A New Disease for Europe of Ficus microcarpa Caused by Botryosphaeriaceae Species

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Abstract: The Indian laurel-leaf fig (Ficus microcarpa) is an important ornamental tree widely distributed in the urban areas of Italy. Surveys conducted in 2019 and 2020 on several tree-lined streets, squares, and public parks in Catania and Siracusa provinces (Sicily, southern Italy) revealed the presence of a new disease on mature trees. About 9% of approximately 450 mature plants showed extensive branch cankers and dieback. Isolations from woody tissues obtained from ten symptomatic plants consistently yielded species belonging to the Botryosphaeriaceae family. The identification of the recovered fungal isolates was based on a multi-loci phylogenetic (maximum parsimony and maximum likelihood) approach of the ITS, tef1-α, and tub2 gene regions. The results of the analyses confirmed the presence of three species: Botryosphaeria dothidea, Neofusicoccum mediterraneum, and N. parvum. Pathogenicity tests were conducted on potted, healthy, 4-year-old trees using the mycelial plug technique. The inoculation experiments revealed that all the Botryosphaeriaceae species identified in this study were pathogenic to this host. Previous studies conducted in California showed similar disease caused by Botryosphaeriaceae spp., and the pathogenic role of these fungi was demonstrated. To our knowledge, this is the first report of Botryosphaeriaceae affecting Ficus microcarpa in Europe.

Keywords: canker; dieback; Indian laurel-leaf fig; Ficus microcarpa; Botryosphaeriaceae; phylogeny

1. Introduction

Ficus microcarpa, commonly known as Chinese or Malayan banyan, Indian laurel-leaf fig, and curtain fig, is a widely distributed evergreen ornamental species belonging to the family Moraceae, native to Ceylon, India, southern China, the Ryukyu Islands, Australia, and New Caledonia [1]. It is considered one of the most common urban trees in warm climates worldwide [2]. Moreover, F. microcarpa is also well known as an invader species due to its ability to grow in inhospitable places, its large fruit production, and its numerous dispersal agents (birds, bats, rodents, and others) [3]. Many Ficus spp. were introduced in southern Italy as ornamental species; they are now common in many urban areas and viewed as an important form of historical heritage [4]. Parks and gardens in urban areas are of significant value for all people living their daily lives in the cities. Urban trees have a positive impact on reducing heat, providing a convenient shelter, reducing wind velocity, and increasing the aesthetic value of the landscape [5–7]. In addition, most people living in cities deal with schedules, work, appointments, meetings, etc., and urban parks and open spaces positively affect mental health [8]. Thus, it is important not to underestimate the health of urban trees.

According to Fungal Database 53 records of fungus association with this host have been reported worldwide [9]. Among these, particular attention is given to species belonging to Botryosphaeriaceae. In fact, diseases caused by Botryosphaeriaceae are drawing the attention of the researchers worldwide, since they are a significant threat to many crops, especially in Mediterranean climates [10]. Botryosphaeriaceae include a large group of diverse fungal
species, distributed all over the world. These fungi are well known as plant pathogens, endophytes, and saprophytes of woody hosts [11]. Due to their role as plant pathogens, these fungal species have been studied for a long time, and their impact on forestry and agricultural production is well known [12]. *Botryosphaeriaceae* induce severe symptoms, such as branch, shoot, and trunk cankers, and blight fruits and leaves. 

*Botryosphaeriaceae* disease studies on *Ficus* spp., including the cultivated common fig (*F. carica*), have been published worldwide, showing that *Botryosphaeriaceae* and *Diaporthe* spp. are involved in complex diseases [13–29]. *Botryosphaeriaceae* cause polyetic epidemics (2–3 cycles per season); thus, the progress of epidemics may extend for several years [10]. In addition, *Botryosphaeriaceae*, characterized by a wide host range, can easily jump from one host to another; this is particularly evident in Mediterranean landscapes, where different crops are cultivated nearby [10]. Especially in the case of urban environments, it must be remembered that dangerous situations are related to the health status of the trees. Therefore, it is important to monitor the health of trees before they become hazardous [30]. Surveys conducted in the metropolitan area of Catania and Siracusa (Sicily), during 2019 and 2020 revealed many *F. microcarpa* distributed among numerous metropolitan areas, including gardens, public parks, tree-lined streets, and squares, showing severe symptoms of branch cankers and dieback. The aims of this study were to (i) investigate the etiology of the disease by (ii) characterizing the fungal isolates recovered from diseased trees based on a multi-loci phylogenetic analysis and (iii) assess their pathogenicity.

2. Results

2.1. Surveys and Fungal Isolations

*Ficus microcarpa* growing in a wide range of site conditions (tree-lined streets, gardens, public parks, and squares) have suffered a widespread dieback in Catania and Siracusa provinces. In the public areas where the research was conducted, more than 40 mature *F. microcarpa* trees (20 to 50 years old) showed cankered twigs and branches on approximately 450 plants. The trees still appeared green in part of the canopy, although this was accompanied by parts of branches and shoots that were defoliated and dead (Figures 1A–D and 2A). Sometimes, it was possible to observe new twigs growing below the damaged branches (Figure 1C). The sample consisted of large portions of branches showing severe internal wood discoloration, including of sapwood and the heartwood (Figure 2B–F). Often, the bark appeared cracked and split along the branches (Figure 2), and internal cankers were sharply demarcated from adjacent, healthy wood (Figure 2B–F). Isolations frequently (>70%) yielded *Botryosphaeriaceae*-like fungi, characterized, as reported by Slippers and Wingfield [11], by a ‘fluffy’ mycelium, either white-to-creamy, pigmented ‘greenish brown’, or gray-to-gray-black. Moreover, with lower frequencies, colonies of *Eutypella*-like species were also isolated from symptomatic tissues.
Figure 1. Symptoms of Botryosphaeriaceae disease observed in urban areas on F. microcarpa. (A) Diseased (left) and healthy (right) plants. (B–D) Defoliation and shoot dieback all over the canopy. (C) New twigs growing below the dead shoots.

Figure 2. Internal symptoms. (A) Dead branch showing cracking of the outer layers of the bark (upper), healthy branch (lower). (B–F) Internal cankers and bark cracked along the branch with diseased tissue sharply demarcated from adjacent, healthy wood. Scale bars: (B) = 15 cm; (C) = 50 cm; (D–F) = 20 cm.
2.2. Morphological Characterization and Phylogenetic Analysis

The PCR amplification of the ITS region, tef1-α, and tub2 generated 577 to 581, 273 to 288, and 422 to 446 bp fragments, respectively. The phylogenetic analyses were performed using a dataset of the three concatenated loci. The sequences generated in this study were deposited in GenBank (Table 1). A preliminary comparison of our sequences in GenBank showed our isolates belonging to the genera Botryosphaeria and Neofusicoccum. The Eutypella-like species showed high similarity with different Eutypella species submitted to GenBank. Since these isolates were excluded from the phylogenetic analyses due to their results in the pathogenicity test, they were identified as Eutypella spp. The phylogenetic analyses were then conducted only for the Botryosphaeriaceae. The results of the partition-homogeneity test indicated no \( (p = 1.00) \) significant differences in the three-gene dataset. The MP analysis of the combined dataset showed that of 2921 total characters, 391 were parsimony-informative, 220 were parsimony-uninformative, and 2310 were constant. In total, 100 trees were retained. Tree length was equal to 1098, CI = 0.707, RI = 0.912, RC = 0.644. The best-fit model of nucleotide evolution based on the AIC was GTR + I + G for ITS, GTR + G for tef1-α, and HKY + G for tub2. The ML analysis showed that of 2921 total characters, 2310 were constant, 475 were parsimony informative, and 136 were autapomorphic. The results of both analyses showed that the isolates FM1-3, FM6 and 7, and FM9 were grouped in the clade of B. dothidea (82/88, MP and ML bootstrap support %, respectively), the isolate FA10 grouped within N. mediterraneum clade (97/97), and FA1-3, FM8, FB4, and FB6 were grouped with the clade of N. parvum (97/98) (Figure 3). The conidia measurements were \((18.66)–22.7–(28.34) \times (3.61)–4.9–(6.38)\) for B. dothidea, \((14.0)–20.0–(27.2) \times (4.3)–5.8–(6.8)\) for N. mediterraneum, and \((12.78)–15.1–(16.9) \times (4.16)–5.3–(7.21)\) for N. parvum.

Table 1. Information on fungal isolates used in the phylogenetic analyses and their corresponding GenBank accession numbers. Isolates in bold are from this study.

| Scheme 1. Isolate ID | ITS           | tef1-α         | tub2          |
|----------------------|---------------|----------------|---------------|
| Botryosphaeria agaves| CBS 133992 = MFLUCC 11-012ST | JX646791 | JX646856 | JX646841 |
| B. agaves            | CBS 141505 = CPC 26299 | KX306750 | MT592030 | MT592463 |
| B. corticis          | CBS 119047T   | DQ299245 | EU017539 | EU637107 |
| B. corticis          | CBS 119048 = CAP 198 | DQ299246 | EU101754 | MT592464 |
| B. dothidea          | CBS 115476 = CMW 8000T | AY236949 | AY236898 | AY236927 |
| B. dothidea          | CBS 110302 = CAP 007 | AY259092 | AY573218 | EU637106 |
| B. dothidea          | FM1           | OM241975 | OM262426 | OM262439 |
| B. dothidea          | FM2           | OM241976 | OM262427 | OM262440 |
| B. dothidea          | FM5           | OM241977 | OM262428 | OM262441 |
| B. dothidea          | FM6           | OM241978 | OM262429 | OM262442 |
| B. dothidea          | FM7           | OM241979 | OM262430 | OM262443 |
| B. dothidea          | FM9           | OM241980 | OM262431 | OM262444 |
| B. dothidea          | FM9           | OM241980 | OM262431 | OM262444 |
| B. fabicerciana      | CBS 118831 = CMW 14009 | DQ316084 | MT592032 | MT592468 |
| B. fabicerciana      | CBS 127193 = CMW 27094T | HQ332197 | HQ332213 | KF779068 |
| B. kuwatsukai        | CGMCC 3.18007 | KX197074 | KX197094 | KX197101 |
| B. kuwatsukai        | CGMCC 3.18008 | KX197075 | KX197095 | KX197102 |
| B. qingyuanensis     | CERC 2946 = CGMCC 3.18742T | KX278000 | KX278105 | KX278209 |
| B. ramosa            | CERC 2947 = CGMCC 3.18743 | KX278001 | KX278106 | KX278210 |
| B. ramosa            | CERC 2001 = CGMCC 3.187396 | KX277989 | KX278094 | KX278198 |

Guignardia philipina
Neofusicoccum arbuti
N. arbuti
N. austral
N. brasiliense
N. brasiliense
N. cordaticola
N. cordaticola
N. cryptostilicola
N. diastema
N. eucalypticola
N. eucalypticola
N. eucalypticola
N. eucalypticola
N. eucalypticola
N. eucalypticola
**Table 1. Cont.**

| Scheme 1. | Isolate ID | ITS | tef1-α | tub2 |
|-----------|------------|-----|--------|------|
| **N. eucalyptorum** | CBS 115791 = CMW 10125 = BOT 24T | AF283686 | AY236891 | AY236920 |
| N. eucalyptorum | CBS 145975 = CPC 29337 | MT587477 | MT592190 | MT592682 |
| **N. hellenicum** | CERC 1947 = CPCF 500678 | MT587103 | MT592206 | MT592606 |
| **N. hongkongense** | CBS 139674 = CMW 41469T | KP860881 | KP860724 | KP860801 |
| **N. luteum** | CBS 110497 = CPC 4594 = CAP 037 | EU673311 | EU673277 | EU673092 |
| **N. macroclavatum** | CBS 118823 = CMW 15955 = WAC 1244T | DQ093196 | DQ093217 | DQ093206 |
| N. mangiferae | CBS 118531 = CMW 7024T | AY615185 | DQ093221 | AY615173 |
| **N. microconidium** | CBS 118821 = CPC 4594 = CAP 002T | EU673277 | EU673092 | EU673092 |
| **N. nonquaesitum** | CBS 126655 = L3IE1 = PD484T | GU251163 | GU251295 | GU251823 |
| **N. occulatum** | CBS 128008 = MUCC 227T | EU339509 | EU339472 | EU339472 |
| **N. parvum** | CBS 138823 = ICMP 8003 | AY259091 | AY573221 | EU673095 |
| **N. pennatisporum** | WAC 13153 = MUCC 510T | EF591925 | EF591969 | EF591975 |
| **N. potato*<sup>α</sup>** | CBS 113178 = CMW 35498 | MT587526 | MT587513 | MT587513 |
| **N. potato** | CBS 113178 = CMW 35499 | MT587509 | MT587526 | MT587513 |
| **N. protearum** | CBS 114176 = CPC 1775 = JT 189T | AF452539 | AF452470 | AF452470 |
| **N. protearum** | CBS 115177 = CPC 4357 | FJ150703 | MT592292 | MT592704 |
| **N. ribis** | CBS 124923 = CMW 28320 | FJ900608 | FJ900645 | FJ900635 |
| **N. ribis** | CBS 124924 = CMW 28320 | FJ900607 | FJ900653 | FJ900634 |
| **N. ribis** | CBS 123645 = CMW 14085T | EU821904 | EU821874 | EU821844 |
| **N. ribis** | CBS 123646 = CMW 14060 | EU821905 | EU821875 | EU821845 |
| **N. sinense** | CGMCC3.18315T | KY350148 | KY871755 | KY350154 |
| **N. sinoeucalypti** | CBS 22005 = CGMCC3.18752T | KX278061 | KX278166 | KX278270 |
| **N. sinicola** | CBS 110864 = CPC 4598 | AY343407 | AY343348 | AY345047 |
| **N. terminaliae** | CBS 125264 = CMW 26683 | GQ471804 | GQ471782 | GQ470535 |
| **N. ussuriensis** | CBS 122811 = CMW 24480T | FJ752746 | FJ752709 | FJ752606 |
| **N. viticola** | CBS 112977 = STE-U 5041 | AY343380 | AY343341 | AY345059 |
| **N. vitifusiforme** | CBS 110887 = CPC 5252 = JM5T | AY343383 | AY343343 | AY345061 |
| **Phyllosticta citricarpa** | CBS 102374 | FJ824776 | FJ538371 | FJ824778 |

T: Type material.
2.3. Pathogenicity Test

The results of the pathogenicity test showed that all three species of Botryosphaeriaceae identified in this study were pathogenic to *F. microcarpa*. Otherwise, the *Eutypella* sp. isolate inoculated did not induce any lesions on the woody tissues, which was similar to the control. For this reason, this species was excluded from the phylogenetic analyses.

External discoloration out of the inoculation point was observed after 7 days and all the inoculated trees showed severe wood discoloration after the outer layer of bark was removed (Figure 4A–D). Moreover, young twigs close to the inoculation point rapidly wilted.
a few days after inoculation. Specifically, among the fungal species, the *N. mediterraneum* isolate FA10 induced the longest lesions (mean 8.10 cm), followed by *N. parvum* isolate FB4 (2.66 cm) and *B. dothidea* isolate FM2 (1.88 cm). All the inoculated species statistically differed from the control (*p* < 0.05) (Figure 5). The colonies that emerged from the re-isolations showed morphological characteristics (color, shape, and mycelium texture) that fulfilled the Koch’s postulates.

![Figure 4. Results of pathogenicity test after two weeks. (A) Neofusicoccum mediterraneum. (B) N. parvum. (C) Botryosphaeria dothidea. (D) Control. Scale bar = 10 cm.](image)

![Figure 5. Comparisons of average lesion length (cm) resulting from pathogenicity test among B. dothidea, N. mediterraneum and N. parvum on potted plants. Columns are the means of 15 inoculation points (five per plant) for each fungal species. Control consisted of 12 inoculation points. Vertical bars represent the standard error of the means. Bars topped with different letters indicate treatments that were significantly different according to Fisher’s protected LSD test (*α* = 0.05).](image)

3. Discussion

The results of our study confirm, for the first time, the presence of three species, *B. dothidea, N. mediterraneum*, and *N. parvum*, affecting *F. microcarpa* in Italy. Regarding *Botryosphaeriaceae*, little is known about its association with *F. microcarpa*. According to the
U.S. National Fungus Collections Fungal Database [9], only a few, old reports describe the association of Lasiodiplodia theobromae (as Botryodiplodia theobromae) in Pakistan [31] and Egypt [29], and Diplodia fici-retusa in Taiwan on Ficus retusa (synonymous of F. microcarpa) [32,33]. A commonly reported disease of F. microcarpa, as well as other Ficus spp., is “sooty canker”, which is caused by Neoscytalidium dimidiatum (traditionally reported also as Hendersonula toruloidea and Natrassia mangiferae). The pathogen, as well as other Botryosphaeriaceae, induces cankers and dieback, often accompanied by a powdery mass of black spores (arthroconidia) produced by this species [14,18–21,26,28,34]. Recently, in California B. dothidea, N. luteum, N. mediterraneum, and N. parvum were reported as causing branch cankers and dieback on F. microcarpa trees in Los Angeles County [25]. In recent years, Botryosphaeriaceae spp. have been reported attacking many different crops in Italy, and, especially in Sicily, it is well known that these species spread from nurseries to the open field, from ornamental plants to the agricultural ones. Specifically, B. dothidea has recently been reported in Sicily on walnut and pistachio [35,36]. Moreover, N. mediterraneum and N. parvum have been reported as highly aggressive pathogens among the Botryosphaeriaceae in Sicily [13,35,37,38]. In addition, N. mediterraneum was the most encountered species in Sicilian pistachio orchards [36]. From this and previous studies conducted in Sicily, it emerged that Botryosphaeriaceae spp., and especially the species described in this study, are easily encountered in different hosts and landscapes. Regarding the ecology of these fungi, it is well known that they are also endophytes on many hosts [11], often coexisting in the same tissues [39] and forming long latent infections [40,41]. This must be taken into serious consideration, since many infections can spread from nurseries (as latent infections) to open fields. Recently, studies conducted in California on latent infections on nut crops helped us to properly quantify these pathogens using real-time PCR assays [40,42,43]. The ability of these fungi to disperse their spores (conidia) by wind, rain, and insects [10] in conjunction with intercontinental human movements with no adequate quarantine strategies led them to easily spread all over the world [44], as demonstrated for N. parvum, the most adapted organism, which is detected from the north to the south, excluding boreal forests and montane grasslands [45]. Many factors can be involved in the ability of some Botryosphaeriaceae species to jump from one host to another, meaning that they are more virulent than other species. Among these, a recent study [46] revealed how some groups of taxa, such as Botryosphaeria, Lasiodiplodia, and Neofusicoccum, show an expansion of certain clades of gene families involved in the pathogenesis. Specifically, in the Botryosphaeria and Neofusicoccum genomes, an expansion of secreted cell-wall-degrading enzymes (CAZymes) was observed [46]. It is no surprise that the species identified in this study also occurred on other taxonomically distant hosts in Sicily. Batista et al. [45] showed that B. dothidea is associated with 403 hosts in 66 countries, and N. parvum with 223 hosts in 50 countries. In recent decades, in Sicily, a relevant increase was observed in Botryosphaeriaceae in nurseries, as well as in open fields (Polizzi G., unpublished data). Botryosphaeriaceae disease expression is strongly related to stresses due to factors other than the Botryosphaeriaceae infection itself [47–49]. Related to this, it should be noticed that climate change contributes to additional stress or pressure on woody plants through extreme weather conditions or the expansion of pathogens’ host ranges [11]. In fact, climate change affects the dynamics of fungal populations, in terms of biology and ecology [49]. Gange et al. [50] conducted a study in the UK on the species Auricularia auricula-judae, demonstrating an alteration in the phenology (the earlier appearance of fruit bodies and a longer fruiting period) and an expansion of the host range consistent with a response to observed warming trends in the climate, also suggesting that climate change affected the interactions between wood-inhabiting fungi. Combative interactions are considered the main drivers of fungal community development in decaying wood [51,52], and these can be strongly affected by temperature, water potential, gaseous regime, and resource size [53–55]. All these factors contribute to making Botryosphaeriaceae disease severe and ubiquitous, compared with otherwise “mild diseases” [56]. Urban areas, which are even less investigated than agricultural ones, must be considered crucial routes of introduction
and dissemination for Botryosphaeriaceae [57]. It is well known that stressed trees are much more predisposed to Botryosphaeriaceae disease [11,58], and this should be taken into careful consideration regarding ornamental trees in urban landscapes. In fact, trees grown in urban areas can also be considered more exposed to stress factors [59], and thus more susceptible to Botryosphaeriaceae disease. This could represent a serious threat in urban areas, not only in terms of aesthetic damage, but mostly in terms of public safety. In relation to these predisposing factors, we ascertained during our investigation that F. microcarpa trees grown in the urban areas of Catania and Siracusa provinces were severely and improperly pruned, especially during the humid seasons. In order to avoid the spread of Botryosphaeriaceae species, some recommendations should be taken into serious consideration. Since it is known that both rainfall and fog [60,61] positively affect the release of Botryosphaeriaceae spores, farmers or pruning crews should not prune when rain is forecasted or with dense fog to avoid the contamination of fresh wounds by Botryosphaeriaceae [62]. Moreover, recommendations as to pruning type depend on the tree species, which is why trained pruning crews should be selected for this crucial practice. As demonstrated on pistachio, Botryosphaeria panicle and shoot blight were reduced by 50–60% by trained pruning crews compared to the disease levels in trees pruned by unspecialized crews [63]. Furthermore, in California, field experiments conducted on F. carica affected by fig limb dieback demonstrated that pruning 5 cm below the canker successfully removed the pathogen from the tissues [36]. Regarding trained pruning crews, it is crucial that workers disinfect their pruning tools, since these could easily transmit inoculum (spores, mycelium, and fruit bodies) from one tree to another. As demonstrated on walnut, pathogen spores were transferred from the chainsaws to the agar media, whereas Botryosphaeriaceae species were not found when the chainsaws were disinfected with a 2% dilution of vinegar or commercial household bleach (T.J. Michailides, unpublished data/personal communication). In addition, the usage of biological control agents as protectants for pruning wounds, especially in urban areas, should be considered. Encouraging results have been obtained on other crops, such as almond and grapevine treated with Trichoderma-based formulants against canker pathogens [64–66]. Further investigations need to be conducted in this direction. Good agronomic practices and, possibly, the usage of biocontrol agents, can help us to control Botryosphaeriaceae disease in urban areas. To our knowledge, this is the first study of Botryosphaeriaceae disease on F. microcarpa in Europe.

4. Materials and Methods

4.1. Surveys and Fungal Isolations

During the years between 2019 and 2020, surveys were carried out in numerous urban areas of the cities of Catania (Catania province), and Siracusa (Siracusa province), Sicily, where F. microcarpa were the most prevalent ornamental trees, including tree-lined streets, gardens, public parks, and squares. Several symptomatic samples obtained from ten plants were collected and brought to the laboratory of the Dipartimento di Agricoltura, Alimentazione e Ambiente, University of Catania, for further investigations. For culture isolation, small sections (0.2 to 0.3 cm²) of symptomatic tissues (branches and shoots) were surface-disinfected for 1 min in 1.5% sodium hypochlorite, rinsed in sterile water, dried on sterile absorbent paper under laminar hood and placed on potato dextrose agar (PDA, Lickson, Vicari, Italy) amended with 100 mg/liter of streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) (PDAS) to prevent bacterial growth, and then incubated at 25 ± 1 °C for 3–5 days until fungal colonies were large enough to be examined. Subsequently, colonies of interest were transferred to fresh PDAS to make pure cultures, and then single-hyphal tip cultures were obtained and maintained on PDAS at 25 ± 1 °C. Isolates characterized in this study were stored in the fungal collection of the laboratory with the labels FA, FB, and FM.
4.2. Morphological and Molecular Characterization

For the morphological characterization of the pathogens, the length and width of 50 conidia from the 21-day-old colonies of the isolates FM1, FA10, and FA1 grown on PDA were measured using a fluorescence microscope (Olympus-BX61) coupled to an Olympus DP70 digital camera; measurements were captured using software analysis 3.2 (Soft Imaging System GmbH, Münster, Germany). Dimensions are reported as the minimum and maximum in parentheses and the average. Total fungal DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), scraping the mycelium with a sterile scalpel from 5-day-old fungal cultures grown on PDA or malt extract agar (MEA, Oxoid LTD. Basingstoke, Hampshire, England) media. The genomic DNA extracted was visualized on 1% agarose gels (90 V for 40 min) stained with GelRed® (Biotium, Fremont, CA, USA). The quality of the DNA was determined through Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The internal transcriber spacer region (ITS) of the nuclear ribosomal RNA operon was amplified with primers ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') [67]; the primers EF1-728F (5'-CAT CGA GAA GTT CGA GAA GG-3') and EF1-986R (5'-TAC TTG AAG GAA CCC TTA CC-3') [68] were used to amplify part of the translation elongation factor 1alpha gene (tef1-α); and primer sets Bt2a (5'-GGT AAC CAA ATC GGT GCT TTC-3') and Bt2b (5'-ACC CTC AGT GTA GTG ACC CTT GGC-3') [69] were used for the partial beta tubulin (tub2). Amplification by polymerase chain reaction (PCR) was performed in a total volume of 25 µL using One Taq® 2X Master Mix with Standard Buffer (BioLabs, New England, NEB), according to the manufacturer’s instructions, on an Eppendorf Mastercycler (AG 22331 Hamburg, Germany). The thermal cycle consisted of initial 30 s at 94 °C, followed by 35 cycles at 94 °C for 30 s, 49 °C (ITS), 57–59 °C (tef1-α), or 52 °C (tub2) for 1 min, 68 °C for 1 min, and 5 min at 68 °C. Regarding Botryosphaeriaceae, in total, 45 isolates were sequenced (tub2) and only 13 representative isolates were considered for further gene sequencing and phylogenetic analyses. Concerning the Eutypella-like species, a total of 7 isolates were sequenced (ITS and tub2). PCR products were visualized on 1% agarose gels (90 V for 40 min), purified, and sequenced by Macrogen Inc. (Seoul, South Korea). Forward and reverse DNA sequences were assembled and edited using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms [70] and submitted to GenBank.

4.3. Phylogenetic Analysis

Chromatograms were viewed using FinchTV Version 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com (accessed on 16 February 2022)). Sequences were read and edited using MEGAX. Before constructing the phylogenetic tree, BLAST searches were performed using the NCBI nucleotide database [71]. ITS, tef1-α, and tub2 DNA sequence datasets were aligned using MEGAX, and manual alignments were performed when necessary. A partition-homogeneity test with heuristic search and 1000 homogeneity replicates was performed using PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0a (Sinauer Associates, Sunderland, MA, USA) [72] to test for discrepancies in the three-gene dataset. For comparison, 79 additional sequences were selected according to the recent literature on the Botryosphaeriaceae [73,74] to be included in the alignment (Table 1). Maximum parsimony analysis (MP) was performed in PAUP v.4.0a. The analysis of the combined dataset (ITS + tef1-α + tub2) was performed with the heuristic search function and tree bisection and reconstruction (TBR) as branch-swapping algorithms with the branch-swapping option set to ‘best trees’ only. Gaps were treated as ‘missing’, the characters were unordered and of equal weight, and Maxtrees were limited to 100. Tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated. To identify the best-fit model of nucleotide evolution for each gene according to the Akaike information criterion (AIC), MrModeltest v. 2.4 [75] was used. The maximum likelihood analysis (ML) of the combined genes was performed in GARLI v.0.951 [76]. For both analyses, clade support was assessed by 1000 bootstrap replicates. Guignardia
Philoprina (CBS 447.68) and Phyllosticta citricarpa (CBS 102374) served as the outgroup in both analyses.

4.4. Pathogenicity Test

Pathogenicity tests were conducted on potted, healthy, 4-year-old *F. microcarpa* plants maintained at room temperature. For each fungal species, one representative isolate was inoculated. Specifically, three plants were used for each isolate, and five inoculation points were chosen along the trunk on each plant (~30 cm distant one from each other). The inoculation site was first surface-disinfected by spraying with 70% ethanol solution, and wounds were made with a sterilized 6-millimeter cork borer after removing the bark, and a mycelium plug (6 mm in diameter) was placed upside down into the plant tissue wound. Wounds were sealed with Parafilm® (Pechney Plastic Packaging Inc., Chicago, IL, USA). In total, 12 additional wounds were inoculated with sterile PDA plugs as controls. Plants were regularly watered. The presence and length of the resulting lesions were recorded two weeks after the inoculation. Lesion length measurements were analyzed in Statistix 10 [77] via analysis of variance (ANOVA), and mean differences were compared with the Fisher’s protected least significant difference (LSD) test at $\alpha = 0.05$. In order to fulfil Koch’s postulates, re-isolations were carried out on PDAS following the procedure described above. Each re-isolated fungus was identified through the observation of colony characteristics.

5. Conclusions

In the present study, three species of *Botryosphaeriaceae* were isolated from symptomatic samples of *F. microcarpa* showing severe symptoms of cankers, wood discoulourations, bark cracking, and dieback. Morphological and molecular tools identified *B. dothidea*, *N. mediterraneum*, and *N. parvum*. Pathogenicity tests fulfilled Koch’s postulates. The results of this study provide new information on this important family of phytopathogenic fungi and its wide host range. This is the first report in Europe of *Botryosphaeriaceae* affecting *F. microcarpa*.

Author Contributions: Conceptualization, D.A., A.F. and G.P.; methodology, A.F., M.B.C. and G.G.; software, G.G.; validation, G.P.; formal analysis, G.G.; investigation, G.P.; resources, G.P.; data curation, G.G.; writing—original draft preparation, G.G.; writing—review and editing, D.A., A.F., G.G. and G.P.; visualization, G.P.; supervision, D.A. and G.P.; project administration, G.P.; funding acquisition, G.P. All authors have read and agreed to the published version of the manuscript.

Funding: Programma Ricerca di Ateneo MEDIT-ECO UNICT 2020–2022 Linea 2-University of Catania (Italy); Starting Grant 2020, University of Catania (Italy); Fondi di Ateneo 2020–2022, University of Catania (Italy), Linea Open Access. Research Project 201–62018, University of Catania 5A722192134.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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