Received Date : 17-Jan-2016

Revised Date : 12-Apr-2016

Accepted Date : 09-May-2016

Article type : Original Article

Characterization of *Botryosphaeriaceae* species associated with diseased loquat (*Eriobotrya japonica* Lindl.) in Spain.

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ppa.12556

Keywords Pathogenicity, Phylogeny, Taxonomy, Virulence

Abstract

Loquat (*Eriobotrya japonica* Lindl.) is an important subtropical fruit crop in Spain and other Mediterranean countries. In recent years, characteristic symptoms of branch canker and dieback have been observed in the main cultivated areas of loquat in Spain. The goal of this study was to identify and characterize the species of *Botryosphaeriaceae* associated with these symptoms. For this, thirty-six affected orchards were surveyed between 2010 and 2011 in six provinces of southeastern Spain. Eighty-four isolates belonging to the family *Botryosphaeriaceae* were recovered from symptomatic samples. These isolates were characterized by means of phenotypical studies, DNA sequence analyses of the internal transcribed spacer (ITS) and part of the translation elongation factor 1- α regions, and pathogenicity tests. Ten fungal species were identified including: *Diplodia malorum*, *D. olivarum*, *D. seriata*, *D. pseudoseriata/D. alatafructa*, *Diplodia* sp., *Dothiorella sarmentorum*, *Neofusicoccum mediterraneum*, *N. parvum*, *Spencermartinsia plurivora* and *S. viticola*. In addition, *Diplodia eriobotryicola* and *Dothiorella eriobotryae* are newly described. The most frequent species isolated from cankers was *D. seriata* and, to our knowledge, this is the first report of *D. malorum*, and species belonging to the complex *D. pseudoseriata/D. alatafructa*, in Spain. All species were pathogenic to one year-old loquat plants cv. Algerie, with *Diplodia* sp. and *S. viticola* as the most virulent.

Introduction

Loquat is an important fruit crop in Spain, which is the second larger producer after China and the world's first exporter (Caballero & Fernandez, 2002). In Spain, over 90% of the production is located in the southeast regions (mainly Alicante, Granada and Valencia)

(Soler *et al.*, 2007). The most important diseases that affect this crop are loquat scab, caused by *Fusicladium eriobotryae* (Cavara) Sacc. (Sánchez-Torres *et al.,* 2009), and loquat decline caused by a complex of soilborne fungal/oomycete pathogens comprising *Armillaria mellea* (Vahl) P. Kumm., *Rosellinia necatrix* Berl. ex Prill., *Phytopththora* spp. and Cylindrocarpon-like spp. (González-Domínguez *et al.,* 2009; Agustí-Brisach *et al.,* 2015).

In recent years, loquat growers and technicians have observed characteristic symptoms of branch cankers and dieback in the main cultivated areas of Spain. When severe, those symptoms impose a significant threat to the production and appear to be caused by a complex of fungal species in the family *Botryosphaeriaceae*.

The family *Botryosphaeriaceae* as currently circumscribed includes a large number of genera and species occurring worldwide as endophytes or pathogens on a wide range of woody hosts (Phillips *et al.,* 2013). Plant pathogens in this family are commonly regarded as stress-related pathogens and have been associated with diseases such as fruit rot, root rot, dieback, and gummosis and cankers on economically and environmentally important plants (Phillips *et al.,* 2013).

Several studies have reported *Botryosphaeriaceae* species as important pathogens associated with fruit crops, such as almond (Gramaje *et al.,* 2012), apple (Phillips *et al.,* 2012), avocado (Twizeyimana *et al.,* 2013), citrus (Linaldeddu *et al.,* 2013), grapevine (Úrbez-Torres, 2011), olive (Lazzizera *et al.,* 2008; Moral *et al.,* 2010), and persimmon (Palou *et al.,* 2013a) among many others. On these hosts, symptoms include leaf spots, bud necrosis, shoot dieback, necrosis of the wood, perennial cankers and fruit rots.

In Spain and other countries of the Mediterranean basin, there are no reports of species of *Botryosphaeriaceae* affecting loquat trees. Species of *Botryosphaeriaceae* causing branch dieback have been reported infecting loquat only in Chile (Besoain & Fuentes, 1998;

Palma *et al.,* 2006). Nevertheless, *Diplodia seriata* De Not. has been recently reported causing postharvest rot of loquat in eastern Spain (Palou *et al.,* 2013b).

Because specific studies have not been performed so far, the lack of information on the etiology of loquat branch cankers and dieback in Spain hampers the development of appropriate control measures to reduce yield loss. Therefore, the aims of this study were to (i) determine the incidence and geographic distribution of loquat branch canker and dieback in Spain, (ii) identify the different *Botryosphaeriaceae* species associated with the disease by means of morphological and molecular studies, (iii) evaluate the effect of temperature on mycelial growth and (iv) evaluate the pathogenicity of the different species to loquat plants.

Materials and methods

Field survey and fungal isolation

Field surveys were conducted during 2010 and 2011 in 36 loquat orchards located in six different provinces of Spain, including Alicante, Castellón, Teruel and Valencia (eastern Spain), and Almeria and Granada (southern Spain) (Fig. 1; Table 1). Wood samples were collected from branches of loquat trees with dieback symptoms, including dead shoots, cankers and internal wood necrosis. Segments were cut from the wood of affected branches, washed under running tap water, surface-disinfected for 1 min in a 1.5% sodium hypochlorite solution, and rinsed twice with sterile distilled water. Small pieces cut from the margin between healthy and discolored or decayed wood tissue were plated on 2% potato dextrose agar (PDA) (Biokar-Diagnostics) amended with 0.5 g l^{-1} streptomycin sulphate (PDAS). Plates were incubated at 25°C in the dark until fungal colonies were large enough to be examined. Preliminary identifications were made from colony characteristics and

micromorphology and isolates corresponding to members of the *Botryosphaeriaceae* were retained. Hyphal tip cultures were transferred twice and stored in 15% glycerol solution at -80°C in 1.5 ml cryovials. Colony colours were determined using the colour charts of Rayner (1970).

Morphological identification and characterization

In order to enhance sporulation, cultures from all the isolates recovered were amended with sterilized pine needles or poplar twigs on 2% water agar (WA; Biokar-Diagnostics) and ¼ strength PDA and incubated at 25°C under near UV light with a 12 h photoperiod (Philips TDL18W/33). Isolates were examined weekly for formation of pycnidia. Pycnidia were mounted in 100% lactic acid and micromorphological characteristics (conidial size, shape, colour, presence or absence of septa, conidiogenous cell morphology and mode of conidiogenesis) of the isolates were determined with a Nikon 80i microscope and images captured with a Nikon DS-Ri1 camera. For each isolate, 100 conidia were observed and measured using the Nikon software NIS-elements D.

DNA isolation, amplification, and phylogenetic analyses

Prior to extraction, pure cultures were transferred separately to a flask containing 100 ml potato dextrose (PD) broth and incubated at room temperature for 7 to 15 days. Liquid cultures were vacuum-filtered and stored at –20ºC. For DNA extraction, freeze-dried fungal tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle. Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek) following manufacturer's instructions. DNA was visualized on 0.7% agarose gels stained with ethidium

bromide and stored at –20ºC. The ITS region was amplified using the primers ITS1 and ITS4 (White *et al.,* 1990) as described by Alves *et al.* (2004). The primers EF1-688F (Alves *et al.,* 2008) and EF1-986R (Carbone & Kohn, 1999) were used to amplify and sequence part of the translation elongation factor 1-alpha (EF1-α) as described by Alves *et al.* (2006). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics) and sequenced by Macrogen Inc., Sequencing Center.

Sequences were aligned with MEGA6 (Tamura *et al.,* 2013) and manual adjustments made where necessary. GenBank sequences of closely related genera were selected based on their high similarity with our sequences using MegaBLAST (Table S1). Sequences from ex-type or ex-epitype isolates of each species were included when available.

Phylogenetic analyses of sequence data were done using PAUPv. 4.0b10 (Swofford, 2003), for Maximum-Parsimony (MP) analyses, and MEGA6 (Tamura *et al.,* 2013), for Maximum-Likelihood (ML) analyses. Trees were rooted to an outgroup and visualized with TreeView (Page, 1996). Separate phylogenetic analyses were performed for each dataset (ITS and EF1-α) and tree topologies were compared.

MP analyses were performed using the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight, and gaps were treated as fifth base (newstate). Maxtrees were set to 10000, branches of zero length were collapsed, and all multiple equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated from 1000 bootstrap replications. Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI).

ML analyses were performed on a MP starting tree generated automatically by the software. The general time reversible model of evolution (Rodriguez *et al.,* 1990), including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories (GTR+G+I) was used. Nearest-Neigbour-Interchange (NNI) was used as the heuristic method for tree inference and 1000 bootstrap replicates were performed. Gaps were treated as a phylogenetically relevant characters.

Temperature growth studies

Representative isolates of the species identified in this study were used for temperature growth studies (Table 1). A 6-mm plug was taken from the margin of a 3-day-old colony and placed in the center of a Petri dish. Three replicates of each isolate were incubated separately at 5, 10, 15, 20, 25, 30, 35, and 40°C in the dark. Colony diameter was measured after 3, 4 and 7 days, and the last data recovered were converted to radial growth rate in millimeters. The experiment was performed twice. A simplified version of the non-linear equation proposed by Duthie (1997) was regressed to the data of each isolate in the form: $Y=((H+1)/H) * H^{1/(H+1)} * \exp(((T-F)*G/(H+1))/(1+\exp((T-F)*G)))$; where Y is the radial growth rate (referred to the maximum observed), T is the temperature (°C) and H, F, and G are the equation parameters. Parameter F is directly proportional to optimal temperature, that was calculated in the form $T_{opt}=F-(1/G)* \ln(H)$ (Duthie 1997). Regression curves were fitted to the data using R ver. 3.1.2 (R Development Core Team) with the nls function of the 'stats' package.

Pathogenicity tests

Pathogenicity of representative isolates belonging to the species identified in this study was evaluated on loquat (Table 1). One year-old loquat plants cv. Algerie were used. The main stem was wounded at the main stem with a 4-mm cork borer at a distance of 10 cm over the substrate, and a 4-mm mycelium plug cut from a 1-week-old culture was placed in the wound. Wounds were wrapped with parafilm. Four plants per fungal isolate were used. Four additional plants were inoculated with 4-mm diam. non colonized PDA agar plugs as controls. After inoculation, plants were placed in a greenhouse at 20–35ºC in a completely randomized design and watered every 3 days or as needed. After five months, stems were sectioned longitudinally and the extent of vascular discoloration was measured upward and downward from the point of inoculation. Although some plants died close to the end of the experiment, it was still possible to differentiate the extent of wood discoloration in the lesions. Thus, the data from these plants were included in the analysis. Small pieces of necrotic tissue from the margin of each lesion were aseptically plated on PDAS in an attempt to complete Koch's postulates. Fungal identity was verified by its colony and conidial morphology. The experiment was performed twice.

For all fungal isolates, a preliminary analysis of variance (ANOVA) was performed to determine if there were significant differences between the two repetitions of the experiments, and if the data could be combined. ANOVA was performed to determine the significance of differences in mean lesion lengths caused by the different fungal isolates. The significance of differences between individual means was determined with Student's Least Significant Difference (LSD) test at the 5% level using Statistix 9 (Analytical Software). To satisfy the assumptions of the ANOVA, the log10 transformation of the data was used.

Results

Fungal collections and isolations

Eighty-four isolates were identified as belonging to the family *Botryosphaeriaceae* based on: (i) colony morphology (isolates with fast growing mycelium, fluffy, white to grey-green), (ii) conidial characteristics (Phillips *et al.*, 2013) and (iii) BLAST searches of ITS sequences (Table 1). The majority of them were recovered from Valencia (30%), Alicante (26%) and Castellón (18%) provinces, while Almería, Teruel, and Granada provinces yielded 13%, 8% and 5% of the isolates, respectively (Fig. 1). From the 84 isolates recovered, 60 were tentatively identified by morphological features as *Diplodia* spp. (70%), 20 as *Spencermartinsia/Dothiorella* spp. and 4 as *Neofusicoccum* spp. (5%). Other fungal genera sporadically isolated from loquat cankers were *Alternaria*, *Phomopsis* and *Cladosporium*.

Phylogenetic analyses

PCR amplifications of the ITS and EF1-α regions gave products of approximately 0.6, and 0.5 kb, respectively. These new sequences were deposited in GenBank (Table 1). Different dataset and analyses were performed for *Diplodia* spp., *Neofusicoccum* spp. and *Dothiorella/Spencermartinsia* spp., using *Lasiodiplodia* spp., *Spencermartinsia viticola* and *Neofusicoccum luteum* as outgroup, respectively. In all cases, the topology of the trees of individual gene regions (i.e., ITS or EF1-α) were consistent between them, with no major conflicts, thus the two loci were combined.

The combined ITS and EF1-α dataset of *Diplodia* spp. included 50 taxa (48 ingroup and 2 outgroup) and contained 844 characters (including gaps). The MP analysis considered 236 parsimony-informative positions, and heuristic searches found 513 equally parsimonious trees (tree length=503, CI=0.736, RI =0.908; HI=0.264). Both, MP and ML analyses

produced trees with the same topology. In the phylogenetic analysis, four main clades were resolved within the ingroup (Fig. 2). The clade 1 was further resolved into two subclades. One of them included the loquat isolate BN-55, that grouped with representative isolates of *D. pseudoseriata* C.A. Pérez, Blanchette, Slippers & M.J. Wingf. / *D. alatafructa* Mehl & Slippers (bootstrap support of 99%). In the same subclade, the loquat isolate BN-67 grouped with a *Diplodia* sp. isolate (92% bootstrap support) previously reported from *Pyracantha coccinea* (Phillips *et al.,* 2012), but not formally described, and referred to here as *Diplodia* sp. Fifty-two loquat isolates clustered with the ex-type isolate of *D. seriata* (Fig. 2). Although 13 of them grouped into a sub-clade within *D. seriata*, this was not well-supported (bootstrap=63%). The other subclade of clade 1 included representative isolates of *D. allocellula* Jami, Gryzenh., Slippers & M.J. Wingf. (bootstrap=99%) and the loquat isolate BN-21 (bootstrap=99%) which is described here as *Diplodia eriobotryicola* sp. nov. (Fig. 2). Four loquat isolates were included in clade 2, three of them grouped with the representative of *D. malorum* Fuckel (BN-30, BN-35 and BN-37) and one with *D. olivarum* A.J.L. Phillips, Frisullo & Lazzizera (BN-43) (Fig. 2).

The combined ITS and EF1-α dataset of *Neofusicoccum* spp. included 33 taxa (31 ingroup and 2 outgroup) and contained 551 characters (including gaps). The MP analysis considered 127 parsimony-informative positions, and heuristic searches found 40 equally parsimonious trees (tree length=190, CI=0.811, RI =0.916; HI=0.189). Both, MP and ML analyses produced trees with the same topology. Loquat isolate BN-12 grouped with the representative isolates of *N. mediterraneum* Crous, M.J. Wingf. & A.J.L. Phillips, and isolates BN-57, BN-65 and BN-66 grouped with those of *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (Fig. S2).

The combined ITS and EF1-α dataset of *Dothiorella* spp. and *Spencermartinsia* spp. included 71 taxa (69 ingroup and 2 outgroup) and contained 796 characters (including gaps). The MP analysis considered 317 parsimony-informative positions, and heuristic searches found 42 equally parsimonious trees (tree length=871, CI=0.642, RI =0.929; HI=0.358). Both, MP and ML analyses produced trees with the same topology. In the phylogenetic analysis, two main clades were resolved in the ingroup; clade 1 contained 26 species of *Dothiorella* and clade 2, contained 6 species of *Spencermartinsia* (Fig. 3). Within clade 1, a well-supported subclade (bootstrap= 100%) was formed by 7 isolates from loquat and two representative isolates of *Do. sarmentorum* (Fr.) A.J.L. Phillips, A. Alves & J. Luque. The other loquat isolate belonging to clade 1, BN-81, formed a subclade with *Do. prunicola* A.J.L. Phillips & Abdollahz., but was well distinct from it (bootstrap=100%), and is described here as *Dothiorella eriobotryae* sp. nov. (Fig. 3). Clade 2 contained 11 loquat isolates that formed a subclade with representative species of *S. viticola* (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous, and one loquat isolate, BN-97, included in the well-supported subclade of *S. plurivora* Abdollahz., Javadi & A.J.L. Phillips (bootstrap=96%) (Fig. 3).

Taking into account the results of the phylogenetic analyses, *Diplodia seriata* was the predominant species isolated from loquat branch cankers with a total of 52 isolates recovered (62% of the total) from all the provinces surveyed, except Teruel. In these provinces, *D. seriata* was the predominant species, especially in Valencia and Alicante, where 18 and 14 isolates were recovered, respectively. Isolates of *S. viticola* represented 13% of the total number of isolates, and were recovered from Alicante (n=4), Castellón (n=4), Almería (n=2) and Valencia (n=1). Following these species, *Do. sarmentorum* represented an 8% of the total, but was recovered only in Alicante (n=3) and Teruel (n=4). *N. parvum* (n=3) and *N. mediterraneum* (n=1) were recovered only from Valencia, and represented less than 5% of

the total. The three isolates of *D. malorum* were all recovered from Teruel, the isolate of *D. olivarum* was recovered from Almeria, and that of *D. pseudoseriata/D. alatafructa* from Castellón. Finally, the two new species described in this paper, *D. eriobotryicola* and *Do. eriobotryae* were recovered from Valencia and Castellón, respectively.

Temperature growth studies

Eighteen isolates, belonging to the different species of *Botryosphaeriaceae* identified in this study, were selected for the temperature growth assessment (Table 1). The non-linear equation selected to describe the mycelial growth at different temperatures fitted the data with R^2>0.95 for all the isolates. All the *Diplodia* spp. isolates grew at temperatures ranging from 5 to 35°C (Table 2). Their optimum temperature for mycelial growth was near to 25°C except for one isolate of *D. malorum* (BN-30), which grew faster at 21.67°C. Most isolates of *Dothiorella* and *Spencermartinsia* spp. were not able to grow at 35°C, except for isolate BN-79 of *S. viticola*. For both species, optimum temperatures for mycelial growth ranged between 23.16 and 26.08°C. Isolates belonging to *N. parvum* and *N. mediterraneum* had higher values of optimum temperatures, from 28.27 to 29.17°C (Table 2). None of the isolates was able to grow at 40ºC. At 25°C, a high variability of mycelial growth rate was observed between isolates belonging to the same species (Table 2).

Pathogenicity tests

Twelve isolates, belonging to the different species of *Botryosphaeriaceae* identified in this study, were selected for the pathogenicity studies (Table 1). ANOVA indicated no significance differences between the two experiments (P>0.05) and, therefore the data from

both experiments were combined. All inoculations resulted in visible lesions in the cambium of loquat plants, and mean lengths of vascular discoloration differed significantly between the isolates ($P<0.05$) among species. Isolates of *D. pseudoseriata/D. alatafructa*, *D. malorum*, *Diplodia* sp. and *N. parvum* produced lesions on the plants significantly higher than those observed in the control (Table 3). All the isolates were able to cause the death of loquat plants, whereas no death was observed in the controls. *Diplodia* sp. caused the death of all inoculated plants (Table 3).

Taxonomy

Diplodia eriobotryicola E. González-Domínguez, J. Armengol, & A. Alves, *sp. nov.*

MycoBank: MB815698 (Fig. 4)

Etymology: Named for the host it was first isolated from, namely *Eriobotrya japonica*.

Sexual morph not seen. Conidiomata pycnidial, produced on pine needles or poplar twigs on WA within 2–3 wk, solitary or aggregated, individual conidiomata globose, superficial or semi-immersed, covered with hyphal hairs, uniloculate, thick-walled. Conidiogenous cells hyaline, thin-walled, smooth, cylindrical to sub-cylindrical, discrete, holoblastic with no evidence of annellations. Conidia ovoid to ellipsoid, apex rounded, base truncate or rounded, aseptate, initially hyaline, becoming brown, thick-walled, wall externally smooth, roughened on the inner surface, (21.9–)23.5–27(–29) x (8.3–)11–13(–14.2) um, 95% confidence limits = 25.6–26.2 x 11.7–12.1 um (av. 100 conidia ± S.D. = 25.9 ± 1.3 x 11.9 ± 1.0 um, l/w ratio = 2.2 ± 0.2).

Culture characteristics — Colonies on PDA initially white turning gray from the middle of colonies, aerial mycelium slightly fluffy, becoming dense, cottony with age, turning smoke gray to dark gray toward the edges, reverse olivaceous-black and with regular margins.

Cardinal temperatures for growth: min < 5 ºC, max > 35ºC, opt. 25ºC.

Type: Spain, Valencia, Godella, from *Eriobotrya japonica* branch cankers, September 2010, E. González-Domínguez, holotype LISE 96306, culture sporulating on pine needles and poplar twig.

Cultures: BN-21 (ex-type)= CBS140851.

Known distribution: Spain

Notes: BN-21 is morphologically and phylogenetically closely related to *D. allocellula*. On average conidia are slightly larger, although the plasticity of morphological characters should be considered. Also, it differs from *D. allocellula* on nucleotide sequences: ITS (1 nucleotide substitution), EF (4 nucleotide substitutions and 1 nucleotide deletion).

Dothiorella eriobotryae E. González-Domínguez, J. Armengol, & A. Alves, *sp. nov.*

MycoBank: MB815699 (Fig. 5).

Etymology: Named for the host it was first isolated from, namely *Eriobotrya japonica*.

Sexual morph not seen. Conidiomata pycnidial, produced on poplar twigs on WA within 2 wk, solitary or aggregated, individual conidiomata globose, superficial or semi-immersed, covered with hyphal hairs, uniloculate, thick-walled. Conidiogenous cells cylindrical to lageniform, discrete or integrated, holoblastic, indeterminate, hyaline, thin-walled, smooth. Conidia subcylindrical to ellipsoid or ovoid, initially hyaline becoming dark brown and 1-

septate while still attached to the conidiogenous cells, occasionally slightly constricted at septum, moderately thick walled, externally smooth, internally finely verruculose, ends rounded, often with a truncate base, (17.2–)18.5–21(–22.6) × (9–)10–11(–12.3) µm, 95% confidence limits = 19.5–19.9 × 10.2–10.4 µm (av. 100 conidia ± S.D. = 19.7 ± 1.2 × 10.3 ± 0.6 µm, l/w ratio = 1.9 ± 0.2). Spermatia hyaline, smooth, aseptate, rod-shaped with rounded ends.

Culture characteristics — Colonies on MEA initially white, mycelium becoming smoke-grey to olivaceous grey at the surface starting from the center and grey-olivaceous to olivaceous-black at the reverse with irregular margins. Poor growth on PDA but good on OMA, MEA and ½ strength PDA.

Cardinal temperatures for growth: min. ≤ 5 °C, max. ≤ 35 °C, opt. 24 °C.

Type: Spain, Castellón, Segorbe, from *Eriobotrya japonica* branch cankers, May 2011, E. González-Domínguez, holotype LISE 96307, culture sporulating on pine needles and poplar twig.

Cultures: BN-81 (ex-type)= CBS140852.

Known distribution: Spain

Notes — Phylogenetically this species is completely different from any known species in *Dothiorella*.

Discussion

This study addresses, for the first time, the presence, diversity and pathogenicity of *Botryosphaeriaceae* species associated with loquat in the major production areas of Spain. Twelve fungal species belonging to four different genera were isolated from branch cankers and dieback of loquat trees in Spain and identified by means of phenotypic characters and DNA sequence analyses. These species included *Diplodia malorum*, *D. olivarum*, *D. seriata*, *D. pseudoseriata/D. alatafructa*, *Diplodia* sp., *Dothiorella sarmentorum*, *Neofusicoccum mediterraneum*, *N. parvum*, *Spencermartinsia plurivora* and *S. viticola*. Moreover, two novel species are described here as *D. eriobotryicola* and *Do. eriobotryae*. To our knowledge, this is the first report of *D. malorum* in Spain. Moreover, none of the species of the complex *D. pseudoseriata/D. alatafructa* have been previously described in Spain.

A great diversity of species from the family *Botryosphaeriaceae* has been previously reported as important pathogens of Mediterranean fruit hosts. Regarding the species identified in this study, *D. seriata*, *Do. sarmentorum*, *N. parvum*, *N. mediterraneum* and *S. viticola* have a worldwide distribution associated with a variety of fruit hosts, including almond (Gramaje *et al.,* 2012), olive (Lazzizera *et al.,* 2008; Moral *et al.,* 2010), citrus (Linaldeddu *et al.*, 2015) and especially grapevine (Martin & Cobos, 2007; Linaldeddu *et al.*, 2015). Specifically in Spain, *D. seriata* is the most common species on olive and grapevine, and was also the most common species in the present study (Martin & Cobos, 2007; Moral *et al.,* 2010). *D. olivarum* has been found in Italy and Spain associated with olive and almond, respectively (Lazzizera *et al.,* 2008; Gramaje *et al.,* 2012), whereas *S. plurivora* have been associated with different fruit hosts, such as citrus, grapevines and apples from Australia, Iran and Spain (Pitt *et al.*, 2014). However, *D. malorum* and *D. pseudoseriata/D. alatafructa* have been previously associated only with forest hosts: *D. malorum* in Portugal and *D.*

pseudoseriata/D. alatafructa in South Africa and Uruguay (Phillips *et al.*, 2012; 2013). In fruit hosts, all these species cause mainly shoot dieback, necrosis of the wood and perennial cankers, but leaf spots, bud necrosis, and fruit rots have been also observed.

This study shows that loquat is one of the many fruit crops that are affected by species in the *Botryosphaeriaceae* in the Mediterranean basin; further surveys should be performed in the main loquat producing countries, such as Italy, Turkey and Pakistan (Caballero & Fernández, 2002), to confirm the role of members of this family as loquat pathogens. Although loquat is an important crop in Asia and the Mediterranean basin (Soler *et al.,* 2007), there are no reports of *Botryosphaeriaceae* affecting loquat trees in these areas. Only in Chile have species of *Botryosphaeriaceae* been reported affecting loquat (Besoain & Fuentes, 1998; Palma, 2006). In both reports the isolates were morphologically characterized as *Botryosphaeria obtusa* (Schwein.) Shoemaker, which is currently known as *D. seriata* (Phillips *et al.,* 2013). However, several new *Diplodia* species have been described recently, including *D. eriobotryicola* described in this study, and therefore *D. seriata* s.l. is considered a complex of species (Phillips *et al.,* 2012; Linaldeddu *et al.,* 2013) and, it is possible that a wider range of species could occur in loquat in Chile.

In this study one isolate was only identified as *Diplodia* sp. Taxonomy of *Diplodia* species has been re-evaluated in the last few years, and most of the misidentifications have been resolved (Phillips *et al.,* 2012; Phillips *et al.,* 2013), but some problems still remain. This *Diplodia* sp. was first collected by Phillips *et al.* (2012), who showed the differences between this isolate from *Pyracantha coccinea* and those of *D. intermedia* A.J.L. Phillips, J. Lopes & Bobev, but they did not describe it as a new species. These differences have been confirmed in the current study, but since this *Diplodia* sp. is being treated elsewhere we have chosen not to formally describe it here.

Temperature growth characterization of isolates causing branch cankers on loquat showed that most species were able to grow in a wide range of temperatures (5-35ºC), with an optimum close to 25ºC for *Diplodia* spp., *Dothiorella* spp. and *Spencermartinsia* spp. In *Neofusicoccum* spp., the optimum temperature was higher at 28–29ºC. These results agree with those reported previously for isolates of *D. seriata*, and *N. mediterraneum* collected from olive in Spain and California (Moral *et al.,* 2010), and *D. seriata* and *N. parvum* collected from grapevines in New Zealand (Baskarathevan *et al.,* 2011). The wide range of temperatures at which these fungi can grow reinforces the idea that species of *Botryosphaeriaceae* are able to growth under a range of different environmental conditions and this can be one of the reasons why they are found in all climatic areas of the world (Phillips *et al.*, 2013).

In this study, the pathogenicity of the species of *Botryosphaeriaceae* to loquat was confirmed. Isolates of all species were able to cause the death of loquat plants, and isolates of *Diplodia* sp. and *S. viticola* were highly virulent. *Do. sarmentorum* has been previously reported as pathogenic on other crops such as grapevines and pistachio, and *Neofusicoccum* spp. have been considered as highly virulent (Carlucci *et al.*, 2015; Chen *et al.*, 2015; Úrbez-Torres *et al.*, 2011). However, species of *Spencermartinsia* and *Diplodia* have been considered as weak pathogens or even as saprophytes (Phillips *et al.,* 2012; Úrbez-Torres *et al.*, 2011). The species of both genera isolated in this study were pathogenic, especially *D. alatafructa*, *D. malorum*, *Diplodia* sp. and *S. viticola*. However, as far as we know, the pathogenicity of *S. plurivora*, *D. malorum* and *D. alatafructa* have never been studied. Thus, regarding *Diplodia* spp., the consideration of them as weak pathogens could come from the results obtained in previous pathogenicity tests using mainly *D. seriata*.

The pathogenicity of *D. seriata* has been studied in detail in the past, with conflicting reports about its virulence in other crops, such as grapevines or apples (Phillips *et al.,* 2012;

Úrbez-Torres *et al.*, 2011). These differences could be due to: (i) the use of different cultivars, (ii) the inoculation of different host tissue, and (iii) variations in the virulence between strains (Phillips *et al.,* 2012; Úrbez-Torres *et al.*, 2011). As *D. seriata* was the most prevalent species in this study, further pathogenicity studies should be performed, including: different loquat varieties, a wider number of isolates, and comparing different inoculations methods (Elena *et al.,* 2015). Moreover, the recent description of *D. seriata* as a postharvest pathogen of loquat (Palou *et al.,* 2013b), emphasizes the importance of this pathogen associated with branch cankers, since these lesions could serve as an inoculum source for fruit infections. In the case of *Diplodia* sp., the high virulence observed in this study encourages further studies of its pathogenicity on the other hosts from which it has been isolated.

The results of this study show a high diversity of *Botryosphaeriaceae* species associated with branch cankers in loquat, two of them newly described here. Loquat is a subtropical evergreen species that grows in latitudes comprised between 20º and 35º North or South (Lin *et al.,* 1999). The wide range of temperatures in which the species of *Botryosphaeriaceae* described here can grow (with optimum ranging from 21 to 29ºC) makes it plausible to hypothesize that these species infect loquat trees throughout all the growing season under Mediterranean conditions. The life cycles of some species of *Botryosphaeriaceae* have been previously described (Úrbez-Torres *et al.,* 2011): the fungus produces pycnidia which, when mature, exude conidia in gelatinous matrices forming cirri; these conidia are splash-borne dispersed during rainfall, arriving to susceptible pruning wounds. In the north hemisphere, loquat is pruned in summer (after harvest). Moreover, to increase the size of loquat fruits, common cultural practices consist of the removal of flowering shoots and fruit thinning (Reig *et al.,* 2011). Thus, control measures against the

infection of *Botryosphaeriaceae* species in these periods should be taken into account in loquat crops, mainly if wounds are produced during rainy periods (Úrbez-Torres *et al.,* 2011).

Acknowledgments

We acknowledge Dr. A.J.L. Phillips (Universidade de Lisboa, Portugal) for valuable discussions, data sharing and the review of the manuscript. This study was funded by the Cooperativa Agrícola de Callosa d'En Sarrià (Alicante, Spain). We would like to thank V. Garrigues, M. Mahiques and A. Ramón-Albalat for technical assistance. Artur Alves acknowledges financing by European Funds through COMPETE and by National Funds through the Portuguese Foundation for Science and Technology (FCT) to the research unit CESAM (UID/AMB/50017/2013 – POCI-01-0145-FEDER-007638) and himself (FCT Investigator Programme – IF/00835/2013).

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Figure captions

Figure 1. Map of Spain, indicating the six provinces surveyed in this study (grey area). The different symbols indicate the species of *Botryosphaeriaceae* found in each province.

Figure 2. Single tree resulting from Maximum Likelihood analysis generated from the combined analysis of ITS and EF1-α sequence data from species of *Diplodia*. Bootstrap values are indicated as Maximum likelihood/Maximum parsimony at each node. The tree was rooted to *Lasiodiplodia* spp. Isolates from loquat are in bold. Ex-type isolates are underlined. Bootstrap values less than 50% are not shown.

Figure 3. Single tree resulting from Maximum Likelihood analysis generated from the combined analysis of ITS and EF1-α sequence data from species of *Dothiorella* and *Spencermartinsia*. Bootstrap values are indicated as Maximum likelihood/Maximum parsimony at each node. The tree was rooted to *Neofusicoccum luteum*. Isolates from loquat are in bold. Ex-type isolates are underlined. Bootstrap values less than 50% are not shown.

Figure 4. *Diplodia eriobotryicola*. A. Pycnidia formed on poplar twigs. B. Conidia developing on conidiogenous cells. C. Conidiogenous layer with conidia developing on conidiogenous cells. D–E. Hyaline aseptate conidia. F. Conidia on initial stage of colour development. G. Conidia in two focal planes to show the finely verruculose inner surface of the wall. H. Conidia showing the several stages of colour development and in two focal planes to show the finely verruculose inner surface of the wall. Scale bars: B–H = 10 μm.

Figure 5. *Dothiorella eriobotryae*. A. Pycnidia formed on poplar twigs. B. Conidia developing on conidiogenous cells. C. Spermatia and 1-septate brown conidia. D, E. Brown 1-septate conidia. G. Conidia in two focal planes to show the finely verruculose inner surface of the wall. Scale bars: B–E = 10 μm; F = 5 μm.

Table 1 Isolates of *Botryosphaeriaceae* spp. from loquat included in this study

Species[a]	Isolate[b]	Origin		GenBank Accession no.	
		Locality	Province	ITS	EF1-α
Diplodia eriobotryicola	**BN-21** [1,2]	Burjassot	Valencia	KT240355	KT240193
D. malorum	BN-30	Olba	Teruel	KT240358	KT240194
	BN-35[1,2]	Olba	Teruel	KT240359	KT240195
	BN-37[1]	Olba	Teruel	KT240360	KT240198
D. olivarum	BN-43[1,2]	La Mojonera	Almería	KT240356	KT240196
	BN-49[1]	La Mojonera	Almería	KT240357	KT240197
D. pseudoseriata/ D. alatafructa	BN-55[1,2]	Almassora	Castellón	KT240361	KT240275
D. seriata	BN-2	Callosa d'En Sarrià	Alicante	KT240300	KT240199
	BN-3[1,2]	Callosa d'En Sarrià	Alicante	KT240301	KT240200
	BN-11	L'Eliana	Valencia	KT240302	KT240201
	BN-16	Valencia	Valencia	KT240303	KT240202
	BN-18	Burjassot	Valencia	KT240304	KT240203
	BN-19	Burjassot	Valencia	KT240305	KT240204
	BN-27	Valencia	Valencia	KT240306	KT240205
	BN-28	Valencia	Valencia	KT240307	KT240206
	BN-29	Valencia	Valencia	KT240308	KT240207
	BN-39	La Mojonera	Almería	KT240309	KT240208
	BN-41	La Mojonera	Almería	KT240310	KT240209
	BN-42	La Mojonera	Almería	KT240311	KT240210
	BN-44	La Mojonera	Almería	KT240312	KT240211
	BN-45	La Mojonera	Almería	KT240313	KT240212
	BN-46	Almuñécar	Granada	KT240314	KT240213
	BN-47	Almuñécar	Granada	KT240315	KT240214
	BN-48	La Mojonera	Almería	KT240316	KT240215
	BN-50	Almuñécar	Granada	KT240317	KT240216
	BN-51	Almuñécar	Granada	KT240318	KT240217
	BN-54	La Mojonera	Almería	KT240319	KT240218
	BN-56	Almassora	Castellón	KT240320	KT240219
	BN-58	Rafelguaraf	Valencia	KT240321	KT240220
	BN-59	Pobla Llarga	Valencia	KT240322	KT240221
	BN-60	Pobla Llarga	Valencia	KT240323	KT240222
	BN-61	Pobla Llarga	Valencia	KT240324	KT240223
	BN-62	Pobla Llarga	Valencia	KT240325	KT240224
	BN-63	Pobla Llarga	Valencia	KT240326	KT240225
	BN-64	Pobla Llarga	Valencia	KT240327	KT240226
	BN-68	Rafelguaraf	Valencia	KT240328	KT240227
	BN-69	Vall de Almonacid	Castellón	KT240329	KT240228
	BN-72	Orihuela	Alicante	KT240330	KT240229
	BN-74	Salinas	Alicante	KT240331	KT240230
	BN-77	Salinas	Alicante	KT240332	KT240231
	BN-82[1]	Segorbe	Castellón	KT240333	KT240232
	BN-83	Segorbe	Castellón	KT240334	KT240233
	BN-84	Segorbe	Castellón	KT240335	KT240234
	BN-85	Segorbe	Castellón	KT240336	KT240235
	BN-86	Pobla Llarga	Valencia	KT240337	KT240236
	BN-87	Pobla Llarga	Valencia	KT240338	KT240237
	BN-88	Segorbe	Castellón	KT240339	KT240238
	BN-89	Segorbe	Castellón	KT240340	KT240239
	BN-90	Segorbe	Castellón	KT240341	KT240240
	BN-92	Callosa d'En Sarrià	Alicante	KT240342	KT240241
	BN-93	Callosa d'En Sarrià	Alicante	KT240343	KT240242
	BN-94	Altea	Alicante	KT240344	KT240243

	BN-96	Callosa d'En Sarrià	Alicante	KT240345	KT240244
	BN-98	Callosa d'En Sarrià	Alicante	KT240346	KT240245
	BN-104	Chiva	Valencia	KT240348	KT240246
	BN-105	Callosa d'En Sarrià	Alicante	KT240349	KT240247
	BN-106	Callosa d'En Sarrià	Alicante	KT240350	KT240248
	BN-108	Callosa d'En Sarrià	Alicante	KT240351	KT240249
	BN-109	Callosa d'En Sarrià	Alicante	KT240352	KT240250
Diplodia sp.	BN-67 [1,2]	Rafelguaraig	Valencia	KT240354	KT240192

Table 1 (continued)

		Origin		GenBank Accession no.	
Species	**Isolate**	**Locality**	**Province**	**ITS**	**EF1-α**
Dothiorella eriobotryae	**BN-81 [1,2]**	Segorbe	Castellón	KT240287	KT240262
Do. sarmentorum	BN-31	Olba	Teruel	KT240280	KT240255
	BN-32 [1,2]	Olba	Teruel	KT240281	KT240256
	BN-33	Olba	Teruel	KT240282	KT240257
	BN-34	Olba	Teruel	KT240283	KT240258
	BN-75	Salinas	Alicante	KT240284	KT240259
	BN-76	Salinas	Alicante	KT240285	KT240260
	BN-95 [1,2]	Callosa d'En Sarrià	Alicante	KT240286	KT240261
Neofusicoccum mediterraneum	BN-12 [1,2]	L'Eliana	Valencia	KT240276	KT240254
N. parvum	BN-57	Rafelguaraf	Valencia	KT240277	KT240251
	BN-65 [1]	Rafelguaraf	Valencia	KT240278	KT240252
	BN-66 [1,2]	Rafelguaraf	Valencia	KT240279	KT240253
Spencermartinsia plurivora	BN-97 [1,2]	Callosa d'En Sarrià	Alicante	KT240299	KT240263
S. viticola	BN-38	Balones	Alicante	KT240288	KT240264
	BN-52 [1]	La Mojonera	Almería	KT240289	KT240265
	BN-53	La Mojonera	Almería	KT240290	KT240266
	BN-78	Segorbe	Castellón	KT240291	KT240267
	BN-79 [1,2]	Segorbe	Castellón	KT240292	KT240268
	BN-80	Segorbe	Castellón	KT240293	KT240269
	BN-91	Segorbe	Castellón	KT240294	KT240270
	BN-101	Altea	Alicante	KT240295	KT240271
	BN-102	Altea	Alicante	KT240296	KT240272
	BN-103	Chiva	Valencia	KT240297	KT240273
	BN-107	Callosa d'En Sarrià	Alicante	KT240298	KT240274

[a] New species strains are in bold.

[b] [1] Indicates isolates used in the temperature growth study; [2] Indicates isolates used in the pathogenicity test.

Table 2. Optimum temperature for mycelial growth and mycelial growth rate at 25°C of *Botryosphaeriaceae* species associated with branch cankers and dieback of loquat trees in Spain.

Species	Isolate	Temperature range[a]	Mycelial growth rate[b]	Optimum temperature[c]
Diplodia eriobotryicola	BN-21	5-35	7.61	25.59
Diplodia malorum	BN-30	5-35	5.56	21.67
	BN-35	5-35	5.31	26.88
Diplodia olivarum	BN-43	5-35	6.47	26.85
	BN-49	5-35	5.80	26.29
Diplodia pseudoseriata	BN-55	5-35	7.12	27.15
Diplodia seriata	BN-3	5-35	9.43	26.99
	BN-82	5-35	8.30	26.52
Diplodia sp.	BN-67	5-35	5.68	27.02
Dothiorella eriobotryae	BN-81	5-30	6.29	24.06
Dothiorella sarmentorum	BN-32	5-30	9.82	25.91
	BN-95	5-30	5.68	23.16
Neofusicoccum mediterraneum	BN-12	5-35	7.16	28.27
Neofusicoccum parvum	BN-65	5-35	7.94	29.17
	BN-66	10-35	10.94	28.36
Spencermartinsia plurivora	BN-97	5-30	7.93	24.66
Spencermartinsia viticola	BN-38	5-30	11.92	23.80
	BN-79	5-35	7.24	26.08

[a] Temperature range at which each *Botryosphaeriaceae* specie was able to grow. Experiments were performed at temperatures from 5 to 40ºC

[b] Mycelial growth rate obtained by dividing the radial growth measured after 3, 4, or 7 days at 25ºC by the number of days.

[c] Optimum temperature calculated in the form $T_{opt}=F-(1/G)*\ln(H)$. Parameters F,G and H were obtained from the non-linear equation proposed by Duthie et al. (1997): $Y=((H+1)/H) * H^{1/(H+1)} * \exp(((T-F)*G/(H+1))/(1+\exp((T-F)*G)))$; where Y is the radial growth rate (referred to the maximum observed), T is the temperature (°C) and H, F, and G are the equation parameters.

Table 3. Pathogenicity of *Botryosphaeriaceae* species to loquat.

Species	Isolate	Lesion length (mm)[a]		Dead plants[c]
Diplodia eriobotryicola	BN-21	8.37 ± 0.46	bc[b]	1
Diplodia malorum	BN-35	13.37 ± 3.57	ab	2
Diplodia olivarum	BN-43	9.25 ± 1.62	bc	1
Diplodia pseudoseriata/ Diplodia alatafructa	BN-55	14.75 ± 6.78	ab	2
Diplodia sp.	BN-67	12.00 ± 4.02	a	6
Diplodia seriata	BN-3	7.00 ± 1.22	bc	1
Dothiorella eriobotryae	BN-81	7.37 ± 1.49	bc	1
Dothiorella sarmentorum	BN-32	6.37 ± 0.46	c	1
Neofusicoccum mediterraneum	BN-12	11.00 ± 2.87	bc	1
Neofusicoccum parvum	BN-66	11.87 ± 4.75	ab	1
Spencermartinsia plurivora	BN-97	7.75 ± 0.65	bc	1
Spencermartinsia viticola	BN-79	6.75 ± 1.10	bc	3
Control		6.12 ± 0.44	c	0

[a] Values represent the average in millimeters of extent of vascular discoloration (4 replications per experiment, 8 plants in total) measured from the point of inoculation ± standard error. [b] Means with the same letter are not significantly different according to LSD test at $P<0.05$.

[c] Number of dead plants after three months of incubation (4 replications per experiment, 8 plants in total).

Fig. 1

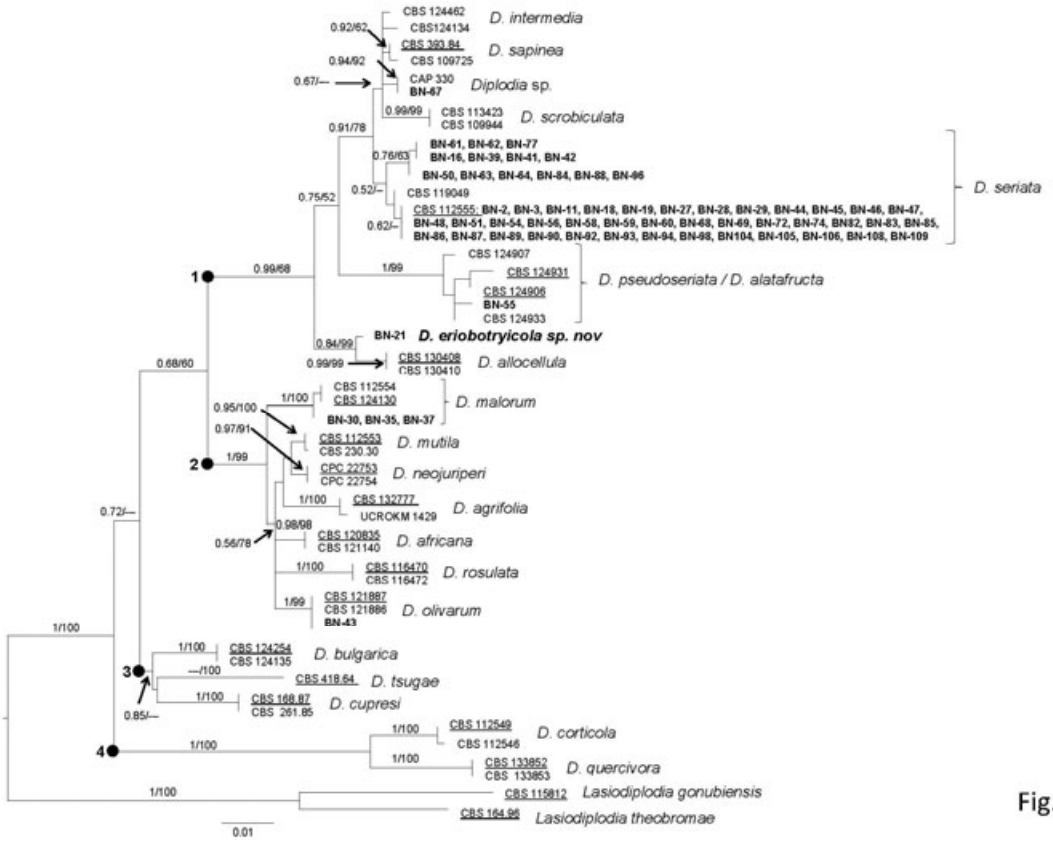

Fig. 2

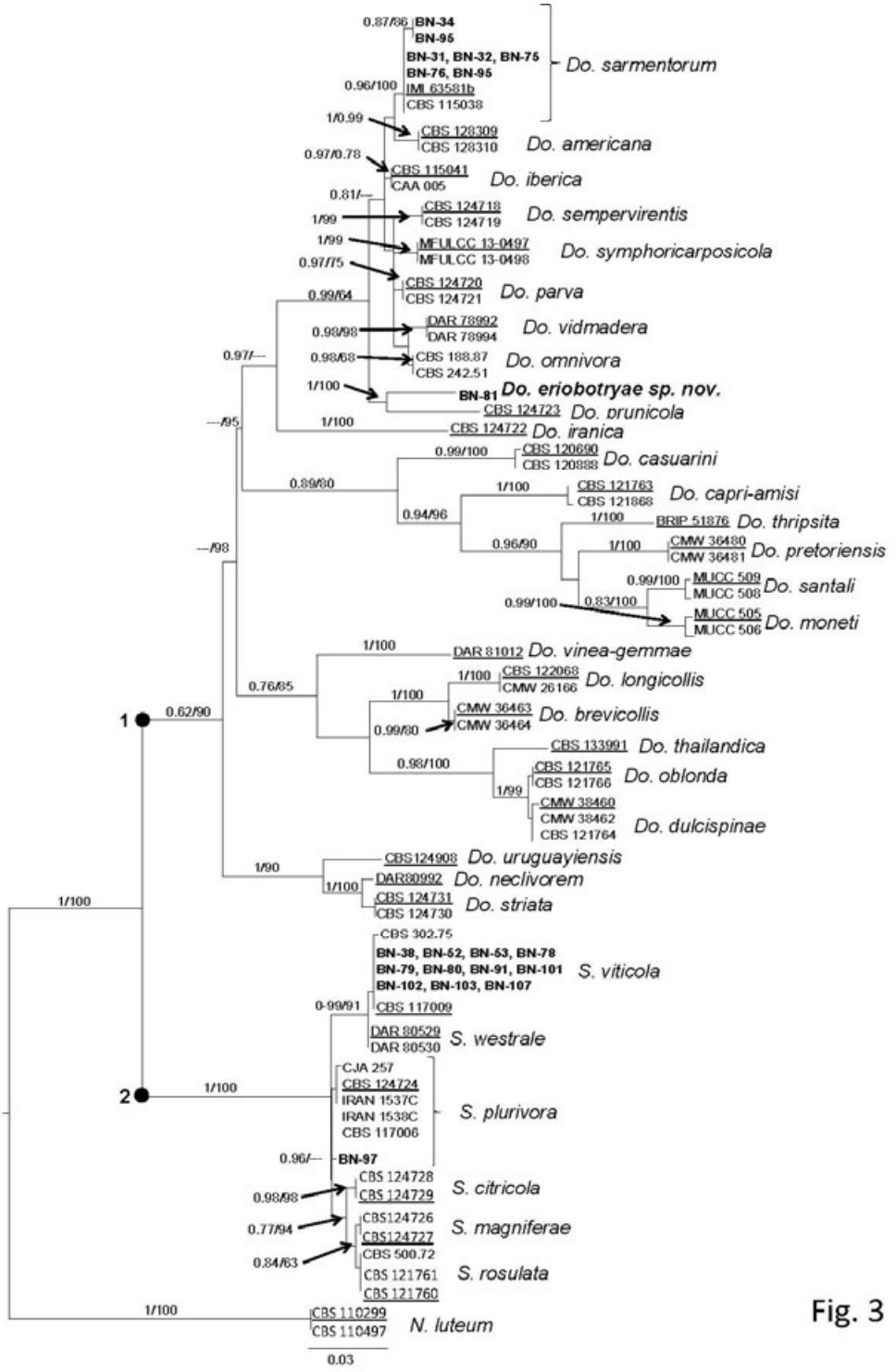

Fig. 3