continuation)

| Isolates | ITS | Host | Maximum Identity | GenBank accession number |
|---|---|---|---|---|
| 141 | *Cylindrocladiella peruviana* CBS 116103 | unknown | 99% | JN943095 |
| 146 | *Clonostachys rosea* 197WS | Open batch fermentation broth | 100% | MG396999 |
| 147 | *Nectriaceae* sp. Mdf-26 | *Paeonia ostii* root | 99% | MF574246 |
| 152 | *Chaetomium globosum* CICR5 | cotton | 100% | KF747364 |
| 158 | *Fusarium proliferatum* 10R-7 | cotton | 100% | MF614934 |
| | *Fusarium fujikuroi* H03S_C1 | grain | 100% | KX681574 |
| | *Fusarium verticillioides* BPS180 | medicinal plant | 100% | KX262965 |
| 161 | *Fusarium solani* seqID3 | Biofilter treating methanol | 100% | MH348945 |
| | *Fusarium keratoplasticum* ATS104 | toenail | 100% | MF411133 |
| 164 | *Fusarium falciforme* ATS98 | toenail and web space | 100% | MF411132 |
| | *Fusarium phaseoli* clone OTU9 | roots of *Dalbergia odorifera* | 100% | KY965401 |
| 166.5 | *Fusarium chlamydosporum* KUSF2102 | soil | 100% | MF136407 |
| | *Fusarium* cf. *equiseti* ATS58 | unknown | 100% | KU900501 |
| 187 | *Fusarium fujikuroi* BJ-1 | *Bletilla striata* | 100% | MF426033 |
| | *Fusarium proliferatum* gss176 | ginseng | 100% | MH290457 |
| 188 | *Fusarium proliferatum* CanR-8 | healthy canola root | 100% | JF817300 |
| 206 | *Fusarium fujikuroi* BJ-1 | *Bletilla striata* | 100% | MF426033 |
| | *Fusarium proliferatum* gss176 | ginseng | 100% | MH290457 |
| 233 | *Clonostachys rosea* 197WS | Open batch fermentation broth | 100% | MG396999 |
| 237 | *Fusarium solani* CCF 4360 | Unknown | 99% | HE974457 |
| | *Fusarium keratoplasticum* FRC S-2478 | Unknown | 99% | JN235273 |
| 241 | *Fusarium* sp. RRA71 | adv. roots | 100% | KX953538 |
| 247 | *Nectriaceae* sp. Mdf-26 | *Paeonia ostii* root | 99% | MF574246 |
| BF2 | *Fusarium phaseoli* clone OTU9 | Roots of *Dalbergia odorifera* | 100% | KY965401 |
| BF19 | *Nectriaceae* sp. E9321c | *Thelypteris lugubriformis* | 99% | JN545764 |
| BF20 | *Fusarium solani* DET-33 | *Aspidosperma polyneuron* | 99% | KX385049 |
| BF21 | *Fusarium keratoplasticum* NRRL:54999 | Unknown | 99% | KC808259 |
| | *Fusarium solani* MLA-9 | Roots of date palm | 99% | MG932644 |
| BF46 | *Fusarium solani* MLA-9 | Roots of date palm | 99% | MG932644 |
| | *Fusarium keratoplasticum* UOA/HCPF AB90 | blood culture | 99% | KC254052 |

Table V - BLAST results of the ITS sequences of the chosen isolates, host information, maximum identity and GenBank accession numbers (Continuation)

| Isolates | ITS | Host | Maximum Identity | GenBank accession number |
|---|---|---|---|---|
| **BF63** | *Fusarium solani* DET-33 | *Aspidosperma polyneuron* | 99% | KX385049 |
| | *Fusarium lateritium* CBPPR0046 | cassava with root rot symptoms | 99% | KT211538 |
| **BF66** | *Fusarium solani* DI16 | Mangrove soil | 99% | KF918581 |
| | *Fusarium keratoplasticum* UOA/HCPF AB90 | blood culture | 99% | KC254052 |

In the Figure 7 we can see the existence genera resulting of the BLAST of the ITS from the isolates.
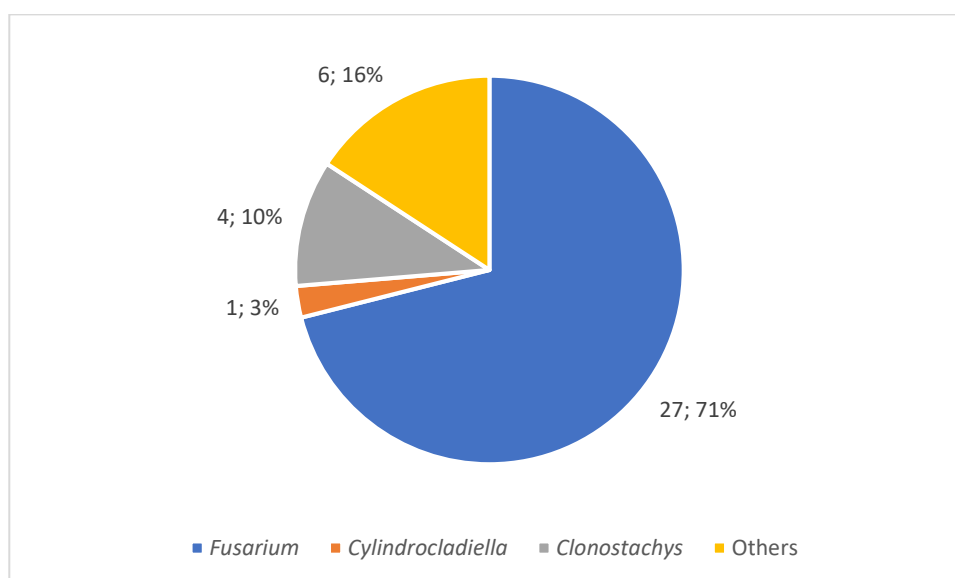


Figure 7- Distribution of the number and percentage of isolates for each genus according to the ITS BLAST results

As can be seen, according to the ITS, the isolates belong to three known genera: *Fusarium* (71%), *Clonostachys* (10%) and *Cylindrocladiella* (3%). There are also 16% of the isolates in the group others. Some of the isolates had more than one BLAST hit with different genera, and so it was impossible to correctly affiliate them. Isolates 152 and 63 belong to the genera *Neurospora* and *Phanerochaete* respectively. Genus *Neurospora* belongs in the order *Sordariales*, and the *Phanerochaete* is a member of the phylum *Basidiomycota,* being both not of interest for the study.

The ITS region of the rDNA is the primary barcode for fungi (Schoch *et al*., 2012; Xu & Adamowicz, 2016). In this study, the use of a second phylogenetic marker or secondary barcode

was necessary due to the existence of genera with high intraspecific diversity of the ITS locus or the possibility of different species having the same ITS sequence, as happens with the genus *Fusarium* (Irinyi *et al*., 2016; O'Donnell *et al*., 2015; Schoch *et al*., 2012).  Although the ITS region is not useful for species delimitation within the genus *Fusarium*, it is important and useful to place *Fusarium* species within the correct species complex (Balajee et al., 2009; O'Donnell et al., 2015).

The chosen secondary phylogenetic marker was *tef1*-α because it is the most frequently used genetic marker for discriminating species in *Fusarium* (Irinyi *et al*., 2016; O'Donnell *et al*., 2010, 2015; Schoch *et al*., 2012; Short *et al*., 2013). Since using only the ITS region for isolates 16, 147, 241, 247, BF19 and BF20 does not discriminate their genera, the utilization of a second marker is necessary to assess it. As for some *Fusarium* isolates whose ITS region retrieves two or more BLAST results with the same coverage and maximum identity but different species, the use of the second marker is necessary to help assess the correct species identity.

Table VI presents the results of the BLAST search using *tef1*-α sequences, showing the maximum identity with the closest relative and corresponding accession numbers.

Table VI - BLAST results of the *tef1*-α sequences of the chosen isolates, host information, maximum identity and GenBank accession numbers

| Isolates | *tef1*-α | Host | Maximum Identity | GenBank accession number |
|---|---|---|---|---|
| 12 | *Fusarium proliferatum* NRRL 25103 | Unknown | 99% | JF740730 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 13 | *Fusarium proliferatum* NRRL 25103 | Unknown | 99% | JF740730 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 14 | *Fusarium proliferatum* NRRL 25103 | Unknown | 99% | JF740730 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 16 | *Pleiocarpon strelitziae* CPC27629 | dry basal rot of *Strelitzia reginae* | 99% | KY304723 |
| 18 | *Fusarium proliferatum* NRRL 25103 | Unknown | 99% | JF740730 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 23 | *Fusarium solani* NRRL 52704 | Unknown | 98% | JF740786 |
| 28 | *Fusarium solani* NRRL 52701 | Unknown | 99% | JF740784 |
| 32 | *Fusarium proliferatum* NRRL 25103 | Unknown | 99% | JF740730 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 35 | *Fusarium proliferatum* NRRL 25103 | Unknown | 99% | JF740730 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 47 | *Clonostachys pseudochroleuca* CML 1983 | soil, Amazon Forest | 99% | KX185015 |
| 52 | *Fusarium solani* NRRL 52701 | Unknown | 99% | JF740784 |
| 54 | *Fusarium proliferatum* NRRL 25103 | Unknown | 99% | JF740730 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 58 | *Fusarium proliferatum* NRRL 25103 | Unknown | 99% | JF740730 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 72 | *Fusarium proliferatum* NRRL 25103 | Unknown | 99% | JF740730 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 139 | *Clonostachys pseudochroleuca* CML 1983 | Amazon Forest soil | 90% | KX185015 |
| 141 | *Cylindrocladiella peruviana* CMW47333 | soil | 99% | MH016995 |
| 146 | *Clonostachys pseudochroleuca* CML 1983 | Amazon Forest soil | 90% | KX185015 |
| 147 | *Pleiocarpon strelitziae* CPC27629 | dry basal rot of *Strelitzia reginae* | 99% | KY304723 |
| 152 | *Neurospora crassa* | Unknown | 97% | BX842622 |
| 158 | *Fusarium proliferatum* NRRL 25103 | Unknown | 99% | JF740730 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 161 | *Fusarium solani* NRRL 52704 | Unknown | 98% | JF740786 |
| 164 | *Fusarium solani* NRRL 52689 | Unknown | 99% | JF740774 |
| 166.5 | *Fusarium* sp. NRRL 25106 | Unknown | 99% | JF740732 |

Table VI - BLAST results of the *tef1*-α sequences of the chosen isolates, host information, maximum identity and GenBank accession numbers (Continuation)

| Isolates | *tef1*-α | Host | Maximum Identity | GenBank accession number |
|---|---|---|---|---|
| 187 | *Fusarium proliferatum* NRRL 52696 | Unknown | 99% | JF740779 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 188 | *Fusarium proliferatum* NRRL 25103 | Unknown | 99% | JF740730 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 206 | *Fusarium proliferatum* NRRL 52696 | Unknown | 99% | JF740779 |
| | *Fusarium fujikuroi* GA1A-11-1 | wilted plant stem tissue | 99% | KY293661 |
| 233 | *Clonostachys pseudochroleuca* CML 1983 | Amazon Forest soil | 90% | KX185015 |
| 241 | *Fusarium solani* NRRL 52701 | Unknown | 99% | JF740784 |
| 247 | *Pleiocarpon strelitziae* CPC27629 | dry basal rot of Strelitzia reginae | 99% | KY304723 |
| BF2 | *Fusarium solani* NRRL 52689 | Unknown | 99% | JF740774 |
| BF19 | *Pleiocarpon strelitziae* ST13 | dry basal rot of Strelitzia reginae | 99% | KY304734 |
| BF20 | *Fusarium solani* MICMW-30.1 | Swietenia macrophylla King | 99% | KX870044 |
| BF21 | *Fusarium solani* Fs-69P | strawberry crown | 93% | KY486677 |
| BF46 | *Fusarium solani* NRRL 52704 | Unknown | 99% | JF740786 |
| BF63 | *Fusarium solani* NRRL 52701 | Unknown | 99% | JF740784 |
| BF66 | *Fusarium solani* AB-G1 | soil | 99% | KY486691 |
| | *Fusarium keratoplasticum* ATS57 | human eye cornea | 99% | MF034510 |

In Figure 8 we can see that through BLAST search of the *tef*1-α sequences from the isolates it was possible to identify four known genera: *Fusarium*, *Clonostachys, Cylindrocladiella* and *Pleiocarpon*.
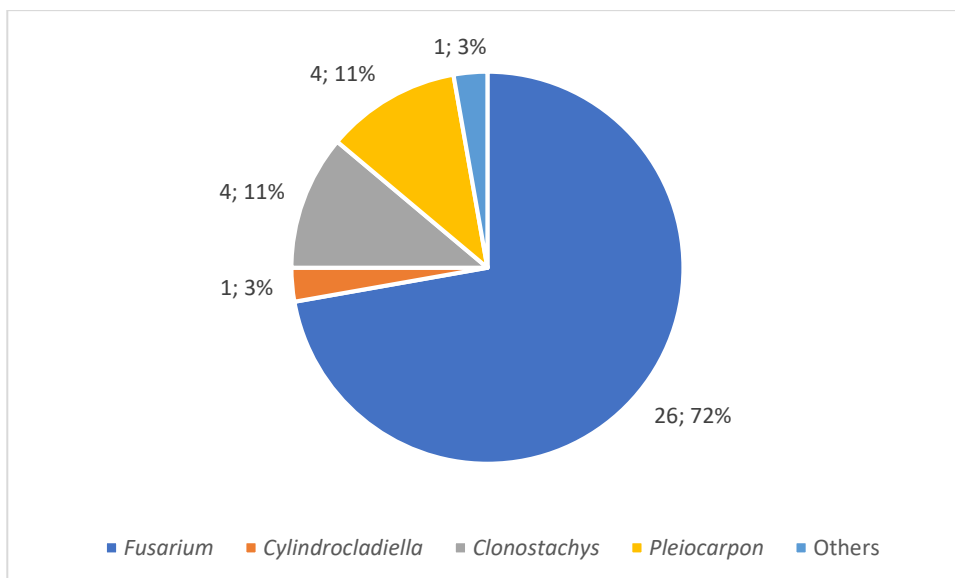
Figure 8 - Distribution of the number and percentage of isolates for each genus according to the *tef*1-α BLAST results

As can be seen in figure 8, using the *tef1*-α region, we can assess the genera of the isolates classified previously as others. Isolates 16, 147, 247 and BF19 belong to the genus *Pleiocarpon* and isolates BF20 and 241 belong to the genus *Fusarium*.

For the genus *Fusarium*, even using the *tef*1-α marker the BLAST search for some isolates retrieved more than one species with the same maximum identity.

For *Clonostachys* and *Cylindrocladiella* species, the *tub2* marker was also used for phylogenetic analyses. For genus *Clonostachys* the number of sequences for *tef*1-α region are low, and the region used for most species is the *tub2*. For the genus *Cylindrocladiella tub2* is the used regions for phylogenetic analyses (Lombard *et al.*, 2017; Pham *et al.*, 2018; Van Coller *et al.*, 2005) .

3.2. Phylogeny and Taxonomy

The phylogeny of the isolates was assessed separately by genus using sequences from other closely related species to determine their phylogenetic relationship, using MEGA7.

3.2.1. Genus *Cylindrocladiella* and Genus *Pleiocarpon*

The Genus *Cylindrocladiella* (Hypocreales, Nectriaceae) was introduced by Boesewinkel in 1982, to accommodate *Cylindrocladium* species with small spores. These species not only differ from *Cylindrocladium* in the shape and size of the conidia, but also in the type of branching of the conidiophores, that can be subverticiliate to penicillate usually arranged around a central

stripe position, and structure of the appendage with vesicle (Boesewinkel, 1982; Crous *et al.*, 1991). The genus *Cylindrocladiella* includes 35 species. These species are known mainly as saprobes but some species are plant pathogens (Pham *et al.*, 2018).

The Genus *Pleiocarpon* is a new genus described for the first time in 2017 (Aiello *et al.*, 2017). Genus *Pleiocarpon* is a new *Cylindrocarpon*-like asexual morph, with abundant aseptate microconidia and macroconidia, with variable conidia shape, phylogenetically closely related to the genus *Thelonectria* (Aiello *et al.*, 2017; Chaverri *et al.*, 2011). There is no known sexual morph. Until now there is only a single known species, *Pleiocarpon strelitziae*. This species was found in association with dry rot of the basal stem of *Strelitziae reginae*, known as "bird of paradise" (Aiello *et al.*, 2017).

The sequences of *Cylindrocladiella* and *Pleiocarpon* species, which were retrieved from GenBank, were used for the phylogenetic analysis, and are shown in Tables VI and VII. The respective phylogenetic trees can be seen in figures 9 and 10.

Table VII - Details of *Cylindrocladiella* species retrieved form GenBank included in the phylogenetic analysis

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | *tub2* (GenBank) | Articles |
|---|---|---|---|---|---|---|---|
| CBS 129567/ CPC 17507 | *Cylindrocladiella australiensis* | Australia | Soil | NR_111652 | JN099060 | JN098747 | Lombard *et al.*, 2012 |
| CPC 234/PPRI 3990/ IMI 346845 | *Cylindrocladiella camelliae* | South Africa | *Eucalyptus grandis* | AF220952 | JN099087 | AY793471 | Lombard *et al.*, 2015 |
| CBS 129564/CPC 17592 | *Cylindrocladiella clavata* | Australia | Soil | NR_111639 | JN098974 | JN098752 | Lombard *et al.*, 2012 |
| CBS 129553/ CPC 17393 | *Cylindrocladiella cymbiformis* | Australia | Soil | NR_111642 | JN098988 | JN098753 | Lombard *et al.*, 2012 |
| CBS 338.92 | *Cylindrocladiella elegans* | South Africa | Leaf litter | NR_103586 | JN099039 | AY793474 | Lombard *et al.* 2012 |
| CBS 129573/ CPC 17560 | *Cylindrocladiella ellipsoidea* | Australia | Soil | NR_111638 | JN098973 | JN098757 | Lombard *et al.*, 2012 |
| CBS 129569/ CPC 12272 | *Cylindrocladiella hawaiiensis* | Hawai | Soil | NR_111651 | JN099057 | JN098761 | Lombard *et al.*, 2012 |
| CBS 129577/ CPC 17551 | *Cylindrocladiella kurandica* | Australia | Soil | NR_111654 | JN099083 | JN098765 | Lombard *et al.*, 2012 |
| CBS 340.92 | *Cylindrocladiella lageniformis* | Brazil | *Eucalyptus* cutting | NR_111054 | JN099003 | AY793481 | Lombard *et al.*, 2015 |
| CBS 129566/CPC 17567 | *Cylindrocladiella lanceolata* | Australia | Soil | NR_111641 | JN098978 | JN098789 | Lombard *et al.*, 2012 |
| CBS 129557/ CPC 18839 | *Cylindrocladiella longiphialidica* | Thailand | Soil | NR_111646 | JN098966 | JN098790 | Lombard *et al.*, 2012 |
| CBS 116075/ CPC 708 | *Cylindrocladiella longistipitata* | China | Soil | AF220958 | JN098969 | AY793506 | Lombard *et al.*, 2012 |
| CBS 111794/ ATCC 38571/ CPC 2375 | *Cylindrocladiella microcylindrica* | Indonesia | *Echeveria elegans* | AY793452 | JN099041 | AY793483 | Lombard *et al.*, 2012 |
| CBS 114943/CPC 456 | *Cylindrocladiella natalensis* | South Africa | Peanut | NR_111647 | JN099016 | JN098794 | Lombard *et al.*, 2012 |

Table VII - Details of *Cylindrocladiella* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | *tub2* (GenBank) | Articles |
|---|---|---|---|---|---|---|---|
| CBS 152.91 | *Cylindrocladiella nederlandica* | Netherlands | Pelargonium | NR_111648 | JN099033 | JN098800 | Lombard *et al.*, 2012 |
| CBS 486.77/ATCC 44815/CPC 2397 | *Cylindrocladiella novaezelandiae* | New Zealand | *Rhododendron indicum* root | NR_111055 | JN099050 | AY793485 | Lombard *et al.*, 2012 |
| CBS 114524/ATCC 28272/CPC 2370 | *Cylindrocladiella parva* | New Zealand | *Telopea speciosissima* | AF220964 | JN099009 | AY793486 | Lombard *et al.*, 2012; Lombard *et al.*, 2015 |
| IMUR 1843/CPC 2404 | *Cylindrocladiella peruviana* | Peru | Ants | NR_119431 | JN098968 | AY793500 | Lombard *et al.*, 2012 |
| CBS 113022/ CPC 4291 | *Cylindrocladiella peruviana* | South Africa | *Eucalyptus* sp. | JN943094 | JN099029 | JN098801 | Lombard *et al.*, 2012 |
| CBS 114697/ CPC 2573 | *Cylindrocladiella peruviana* | South Africa | *Vitis vinifera* | JN943092 | JN099007 | JN098802 | Lombard *et al.*, 2012 |
| CBS 114942/ CPC 267 | *Cylindrocladiella peruviana* | South Africa | *Acacia mearnsii* | JN943093 | JN099015 | JN098803 | Lombard *et al.*, 2012 |
| CBS 114952/ CPC 398 | *Cylindrocladiella peruviana* | South Africa | *Eucalyptus* sp. | JN100572 | JN099089 | JN098804 | Lombard *et al.*, 2012 |
| CBS 114953/ CPC 399 | *Cylindrocladiella peruviana* | South Africa | *Eucalyptus* sp. | JN099123 | JN099006 | JN098805 | Lombard *et al.*, 2012 |
| CBS 116103/CPC 637/ UFO 197 | *Cylindrocladiella peruviana* | Brazil | *Psidium guajava* | JN943095 | JN099031 | JN098807 | Lombard *et al.*, 2012 |
| CPC 17517 | *Cylindrocladiella peruviana* | Australia | Soil | JN100637 | JN099074 | JN098808 | Lombard *et al.*, 2012 |
| CPC 17532 | *Cylindrocladiella peruviana* | Australia | Soil | JN099091 | JN098970 | JN098809 | Lombard *et al.*, 2012 |
| CPC 17533 | *Cylindrocladiella peruviana* | Australia | Soil | JN100633 | JN099070 | JN098810 | Lombard *et al.*, 2012 |
| CPC 17534 | *Cylindrocladiella peruviana* | Australia | Soil | JN099110 | JN098984 | JN098811 | Lombard *et al.*, 2012 |

Table VII - Details of *Cylindrocladiella* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1α* (GenBank) | *tub2* (GenBank) | Articles |
|---|---|---|---|---|---|---|---|
| CPC 17535 | *Cylindrocladiella peruviana* | Australia | Soil | JN100638 | JN099075 | JN098812 | Lombard *et al.*, 2012 |
| CPC 17556 | *Cylindrocladiella peruviana* | Australia | Soil | JN100569 | JN099084 | JN098813 | Lombard *et al.*, 2012 |
| CBS 129555/ CPC 18825 | *Cylindrocladiella pseudocamelliae* | Thailand | Soil | NR_111644 | JN098958 | JN098814 | Lombard *et al.*, 2012 |
| CBS 210.94/PPRI 4450/UFV 125 | *Cylindrocladiella pseudohawaiiensis* | Brazil | *Eucalyptus* cuttings | NR_111643 | JN099012 | JN098819 | Lombard *et al.*, 2012 |
| CBS 129560/CPC 18149 | *Cylindrocladiella pseudoparva* | Netherlands | Soil | NR_111650 | JN099056 | JN098824 | Lombard *et al.*, 2012 |
| CBS 129574/CPC 17568 | *Cylindrocladiella queenslandica* | Australia | Soil | NR_111640 | JN098977 | JN098826 | Lombard *et al.*, 2012 |
| CBS 110668/CPC 517 | *Cylindrocladiella stellenboschensis* | South Africa | Soil | NR_111649 | JN099051 | JN098829 | Lombard *et al.*, 2012 |
| CBS 129571/CPC 18835 | *Cylindrocladiella thailandica* | Thailand | Soil | NR_111645 | JN098963 | JN098834 | Lombard *et al.*, 2012 |
| CBS 129561/CPC 17505 | *Cylindrocladiella variabilis* | Australia | Soil | NR_111653 | JN099080 | JN098719 | Lombard *et al.*, 2012 |
| CBS 112897/CPC 5606 | *Cylindrocladiella viticola* | South Africa | *Vitis vinifera* | AY793468 | JN099064 | AY793504 | Lombard *et al.*, 2012 |

Table VIII- Details of *Thelonectria* and *Pleiocarpon* species retrieved form GenBank included in the phylogenetic analysis

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| CBS 134034; AR 4742 | *Thelonectria discophora* | Chile | *Tepualia stipularis* | KC153714 | KC153843 | Aiello *et al.*, 2017 |
| CBS 142255 | *Thelonectria olida* | Italy | *Strelitzia reginae* | KY304659 | KY304737 | Aiello *et al.*, 2017 |
| CBS 113.12; IMI 113918 | *Thelonectria rubi* | Unknown | *Rubus idaeus* | KC153718 | KC153847 | Aiello *et al.*, 2017 |
| CBS 112467; IMI 352560 | *Thelonectria trachosa* | Scotland | Bark of conifer | NR_145027 | KM231896 | Aiello *et al.*, 2017 |
| CBS 132341; AR 1751 | *Thelonectria veuillotiana* | Portugal | Bark of *Eucalyptus* sp. | JQ403305 | JQ394734 | Aiello *et al.*, 2017 |
| CBS 142251/ CPC 27628 | *Pleiocarpon strelitziae* | Italy | *Strelitzia reginae* | KY304644 | KY304722 | Aiello *et al.*, 2017 |
| CPC 27629 | *Pleiocarpon strelitziae* | Italy | *Strelitzia reginae* | KY304645 | KY304723 | Aiello *et al.*, 2017 |

Figure 9 - Phylogenetic tree based on Maximum Likelihood (ML) analysis of a combined data set of ITS and *tef1*-α sequence alignments. The applied evolutionary model was the Hasakawa-Kishino-Yana model with Gama distribution and allowing invariable sites. The bootstrap values are presented (%) resulting of 1000 replicates. The samples used in the study are represented in bold with a black box.
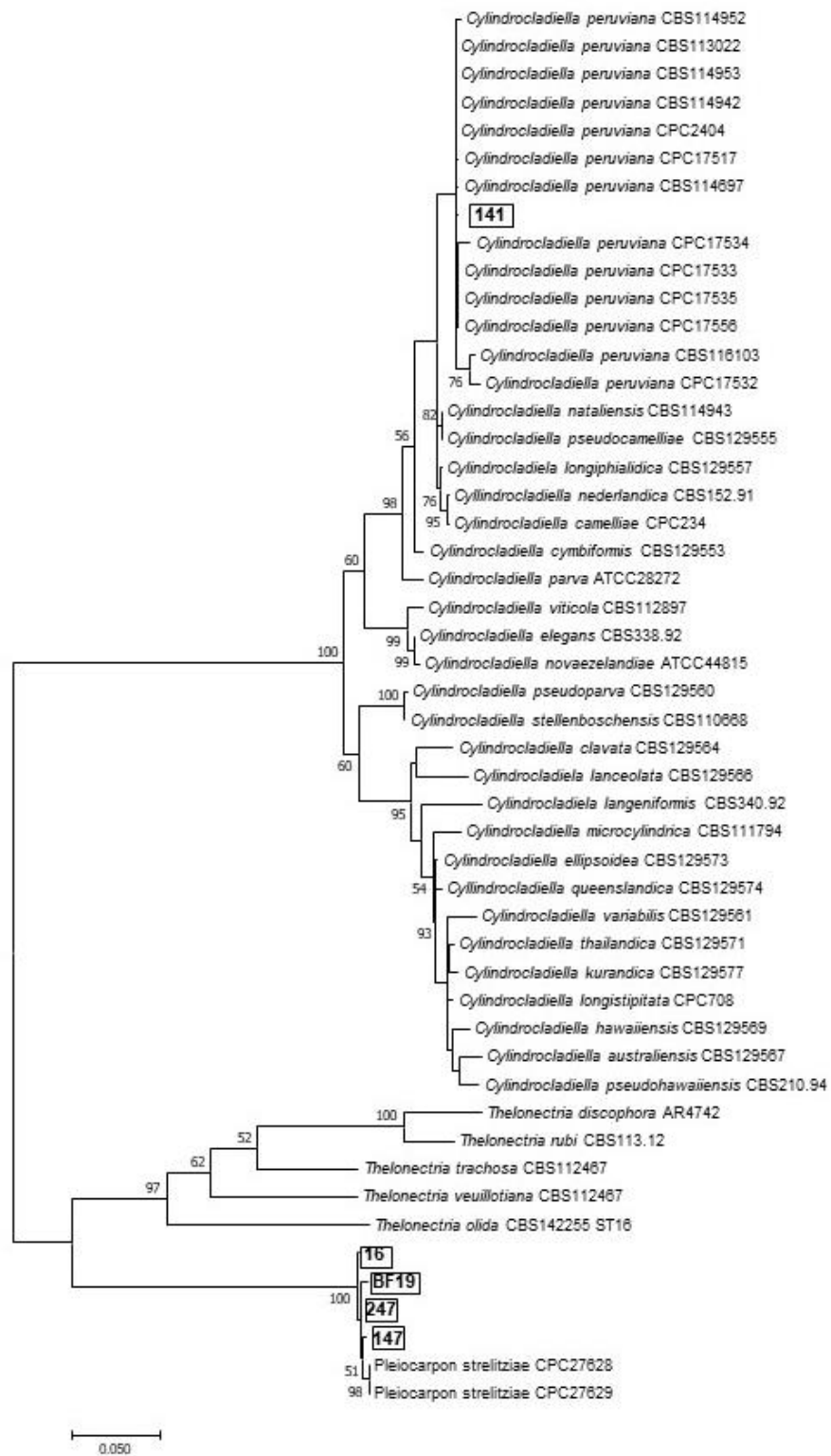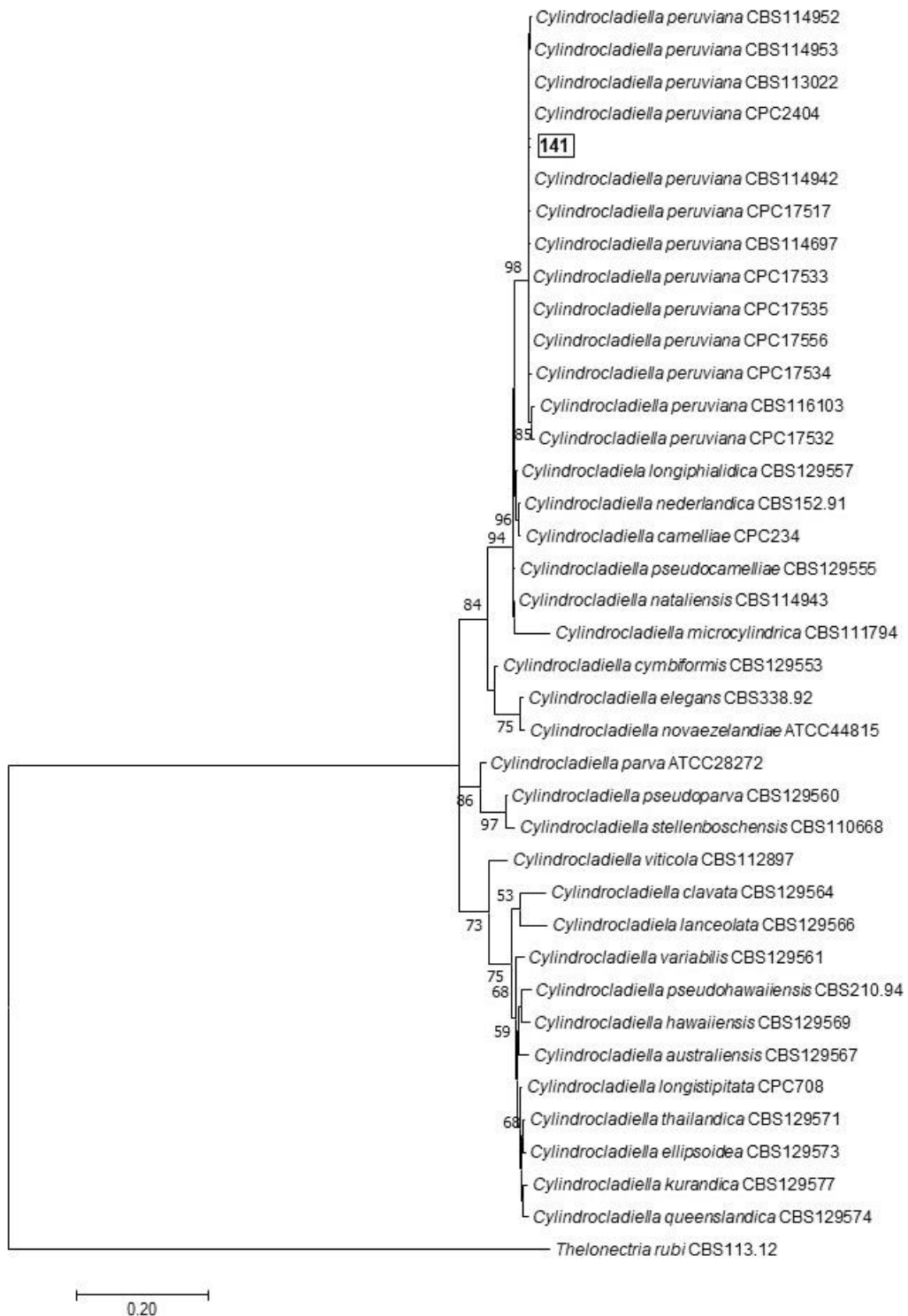
Figure 10 - Phylogenetic tree based on Maximum Likelihood (ML) analysis of a combined data set of ITS, *tef1*-α and *tub2* sequence alignments. The applied evolutionary model was the Tamura-Nei model with gamma distribution. The bootstrap values are presented (%) resulting of 1000 replicates. The samples used in the study are represented in bold with a black box.

For the alignment were used amplicons with 540 – 524 bp for ITS, 546-487 bp for EF and 544-535 bp for BT.

For the genus *Pleiocarpon*, we can see a well-supported group (100%) with the isolates 16, 147, 247 and BF19 related with the available sequences belonging to the species *Pleiocarpon strelitziae*. The differences between the isolates and the available sequences are most likely result of intraspecific variability, and they belong to the same species.

*Pleiocarpon strelitziae* has been related with the basal stem rot of *Strelitzia reginae* (Aiello *et al.*, 2017). In this study, authors show that after 10 days, the plants presented symptoms of disease and after 2 months they died. So far *P. strelitziae* has never been reported from grapevines. Although our isolates were obtained from symptomatic plants it is necessary to carry out pathogenicity studies to assess if this known pathogen of *Strelitzia reginae* also develops disease in *Vitis vinifera*.

For the genus *Cylindrocladiella*, we can see that isolate 141 groups with the species *Cylindrocladiella peruviana* both in ITS+*tef1*-α tree (Figure 9) and ITS+*tef1*-α+*tub2* tree (Figure 10). In the first tree, Figure 9, the isolate 141 groups with *C. peruviana*, showing a low bootstrap support (41%). In the Figure 10 we can see that, adding the *tub2* to the analysis, the resolution in some species is reduced, but for the *Cylindrocladiella peruviana* group the support was higher than with only ITS+EF, being the support of the group 98%. One of the latest studies of *Cylindrocladiella* (Pham *et al.*, 2018) showed that *tub2* was not a very informative site for the phylogenetic resolution of some species and recommends the use of his3 (histone) marker since it alone resolves better the phylogenetic relationship between species amongst all used genetic regions but, in this study, the addition of *tub2* helped giving a better support for the group.

In Van Coller *et al*. (2005) *Cylindrocladiella lageniformis* and *C. peruviana* are reported to be associated with black foot disease although they could not assess if they were causing the symptoms in the grapevines. In 2012, *C. peruviana* was also associated for the first time with grapevine with GTD symptoms in Peru (Álvarez *et al*., 2012).

Agustí-Brisach *et al*. (2012) described for the first time *Cylindrocladiella parva* and *C. peruviana* as pathogenic agents causing BFD in grapevines in Spain. After 20 days of the inoculation of *C. peruviana* in grapevines with 1 month, they presented symptoms of BFD, like reduced vigour, necrotic lesions, and occasional mortality.

Koike et al. (2016) reported for the first time *C. lageniformis* and *C. peruviana* causing black foot disease in grapevines in California, by testing their pathogenicity by inoculating the isolates in canes and after 6 months they presented brown streaking above and below the inoculation site and the isolates were re-isolated from discoloration in the canes while control canes did not developed symptoms.

Isolate 141 affiliated to *C. peruviana,* and thus can be a probable causing agent of Black foot disease in Peru but pathogenicity tests must be made to be certain about their role in the disease, and to see if it fulfils the Koch's postulates.

### 3.2.2. Genus *Fusarium/Neocosmospora*

*Fusarium* (Hypocreales, Nectriacea) was described for the first time by Link, 1809. It is one of the most renowned genera in the kingdom Fungi (Sandoval-Denis *et al*., 2017). This genus has approximately 300 phylogenetic species with at least half of them lacking formal description (Aoki *et al*., 2014; Ramdial *et al*., 2016).

Many *Fusarium* species are found as air-, soil- and water-borne saprobic organisms, found also in dead or living plant material as endophytes or epiphytes. They can also be pathogens of plants, humans, and other animals (Aoki *et al*., 2014; Leslie & Summerell, 2006; Sandoval-Denis *et al*., 2017). Some species of *Fusarium* are mycotoxin-producers capable of contaminating important agricultural products turning them unsuitable for consumption, both for humans and for animals (Aoki *et al*., 2014; Ramdial *et al*., 2016; Sandoval-Denis *et al*., 2017;).

The systematics and taxonomy of *Fusarium* species was primarily based on phenotypic characters observed from isolates from diverse substrates. Wollenweber & Reinking (1935) published a taxonomic study entitled "Die Fusarien" in which they organized the genus in 16 subgeneric sections, 65 species, 55 varieties and 22 forms using detailed morphological characters. Following these publication, it was modified by Booth (1971) that proposed an intermediate taxonomy with special emphasis in the conidiophores and conidiogenous cells. The Wollenweber & Reinking system was amplified by Gerlach & Nirenberg (1982) to 78 morphologically distinct species, and other systems appeared like the Snyder & Hansen (1940, 1941, 1945) system in which only 9 species of *Fusarium* were recognised by visual inspection of agar cultures in petri dishes. Nelson *et al.* (1983) expanded the Snyder and Hansen system up to 30 species by mixing it with both Booth and Wollenweber & Reinking systems (Aoki *et al.*, 2014; Leslie & Summerell, 2006; Nelson *et al*., 1994).

The genealogic concordance phylogenetic species recognition (GCPSR), implemented by Taylor *et al*. (2000), had a big impact on *Fusarium* systematics. The phylogenetic studies have shown that the previous taxonomic studies based on morphology like Wollenweber & Reinking (1935), Booth (1971) and Nelson *et al.* (1983) are artificial so they were replaced by monophyletic species complexes based on molecular phylogeny (Aoki *et al*., 2014).

Aoki *et al*. (2014) reported four species complexes as the most important phytophatogenic-containing species complexes within the genus *Fusarium*: *Fusarium fujikuroi* species complex (FFSC), *Fusarium graminearum* species complex (FGSC), *Fusarium oxysporum* species complex (FOSC) and *Fusarium/Neocosmospora solani* species complex (FSSC).

In this study, there will be referred two of the species complexes appointed by Aoki *et al*., 2014, *Fusarium fujikuroi* species complex and *Fusarium/Neocosmospora solani* species complex, and there will be referred another species complex, the *Fusarium incarnatum-equiseti* species complex (FIESC).

The sequences of *Fusarium and Neocosmospora* species which were retrieved from the GenBank and used for the phylogenetic analyses are shown in Tables VIII, IX and X. The respective phylogenetic trees can be seen in figures 11, 12 and 13.

Table IX - Details of *Fusarium/Neocosmospora solani species complex* species retrieved form GenBank included in the phylogenetic analysis

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| NRRL 20438 | *Fusarium ambrosium /Neocosmospora abrosium*(FSSC 19) | India | *Xyleborus fornicatus* | DQ094315 | AF178332 | Sandoval-Denis *et al*., 2017 |
| CBS 115.40 | *Fusarium ershadii* (FSSC 9c) | Vietnam | *Musa sapientum* | KX503267 | KX503269 | Papizadeh *et al*., 2018 |
| NRRL 22938 | *Fusarium falciforme/ Neocosmospora falsiformis* (FSSC 3+4) | Indonesia | Human eye | DQ094338 | DQ246855 | O'Donnell *et al*., 2008 |
| CBS 119605 | *Fusarium illudens/ Neocosmospora illudens* (FSSC clade 1) | New Zealand | *Metrosideros* sp. | KM231806 | KM231935 | Lombard *et al*., 2015 |
| NRRL 22090 | *Fusarium illudens/Neocosmospora illudens* (FSSC clade 1) | New Zealand | *Beilschmiedia tawa* | AF178393 | AF178326 | Sandoval-Denis & Crous, 2018; Schroers *et al*., 2016 |
| NRRL 22661/ CBS 490.63 | *Fusarium keratoplasticum/ Neocosmospora keratoplastica* (FSSC 2) *(T)* | Japan | Human eye | DQ094331 | DQ246846 | Sandoval-Denis & Crous, 2018; Sandoval-Denis *et al*., 2017 |
| FRC S-2477 | *Fusarium keratoplasticum/ Neocosmospora Keratoplastica* (FSSC 2) | USA | Sink drain | NR_130690 | JN235712 | Short *et al*., 2013 |
| NRRL 28030 | *Fusarium lichenicola/ Neocosmospora lichenicola* (FSSC 16) | USA | Unknown | DQ094355 | DQ246877 | Sandoval-Denis & Crous, 2018; Schroers *et al*., 2016 |
| NRRL 43467 | *Fusarium neocosmosporiellum/ Neocosmspora vasinfecta* (FSSC 8) | USA | Left eye | EF453092 | EF452940 | Schroers *et al*., 2016 |

Table IX - Details of *Fusarium/Neocosmospora solani species complex* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| NRRL 46440 | *Fusarium pethrophilum/ Neocosmospora pethrophila* (FSSC 1) | Italy | Finger | GU170645 | GU170625 | Migheli *et al.*, 2010; Papizadeh *et al.*, 2018 |
| NRRL 46517 | *Fusarium pseudensiforme/ Neocosmospora pseudoensiformis* (FSSC 33) | Sri Lanka | Recently dead tree | KC691584 | KC691555 | Kasson *et al.*, 2013; Sandoval-Denis & Crous, 2018 |
| NRRL 66304/G.J.S 09-1466/ CBS 140079 | *Fusarium solani/ Neocosmospora solani* (FSSC 5) *(ET)* | Slovenia | Decaying tuber of *Solanum tuberosum* | KT313633 | KT313611 | Sandoval-Denis & Crous, 2018; Sandoval-Denis *et al.*, 2017; Schroers *et al.*, 2016 |
| NRRL 22400 | *Fusarium solani f. sp. batatas* (FSSC 23) | Unknown | Unknown | AF178407 | AF178343 | Sandoval-Denis & Crous, 2018; Schroers *et al.*, 2016 |
| NRRL 22153 | *Fusarium solani f. sp. Cucurbitae* (FSSC 10) | Unknown | Unknown | DQ094302 | AF178346 | Schroers *et al.*, 2016 |
| NRRL 22157 | *Fusarium solani f. sp. mori* (FSSC 17) | Japan | Mulberry tree branch | DQ094306 | AF178359 | Schroers *et al.*, 2016 |
| NRRL 22278 | *Fusarium solani f. sp. pisi* (FSSC 11) | Unknown | *Pisum sativum* | DQ094309 | AF178337 | Sandoval-Denis & Crous, 2018; Schroers *et al.*, 2016 |
| NRRL 22161 | *Fusarium solani f. sp. robiniae* (FSSC 13) | Unknown | Unknown | DQ094311 | AF178330 | Schroers *et al.*, 2016 |
| NRRL 22163 | *Fusarium solani f. sp. Xanthoxyli* (FSSC 22) | Japan | Branch of *Xanthoxylum piperitum* | AF178394 | AF178328 | Schroers *et al.*, 2016 |

Table IX - Details of *Fusarium/Neocosmospora solani species complex* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| NRRL 22101 | *Fusarium striatum* (FSSC 21) | Unknown | Unknown | AF178398 | AF178333 | Papizadeh *et al.*, 2018; Sandoval-Denis & Crous, 2018 |
| NRRL 36604 | *Fusarium virguliforme* | Unknown | Unknown | EF408543 | EF408438 | ARS collection |
| NRRL 22782 | *Fusarium metagrovorans/ Neocosmospora metavorans* FSSC 6 | Spain | Human eye | EU329670 | DQ246850 | Sandoval-Denis & Crous, 2018; Sandoval-Denis *et al.*, 2017 |
| NRRL 32770 | *Neocosmospora gamsii* FSSC 7 | USA | Human eye | DQ094544 | DQ247083 | Sandoval-Denis *et al.*, 2017 |
| NRRL 32755 | *Neocosmospora tonkinensis* FSSC 9a | USA | Turtle | DQ094534 | DQ247073 | Papizadeh *et al.*, 2018 |
| NRRL 46615 | FSSC 9b | Italy | Soil | GU250666 | GU250543 | Papizadeh *et al.*, 2018 |
| CBS 222.49 | FSSC 9e | Netherlands | *Euphorbia fulgens* | JX435209 | JX435159 | Papizadeh *et al.*, 2018 |
| NRRL 22642/ATCC 38341 | FSSC 12 | Japan | Gill of *Penaeus japonicus* | DQ094329 | DQ246844 | Sandoval-Denis *et al.*, 2017 |
| NRRL 32705 | FSSC 14 | USA | Human | DQ094488 | DQ247025 | Sandoval-Denis *et al.*, 2017 |
| NRRL 28009 | *Fusarium ensiforme* (FSSC 15) | USA | Human eye | DQ094351 | DQ246869 | Sandoval-Denis *et al.*, 2017 |
| NRRL 32792 | *Fusarium ensiforme* (FSSC 15) | Japan | Human | DQ094561 | DQ247101 | Sandoval-Denis *et al.*, 2017 |
| NRRL 31158 | FSSC 18 | USA | Human wound | DQ094389 | DQ246916 | Sandoval-Denis *et al.*, 2017 |
| NRRL 28001 | FSSC 20 | USA | Human | DQ094348 | DQ246866 | Sandoval-Denis *et al.*, 2017 |
| NRRL 22389 | FSSC 24 | USA | *Liriodendron tulipifera* | AF178404 | AF178340 | Sandoval-Denis *et al.*, 2017 |
| NRRL 31169 | FSSC 25 | USA | Human oral wound | DQ094396 | KR673963 | Sandoval-Denis *et al.*, 2017 |

Table IX - Details of *Fusarium/Neocosmospora solani species complex* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| NRRL 28541 | FSSC 26 | USA | Synuvial fluid | EU329674 | DQ246882 | Papizadeh *et al.*, 2018; Sandoval-Denis *et al.*, 2017; Zhang *et al*., 2006 |
| NRRL 37625 | FSSC 27 | Netherlands | Human | EU329684 | FJ240353 | O'Donnell *et al.*, 2008; Papizadeh *et al.*, 2018; Sandoval-Denis *et al.*, 2017 |
| NRRL 32437/ CBS 109028 | FSSC 28 | Switzerland | Human subcutaneous nodule | DQ094446 | DQ246979 | Papizadeh *et al.*, 2018; Sandoval-Denis *et al.*, 2017; Zhang *et al*., 2006 |
| NRRL 28008 | FSSC 29 | USA | Unknown | DQ094350 | DQ246868 | Papizadeh *et al.*, 2018; Sandoval-Denis *et al.*, 2017; Zhang *et al*., 2006 |
| NRRL 22579 | FSSC 30 | Indonesia | Bark | AF178415 | AF178352 | O'Donnell *et al.*, 2008; Papizadeh *et al.*, 2018; Sandoval-Denis *et al.*, 2017 |
| NRRL 22570 | *Fusarium solani f.sp. piperis* (FSSC 31) | Brazil | *Piper nigrum* | AF178422 | AF178360 | Sandoval-Denis et al., 2018 |
| NRRL 22178 | FSSC 32 | Venezuela | Dicot tree | AF178399 | AF178334 | Sandoval-Denis et al., 2018 |
| NRRL 22354 | FSSC 33 | French Guiana | Bark | AF178402 | AF178338 | Sandoval-Denis et al., 2018 |
| NRRL 46703 | FSSC 34 | Spain | Nematode | EU329712 | HM347126 | Sandoval-Denis et al., 2018 |
| NRRL 46707 | FSSC 35 | Brazil | Human eye | EU329716 | HM347127 | Sandoval-Denis et al., 2018 |
| NRRL 54723/CBS 135855 | *Fusarium euwallacea* (FSSC 36) | Israel | Beetle from avocado tree | JQ038015 | JQ038008 | Sandoval-Denis et al., 2018 |
| NRRL 25137 | FSSC 37 | Papua New Guinea | Diseased cocoa pods | JF740899 | JF740757 | Sandoval-Denis et al., 2018 |

Table IX - Details of *Fusarium/Neocosmospora solani species complex* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| NRRL 52781 | FSSC 38 | Benin | *Hypothenemus hampei* | N/A | JF740849 | Sandoval-Denis *et al.*, 2017 |
| FRC S-2432 | FSSC 39 | USA | University building | JN235326 | JN235756 | Sandoval-Denis *et al.*, 2017 |
| NRRL 54992 | FSSC 43 | USA | Zebra Shark | KC808255 | KC808213 | Sandoval-Denis *et al.*, 2017 |
| NRRL 62797 | FSSC 45 | USA | Unknown | KF906130 | KF906129 | Sandoval-Denis *et al.*, 2017 |
| CBS 142423/CPC 27186 | *Neocosmospora croci* (T) | Italy | *Citrus sinensis* | LT746264 | LT746216 | Sandoval-Denis *et al.*, 2017 |
| CML 1830 | *Fusarium paranaense* (T) | Brazil | Soybean root | N/A | KF597797 | Sandoval-Denis *et al.*, 2017 |
| CBS 142424/CPC 28191 | *Neocosmospora macrospora* (T) | Italy | *Citrus sinensis* | LT746266 | LT746218 | Sandoval-Denis & Crous, 2018; Sandoval-Denis *et al.*, 2017 |
| NRRL 22743 | *Fusarium brasiliense* (FSSC clade 2) | Brazil | *Clycine max* | EF408512 | EF408407 | Sandoval-Denis *et al.*, 2017 |
| NRRL 31104 | *Fusarium cuneirostrum* (FSSC clade 2) | Japan | *Phaseolus vulgaris* | EF408518 | EF408413 | Sandoval-Denis *et al.*, 2017 |
| NRRL 22632 | *Fusarium plagianthi/ Neocosmospora plagianthi* (FSSC clade 1) | New Zealand | *Hoheria glabrata* | AF178417 | AF178354 | Sandoval-Denis *et al.*, 2017 |
| NRRL 22316 | *Fusarium staphyleae/ Geejayeesia atrofusca* (Outgroup) | USA | *Staphylea trifolia* | AF178423 | AF178361 | O'Donnell, 2000; Sandoval-Denis & Crous, 2018; Schroers *et al.*, 2016 |
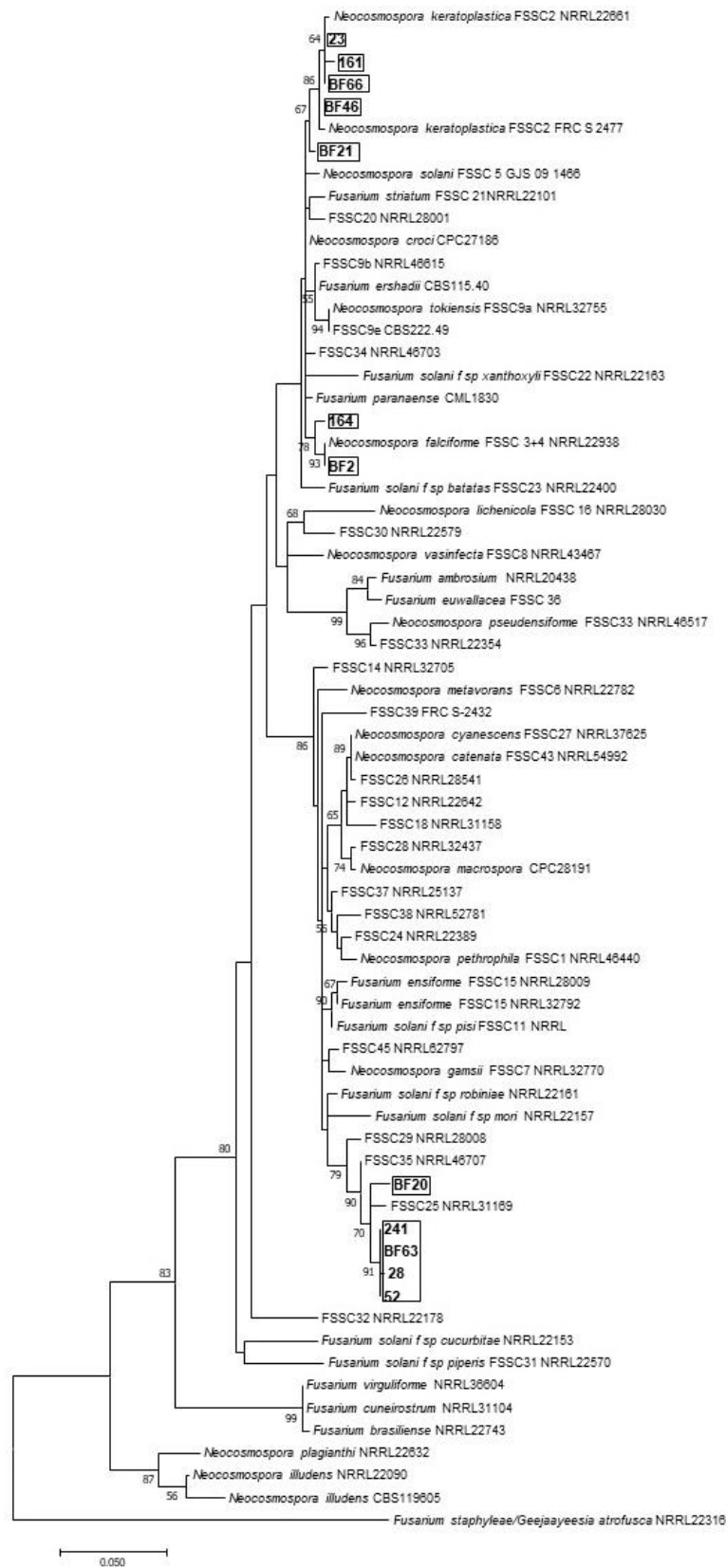
Figure 11- Phylogenetic tree for *Fusarium/Neocosmospora solani species complex*, based on Maximum Likelihood (ML) analysis of *tef1*-α sequences alignment. The applied evolutionary model was the Kimura-2-parameter model with Gamma distribution. The bootstrap values resulting of 1000 replicates are presented (%). The samples used in the study are represented in bold with a black box.

The *Fusarium/Neocosmospora solani* species complex contains important plant pathogenic species, causing fruit rot, root rot and seedling damping-off on diverse plant hosts (Sandoval-Denis & Crous, 2018). *Fusarium/Neocosmospora solani* species complex is also an important human pathogen associated with opportunistic infections and mycotic keratitis (Al-Hatmi *et al*., 2016; Sandoval-Denis & Crous, 2018; Short *et al*., 2013; Short *et al*., 2011).

In this study, both *Fusarium and Neocosmospora* names are used for the species complex since both names are correct for the genera. Since the most cited name in the recent literature (Sandoval-Denis & Crous, 2018; Sandoval-Denis *et al*., 2017) is *Neocosmospora*, this was the one used for the genus.

For the alignment of the *Fusarium/Neocosmospora solani* species complex *tef1*-α amplicons with approximately 508 – 523 bp were used.

As can be seen in Figure 11, the isolates are dispersed by 3 groups: first group related with *Neocosmospora keratoplastica* (FSSC 2), the second one related with *Neocosmospora falsiforme* (FSSC 3+4) and the third one related with FSSC 25.

The first group is composed by isolates BF46, BF66, 23 and 161 in a well-supported group with bootstrap support of 86%, grouped with *N. keratoplastica*. Isolate BF 21 is related with the *N. keratoplastica* but it is in a different branch with a bootstrap support of 67% (Figure 11).

The second group is composed by isolates BF2 and 164. Isolate BF2 is placed in a well-supported group with bootstrap support of 93% with *N. falsiforme*. Isolate 164 is separated from the group with a well-supported branch, with a bootstrap support of 78%, meaning that it is related with the *N. falsiforme* but maybe a new species (Figure 11).

The last group is composed by isolates BF20, BF63, 28, 52, and 241 (Figure 11). Isolate BF20 groups with FSSC 25, with a bootstrap support of 70%, but in a different branch. Isolates BF 63, 28, 52 and 241 form a well-supported different group, related with FSSC 25.

For all the groups formed, the possibility of some species being novelties for the *N. solani* species complex is high for isolates BF21, 164, BF20 and the group formed by isolates BF63, 28, 52 and 241. For the confirmation of  phylogenetic relationship of these species with the related known *Neocosmospora* species, more genetic markers should to be used, like the *rpb2* and *rpb1* genes (Al-Hatmi *et al*., 2018; O'Donnell *et al*., 2010, 2013, 2015; Short *et al*., 2013) and more sequences of each species should be used for the construction of the phylogenetic tree, if available.

Several studies show *N. solani* species associated with different plant diseases, like *N. solani* FSSC 5 is associated with potato tuber rot (Schroers *et al*., 2016b) and with dry root rot of citrus (Sandoval-Denis *et al*., 2017). Several species also cause soybean sudden death syndrome

(Aoki *et al*., 2014; Aoki *et al*., 2003) and *Neocosmospora perseae* is the causing agent of trunk cankers on avocado (Guarnaccia *et al*., 2018), among others.

Up to date, to the best of our knowledge there is no study relating the *N. solani* species complex with BFD of the grapevine.

Table X - Details of *Fusarium incarnatum-equiseti species complex* species retrieved form GenBank included in the phylogenetic analysis

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| NRRL 26419/ CBS 307.94 | *Fusarium equiseti* FIESC 14a | Braunschweig | Soil | NR_121457 | GQ505599 | O'Donnell *et al.*, 2009 |
| NRRL 20423 | *Fusarium lacertarum* FIESC 4a | India | Lizard skin | GQ505682 | GQ505593 | O'Donnell *et al.*, 2009 |
| NRRL 13402 | *Fusarium sciripi* FIESC 9b | Australia | Pine nursery soil | GQ505681 | GQ505592 | O'Donnell *et al.*, 2009 |
| NRRL 43640 | FIESC 1a | USA | Dog nose | GQ505756 | GQ505667 | O'Donnell *et al.*, 2009 |
| NRRL 34039 | FIESC 1b | USA | Human | GQ505728 | GQ505639 | O'Donnell *et al.*, 2009 |
| NRRL 34034 | FIESC 1c | USA | Human leg | GQ505725 | GQ505636 | O'Donnell *et al.*, 2009 |
| NRRL 36401/ CBS 264.50 | FIESC 2a | Mozambique | Cotton | GQ505740 | GQ505651 | O'Donnell *et al.*, 2009 |
| NRRL 36448/ CBS 384.92 | FIESC 2b | Sudan | *Phaseolus vulgaris* seed | GQ505741 | GQ505652 | O'Donnell *et al.*, 2009 |
| NRRL 36323/ CBS 186.31 | FIESC 3a | England | Cotton yarn | GQ505737 | GQ505648 | O'Donnell *et al.*, 2009 |
| NRRL 28029 | FIESC 3b | USA | Human eye | GQ505691 | GQ505602 | O'Donnell *et al.*, 2009 |
| NRRL 36123/ CBS 102300 | *Fusarium laceratum* FIESC 4b | Unknown | Unknown | GQ505732 | GQ505643 | O'Donnell *et al.*, 2009 |
| NRRL 32871 | FIESC 5a | USA | Human abcess | GQ505708 | GQ505619 | O'Donnell *et al.*, 2009 |
| NRRL 45995 | FIESC 5b | USA | Human abcess | GQ505759 | GQ505670 | O'Donnell *et al.*, 2009 |
| NRRL 25795/ CBS 394.93 | FIESC 5c | Germany | *Disphyma crassifolium* seed | GQ505686 | GQ505597 | O'Donnell *et al.*, 2009 |
| NRRL 34035 | FIESC 5d | USA | Human sinus | GQ505726 | GQ505637 | O'Donnell *et al.*, 2009 |
| NRRL 43623 | FIESC 5e | USA | Human maxillary sinus | GQ505750 | GQ505661 | O'Donnell *et al.*, 2009 |
| NRRL 45997 | FIESC 5f | USA | Human sinus | GQ505761 | GQ505672 | O'Donnell *et al.*, 2009 |
| NRRL 43694 | FIESC 6a | USA | Human eye | GQ505757 | GQ505668 | O'Donnell *et al.*, 2009 |

Table X - Details of *Fusarium incarnatum-equiseti species complex* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| NRRL 45998 | FIESC 6b | USA | Human toe | GQ505762 | GQ505673 | O'Donnell *et al.*, 2009 |
| NRRL 32997 | FIESC 7a | USA | Human toenail | GQ505713 | GQ505624 | O'Donnell *et al.*, 2009 |
| NRRL 5537/ ATCC 28805 | FIESC 8a | USA | Fescue hay | GQ505677 | GQ505588 | O'Donnell *et al.*, 2009 |
| NRRL 43498 | FIESC 8b | USA | Human eye | GQ505747 | GQ505658 | O'Donnell *et al.*, 2009 |
| NRRL 36478/ CBS 447.84 | *Fusarium scirpi* FIESC 9a | Australia | Pasture soil | GQ505743 | GQ505654 | O'Donnell *et al.*, 2009 |
| NRRL 26922/ CBS 610.95 | *Fusarium scirpi* FIESC 9c | France | soil | GQ505690 | GQ505601 | O'Donnell *et al.*, 2009 |
| NRRL 3020 | FIESC 10a | Unknown | Unknown | GQ505675 | GQ505586 | O'Donnell *et al.*, 2009 |
| NRRL 36372/ CBS 235.79 | FIESC 11a | Netherlands Antilles | Air | GQ505738 | GQ505649 | O'Donnell *et al.*, 2009 |
| NRRL 31011 | FIESC 12a | Germany | *Thuja* sp. | GQ505695 | GQ505606 | O'Donnell *et al.*, 2009 |
| NRRL 36269/ CBS 162.57 | FIESC 12b | Croatia | *Pinus nigra* seedling | GQ505734 | GQ505645 | O'Donnell *et al.*, 2009 |
| NRRL 36392/ CBS 259.54 | FIESC 12c | Germany | Seedling | GQ505739 | GQ505650 | O'Donnell *et al.*, 2009 |
| NRRL 43635 | FIESC 13a | USA | Horse | GQ505751 | GQ505662 | O'Donnell *et al.*, 2009 |
| NRRL 20697/ CBS 245.61 | *Fusarium equiseti* FIESC 14b | Chile | Beet | GQ505683 | GQ505594 | O'Donnell *et al.*, 2009 |
| NRRL 43636 | *Fusarium equiseti* FIESC 14c | USA | Dog | GQ505752 | GQ505663 | O'Donnell *et al.*, 2009 |
| NRRL 34006 | FIESC 15a | USA | Human eye | GQ505719 | GQ505630 | O'Donnell *et al.*, 2009 |
| NRRL 32182 | FIESC 15b | USA | Human blood | GQ505700 | GQ505611 | O'Donnell *et al.*, 2009 |
| NRRL 32994 | FIESC 15c | USA | Human ethmoid sinus | GQ505710 | GQ505621 | O'Donnell *et al.*, 2009 |
| NRRL 34008 | FIESC 15d | USA | Human lung | GQ505721 | GQ505632 | O'Donnell *et al.*, 2009 |

Table X - Details of *Fusarium incarnatum-equiseti species complex* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| NRRL 34001 | FIESC 15e | USA | Human foot wound | GQ505714 | GQ505625 | O'Donnell *et al.*, 2009 |
| NRRL 34004 | FIESC 16a | USA | Human BAL | GQ505717 | GQ505628 | O'Donnell *et al.*, 2009 |
| NRRL 34056 | FIESC 16b | USA | Human bronchial wash | GQ505729 | GQ505640 | O'Donnell *et al.*, 2009 |
| NRRL 43730 | FIESC 16c | USA | Contact lens | EF453193 | GQ505669 | O'Donnell *et al.*, 2009 |
| NRRL 32864 | FIESC 17a | USA | Human | GQ505702 | GQ505613 | O'Donnell *et al.*, 2009 |
| NRRL 36548/ CBS 190.60 | FIESC 17b | Congo | Banana | GQ505744 | GQ505655 | O'Donnell *et al.*, 2009 |
| NRRL 34070 | FIESC 17c | USA | Tortoise | GQ505731 | GQ505642 | O'Donnell *et al.*, 2009 |
| NRRL 31167 | FIESC 18a | USA | Human sputum | GQ505697 | GQ505608 | O'Donnell *et al.*, 2009 |
| NRRL 32522 | FIESC 18b | USA | Human diabetic cellulitis | GQ505701 | GQ505612 | O'Donnell *et al.*, 2009 |
| NRRL 43639 | FIESC 19a | USA | Manatee | GQ505755 | GQ505666 | O'Donnell *et al.*, 2009 |
| NRRL 34003 | FIESC 20a | USA | Human sputum | GQ505716 | GQ505627 | O'Donnell *et al.*, 2009 |
| NRRL 36575/ CBS 976.97 | FIESC 20b | Hawaii | *Juniperus chinensis* leaf | GQ505745 | GQ505656 | O'Donnell *et al.*, 2009 |
| NRRL 13335 | FIESC 21a | Australia | Alfalfa | GQ505679 | GQ505590 | O'Donnell *et al.*, 2009 |
| NRRL 32865 | FIESC 21b | Brazil | Human endocarditis | GQ505703 | GQ505614 | O'Donnell *et al.*, 2009 |
| NRRL 34002 | FIESC 22a | USA | Human ethmoid sinus | GQ505715 | GQ505626 | O'Donnell *et al.*, 2009 |
| NRRL 32866 | FIESC 23a | USA | Human cancer patient | GQ505704 | GQ505615 | O'Donnell *et al.*, 2009 |
| NRRL 13379 | FIESC 23b | India | *Oryza sativa* | GQ505680 | GQ505591 | O'Donnell *et al.*, 2009 |

Table X - Details of *Fusarium incarnatum-equiseti species complex* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| NRRL 34005 | FIESC 24a | USA | Human intravitreal fluid | GQ505718 | GQ505629 | O'Donnell *et al.*, 2009 |
| NRRL 43297 | FIESC 24b | USA | *Spartina rhizomes* | GQ505746 | GQ505657 | O'Donnell *et al.*, 2009 |
| NRRL 22244 | FIESC 25a | China | Rice | GQ505685 | GQ505596 | O'Donnell *et al.*, 2009 |
| NRRL 32993 | FIESC 25b | USA | Human nasal tissue | GQ505709 | GQ505620 | O'Donnell *et al.*, 2009 |
| NRRL 32868 | FIESC 25c | USA | Human blood | GQ505706 | GQ505617 | O'Donnell *et al.*, 2009 |
| NRRL 26417/ CBS 544.96 | FIESC 26a | Cuba | Leaf litter | GQ505687 | GQ505598 | O'Donnell *et al.*, 2009 |
| NRRL 28714/ ATCC 74289 | FIESC 26b | Costa Rica | *Acacia* sp. branch | GQ505693 | GQ505604 | O'Donnell *et al.*, 2009 |
| NRRL 20722 | FIESC 27a | Kenya | *Chrysanthemum* sp. | GQ505684 | GQ505595 | O'Donnell *et al.*, 2009 |
| NRRL 28577/ CBS 430.81 | FIESC 28a | Romania | Grave stone | GQ505692 | GQ505603 | O'Donnell *et al.*, 2009 |
| NRRL 13459/ CBS 691.87 | *Fusarium concolor* (Outgroup) | South Africa | Plant debris | GQ505763 | GQ505674 | O'Donnell *et al.*, 2009 |
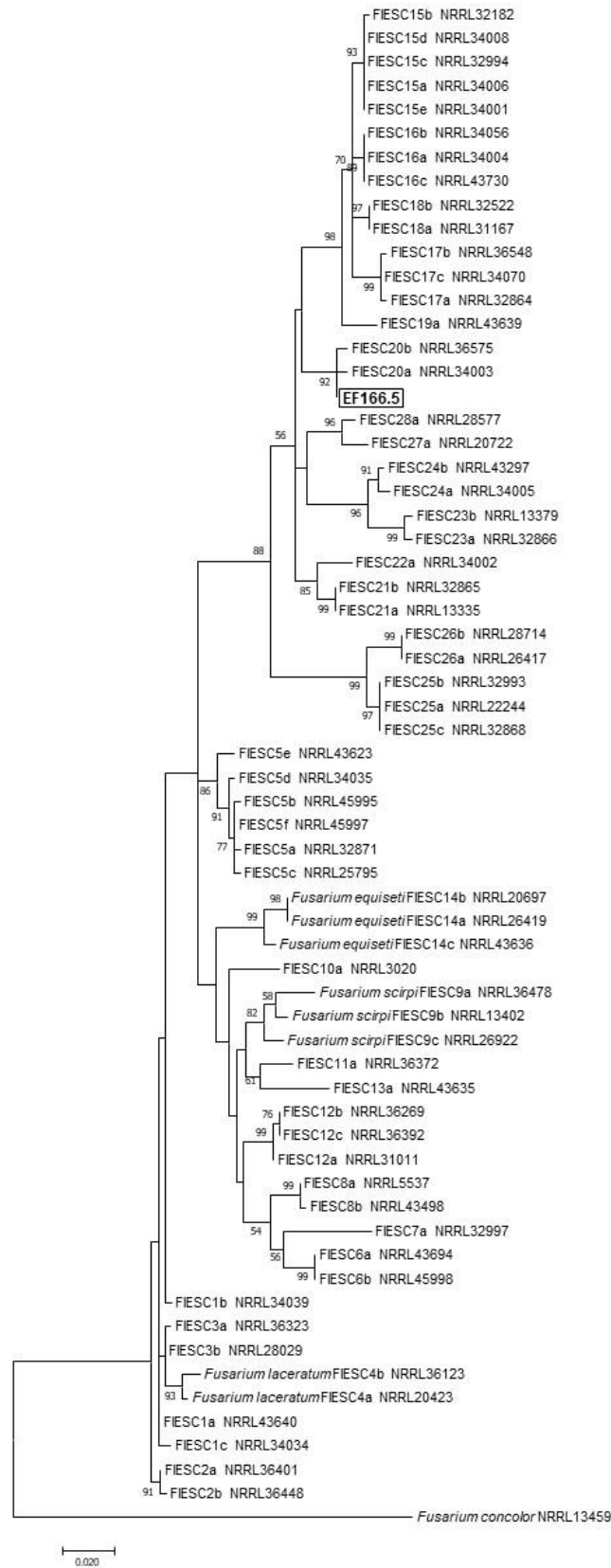
Figure 12- Phylogenetic tree for *Fusarium incarnatum-equiseti species complex*, based on Maximum Likelihood (ML) analysis of *tef1*-α sequences alignment. The applied evolutionary model was the Kimura-2-parametre model with Gamma distribution. The bootstrap values are presented (%) resulting of 1000 replicates. The samples used in the study are represented in bold with a black box.

The *Fusarium incarnatum-equiseti* species complex (FIESC) is known by their ability to produce secondary metabolites, like mycotoxins and other metabolites (Desjardins, 2006; Villani *et al*., 2016). Their species are also identified as co-occurring with plant and human infections (Jacobs *et al*., 2018; Villani *et al*., 2016). This species complex has a high level of cryptic speciation and phylogenetic analyses have shown that it comprises 30 phylogenetically distinct species (Castellá & Cabañes, 2014; Jacobs *et al*., 2018; O'Donnell *et al*., 2009, 2012; Villani *et al*., 2016).

*Fusarium equiseti* species complex is composed by species that are cosmopolitan soil inhabitants that have been isolated from roots and plant tissues from many parts of the world and *Fusarium incarnatum* species complex is composed by species that are widespread and common in tropics and subtropics but are also found in Mediterranean and occasionally in temperate regions (Castellá & Cabañes, 2014; Leslie & Summerell, 2006; Villani *et al*., 2016).

For the alignment of the *Fusarium incarnatum-equiseti* species complex were used *tef1-*α amplicons with 495 bp.

As can be seen in Figure 12, there is only one isolate, 166.5, that grouped in a well-supported group with bootstrap value of 92% with members of the FIESC 20, belonging to the *F. incarnatum* Clade.

Ramdial *et al*. (2016) and Jacobs *et al*. (2018) show that *tef1-*α enables a well-supported phylogenetic inference with highly resolved branching patterns of the phylogenetic species within the FIESC. Besides that information, for the confirmation of phylogenetic relationship of these species with the related known *Fusarium incarnatum-equiserti* species, more genetic markers, like the *rpb2* and *rpb1* genes (Al-Hatmi *et al*., 2018; O'Donnell *et al*., 2010, 2013, 2015; Ramdial *et al*., 2016; Short *et al*., 2013), as well as more sequences of each species should be use for the construction of the phylogenetic tree, when available.

Some studies like Ramdial *et al*. (2016) show the relationship between *Fusarium incarnatum-equiseti* and plants diseases. The authors reported for the first time the association of FIESC to bell pepper fruit disease. Villani *et al*. (2016) used a polyphasic approach to characterise FIESC species associated with several cereals like maize, oat, and wheat.

Castellá & Cabañes (2014) studied for the first time FIESC in Spanish wheat with no symptoms of disease. Botha *et al*. (2014) isolated FIESC species from toxic Kikuyu grass pastures, often associated with cattle poisoning, in South Africa without the assessment of their role in the disease and Jacobs *et al*. (2018) studied FIESC isolates associated with undisturbed soil in the grassland biome of South Africa.

Until now, there is no study with FIESC associated with grapevine disease or associated with grapevine but since Jacobs *et al.* (2018) found FIESC isolates associated with soil, they may be soil inhabitants or they can have other role in the plant.

Table XI - Details of *Fusarium fujikuroi species complex* species retrieved form GenBank included in the phylogenetic analysis

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| NRRL 54463 | *Fusarium agapanthi* (T) | Australia | *Agapanthus* sp. | N/A | KU900630 | Sandoval-Denis *et al.*, 2017 |
| NRRL 22945/ CBS 184.29 | *Fusarium ananatum* | England | *Ananas comosus* | U34562 | KR071762 | Sandoval-Denis *et al.*, 2017 |
| NRRL 53131 | *Fusarium ananatum* | Italy | Human | N/A | HM347128 | Sandoval-Denis *et al.*, 2017 |
| NRRL 31727/ CBS 119857 | *Fusarium andiyazi (T)* | South Africa | *Sorgum bicolor* soil debris | KR071651 | KR071718 | Sandoval-Denis *et al.*, 2017 |
| NRRL 13602/ CBS 737.97 | *Fusarium anthophilum* | Germany | *Hippeastrum* sp. | N/A | AF160292 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 25214 | *Fusarium anthophilum* | Germany | Hippeastrum sp. | N/A | KF466414 | Sandoval-Denis *et al.*, 2017 |
| NRRL 25300/ CBS 403.97 | *Fusarium begoniae (T)* | Germany | *Begonia elatior* hybrid plant | N/A | AF160293 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 13618/ CBS 220.76 | *Fusarium bulbicola (T)* | Germany | *Nerine bowdenii* | U61676 | AF160294 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 25331/ CBS 405.97 | *Fusarium circinatum* (T) | USA | Monterrey pine tree | NR_120263 | AF160295 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 25181/ CBS 450.97 | *Fusarium concentricum (T)* | Costa Rica | *Musa sapientum* | NR_111886 | AF160282 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 25302/ CBS 735.97 | *Fusarium denticulatum* | USA | *Ipomoea batatas* | N/A | AF160269 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 28852 | *Fusarium fractiflexum* (T) | Japan | *Cymbidium* sp. | AF158304 | AF160288 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 13566/ ATCC 38941 | *Fusarium fujikuroi* | China | *Oryza sativa* | AF158304 | AF160279 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 26131/ CBS 428.97 | *Fusarium globosum* (T) | South Africa | Corn seed | N/A | AF160285 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |

Table XI - Details of *Fusarium fujikuroi species complex* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| NRRL 20433/ CBS 716.74 | *Fusarium inflexum (outgroup) (T)* | Germany | *Vicia faba* | U34577 | AF008479 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 25200/ CBS 411.97 | *Fusarium lactis (NT)* | USA | *Ficus carica* | NR_111887 | AF160272 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 25226/ BBA 69662 | *Fusarium mangiferae (T)* | India | *Mangifera indica* | U61691 | AF160281 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 13604/ CBS 748.97 | *Fusarium napiforme (T)* | Namibia | *Pennisetum typhoides* | U34570 | AF160266 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 13448/ CBS 749.97 | *Fusarium nygamai (T)* | Australia | necrotic sorghum root | NR_130698 | AF160273 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 22902/ IMI 375335 | *Fusarium oxysporum (outgroup)* | USA | Douglas fir seedling root | U34566 | AF160312 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 13617/ CBS 216.76 | *Fusarium phyllophilum (T)* | Italy | *Dracaena deremensis* | U34574 | AF160274 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 22944/ CBS 217.76 | *Fusarium proliferatum* | Germany | *Cymbidium* sp. | U34574 | AF160280 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 22496/ CBS 126.73 | *Fusarium pseudocircinatum (T)* | Ghana | *Solanum* sp. | U34569 | AF160271 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 13592/ CBS 417.97 | *Fusarium pseudonygamai (T)* | Nigeria | *Pennisetum typhoides* | NR_137162 | AF160263 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 25208/ CBS 418.97 | *Fusarium ramigenum (T)* | USA | *Ficus carica* | NR_111888 | AF160267 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| NRRL 13999/ CBS 223.76 | *Fusarium sacchari* | India | *Saccharum officinarum* | U34556 | AF160278 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| CBS 142422/ CPC 27188 | *Fusarium siculi (T)* | Italy | Dry root rot *Citrus sinensis* | LT46262 | LT746214 | Sandoval-Denis *et al.*, 2017 |

Table XI - Details of *Fusarium fujikuroi species complex* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | Articles |
|---|---|---|---|---|---|---|
| **NRRL 26756** | *Fusarium sp.* | South Africa | ornamental grass | AF158310 | AF160307 | Sandoval-Denis *et al.*, 2017 |
| **NRRL 25623** | *Fusarium sterilihyphosum (T)* | South Africa | mango | F158305 | AF160300 | Sandoval-Denis *et al.*, 2017 |
| **NRRL 22016/ CBS 747.97** | *Fusarium subglutinans (T)* | USA | Corn | U34559 | AF160289 | Sandoval-Denis *et al.*, 2017 |
| **NRRL 13613/ CBS 219.76** | *Fusarium succisae* | Germany | *Succisa pratensis* | U34561 | AF160291 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| **NRRL 22045/ CBS 733.97** | *Fusarium thapsinum* | South Africa | *Sorgum bicolor* | U34560 | AF160270 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| **NRRL 53984** | *Fusarium tupiense (T)* | Brazil | *Mangifera indica* | N/A | DQ452859 | Sandoval-Denis *et al.*, 2017 |
| **NRRL 22949/ CBS 178.32** | *Fusarium udum* | Germany | Unknown | U34575 | AF160275 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
| **NRRL 22172/ CBS 734.97** | *Fusarium verticilioides* | Germany | *Corn* | U34555 | AF160262 | Sandoval-Denis *et al.*, 2017; Scauflaire *et al.*,2011 |
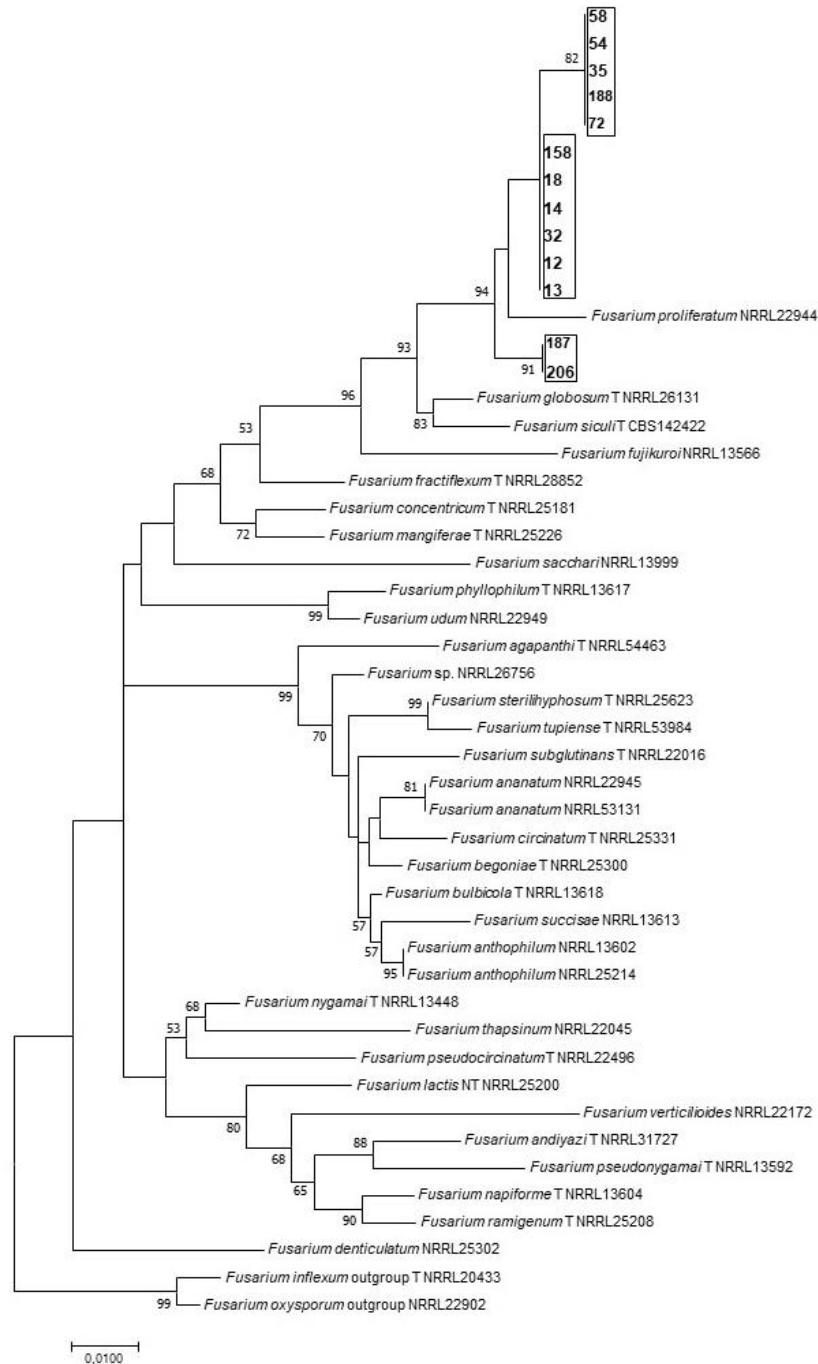
Figure 13 - Phylogenetic tree for *Fusarium fujikuroi* species complex, based on Maximum Likelihood (ML) analysis of *tef1*-α sequences alignment. The applied evolutionary model was the Kimura-2-parametre model with Gamma distribution. The bootstrap values resulting of 1000 replicates are presented (%). The samples used in the study are represented in bold with a black box.

The *Fusarium fujikuroi species complex* (FFSC) is a group of fungi that occurs on a wide range of plants and has different host specificity. It is composed of at least 50 distinct species and groups in 3 different clades: The American, African and Asian Clades (Al-Hatmi *et al*., 2015; Kvas *et al*., 2009; Niehaus *et al*., 2016; O'Donnell *et al*., 1998).

Some species within FFSC are known mycotoxin producers, like *Fusarium verticillioides* and *Fusarium proliferatum* (Desjardins, 2006; Leslie & Summerell, 2006; Niehaus *et al*., 2016).

For the alignment of the *Fusarium fujikuroi* species complex were used *tef1*-α amplicons with approximately 463 – 672 bp.

The isolates formed a well-supported group with bootstrap 94% related with *Fusarium proliferatum*. As we can see, the isolates form 3 subgroups, with two of them well-supported with a high bootstrap value: one group with 91% constituted by isolates 187 and 206, the second one related with *F. proliferatum* but without a good bootstrap support (<70%) with the isolates 12, 13, 14, 18, 32 and 158, and the third one, related with the second group but different, well-supported with 82% bootstrap, with isolates 35, 54, 58, 72 and 188 (Figure 13).

For a better understanding of the phylogenetic relationship between the isolates and *F. proliferatum*, more genetic markers should be used like the *rpb2* and *rpb1* genes (Al-Hatmi *et al*., 2018; O'Donnell *et al*., 2010, 2013, 2015; Ramdial *et al*., 2016; Short *et al*., 2013b ). Gálvez *et al*. (2017) used *tef1*-α and fumonisin gene *fum1* for phylogenetic analyses of *F. proliferatum* causing garlic bulb rot in Spain. Since these authors showed that *F. proliferatum* presents a high intraspecific variability, more sequences of *F. proliferatum* should be use for the construction of the phylogenetic tree and for the assessment of the relationship between the available sequences and our isolates.

Various studies have shown the *F. proliferatum* as a causal agent of diverse plant diseases (Gálvez *et al*., 2017; Proctor *et al*., 2010), including wheat – based products (Cendoya *et al*., 2018). The diseases caused by *F. proliferatum* includes blights, dieback, wilt and roots, bulbs, crowns, stems, shoots, seeds, and fruit rots (Desjardins, 2006).

Wang *et al*. (2015) reported for the first time *F. proliferatum* as causing agent of fruit rot on grapes (*Vitis vinifera*) in China. After 10 days of inoculation of the fungus in the fruit, they presented symptoms of disease. Besides this study, there was not any reference to *Fusarium proliferatum* on grapevine especially causing BFD.

To establish a relation between the *Fusarium* isolates from all species complexes retrieved from the root of diseased grapevine and the symptoms of the disease, pathogenicity tests should be performed to see if it fulfils Koch's postulates.

### 3.2.3. Genus *Clonostachys*

The genus *Clonostachys* (Hypocreales, Bionectriaceae), described by Corda in 1839, is constituted by fungi species common in soils, endo- and epiphytes, saprobes, mycoparasites and nematodes and insects parasites (Moreira *et al*., 2016; Schroers, 2001; Toledo *et al*., 2006). They are found in forests, cultivated soils, estuarine sediments or desertic soils as well as in temperate and tropical regions (Domsch *et al*., 2007; Gláucia Mara Moreira, 2012; Rossman, 1996).

*Clonostachys* species are characterized by the existence of dimorphic conidiophores: the primary conidiophores verticillium-like and secondary conidiophores penicillium-like (Schroers, 2001; Schroers *et al*., 1999).

There are approximately 50 species of *Clonostachys*, being the majority of the species described by Schroers (2001) using genetic markers and morphologic characters. Moreira *et al*. (2016) described *Clonostachys chloroleuca* using genetic markers and MALDI-TOF, and 5 species have been described by Chinese and Japanese investigators (Dao *et al*., 2016; Hirooka & Kobayashi, 2007; Luo & Zhuang, 2010; Wan-Hao *et al*., 2016). In Index Fungorum (http://www.indexfungorum.org/names/names.asp) there are more than 80 entries for the genus *Clonostachys*.

One of the most known species of the genus is *Clonostachys rosea*, because of the biocontrol proprieties presented (Alvindiaa & Hirookab, 2011; Moreira *et al*., 2016; Gláucia Mara Moreira, 2012). *Clonostachys rosea* is a known biocontrol agent against *Botrytis cinerea* and other plant pathogenic fungi (Cota *et al*., 2008; Gan *et al*., 2007; Moreira, 2012; Silvera-Pérez *et al*., 2010). Moreira (2012) showed that other species of *Clonostachys* have biocontrol potentialities against the *B. cinerea*, being possible that they can also act as biocontrol agents against other plant pathogenic fungi.

The sequences of *Clonostachys* species, which were retrieved from GenBank, were used for the phylogenetic analysis, and are shown in Table XI, and the respective phylogenetic trees can be seen in figures 14 e 15.

Table XII - Details of *Clonostachys* species retrieved form GenBank included in the phylogenetic analysis

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | *tub2* (GenBank) | Articles |
|---|---|---|---|---|---|---|---|
| CBS 533.81 | *Clonostachys agrawallii* | India | Decomposing buffalo horn | AF358241 | N/A | AF358187 | Schroers, 2001 |
| CBS 130.87 | *Clonostachys* apocyni (macrospora) | USA | Dead steam of *Apocynum cannabinum* | AF210688 | N/A | AF358168 | Lombard *et al*., 2015 |
| WC-2015 | *Clonostachys* aranearum | China | Unknown | KT895417 | N/A | KU212400 | Wan-Hao *et al*., 2016 |
| CBS 195.93 | *Clonostachys aureofulvella* | New Zealand | Root of tree | AF358226 | N/A | AF358181 | Schoers, 2001 |
| CBS 696.93 | *Clonostachys* buxi | France | *Buxus sempervirens* | KM231840 | KM231977 | KM232111 | Lombard *et al*., 2015 |
| CBS 364.78/ CML 2510 | *Clonostachys byssicola* | Venezuela | Bark | KJ499907 | KX184967 | AF358153 | Moreira *et al*., 2016; CBS |
| CBS 504.67/CML 2512 | *Clonostachys candelabrum* (Outgroup) | Netherlands | Wheat field soil | AF210668 | KX185029 | KF871189 | Moreira *et al*., 2016; CBS; Schroers, 2001 |
| CBS 218.93 | *Clonostachys capitate* | Japan | Bark | AF358240 | N/A | AF358188 | Schroers, 2001 |
| CBS 287.90 | *Clonostachys chlorine* | Brazil | Soil | NR_137651 | N/A | N/A | Schroers, 2001 |
| CBS 141588/ CML 1941 | *Clonostachys chloroleuca* | Brazil | Native soil | KC806286 | KX184988 | KF871172 | Moreira *et al*., 2016; CBS |
| CBS 729.87 | *Clonostachys compactiuscula* | Germany | Soil | AF358242 | N/A | AF358193 | Schroers, 2001 |
| CBS 967.73b | *Clonostachys divergens* | Germany | Soil | NR_137532 | N/A | AF358191 | Schroers, 2001 |
| CBS 101037 | *Clonostachys* epichloë | Japan | *Sasa* sp. | AF210675 | N/A | AF358209 | Schroers, 2001 |
| CBS 209.93 | *Clonostachys grammicospora* | French Guiana | Standing dead tree | NR_137650 | N/A | AF358206 | Schroers, 2001 |

Table XII - Details of *Clonostachys* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | *tub2* (GenBank) | Articles |
|---|---|---|---|---|---|---|---|
| CBS 115.87 | *Clonostachys grammicosporopsis* | New Zealand | Bark of *Metrosideros* sp. | AF210679 | N/A | AF358204 | Schroers, 2001 |
| CBS 508.82 | *Clonostachys intermedia* | Netherlands | Soil | NR_137652 | N/A | AF358205 | Schroers, 2001 |
| CBS 461.95 | *Clonostachys kowhai* | New Zealand | Bark of *Sophora microphylla* | AF358250 | N/A | AF358170 | Schroers, 2001 |
| CBS 948.97 | *Clonostachys levigate* | France | Branch of dead *Buxus sempervirens* | AF210680 | N/A | AF358196 | Schroers, 2001 |
| CBS 100008 | *Clonostachys lucifer* | USA | Bark of recently dead *Casearia arborea* | AF210683 | N/A | AF358208 | Schroers, 2001 |
| CBS 997.69 | *Clonostachys* miodochialis | Netherlands | Soil | NR_137649 | N/A | AF358210 | Schroers, 2001 |
| CBS 100285 | *Clonostachys obligispora* | Japan | Bark of dying *Orixa japonica* | AF358248 | N/A | AF358169 | Schroers, 2001 |
| CBS 921.97 | *Clonostachys phyllophila* | France | Leaves of *Viscum album* | NR_137531 | N/A | N/A | Schroers, 2001 |
| CBS 102033 | *Clonostachys* pityrodes | Mauritius | Bark | AF210672 | N/A | AF358212 | Schroers, 2001 |
| CBS 187.94/CML 2513 | *Clonostachys pseudochroleuca* | French Guiana | Base of decaying palm frond | KJ499909 | KX185003.1 | KF871188 | Moreira *et al*., 2016 |
| CBS 119.87 | *Clonostachys pseudostriata* | Indonesia | Bark | AF358251 | N/A | AF358183 | Schroers, 2001 |
| CBS 102845 | *Clonostachys ralfsii* | Australia | Bark | AF358253 | N/A | AF358219 | Schroers, 2001 |

Table XII - Details of *Clonostachys* species retrieved form GenBank included in the phylogenetic analysis (Continuation)

| Database number | Species name | Country | Source | ITS (GenBank) | *tef1*-α (GenBank) | *tub2* (GenBank) | Articles |
|---|---|---|---|---|---|---|---|
| CBS 361/CML 2514 | *Clonostachys* rhizophaga | Switzerland | Culture contaminant | AF358228.1 | KX184993 | AF358158 | Moreira *et al.*, 2016 |
| CBS 920.97/ CML 2557 | *Clonostachys rogersoniana* | Brazil | Soil under Araucaria | N/A | KX185022 | KX185047 | Moreira *et al.*, 2016 |
| CML 1926 | *Clonostachys rogersoniana* | Brazil | Native soil from Cerrado | KC806293 | KX185020 | KF871176 | Moreira *et al.*, 2016 |
| CBS 154.27/ CML 2516 | *Clonostachys rosea f. cantenulata* | USA | Soil | NR_145021 | KX184995 | AF358160 | Moreira *et al.*, 2016 |
| CBS 710.86/ CML 2518 | *Clonostachys rosea f. rosea* | Netherlands | Soil | AF358235 | KX184999 | AF358161 | Moreira *et al.*, 2016 |
| CBS 210.93 | *Clonostachys rossmaniae* | French Guiana | Bark of twings | AF358227 | N/A | AF358213 | Schroers, 2001 |
| CBS 699.97 | *Clonostachys samuelsii* | Venezuela | Bark | AF358236 | N/A | AF358190 | Schroers, 2001 |
| CBS 180.88 | *Clonostachys sesquicillii* | Guyana | Twigs and lichen | AF210666 | N/A | AF358214 | Schroers, 2001 |
| CBS 834.91 | *Clonostachys setosa* | Cuba | *Trophis racemosa* | AF210670 | N/A | AF358211 | Schroers, 2001 |
| CBS 101926 | *Clonostachys solani* | Venezuela | Decaying palm inflorescence | AF358230 | N/A | AF358179 | Schroers, 2001 |
| CBS 142.91 | *Clonostachys solani f.nigrovirens* | Germany | Egg of *Arion ater* | AF358244 | N/A | AF358178 | Schroers, 2001 |
| CBS 101921 | *Clonostachys sporondochialis* | USA | Bark | AF210685 | N/A | AF358149 | Schroers, 2001 |
| CBS 107.87 | *Clonostachys subquaternata* | Venezuela | Wood | Don't have | N/A | AF358207 | Schroers, 2001 |
| HMAS 183151 | *Clonostachys* vesiculosa | China | Unknown | NR_119828 | N/A | N/A | Luo & Zhuang, 2010 |
| HMAS 172156 | *Clonostachys wenpingii* | China | Dead leaves | NR_119651 | HM054097 | HM054127 | Luo & Zhuang, 2007 |
| CBS 232.80 | *Clonostachys zelandiaenovae* | New Zealand | Bark of *Coprosma* sp. | AF210684 | N/A | AF358185 | Schroers, 2001 |
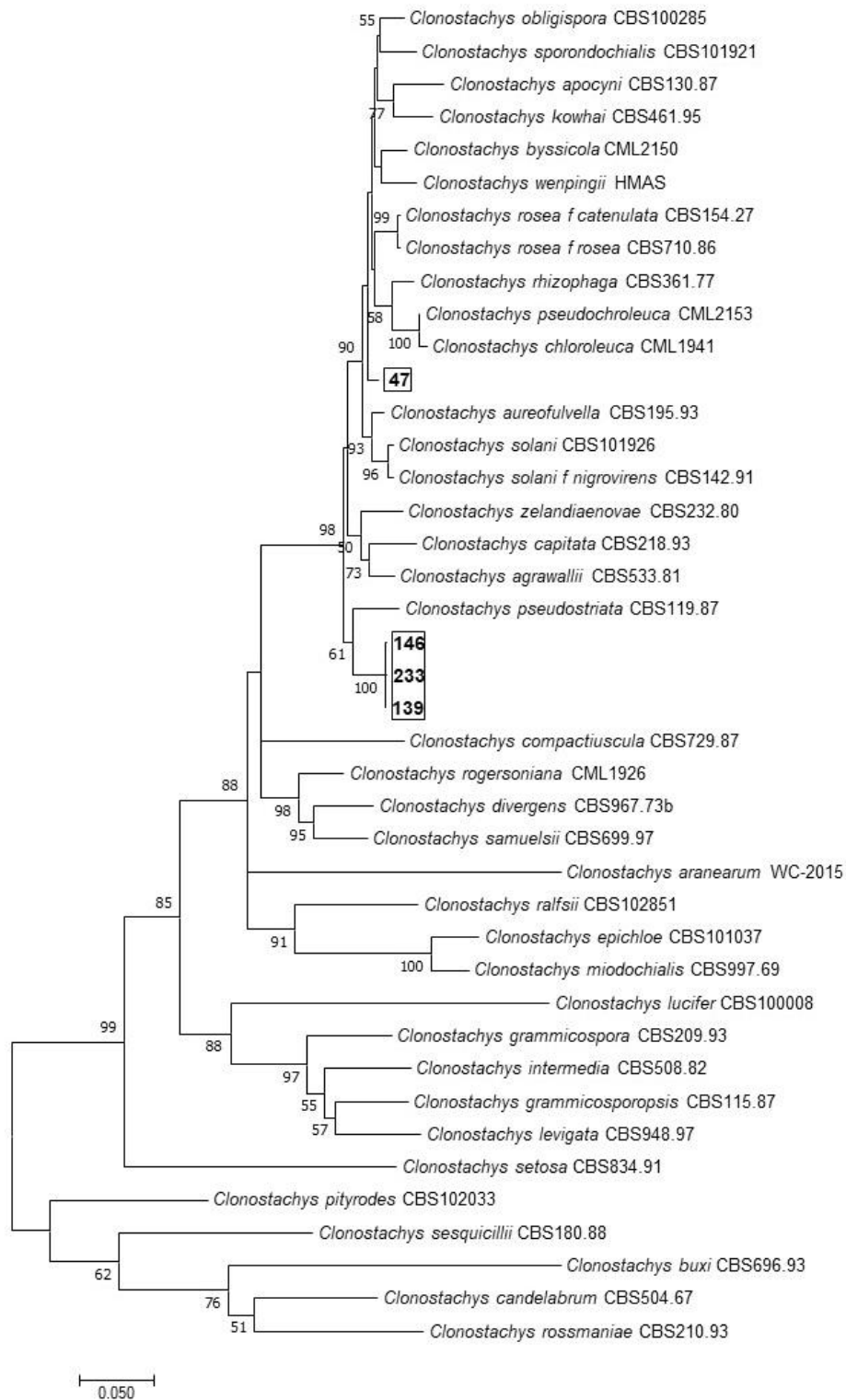
Figure 14 - Phylogenetic tree based on Maximum Likelihood (ML) analysis of a combined data set of ITS and *tub2* sequence alignments. The applied evolutionary model was the Kimura-2-parameter with Gamma distribution permitting invariable sites. The bootstrap values resulting of 1000 replicates are presented (%). The samples used in the study are represented in bold with a black box.
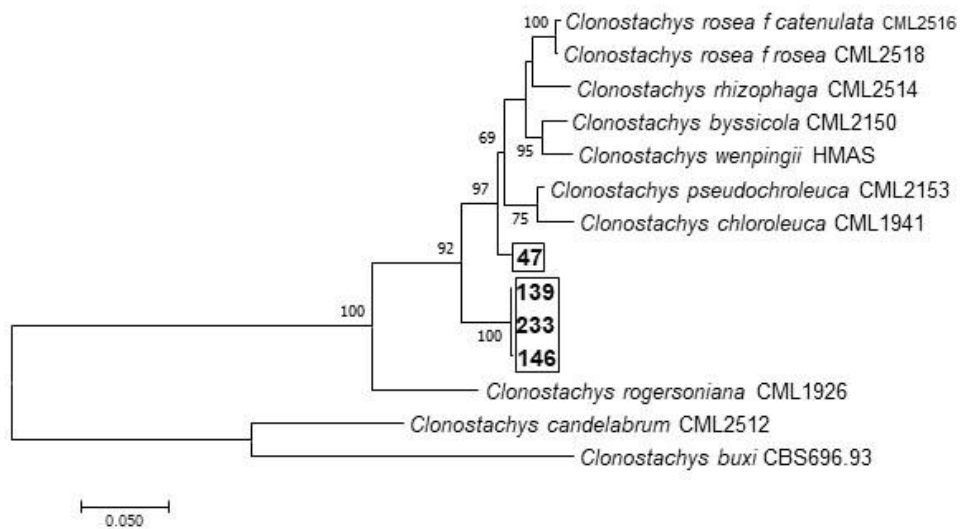
Figure 15 - Phylogenetic tree based on Maximum Likelihood (ML) analysis of a combined data set of ITS, *tef1*-α and *tub2* sequence alignments. The applied evolutionary model was the Jukes-Cantor model with Gamma distribution. The bootstrap values resulting of 1000 replicas are presented (%). The samples used in the study are represented in bold with a black box.

For the alignments of the *Clonostachys* species sequences, amplicons of ITS, *tef1*-α and *tub2* were used, with 481 bp, 502 bp and in between 343 bp and 598 bp, respectively.

In the phylogenetic trees, figures 14 and 15, it can be seen that the isolates are different from the existent sequences available. For isolate 47, the closest relative are the *Clonostachys chloroleuca* and *Clonostachys pseudochroleuca*. For isolates 139, 146 and 233, they create a separate group related to *Clonostachys pseudostriata*, as can be seen in the Figure 14, and a complete separate group in Figure 15, since we don't have *tef1*-α sequence available for the majority of species of *Clonostachys* genus. Since the phylogenetic analysis show that the isolates are different from the currently described species, these are described here as 2 new species, *Clonostachys viticola* (sp. 1) and *Clonostachys peruviana* (sp. 2).

For this genus, there are no known plant pathogenic species (Moreira, 2012; Schroers, 2001; Sutton *et al.*, 1997). As for the other species studied in this work, these new ones should be as well tested for their pathogenicity and fulfilment of Koch's postulates.

Since some of the species of this genus are known for being mycoparasites, it would be useful in the future to evaluate if these novel species described here exhibit the same behavior, and if so, against which fungi they could be used as biocontrol agents.

### 3.2.3.1. Description of the new species of the genus *Clonostachys*

#### 3.2.3.1.1. Growth rates

The isolates were inoculated in 3 types of medium, PDA and OA for 14 days for the assessment of macroscopic and microscopic characters, and MEA 3% during 14 days for assessment of growth rates.

Figures 16 and 17 show the growth rates of the species at different temperatures.
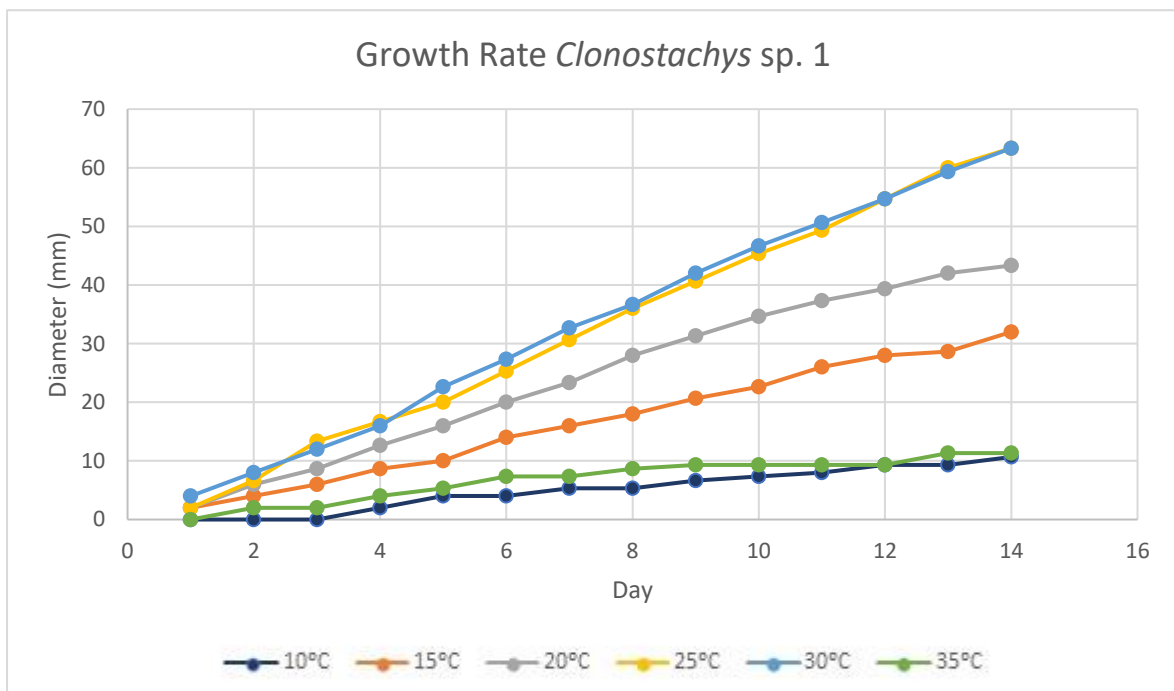


Figura 16 – Growth rate for *Clonostachys* sp.1 (*viticola*) for 10℃, 15℃, 20℃, 25℃, 30℃ and 35℃

In figure 16, it can be seen that a range between 25°C and 30°C is the optimum growth temperature for isolate 47, *Clonostachys* sp. 1, with colonies reaching 60mm in diameter. Also, the extreme temperatures of the test, 10°C and 35°C, are the least favourable temperatures for the species, with colonies reaching only 11mm in diameter.
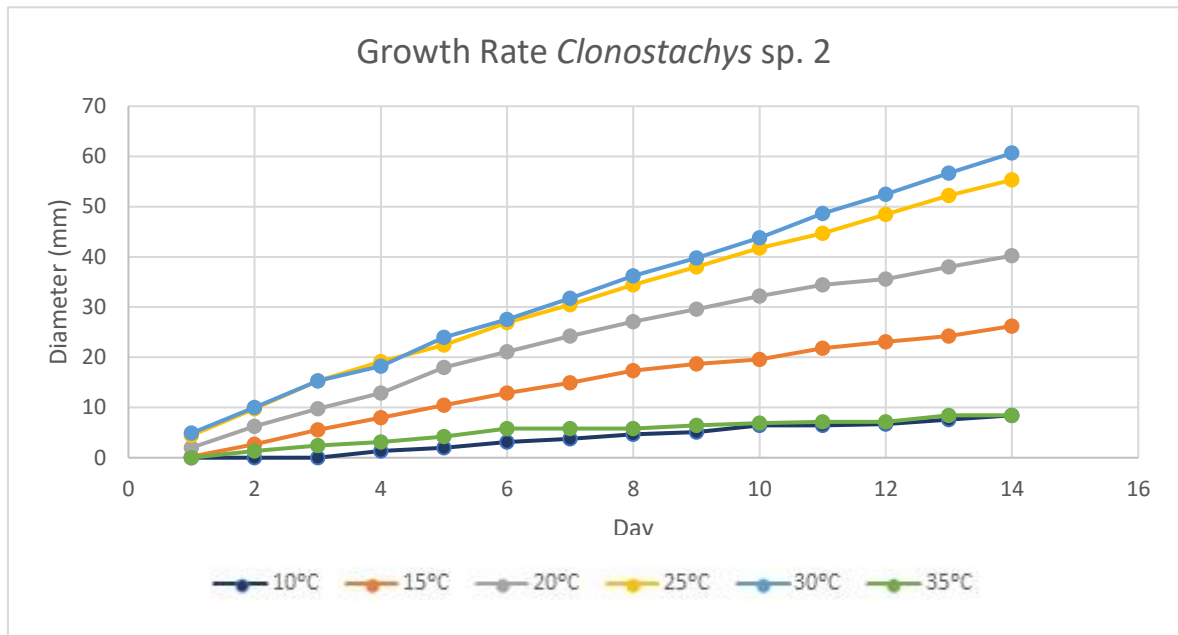
Figura 17 – Average growth rates for *Clonostachys* sp. 2 (*peruviana*) for 10℃, 15℃, 20℃, 25℃, 30℃ and 35℃

In figure 17, the average growth rates at different temperatures of the isolates 139, 146 and 233, *Clonostachys* sp. 2, are presented. It is possible to see that the growth rates at the temperatures 25°C and 30°C are similar but with colonies growing better at 30°C, reaching approximately 63mm in diameter. The difference between 25°C and 30°C is small, and thus the optimum range of growth for the species is considered to lie between 25°C and 30°C. The extreme temperatures of the test, 10°C and 35°C, are the least favourable for the species growth, with colonies reaching only 9mm in diameter.

For a more correct measurement of growth rates it would be necessary to use more temperatures, both below 10°C and above 35°C, since we do not have a minimum nor a maximum growth temperature for both fungi.

### 3.2.3.2. Brief micro- and macromorphological description of the species

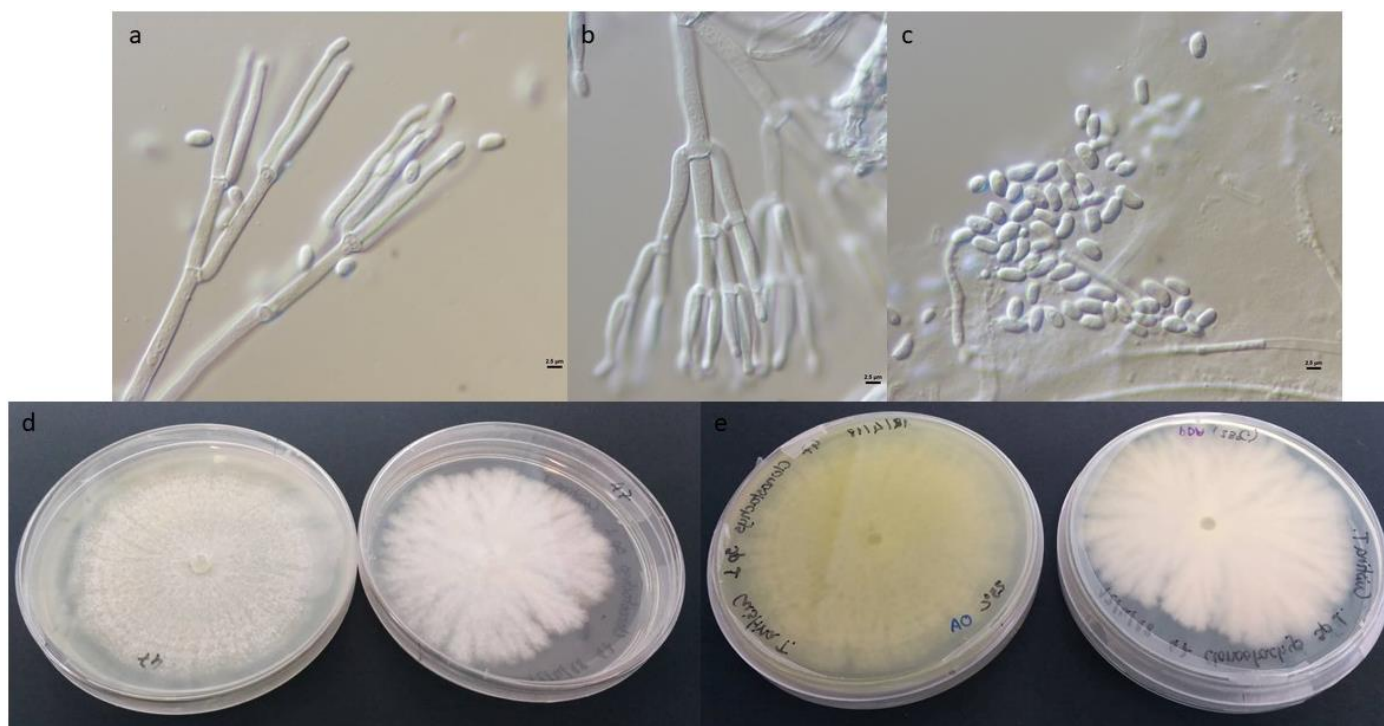**_Clonostachys_ sp. 1 (47) – _Clonostachys viticola_ sp. Nov (Figure 18)**



Figure 18 – _Clonostachys viticola_ – sample 47. Primary Conidiophores with adpressed branches and phialides (A); Secondary Conidiophores with adpressed branches and phialides (B); Conidia (C); Colonies of _Clonostachys viticola_ (47) in OA and PDA respectively (d) and reverse colonies of _Clonostachys viticola_ (47) in OA and PDA respectively (e)

Etymology: _viticola_, referring to the host _Vitis vinifera_ L.

Colonies reaching 63-mm diameter in 14 days at 25 °C on MEA, optimum for growth 25–30 °C (average 63-mm diameter). Average growth of 10.7-mm diameter at 10 °C, 32-mm at 15 °C, 43.3-mm at 20 °C, 11-mm at 35 °C. On OA colony, reverse pale yellow to white; surface unpigmented, white, felty due to strands of aerial mycelium or granular due to sporulation. On PDA incubated in darkness, colony reverse yellowish white to white; surface white cottony to felty due to aerial mycelium. On PDA and OA, during the incubation to room temperature after being incubated at 25°C in the dark, the mycelium changed colour to pale orange. Conidiophores dimorphic. Primary conidiophores verticillium-like, $16.77 \pm 7.82$ µm x $0.68 \pm 0.32$ µm, sometimes with short side branches arising from the upper part. Phialides adpressed, $4.79 \pm 2.42$ µm x $0.39 \pm 0.19$ µm, straight, cylindrical, slightly tapering towards tip. Secondary conidiophores solitary or aggregated, arising from strands of aerial mycelium or directly from medium; bi- to quaterverticillate, measurement not available, typically with two to three primary branches,

divergent, terminating in moderately divergent metulae and adpressed phialides. Phialides 6.88 ± 1.37 µm x 0.75 ± 0.15 µm, in whorls of 2–6, almost cylindrical tapering in upper part, straight to slightly curved; metulae 2.86 ± 1.30 µm x 0.53 ± 0.23 µm. Intercalary phialides not observed. Conidia 1.91 ± 0.38 µm x 0.98 ± 0.19 µm hyaline to pale yellow, ellipsoidal, hilum typically laterally displaced.

Type for *Clonostachys viticola*: 47

Known distribution: Peru

Habitat: Root of *Vitis vinifera* L.

Additional specimens/strains examined: None

### *Clonostachys* sp. 2 (139, 146, 233) – *Clonostachys peruviana* sp. nov (Figure 19)
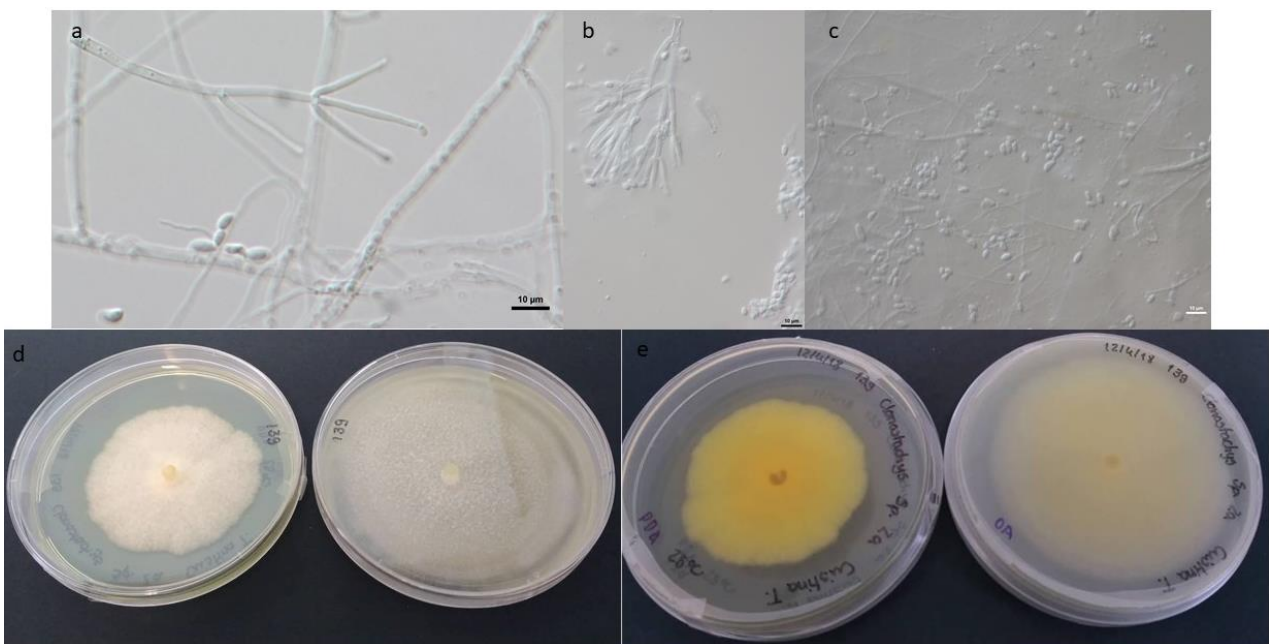


Figure 19 – *Clonostachys peruviana* – sample 139. Primary Conidiophores with divergent branches and phialides (A); Secondary Conidiophores with adpressed branches and phialides (B); Conidia (C); Colonies of *Clonostachys viticola* (47) in OA and PDA respectively (d) and reverse colonies of *Clonostachys viticola* (47) in OA and PDA respectively (e)

Etymology: *peruviana*, referring to country from where the specie was isolated, Peru.

Colonies reaching 46–70mm diameter in 14 d at 25°C on MEA; optimum for growth 25–30°C (average 57mm). Average growth of 8.4-mm diameter at 10 °C, 26.2-mm at 15 °C, 40.2-mm at 20 °C, 8.4-mm at 35 °C.

On OA colony, reverse pale yellow to strong yellow; surface unpigmented, white, felty due to strands of aerial mycelium or granular due to sporulation. On PDA incubated in darkness, reverse pale yellow to strong yellow; surface unpigmented, white, cottony to felty due to aerial mycelium. On PDA and OA, during the incubation to room temperature after being incubated at 25°C in the dark, the mycelium changed colour to pale orange.

Conidiophores dimorphic. Primary conidiophores verticillium-like 3.52 ± 7.09 µm x 0.14 ± 0.27 µm (5 measurements), with branches at somewhat acute angles, mono- to three – verticillate, phialides 3.86 ± 2.62 µm (14 measurements) straight, cylindrical, slightly tapering towards tip. Secondary conidiophores broadly penicillate, quater- or higherverticillate, branches, phialides and metulae divergent only observed once and without measurements.

Conidia 2.22 ± 0.45 µm x 1.16 ± 0.23 µm hyaline to pale yellow, ellipsoidal/oval, slightly curved, with a laterally displaced hilum.

Type for *Clonostachys peruviana*: 139

Known distribution: Peru

Habitat: Root of *Vitis vinifera* L.

Additional specimens/strains examined: 146, 233


For *Clonostachys viticola* (sample 47), there were used forty measurements for secondary phialides and conidia, twenty-one measurements to secondary metulae and twenty measurements for primary phialides.

For *Clonostachys peruviana* (sample 139), some measurements were impossible to do, like secondary conidiophores and secondary phialides, since it was only observed one secondary structure (figure 19b). There were also few measurements for primary conidiophores and primary phialides: 5 and 14 measurements, respectively.

For more measurements, more incubation time was necessary for *Clonostachys peruviana* (sample 139).

# 4. Conclusions and Future Perspectives

In this study, a collection of isolates associated with BFD from Peru was characterised.

Species belonging to four different genera were identified: *Clonostachys*, *Cylindrocladiella*, *Fusarium/Neocosmospora* and *Pleiocarpon*.

For the assessment of the role of the isolates in BFD, pathogenicity tests should be done for the representative isolates of each species. Since most of the isolates do not belong to the most common genera involved in BFD, it is unclear if these isolates are involved or not in the disease process and it's important to know their role in the development of the disease.

Since there are different kinds of cultivars of *Vitis vinifera* L., these pathogenicity tests should be made in different varieties to evaluate their susceptibilities to the different isolates/species.

If the fungi isolated from grapevine root with disease are not pathogenic, we can try to evaluate their role on the plant or for the other fungi present, or even if they are only there because of the proximity of their habitat (for example soil or root surface) with the lesion site.

One of the genus present in this study, genus *Clonostachys*, is known for the presence of species with biocontrol proprieties against *Botrytis cinerea* and other phytopathogenic fungi (Cota *et al*., 2008; Gan *et al*., 2007; Moreira, 2012; Silvera-Pérez *et al*., 2010). This property should be evaluated in the new species described in this study, *Clonostachys vitcola* (sp.1) and *Clonostachys peruviana* (sp.2), against the most common pathogens associated with GTDs to evaluate the possible development of a new biological control agent as an alternative or a complement to chemical control (Bertsch *et al*., 2013).

For the genus, *Fusarium/Neocosmospora*, besides the pathogenicity tests, is necessary to use more molecular markers like *rbp1* and *rb2* (Al-Hatmi *et al*., 2018; O'Donnell *et al*., 2010, 2013, 2015; Short *et al*., 2013) since its necessary more than one marker to identify correctly the isolates and to provide a better understanding of their phylogeny. Within some species complex, like FIESC and FSSC, some species do not have a formal nomination. It would be important to give them a name for an easier identification and better understanding of their role in the environment.

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# 6. Attachment 1

Table XIII - List of isolates used in this study

| Older samples | 1st samples | | | | 2nd samples | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| BF2 | 6 | 24 | 54 | 70 | 130 | 166.2 | 192 | 231 | 245 | 260 |
| BF19 | 7 | 27 | 55 | 72 | 136 | 166.3 | 194 | 232 | 246 | 261 |
| BF20 | 8 | 28 | 56 | 138 | 139 | 166.4 | 198 | 233 | 247 | 262 |
| BF21 | 9 | 29 | 57 | 140 | 142 | 166.5 | 202 | 234 | 248 | 263 |
| BF25 | 10 | 30 | 58 | 141 | 153 | 166.6 | 203 | 235 | 249 | 265 |
| BF33 | 11 | 32 | 60 | 144 | 153.4 | 166.7 | 204 | 236 | 250 | 266 |
| BF37 | 12 | 35 | 61 | 146 | 154 | 166.8 | 206 | 237 | 251 | 267 |
| BF43 | 13 | 36 | 63 | 147 | 156 | 169 | 210 | 238 | 252 | 268 |
| BF46 | 14 | 38 | 64 | 148 | 157 | 174 | 216 | 239 | 253 | 270 |
| BF49 | 15 | 47 | 65 | 149 | 158 | 185 | 221 | 240 | 254 | 273 |
| BF53 | 16 | 48 | 66 | 150 | 161 | 186 | 226 | 241 | 255 | |
| BF62 | 17 | 49 | 67 | 152 | 163 | 187 | 227 | 242 | 256 | |
| BF63 | 18 | 50 | 68 | 162 | 166 | 188 | 229 | 243 | 257 | |
| BF66 | 23 | 52 | 69 | 164 | 166.1 | 189 | 230 | 244 | 259 | |

# 7. Attachment 2

Table XIV - List of primers used in this study

| Primers | Primer sequence | Fornecedor | References |
|---------|-----------------|------------|------------|
| **GTG5** | 5' - GTGGTGGTGGTGGTG - 3' | MWG - Biotech AG | Meyer *et al.*, 1993 |
| **ITS5** | 5' - GGAAGTAAAAGTCGTAACAAGG - 3' | Invitrogen | White *et al.*, 1990 |
| **NL4** | 5' - GGTCCGTGTTTCAAGACGG - 3' | STAB Vida | |
| **688F** | 5' - CGGTCACTTGATCTACAAGTGC - 3' | Invitrogen | Alves *et al.*, 2008 |
| **1251R** | 5' - CCTCGAACTCACCAGTACCGT - 3' | Invitrogen | |
| **T1** | 5' - AACATGCGTGAGATTGTAAGT - 3' | STAB Vida | O'Donnell & Cigelnik, 1997 |
| **BT-2B** | 5' - ACCCTCAGTGTAGTGACCCTTGGC - 3' | MWG - Biotech AG | Glass & Donaldson, 1995 |