Identification and Characterization of *Diaporthe* spp. Associated with Twig Cankers and Shoot Blight of Almonds in Spain

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Abstract: Two hundred and twenty-five *Diaporthe* isolates were collected from 2005 to 2019 in almond orchards showing twig cankers and shoot blight symptoms in five different regions across Spain. Multilocus DNA sequence analysis with five loci (ITS, tub, tef-1α, cal and his), allowed the identification of four known *Diaporthe* species, namely: *D. amygdali*, *D. eres*, *D. foeniculina* and *D. phaseolorum*. Moreover, a novel phylogenetic species, *D. mediterranea*, was described. *Diaporthe amygdali* was the most prevalent species, due to the largest number of isolates (85.3%) obtained from all sampled regions. The second most frequent species was *D. foeniculina* (10.2%), followed by *D. mediterranea* (3.6%), *D. eres* and *D. phaseolorum*, each with only one isolate. Pathogenicity tests were performed using one-year-old almond twigs cv. Vayro and representative isolates of the different species. Except for *D. foeniculina* and *D. phaseolorum*, all *Diaporthe* species were able to cause lesions significantly different from those developed on the uninoculated controls. *Diaporthe mediterranea* caused the most severe symptoms. These results confirm *D. amygdali* as a key pathogen of almonds in Spain. Moreover, the new species, *D. mediterranea*, should also be considered as a potential important causal agent of twig cankers and shoot blight on this crop.
Keywords: Diaporthe amygdali; D. mediterranea; multilocus DNA sequence analysis; pathogenicity; Prunus dulcis

1. Introduction

The worldwide cultivated area for almond (Prunus dulcis (Mill.) D.A. Webb) is over 2,000,000 ha. Spain, with 657,768 ha, is the country with the largest area for almond production in the world, followed by the United States, with 441,107 ha [1]. Almond is the second largest tree crop in Spain, after olive, and it is widely distributed in all regions of the country [2]. Nevertheless, Spain only contributes approximately 10% to world almond production, because the trees have been traditionally grown under rain-fed conditions and planted in marginal areas with poor soils, low rainfall and a high incidence of frost [3], thus presenting low average yields (5154 kg ha$^{-1}$) [1].

In recent years, almond production in Spain has been experiencing a highly favorable period, in which crop intensification, with the introduction of drip irrigation and the use of new highly productive cultivars, has increased the yield in new plantations [4]. However, the incidence of almond-associated fungal diseases, such as twig cankers and shoot blight caused by Diaporthe spp., is increasing and compromises crop productivity, especially in coastal areas with higher humidity and milder temperatures [5,6].

Diaporthe amygdali (Delacr.) Udayanga, Crous and K.D. Hyde is considered the causal agent of twig canker and shoot blight of almond and peach (Prunus persica (L.) Batsch) [7,8]. Symptoms of this disease are characterized by the quick desiccation of buds, flowers and leaves in late winter or early spring. Brown lesions (1 to 5 cm diameter), initially formed around buds on green shoots, further develop into annual sunken cankers, sometimes with a gummy exudate, as well as withering of twigs. As a result, leaves wilt and, when the disease is severe, defoliation can occur. In summer, pycnidia develop just under the dry canker epidermis [7,9,10].

The species D. amygdali was first described as Fusicoccum amygdali Delacr., associated with almond cankers in France [11]. Tuset and Portilla [9] re-examined the type specimen of F. amygdali and, based on morphology and symptomatology, they re-classified this fungus into Phomopsis as P. amygdali (Delacr.) J.J Tuset and M.T. Portilla. Additionally, they also considered P. amygdalina Canonaco to be a synonym of P. amygdali. Diogo et al. [8] used morphological, molecular and pathogenicity data to clarify the identity of a collection of Phomopsis isolates obtained from almond in Portugal. In this research, as no cultures of P. amygdali were linked unequivocally to any existing type, the authors proposed the fungus in voucher CBS-H 20420 (from Portugal) as the epitype for this species (isolate CBS 126679). Udayanga et al. [12] re-evaluated the phylogenetic species recognition in the genus Diaporthe using a multi-locus phylogeny based on the internal transcribed spacer (ITS) region of the nuclear rDNA, and partial sequences from translation elongation factor 1-α (tef-1α), β-tubulin (tub) and calmodulin (cal) genes. In this study, P. amygdali was transferred into Diaporthe as D. amygdali based on multi-locus DNA sequence data.

In recent years, the taxonomy of the genus Diaporthe has been deeply revised. The generic names Diaporthe and Phomopsis are no longer used to distinguish different morphs of this genus, as Rossman et al. [13] proposed that the genus name Diaporthe should be retained over Phomopsis because: (i) it was introduced before Phomopsis and (ii) Diaporthe represents the majority of species described, and therefore it has priority over Phomopsis. Diaporthe was historically considered as monophyletic based on its typical sexual morph and Phomopsis’s asexual morph [14]. However, Gao et al. [15] revealed its paraphyletic nature. Recent studies have demonstrated that morphological characters are inadequate to define species in this genus [16], due to their variability under changing environmental conditions [14]. Therefore, genealogical concordance methods based on multi-gene DNA sequence data provide a better approach to resolving the taxonomy for Diaporthe [17].

Literature about recent characterization studies of collections of Diaporthe isolates, obtained exclusively from almonds or including them together with isolates from other fruit or nut crops, is very scarce. Diogo et al. [8] examined Diaporthe isolates from almond and other Prunus species.
in Portugal through combining morphology, pathogenicity data and a phylogenetic study based only on ITS sequences. These authors concluded that *D. amygdali* was the main species on almond, reported *D. neotheicola* for the first time on this host and a third species represented by a single isolate, which could not be unequivocally identified. Later, Lawrence et al. [18] characterized morphologically different *Diaporthe* isolates associated with wood cankers of fruit and nut crops in northern California, including three almond isolates, which were assigned to the species *D. australafricana* and *D. novem*, based on multi-gene, ITS, tef-1a and cal sequence analyses.

In Spain, the studies of Tuset and Portilla [9] and Tuset et al. [10] described almond diseases and their associated pathogens, including *D. amygdali*. These studies were based solely on the morphological characterization of the isolates. Additional studies using molecular tools to ascertain the identity of representative sets of *Diaporthe* isolates from almond in this country are lacking. Gramaje et al. [19] reported only one isolate of *D. amygdali*, which was collected from a survey of wood-associated fungal trunk pathogens of almond trees on the island of Mallorca. Thus, the objectives of the present study were: (i) to characterize a wide collection of *Diaporthe* isolates collected from almond trees in Spain by means of phenotypical characterization (fungal morphology and temperature growth) and DNA sequence analyses and (ii) to evaluate the pathogenicity of these *Diaporthe* isolates to almond twigs. The final goal was to obtain updated and more complete information about the *Diaporthe* species causing twig cankers and shoot blight of almonds in Spain.

2. Materials and Methods

2.1. Sampling and Isolation

A total of 225 *Diaporthe* isolates were collected from 2005 to 2019 in almond orchards showing twig cankers and shoot blight symptoms (Figure 1) in five different regions across Spain (Andalucía (n = 56), Islas Baleares (n = 39), Cataluña (n = 43), Comunidad Valenciana (n = 76) and La Rioja (n = 11)). For isolation, wood segments with cankers were cut from the affected branches, washed under running tap water, surface disinfected for 1 min in a 1.5% sodium hypochlorite solution and rinsed twice in sterile distilled water. Small pieces of affected tissues taken from the margin of the lesions were plated on potato dextrose agar (PDA; Biokar-Diagnostics, Zac de Ther, France) supplemented with 0.5 g/L of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA) (PDAS). Plates were incubated at 25 °C in the dark for 7 to 10 d, and all colonies were transferred to PDA. All isolates were hyphal-tipped and maintained in 15% glycerol solution at −80 °C in 1.5 mL cryovials in the fungal collection of the Instituto Agroforestal Mediterráneo–Universitat Politècnica de València (IAM-UPV) (Spain) (Table 1).

![Figure 1. Twig canker and shoot blight symptoms caused by *Diaporthe* spp. on almond.](image-url)
### Table 1. Collection details and GenBank accession numbers of isolates included in this study.

| Species    | Strain Number | Year | Location            | Province/Region      | GenBank Accession Numbers |
|------------|---------------|------|---------------------|----------------------|---------------------------|
|            |               |      |                     |                      | **ITS** | **tef-1α** | **tub** | **his** | **cal** |
| *D. amygdali* |               |      |                     |                      |              |            |        |        |        |
| DAL-1      | 2014          | Sant Joan Mallorca/Islas Baleares | MT007292 MT006769 MT006466 | - | - |
| DAL-2      | 2014          | Sant Joan Mallorca/Islas Baleares | MT007293 MT006770 MT006467 | - | - |
| DAL-3      | 2014          | Santa Margalida Mallorca/Islas Baleares | MT007294 MT006771 MT006468 MT006997 MT006694 | - | - |
| DAL-4      | 2014          | Santa Margalida Mallorca/Islas Baleares | MT007295 MT006772 MT006469 MT006998 MT006695 | - | - |
| DAL-5      | 2014          | Calvia Mallorca/Islas Baleares | MT007296 MT006773 MT006470 | - | - |
| DAL-7      | 2014          | Calvia Mallorca/Islas Baleares | MT007297 MT006774 MT006471 MT006999 | - | - |
| DAL-9      | 2014          | Calvia Mallorca/Islas Baleares | MT007298 MT006775 MT006472 MT007000 MT006696 | - | - |
| DAL-12     | 2014          | Binissalem Mallorca/Islas Baleares | MT007299 MT006776 MT006473 MT007001 | - | - |
| DAL-13     | 2014          | Llucmajor Mallorca/Islas Baleares | MT007300 MT006777 MT006474 | - | - |
| DAL-14     | 2014          | Llucmajor Mallorca/Islas Baleares | MT007301 MT006778 MT006475 | - | - |
| DAL-15     | 2014          | Marratxi Mallorca/Islas Baleares | MT007302 MT006779 MT006476 MT007002 | - | - |
| DAL-16     | 2014          | Sa Pobla Mallorca/Islas Baleares | MT007303 MT006780 MT006477 MT007003 MT006697 | - | - |
| DAL-17     | 2014          | Sa Pobla Mallorca/Islas Baleares | MT007304 MT006781 MT006478 | - | - |
| DAL-18     | 2014          | Inca Mallorca/Islas Baleares | MT007305 MT006782 MT006479 MT007004 | - | - |
| DAL-19     | 2014          | Binissalem Mallorca/Islas Baleares | MT007306 MT006783 MT006480 MT007005 | - | - |
| DAL-20     | 2014          | Palma Mallorca/Islas Baleares | MT007307 MT006784 MT006481 | - | - |
| DAL-21     | 2014          | Binissalem Mallorca/Islas Baleares | MT007308 MT006785 MT006482 | - | - |
| DAL-22     | 2014          | Llucmajor Mallorca/Islas Baleares | MT007309 MT006786 MT006483 MT007006 | - | - |
| DAL-23     | 2014          | Inca Mallorca/Islas Baleares | MT007310 MT006787 MT006484 | - | - |
| DAL-32     | 2017          | Alcalali Alicante/Comunidad Valenciana | MT007313 MT006790 MT006487 | - | - |
| DAL-33     | 2017          | Alcalali Alicante/Comunidad Valenciana | MT007314 MT006791 MT006488 | - | - |
| DAL-35     | 2017          | Alcalali Alicante/Comunidad Valenciana | MT007315 MT006792 MT006489 | - | - |
| DAL-36     | 2017          | Alcalali Alicante/Comunidad Valenciana | MT007316 MT006793 MT006490 | - | - |
| DAL-37     | 2017          | Alcalali Alicante/Comunidad Valenciana | MT007317 MT006794 MT006491 | - | - |
| DAL-38     | 2017          | Alcalali Alicante/Comunidad Valenciana | MT007318 MT006795 MT006492 | - | - |
| DAL-39     | 2017          | Alcalali Alicante/Comunidad Valenciana | MT007319 MT006796 MT006493 | - | - |
| DAL-40     | 2017          | Alcalali Alicante/Comunidad Valenciana | MT007320 MT006797 MT006494 | - | - |
| DAL-41     | 2017          | Alcalali Alicante/Comunidad Valenciana | MT007321 MT006798 MT006495 | - | - |
| DAL-42     | 2017          | Alcalali Alicante/Comunidad Valenciana | MT007322 MT006799 MT006496 MT007008 MT006699 | - | - |
| DAL-43     | 2017          | Bunyola Mallorca/Islas Baleares | MT007323 MT006800 MT006497 MT007009 MT006700 | - | - |
| DAL-44     | 2017          | Bunyola Mallorca/Islas Baleares | MT007324 MT006801 MT006498 | - | - |
| DAL-45     | 2017          | Bunyola Mallorca/Islas Baleares | MT007325 MT006802 MT006499 MT007010 MT006701 | - | - |
| DAL-46     | 2017          | Bunyola Mallorca/Islas Baleares | MT007326 MT006803 MT006500 | - | - |
| DAL-47     | 2017          | Bunyola Mallorca/ Islas Baleares | MT007327 MT006804 MT006501 | - | - |
| DAL-48     | 2017          | Bunyola Mallorca/ Islas Baleares | MT007328 MT006805 MT006502 MT007011 MT006702 | - | - |
Table 1. Cont.

| Species Strain Number | Year | Location | Province/Region | GenBank Accession Numbers |
|-----------------------|------|----------|----------------|--------------------------|
|                       |      |          |                | ITS | tef-1α | tub | his | cal |
| **D. amygdali** (cont.) | DAL-49 | 2017 | Bunyola | Mallorca/Islas Baleares | MT007329 | MT006806 | MT006503 | - | - |
| | DAL-50 | 2017 | Bunyola | Mallorca/Islas Baleares | MT007330 | MT006807 | MT006504 | MT007012 | - | - |
| | DAL-51 | 2017 | Bunyola | Mallorca/Islas Baleares | MT007331 | MT006808 | MT006505 | - | - |
| | DAL-52 | 2017 | Palma | Mallorca/Islas Baleares | MT007332 | MT006809 | MT006506 | - | - |
| | DAL-53 | 2017 | Palma | Mallorca/Islas Baleares | MT007333 | MT006810 | MT006507 | - | - |
| | DAL-54 | 2017 | Palma | Mallorca/Islas Baleares | MT007334 | MT006811 | MT006508 | - | - |
| | DAL-55 | 2017 | Palma | Mallorca/Islas Baleares | MT007335 | MT006812 | MT006509 | - | - |
| | DAL-56 | 2017 | Palma | Mallorca/Islas Baleares | MT007336 | MT006813 | MT006510 | - | - |
| | DAL-57 | 2017 | Palma | Mallorca/Islas Baleares | MT007337 | MT006814 | MT006511 | MT007013 | - | - |
| | DAL-65 | 2017 | La Rinconada | Sevilla/Andalucía | MT007338 | MT006815 | MT006512 | MT007014 | - | - |
| | DAL-70 | 2018 | Godelleta | Valencia/Comunidad Valenciana | MT007339 | MT006816 | MT006513 | MT007015 | MT006703 | - | - |
| | DAL-71 | 2018 | Godelleta | Valencia/Comunidad Valenciana | MT007340 | MT006817 | MT006514 | - | - |
| | DAL-72 | 2018 | Godelleta | Valencia/Comunidad Valenciana | MT007341 | MT006818 | MT006515 | - | - |
| | DAL-73 | 2018 | Godelleta | Valencia/Comunidad Valenciana | MT007342 | MT006819 | MT006516 | - | - |
| | DAL-74 | 2018 | Godelleta | Valencia/Comunidad Valenciana | MT007343 | MT006820 | MT006517 | - | - |
| | DAL-75 | 2018 | Godelleta | Valencia/Comunidad Valenciana | MT007344 | MT006821 | MT006518 | - | - |
| | DAL-76 | 2018 | Montserrat | Valencia/Comunidad Valenciana | MT007345 | MT006822 | MT006519 | MT007016 | MT006704 | - | - |
| | DAL-77 | 2018 | Montserrat | Valencia/Comunidad Valenciana | MT007346 | MT006823 | MT006520 | - | - |
| | DAL-78 | 2018 | Montserrat | Valencia/Comunidad Valenciana | MT007347 | MT006824 | MT006521 | - | - |
| | DAL-79 | 2018 | Montserrat | Valencia/Comunidad Valenciana | MT007348 | MT006825 | MT006522 | - | - |
| | DAL-80 | 2018 | Montserrat | Valencia/Comunidad Valenciana | MT007349 | MT006826 | MT006523 | - | - |
| | DAL-81 | 2018 | Montserrat | Valencia/Comunidad Valenciana | MT007350 | MT006827 | MT006524 | - | - |
| | DAL-82 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007351 | MT006828 | MT006525 | MT007017 | MT006705 | - | - |
| | DAL-83 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007352 | MT006829 | MT006526 | - | - |
| | DAL-84 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007353 | MT006830 | MT006527 | - | - |
| | DAL-85 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007354 | MT006831 | MT006528 | MT007018 | MT006706 | - | - |
| | DAL-86 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007355 | MT006832 | MT006529 | - | - |
| | DAL-87 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007356 | MT006833 | MT006530 | - | - |
| | DAL-88 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007357 | MT006834 | MT006531 | - | - |
| | DAL-89 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007358 | MT006835 | MT006532 | - | - |
| | DAL-90 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007359 | MT006836 | MT006533 | - | - |
| | DAL-91 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007360 | MT006837 | MT006534 | - | - |
| | DAL-92 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007361 | MT006838 | MT006535 | - | - |
| | DAL-93 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007362 | MT006839 | MT006536 | - | - |
| | DAL-94 | 2018 | Viver | Castellón/Comunidad Valenciana | MT007363 | MT006840 | MT006537 | MT007019 | - | - |
Table 1. Cont.

| Species          | Strain Number | Year | Location          | Province/Region                  | GenBank Accession Numbers         |
|------------------|---------------|------|-------------------|----------------------------------|-----------------------------------|
|                  |               |      |                   |                                  | ITS                              |
| *D. amygdali*    | DAL-95        | 2018 | Viver             | Castellón/Comunidad Valenciana   | MT007364 MT006841 MT006538 MT007020 - |
|                  | DAL-96        | 2018 | Viver             | Castellón/Comunidad Valenciana   | MT007365 MT006842 MT006539 - -    |
|                  | DAL-97        | 2018 | Fuente la Higuera | Valencia/Comunidad Valenciana    | MT007366 MT006843 MT006540 - -    |
|                  | DAL-98        | 2018 | Fuente la Higuera | Valencia/Comunidad Valenciana    | MT007367 MT006844 MT006541 - -    |
|                  | DAL-103       | 2017 | Gibraleón         | Huelva/Andalucía                | MT007368 MT006845 MT006542 MT007021 MT006707 |
|                  | DAL-104       | 2016 | El Contador       | Almería/Andalucía               | MT007369 MT006848 MT006543 MT007022 MT006708 |
|                  | DAL-105       | 2017 | Alcalá del Río    | Sevilla/Andalucía               | MT007370 MT006846 MT006544 MT007023 MT006709 |
|                  | DAL-108       | 2018 | Biar              | Alicante/Comunidad Valenciana   | MT007371 MT006847 MT006545 MT007024 MT006710 |
|                  | DAL-109       | 2018 | Biar              | Alicante/Comunidad Valenciana   | MT007372 MT006849 MT006546 - -    |
|                  | DAL-110       | 2018 | Fuente la Higuera | Valencia/Comunidad Valenciana    | MT007373 MT006850 MT006547 - -    |
|                  | DAL-111       | 2018 | Fuente la Higuera | Valencia/Comunidad Valenciana    | MT007374 MT006851 MT006548 - -    |
|                  | DAL-112       | 2018 | Fuente la Higuera | Valencia/Comunidad Valenciana    | MT007375 MT006852 MT006549 - -    |
|                  | DAL-113       | 2018 | Fontanars dels Alforins | Valencia/Comunidad Valenciana | MT007376 MT006853 MT006550 MT007025 MT006711 |
|                  | DAL-114       | 2018 | Fontanars dels Alforins | Valencia/Comunidad Valenciana | MT007377 MT006854 MT006551 MT007026 MT006712 |
|                  | DAL-116       | 2018 | Alcublas          | Valencia/Comunidad Valenciana    | MT007378 MT006855 MT006552 MT007027 - |
|                  | DAL-117       | 2018 | Alcublas          | Valencia/Comunidad Valenciana    | MT007379 MT006856 MT006553 - -    |
|                  | DAL-118       | 2018 | Casinos           | Valencia/Comunidad Valenciana    | MT007380 MT006857 MT006554 - -    |
|                  | DAL-119       | 2018 | Casinos           | Valencia/Comunidad Valenciana    | MT007381 MT006858 MT006555 - -    |
|                  | DAL-120       | 2018 | Casinos           | Valencia/Comunidad Valenciana    | MT007382 MT006859 MT006556 - -    |
|                  | DAL-121       | 2018 | Vall d’Alba       | Castellón/Comunidad Valenciana   | MT007383 MT006860 MT006557 MT007028 - |
|                  | DAL-122       | 2018 | Vall d’Alba       | Castellón/Comunidad Valenciana   | MT007384 MT006861 MT006558 - -    |
|                  | DAL-125       | 2018 | Vall d’Alba       | Castellón/Comunidad Valenciana   | MT007385 MT006862 MT006559 - -    |
|                  | DAL-126       | 2018 | Vall d’Alba       | Castellón/Comunidad Valenciana   | MT007386 MT006863 MT006560 - -    |
|                  | DAL-128       | 2018 | Godelleta         | Valencia/Comunidad Valenciana    | MT007387 MT006864 MT006561 - -    |
|                  | DAL-129       | 2018 | Godelleta         | Valencia/Comunidad Valenciana    | MT007388 MT006865 MT006562 - -    |
|                  | DAL-130       | 2018 | Torremendo        | Alicante/Comunidad Valenciana    | MT007389 MT006866 MT006563 MT007029 - |
|                  | DAL-131       | 2018 | Torremendo        | Alicante/Comunidad Valencian     | MT007390 MT006867 MT006564 - -    |
|                  | DAL-132       | 2018 | Requena           | Valencia/Comunidad Valenciana    | MT007391 MT006868 MT006565 MT007030 MT006713 |
|                  | DAL-133       | 2018 | Requena           | Valencia/Comunidad Valenciana    | MT007392 MT006869 MT006566 MT007031 - |
|                  | DAL-134       | 2018 | Requena           | Valencia/Comunidad Valenciana    | MT007393 MT006870 MT006567 MT007032 - |
|                  | DAL-135       | 2018 | L’Elíana          | Valencia/Comunidad Valenciana    | MT007394 MT006871 MT006568 MT007033 - |
|                  | DAL-136       | 2018 | L’Elíana          | Valencia/Comunidad Valenciana    | MT007395 MT006872 MT006569 - -    |
|                  | DAL-138       | 2005 | Constanti         | Tarragona/Cataluña               | MT007396 MT006873 MT006570 - -    |
|                  | DAL-139       | 2005 | Constanti         | Tarragona/Cataluña               | MT007397 MT006874 MT006571 MT007034 - |
|                  | DAL-140       | 2012 | Ulledecona        | Tarragona/Cataluña               | MT007398 MT006875 MT006572 MT007035 MT006714 |
Table 1. Cont.

| Species        | Strain Number | Year | Location                  | Province/Region     | GenBank Accession Numbers |
|----------------|---------------|------|---------------------------|---------------------|---------------------------|
|                |               |      |                           |                     |                           |
| *D. amygdali* (cont.) |               |      |                           |                     |                           |
| DAL-141        | 2016          | Gandesa | Tarragona/Cataluña       | MT007399 MT006876 MT006573 |                           |
| DAL-143        | 2018          | Gandesa | Tarragona/Cataluña       | MT007400 MT006877 MT006574 |                           |
| DAL-144        | 2018          | Gandesa | Tarragona/Cataluña       | MT007401 MT006878 MT006575 |                           |
| DAL-145        | 2018          | Gandesa | Tarragona/Cataluña       | MT007402 MT006879 MT006576 |                           |
| DAL-146        | 2018          | Gandesa | Tarragona/Cataluña       | MT007403 MT006880 MT006577 MT007036 | -                           |
| DAL-147        | 2018          | Constantí | Tarragona/Cataluña   | MT007404 MT006881 MT006578 MT007037 | -                           |
| DAL-148        | 2018          | Constantí | Tarragona/Cataluña   | MT007405 MT006882 MT006579 MT007038 | -                           |
| DAL-149        | 2018          | Constantí | Tarragona/Cataluña   | MT007406 MT006883 MT006580 MT007039 MT006715 | -                           |
| DAL-151        | 2018          | Constantí | Tarragona/Cataluña   | MT007407 MT006884 MT006581 | -                           |
| DAL-152        | 2018          | Constantí | Tarragona/Cataluña   | MT007408 MT006885 MT006582 MT007040 MT006716 | -                           |
| DAL-153        | 2018          | Constantí | Tarragona/Cataluña   | MT007409 MT006886 MT006583 | -                           |
| DAL-154        | 2018          | La Selva del Camp | Tarragona/Cataluña | MT007410 MT006887 MT006584 MT007041 MT006717 | -                           |
| DAL-155        | 2018          | La Selva del Camp | Tarragona/Cataluña | MT007411 MT006888 MT006585 MT007042 MT006718 | -                           |
| DAL-156        | 2018          | La Selva del Camp | Tarragona/Cataluña | MT007412 MT006889 MT006586 | -                           |
| DAL-158        | 2018          | La Selva del Camp | Tarragona/Cataluña | MT007413 MT006890 MT006587 | -                           |
| DAL-159        | 2018          | La Selva del Camp | Tarragona/Cataluña | MT007414 MT006891 MT006588 | -                           |
| DAL-160        | 2018          | La Selva del Camp | Tarragona/Cataluña | MT007415 MT006892 MT006589 | -                           |
| DAL-161        | 2018          | Constantí | Tarragona/Cataluña   | MT007416 MT006893 MT006590 | -                           |
| DAL-162        | 2018          | Constantí | Tarragona/Cataluña   | MT007417 MT006894 MT006591 | -                           |
| DAL-163        | 2018          | Estepa  | Sevilla/Andalucía       | MT007418 MT006895 MT006592 | -                           |
| DAL-164        | 2018          | Estepa  | Sevilla/Andalucía       | MT007419 MT006896 MT006593 MT007043 MT006719 | -                           |
| DAL-167        | 2018          | Los Palacios | Sevilla/Andalucía | MT007420 MT006897 MT006594 MT007044 MT006720 | -                           |
| DAL-168        | 2018          | Los Palacios | Sevilla/Andalucía | MT007421 MT006898 MT006595 | -                           |
| DAL-169        | 2018          | Los Palacios | Sevilla/Andalucía | MT007422 MT006899 MT006596 | -                           |
| DAL-170        | 2018          | Los Palacios | Sevilla/Andalucía | MT007423 MT006900 MT006597 | -                           |
| DAL-171        | 2018          | Los Palacios | Sevilla/Andalucía | MT007424 MT006901 MT006598 | -                           |
| DAL-172        | 2018          | Los Palacios | Sevilla/Andalucía | MT007425 MT006902 MT006599 MT007045 | -                           |
| DAL-181        | 2018          | Córdoba  | Córdoba/Andalucía       | MT007426 MT006903 MT006600 MT007046 MT006721 | -                           |
| DAL-182        | 2018          | Córdoba  | Córdoba/Andalucía       | MT007427 MT006904 MT006601 | -                           |
| DAL-183        | 2018          | Córdoba  | Córdoba/Andalucía       | MT007428 MT006905 MT006602 | -                           |
| DAL-184        | 2018          | Mairena del Alcor | Sevilla/Andalucía | MT007429 MT006906 MT006603 MT007047 | -                           |
| DAL-185        | 2018          | Mairena del Alcor | Sevilla/Andalucía | MT007430 MT006907 MT006604 | -                           |
| DAL-186        | 2018          | Mairena del Alcor | Sevilla/Andalucía | MT007431 MT006908 MT006605 | -                           |
| DAL-187        | 2018          | Mairena del Alcor | Sevilla/Andalucía | MT007432 MT006909 MT006606 | -                           |
| Species          | Strain Number | Year | Location          | Province/Region   | GenBank Accession Numbers |
|------------------|---------------|------|-------------------|-------------------|---------------------------|
| *D. amygdali*    | DAL-188       | 2018 | Mairena del Alcor | Sevilla/Andalucía | MT007433 MT006910 MT006607 |
|                  | DAL-189       | 2018 | Mairena del Alcor | Sevilla/Andalucía | MT007434 MT006911 MT006608 |
|                  | DAL-190       | 2018 | Mairena del Alcor | Sevilla/Andalucía | MT007435 MT006912 MT006609 |
|                  | DAL-191       | 2018 | Mairena del Alcor | Sevilla/Andalucía | MT007436 MT006913 MT006610 |
|                  | DAL-192       | 2018 | Mairena del Alcor | Sevilla/Andalucía | MT007437 MT006914 MT006611 |
|                  | DAL-193       | 2018 | Ronda             | Málaga/Andalucía  | MT007438 MT006915 MT007050 |
|                  | DAL-194       | 2018 | Ronda             | Málaga/Andalucía  | MT007439 MT006916 MT006613 |
|                  | DAL-195       | 2018 | Ronda             | Málaga/Andalucía  | MT007440 MT006917 MT006614 |
|                  | DAL-196       | 2018 | Ronda             | Málaga/Andalucía  | MT007441 MT006918 MT006615 |
|                  | DAL-197       | 2018 | Ronda             | Málaga/Andalucía  | MT007442 MT006919 MT006616 |
|                  | DAL-198       | 2018 | Ronda             | Málaga/Andalucía  | MT007443 MT006920 MT006617 |
|                  | DAL-199       | 2018 | Ronda             | Málaga/Andalucía  | MT007444 MT006921 MT006618 |
|                  | DAL-200       | 2018 | Ronda             | Málaga/Andalucía  | MT007445 MT006922 MT006619 |
|                  | DAL-201       | 2018 | Ronda             | Málaga/Andalucía  | MT007446 MT006923 MT006620 |
|                  | DAL-202       | 2018 | Ronda             | Málaga/Andalucía  | MT007447 MT006924 MT006621 |
|                  | DAL-203       | 2018 | Reus              | Tarragona/Cataluña| MT007448 MT006925 MT006622 |
|                  | DAL-204       | 2018 | Reus              | Tarragona/Cataluña| MT007449 MT006926 MT007053 |
|                  | DAL-205       | 2018 | Reus              | Tarragona/Cataluña| MT007450 MT006927 MT007054 |
|                  | DAL-206       | 2018 | Riudoms           | Tarragona/Cataluña| MT007451 MT006928 MT006625 |
|                  | DAL-207       | 2018 | Riudoms           | Tarragona/Cataluña| MT007452 MT006929 MT006626 |
|                  | DAL-208       | 2018 | Riudoms           | Tarragona/Cataluña| MT007453 MT006930 MT006627 |
|                  | DAL-209       | 2018 | Riudoms           | Tarragona/Cataluña| MT007454 MT006931 MT006628 |
|                  | DAL-210       | 2018 | Riudoms           | Tarragona/Cataluña| MT007455 MT006932 MT006629 |
|                  | DAL-211       | 2018 | Riudoms           | Tarragona/Cataluña| MT007456 MT006933 MT006630 |
|                  | DAL-212       | 2018 | Riudoms           | Tarragona/Cataluña| MT007457 MT006934 MT006631 |
|                  | DAL-213       | 2018 | Riudoms           | Tarragona/Cataluña| MT007458 MT006935 MT006632 |
|                  | DAL-214       | 2018 | Botarell          | Tarragona/Cataluña| MT007459 MT006936 MT006633 |
|                  | DAL-215       | 2018 | Botarell          | Tarragona/Cataluña| MT007460 MT006937 MT006634 |
|                  | DAL-216       | 2018 | Botarell          | Tarragona/Cataluña| MT007461 MT006938 MT006635 |
|                  | DAL-219       | 2018 | Les Borges Blanques| Lérida/Cataluña  | MT007462 MT006939 MT006636 |
|                  | DAL-220       | 2018 | Isona i Conca Dellà| Lérida/Cataluña  | MT007463 MT006940 MT006637 |
|                  | DAL-221       | 2018 | Isona i Conca Dellà| Lérida/Cataluña  | MT007464 MT006941 MT006638 |
|                  | DAL-225       | 2019 | Murillo           | Logroño/La Rioja | MT007465 MT006942 MT006639 |
|                  | DAL-226       | 2019 | Murillo           | Logroño/La Rioja | MT007466 MT006943 MT006640 |
| Species          | Strain Number | Year | Location                  | Province/Region | GenBank Accession Numbers |
|------------------|---------------|------|---------------------------|-----------------|---------------------------|
|                  |               |      |                           |                 | **ITS**       | **tef-1α** | **tub** | **his** | **cal** |
| **D. amygdali**  | DAL-227       | 2019 | Santa Engracia de Jubera  | Logroño/La Rioja | MT007467     | MT006944 | MT006641 | MT007064 | MT006730 |
|                  | DAL-228       | 2019 | Santa Engracia de Jubera  | Logroño/La Rioja | MT007468     | MT006945 | MT006642 |           |           |
|                  | DAL-229       | 2019 | Santa Engracia de Jubera  | Logroño/La Rioja | MT007469     | MT006946 | MT006643 |           |           |
|                  | DAL-230       | 2019 | Santa Engracia de Jubera  | Logroño/La Rioja | MT007470     | MT006947 | MT006644 |           |           |
|                  | DAL-231       | 2019 | Santa Engracia de Jubera  | Logroño/La Rioja | MT007471     | MT006948 | MT006645 |           |           |
|                  | DAL-232       | 2019 | Santa Engracia de Jubera  | Logroño/La Rioja | MT007472     | MT006949 | MT006646 |           |           |
|                  | DAL-233       | 2019 | Santa Engracia de Jubera  | Logroño/La Rioja | MT007473     | MT006950 | MT006647 | MT007065 | MT006731 |
|                  | DAL-234       | 2019 | Santa Engracia de Jubera  | Logroño/La Rioja | MT007474     | MT006951 | MT006648 |           |           |
|                  | DAL-236       | 2019 | Alcalá del Río            | Sevilla/Andalucía| MT007475     | MT006952 | MT006649 | MT007066 |           |
|                  | DAL-237       | 2019 | Alcalá del Río            | Sevilla/Andalucía| MT007476     | MT006953 | MT006650 |           |           |
|                  | DAL-238       | 2019 | Alcalá del Río            | Sevilla/Andalucía| MT007477     | MT006954 | MT006651 |           |           |
|                  | DAL-239       | 2019 | Córdoba                   | Córdoba/Andalucía| MT007478     | MT006955 | MT006652 |           |           |
|                  | DAL-240       | 2019 | Córdoba                   | Córdoba/Andalucía| MT007479     | MT006956 | MT006653 | MT007067 | MT006732 |
|                  | DAL-241       | 2019 | Córdoba                   | Córdoba/Andalucía| MT007480     | MT006957 | MT006654 |           |           |
|                  | DAL-242       | 2019 | Santa Cruz                | Córdoba/Andalucía| MT007481     | MT006958 | MT006655 |           |           |
|                  | DAL-243       | 2019 | Santa Cruz                | Córdoba/Andalucía| MT007482     | MT006959 | MT006656 |           |           |
|                  | DAL-244       | 2019 | Villamorrique de la Condesa| Sevilla/Andalucía| MT007483     | MT006960 | MT006657 | MT007068 | MT006733 |
|                  | DAL-245       | 2019 | Villamorrique de la Condesa| Sevilla/Andalucía| MT007484     | MT006961 | MT006658 |           |           |
|                  | DAL-246       | 2019 | Santa Engracia de Jubera  | Logroño/La Rioja | MT007485     | MT006962 | MT006659 |           |           |
|                  | DAL-102       | 2016 | Córdoba                   | Córdoba/Andalucía| MN997106     | MT007104 | MT006642 | MT007106 | MT006465 |
| **D. eres**      | DAL-10        | 2014 | Santa Margalida i Calvià  | Mallorca/Islas Baleares | MT007497     | MT006963 | MT006660 | MT007069 | MT006734 |
|                  | DAL-11        | 2014 | Santa Margalida i Calvià  | Mallorca/Islas Baleares | MT007498     | MT006964 | MT006661 | MT007070 | MT006735 |
|                  | DAL-27        | 2017 | Alcalá                    | Alicante/Comunidad Valenciana | MT007499     | MT006965 | MT006662 | MT007071 | MT006736 |
|                  | DAL-28        | 2017 | Alcalá                    | Alicante/Comunidad Valenciana | MT007500     | MT006966 | MT006663 | MT007072 | MT006737 |
|                  | DAL-30        | 2017 | Alcalá                    | Alicante/Comunidad Valenciana | MT007501     | MT006967 | MT006664 | MT007073 | MT006738 |
|                  | DAL-31        | 2017 | Alcalá                    | Alicante/Comunidad Valenciana | MT007502     | MT006968 | MT006665 | MT007074 | MT006739 |
|                  | DAL-61        | 2016 | Alcalá del Río            | Sevilla/Andalucía    | MT007503     | MT006969 | MT006666 | MT007075 | MT006740 |
|                  | DAL-62        | 2016 | Alcalá del Río            | Sevilla/Andalucía    | MT007504     | MT006970 | MT006667 | MT007076 | MT006741 |
|                  | DAL-63        | 2016 | Alcalá del Río            | Sevilla/Andalucía    | MT007505     | MT006971 | MT006668 | MT007077 | MT006742 |
|                  | DAL-64        | 2016 | Alcalá del Río            | Sevilla/Andalucía    | MT007506     | MT006972 | MT006669 | MT007078 | MT006743 |
|                  | DAL-66        | 2017 | La Rinconada              | Sevilla/Andalucía    | MT007507     | MT006973 | MT006670 | MT007079 | MT006744 |
|                  | DAL-67        | 2017 | La Rinconada              | Sevilla/Andalucía    | MT007508     | MT006974 | MT006671 | MT007080 | MT006745 |
|                  | DAL-68        | 2017 | La Rinconada              | Sevilla/Andalucía    | MT007509     | MT006975 | MT006672 | MT007081 | MT006746 |
|                  | DAL-69        | 2017 | La Rinconada              | Sevilla/Andalucía    | MT007510     | MT006976 | MT006673 | MT007082 | MT006747 |
|                  | DAL-99        | 2018 | Fuente la Higuera         | Valencia/Comunidad Valenciana | MT007511     | MT006977 | MT006674 | MT007083 | MT006748 |
|                  | DAL-100       | 2018 | Fuente la Higuera         | Valencia/Comunidad Valenciana | MT007512     | MT006978 | MT006675 | MT007084 | MT006749 |
Table 1. Cont.

| Species            | Strain Number | Year | Location         | Province/Region               | GenBank Accession Numbers |
|--------------------|---------------|------|------------------|-------------------------------|---------------------------|
|                    |               |      |                  |                               | **ITS**       | **tef-1α**  | **tub**    | **his**   | **cal** |
| *D. foeniculina* (cont.) | DAL-101      | 2018 | Fuente la Higuera | Valencia/Comunidad Valenciana | MT007513   | MT006979   | MT006676   | MT007085  | MT006750 |
|                    | DAL-107      | 2018 | Marchena         | Sevilla/Andalucía            | MT007514   | MT006980   | MT006677   | MT007086  | MT006751 |
|                    | DAL-142      | 2018 | Cabrils          | Barcelona/Cataluña           | MT007515   | MT006981   | MT006678   | MT007087  | MT006752 |
|                    | DAL-150      | 2018 | Constantí        | Tarragona/Cataluña           | MT007516   | MT006982   | MT006679   | MT007088  | MT006753 |
|                    | DAL-157      | 2018 | La Selva del Camp| Tarragona/Cataluña           | MT007517   | MT006983   | MT006680   | MT007089  | MT006754 |
|                    | DAL-165      | 2018 | Estepa           | Sevilla/Andalucía            | MT007518   | MT006984   | MT006681   | MT007090  | MT006755 |
|                    | DAL-217      | 2018 | Les Borges Blanques | Lérida/Comunidad Valenciana | MT007519   | MT006985   | MT006682   | MT007091  | MT006756 |
| *D. mediterranea*   | DAL-6        | 2014 | Calvià           | Mallorca/Comunidad Valenciana| MT007486   | MT006986   | MT006683   | MT007092  | MT006758 |
|                    | DAL-8        | 2014 | Consell          | Mallorca/Isles Baleares      | MT007487   | MT006987   | MT006684   | MT007093  | MT006759 |
|                    | DAL-24       | 2014 | Sant Llorenç d’Escardassar | Mallorca/Isles Baleares | MT007488   | MT006988   | MT006685   | MT007094  | MT006760 |
|                    | DAL-34       | 2017 | Alcalali         | Alicante/Comunidad Valenciana| MT007489   | MT006989   | MT006686   | MT007095  | MT006761 |
|                    | DAL-173      | 2018 | Altea la Vella   | Alicante/Comunidad Valenciana| MT007493   | MT006993   | MT006691   | MT007099  | MT006765 |
|                    | DAL-174      | 2018 | Altea la Vella   | Alicante/Comunidad Valenciana| MT007494   | MT006994   | MT006690   | MT007100  | MT006766 |
|                    | DAL-175      | 2018 | Altea la Vella   | Alicante/Comunidad Valenciana| MT007495   | MT006995   | MT006692   | MT007101  | MT006767 |
|                    | DAL-176      | 2018 | Altea la Vella   | Alicante/Comunidad Valenciana| MT007496   | MT006996   | MT006693   | MT007102  | MT006768 |
| *D. phaseolorum*    | DAL-222      | 2016 | Alcalá del Río  | Sevilla/Andalucía            | MN997107   | MT007103   | MT006463   | MT007105  | MT006464 |
2.2. DNA Extraction, PCR Amplification and Sequencing

Mycelium was scraped from 10-day-old fungal cultures grown on PDA medium. Total fungal DNA was extracted using the E.Z.N.A. Plant DNA Kit (Omega Bio-tek, Norcross, GA, USA), following the manufacturer’s short protocol instructions.

The ITS region and fragments of tub and tef-1α genes were amplified and sequenced. Based on these preliminary results, representative isolates were selected for amplifying and sequencing cal and histone H3 (his) genes. Amplification by polymerase chain reaction (PCR) was performed in a total volume of 25 µL using HotBegan™ Taq DNA Polymerase (Canvax Biotech SL, Córdoba, Spain), according to the manufacturer’s instructions on a Peltier Thermal Cycler-200 (MJ Research). One reaction was composed of 2.5 µL of 10× PCR Buffer B, 2.5 µL of MgCl2 (25 mM), 2.5 µL of dNTPs (8 mM), 1 µL of each primer (10 µM), 0.2 µL of HotBegan Taq DNA Polymerase (5 U/µL), 1 µL of purified template DNA and 14.3 µL of nuclease-free water. The thermal cycle consisted of an initial step of 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s and elongation at 72 °C for 45 s. A final extension was performed at 72 °C for 5 min. The primers pairs and the annealing temperatures (Ta) for each locus were as follows: ITS1-F and ITS4 for ITS (Ta = 55 °C) [20,21], EF1-688F and EF1-1251R for tef-1α (Ta = 55 °C) [22], BtCadF and BtCadR or T1 and BT2b for tub (Ta = 55 °C for both pairs) [23–25], CYLH3F and H3-1b for his (Ta = 58 °C) [25,26], CL1C and CL2C or CAL-563F and CL2C for cal (Ta = 58 °C for both pairs) [27,28]. PCR products were analyzed by 1% agarose gel electrophoresis, purified and sequenced by Macrogen Inc. (Madrid, Spain) using both PCR primers. Each consensus sequence was assembled using Sequencher software 5.0 (Gene Codes Corp., Ann Arbor, Michigan).

2.3. Phylogenetic Analyses

Sequences generated in this study were compared with reference sequences in the GenBank nucleotide database to determine the closest relatives for the phylogenetic studies. For each of the five loci (ITS, tub, tef-1α, cal and his), the DNA sequences obtained in this study (Table 1), together with those retrieved from GenBank (Table 2), were aligned using the ClustalW algorithm included in the MEGAX software package [29,30]. The alignments were analyzed and adjusted manually when necessary. Ambiguous sequences at either end of the alignments were excluded prior to analyses. Concatenated datasets were built in Sequence Matrix v.1.8 [31].
Table 2. Additional *Diaporthe* species used in the phylogenetic analyses.

| Species          | Strain      | Host                 | Country          | GenBank Accession Numbers |
|------------------|-------------|----------------------|------------------|---------------------------|
|                  |             |                      |                  | ITS | tef-1α | tub | his | cal  |
| *D. acaciigena*  | CBS 129521  | *Acacia retinodes*   | Australia        | KC343005 | KC343731 | KC343973 | KC343489 | KC343247 |
|                  | CBS 126679  | *Prunus dulcis*      | Portugal         | KC343022 | KC343748 | KC343990 | KC343506 | KC343264 |
|                  | CBS 111811  | *Vitis vinifera*     | South Africa     | KC343019 | KC343745 | KC343987 | KC343503 | KC343261 |
| *D. amygdali*    | CBS 139.27  | *Celastrus scandens*  | USA              | KC343047 | KC343773 | KC344015 | KC343531 | KC343289 |
|                  | CBS 143349  | *Vitis vinifera*     | UK               | MG281017 | MG281538 | MG281190 | MG281363 | MG281712 |
|                  | CBS 143350  | *Vitis vinifera*     | UK               | MG281018 | MG281539 | MG281191 | MG281364 | MG281713 |
| *D. chamaeropis* | CBS 454.81  | *Chamaecyparis humilis* | Greece          | KC343048 | KC343774 | KC344016 | KC343532 | KC343290 |
|                  | CBS 753.70  | *Spartium junceum*   | Croatia          | KC343049 | KC343775 | KC344017 | KC343533 | KC343291 |
| *D. chongqingensis* | PSCG 435 | *Pyrus pyrifolia*    | China            | MK626916 | MK654866 | MK691321 | MK726257 | MK691209 |
|                  | PSCG 436   | *Pyrus pyrifolia*    | China            | MK626917 | MK654867 | MK691322 | MK726256 | MK691208 |
| *D. cinerascens* | CBS 719.96  | *Ficus carica*       | Bulgaria         | KC343050 | KC343776 | KC344018 | KC343534 | KC343292 |
| *D. endophytica* | CBS 133811  | *Schinus terebinthifolius* | Brazil         | KC343065 | KC343791 | KC344033 | KC343549 | KC343307 |
|                  | CBS 138599  | *Ulmus laevis*       | Germany          | KJ210529 | KJ210550 | KJ420799 | KJ420850 | KJ434999 |
| *D. foeniculina* | CBS 109767  | *Acer campestre*     | Austria          | KC343075 | KC343801 | KC344043 | KC343559 | KC343317 |
|                  | CBS 111553  | *Foeniculum vulgare* | Spain            | KC343101 | KC343827 | KC344069 | KC343585 | KC343343 |
|                  | CBS 187.27  | *Camellia sinensis*  | Italy            | KC343107 | KC343833 | KC344075 | KC343591 | KC343349 |
| *D. fuscola*     | CGMCC 3.17087 | *Lithocarpus glabra* | China          | KF576281 | KF576256 | KF576305 | -        | KF576233 |
|                  | CGMCC 3.17088 | *Lithocarpus glabra* | China          | KF576263 | KF576238 | KF576287 | -        | KF576221 |
| *D. garethjonesii* | MFLUCC 12-0542A | Unknown dead leaf | Thailand       | KT459423 | KT459457 | KT459441 | -        | KT459470 |
| *D. helicis*     | CBS 138596  | *Hedera helix*       | Germany          | KJ210538 | KJ210559 | KJ420828 | KJ420875 | KJ435043 |
| *D. kadsurae*    | CFCC 52586  | *Kadsura longipedunculata* | China     | MH121521 | MH121563 | MH121600 | MH121479 | MH121439 |
|                  | CFCC 52587  | *Kadsura longipedunculata* | China     | MH121522 | MH121564 | MH121601 | MH121480 | MH121440 |
| *D. masirevicii* | BRIP 54120c | *Zea mays*           | Australia        | KJ197278 | KJ197240 | KJ197258 | -        | -        |
|                  | BRIP 57892a | *Helianthus annuus*  | Australia        | KJ197276 | KJ197239 | KJ197257 | -        | -        |
| *D. ovalispora*  | ICMP20659  | *Citrus limon*       | China           | KJ490628 | KJ490507 | KJ490449 | KJ490570 | KJ490570 |
| *D. oovicola*    | CGMCC 3.17092 | *Lithocarpus glabra* | China          | KF576264 | KF576239 | KF576288 | -        | KF576222 |
|                  | CGMCC 3.17093 | *Citrus sp.*        | China           | KF576265 | KF576240 | KF576289 | -        | KF576223 |
| *D. phaseolorum* | CBS 113425  | *Olearia cf. rani*   | New Zealand      | KC343174 | KC343900 | KC344142 | KC343658 | KC343416 |
|                  | CBS 116019  | *Caperonia palustris* | USA             | KC343175 | KC343901 | KC344143 | KC343659 | KC343417 |
| *D. pulla*       | CBS 338.89  | *Hedera helix*       | Croatia          | KC343152 | KC343878 | KC344120 | KC343636 | KC343394 |
|                  | CBS 109742  | *Acer pseudoplatanus* | Austria         | KC343185 | KC343911 | KC344153 | KC343669 | KC343427 |
| *D. pustulata*   | CBS 109784  | *Prunus padus*       | Austria          | KC343187 | KC343913 | KC344155 | KC343671 | KC343429 |
Table 2. Cont.

| Species          | Strain          | Host                | Country | GenBank Accession Numbers | GenBank Accession Numbers |
|------------------|-----------------|---------------------|---------|---------------------------|---------------------------|
|                  |                 |                     |         | ITS                       | tef-1α                    |
| D. sojae         | CBS 100.87      | Glycine soja        | Italy   | KC343196                  | KC343922                  |
|                  | CBS 116017      | Euphorbia nutans    | USA     | KC343197                  | KC343923                  |
|                  | D. sterilis     | Vaccinium corymbosum| Italy   | KC343197                  | KC343923                  |
|                  | CBS 136969      | Vaccinium corymbosum| Italy   | KC343197                  | KC343923                  |
|                  | CBS 136970      | Vaccinium corymbosum| Italy   | KC343197                  | KC343923                  |
|                  | D. subellipcola | On dead wood        | China   | MG746632                  | MG746632                  |
| Diaporthella corylina | MFLUCC 17-1197 | Corylus sp.         | China   | MG746632                  | MG746632                  |
| Phomopsis sp. 5  | PMM1657         | Vitis vinifera      | South Africa | KY511331                | KY511331                  |
|                  | PMM1660         | Vitis vinifera      | South Africa | KY511333                | KY511333                  |

Note. BRIP: Queensland Plant Pathology Herbarium, Brisbane, Queensland, Australia; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CFCC: China Forestry Culture Collection Center; CGMCC: China General Microbiological Culture Collection, Beijing, China; ICMP: International Collection of Microorganisms from Plants, Auckland, New Zealand; LGMF: Culture collection of the Laboratory of Genetics of Microorganisms, Federal University of Parana, Curitiba, Brazil; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; PMM: Lesuthu et al., 2019. Ex-type isolates are indicated in bold.
Phylogenetic analyses were based on Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP). Bayesian analyses were performed using MrBayes v 3.2 on the CIPRES Science Gateway v 3.3 [32,33]. The best-fitting model of nucleotide evolution for each partition was determined by MrModeltest 2.3 using the Akaike information criterion (AIC) [34]. Four simultaneous analyses were run for 100 million generations, sampling every 10,000, with four Markov chain Monte Carlo (MCMC) chains. The first 25% of saved trees were discarded and posterior probabilities were determined from the remaining trees. The ML analyses were done with the tool Randomized Axelerated Maximum Likelihood RAxML-HPC2 on XSEDE implemented on CIPRES Science Gateway v 3.3 [35]. ML tree searches were performed under the generalized time-reversible with gamma correction (GTR + Γ) nucleotide substitution model using 1000 pseudoreplicates. The other parameters were used as default settings. MP analyses were performed in MEGA X with the tree Bisection and reconnection (TBR) algorithm, where gaps were treated as missing data. The robustness of the topology was evaluated by 1000 bootstrap replications [36]. Measures for the maximum parsimony as tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were also calculated.

New sequences obtained in this study were deposited in GenBank (Table 1) and the multilocus alignment in was deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S26453).

2.4. Taxonomy

Agar plugs (6-mm diameter) were taken from the edge of actively growing cultures on PDA and transferred onto the center of 9-cm diameter Petri dishes containing one of the following culture media: malt extract agar (MEA; Sigma-Aldrich Laboratories), PDA, 2% tap water agar supplemented with sterile pine needles (PNA) or oatmeal agar (OA; 60 g oatmeal, 12.5 g agar, Difco, Le Pont de Claix, France). Plates were then incubated at 21–22 °C under a 12 h/12 h near-ultraviolet light/darkness cycle to induce sporulation as described by Guarnaccia et al. (2018). Cultures were examined periodically for the development of ascomata and conidiomata. Colony colors were rated only on PDA after 15 days of incubation according to Rayner [37]. Morphological characteristics were examined using an Axio Scope A.1 microscope (Zeiss, manufacturer data) after mounting single pycnidia in lactic acid. Fungal structures were measured (30 measurements per type of structure) using the Zeiss AxioVision LE imaging device. Photos were captured using a Zeiss AxioCam MRm digital camera from images recorded with the 40× objective. Descriptions, nomenclature and illustrations of taxonomic novelties were deposited in MycoBank (MB 836048).

The effect of temperature on the mycelial growth of selected isolates of the species D. mediterranea (DAL24, DAL34 and DAL174) was measured on PDA. For this purpose, agar plugs (6-mm diameter) obtained from the growing edge of colonies were transferred to the center of PDA plates, which were incubated at 5, 10, 15, 20, 25, 30, 35 or 40 °C in darkness. Four replicates for each isolate and temperature combination were used. Growth was determined after 7 days in two orthogonal directions, and the mean growth rate was calculated in mm/day using a simplified version of the non-linear equation proposed by Duthie et al. [38]. Regression curves were fitted to the data using the R function “nls” included in the “stats” package [39,40].

2.5. Pathogenicity Tests

Pathogenicity tests were conducted as described by Diogo et al. [8]. One-year-old twigs of almond cv. Vayro, about 30 cm long, were inoculated with a set of 14 representative isolates of the five Diaporthe species found associated with P. dulcis in this study: D. amygdali (isolates DAL-3, DAL-4, DAL-45, DAL-70, DAL-105, DAL-140 and DAL-159), D. eres (DAL-102), D. foeniculina (DAL-27 and DAL-61), D. phaseolorum (DAL-222) and D. mediterranea (DAL-24, DAL-34 and DAL-174). These isolates were selected to represent diverse geographical origins. The twigs were surface sterilized by immersion in 70% ethanol for 30 s, 1.5% sodium hypochlorite solution for 1 min and ethanol for 30 s. Then, they were air dried in a laminar flow cabinet.
Wounds were made in the center of each twig with a 6-mm cork borer. Colonized agar plugs with mycelium of about the same size, which were obtained from active 10-day-old colonies growing on PDA, were inserted underneath the epidermis and the wounds were sealed with Parafilm. Inoculated twigs were kept in an upright position with their lower ends immersed in 1 L jars with 500 mL of sterile water in a growth chamber at 23 °C with 12 h of light per day. The twigs were covered with a plastic bag during the first 4 days to keep a moist environment. Six twigs per isolate were used and a negative control was prepared using uncolonized PDA plugs. Jars were arranged in a completely randomized design and the water was changed every 3 days. The experiment was repeated once.

Lesion lengths were measured 15 days after inoculation. Immediately after lesion measurements, two representative shoots per inoculated isolate and replicate were surface sterilized as described above. Small internal fragments were cut from the margin of the healthy and necrotic tissue and placed onto PDA. Plates were incubated at 25 °C in the dark for 7 to 10 d, and all fungal growths resembling Diaporthe were transferred to PDA. A representative subsample, one culture from each of the 14 isolates and replicates, were subjected to DNA extraction and molecular identification as described above to satisfy Koch’s postulates.

Significance levels for mean values of lesion length (cm), corresponding to different Diaporthe spp. isolates inoculated and control detached twigs, were determined. The analyses were performed considering individual isolates and groups of isolates from each Diaporthe spp. ANOVA assumptions were verified using Shapiro–Wilk and Levene’s tests. The datasets did not meet ANOVA assumptions, thus the analysis was performed using the Kruskal–Wallis test. Control twigs were compared with the inoculated ones considering individual isolates, and different species were compared with D. amygdali using the Wilcoxon rank sum test ($p < 0.01$). The analyses were performed in R using the agricolae and stats packages [39,40].

3. Results

3.1. Phylogenetic Analyses

Three loci (ITS region and fragments of tub and tef-1α genes) were sequenced in all Diaporthe isolates ($n = 225$) obtained in this study and compared with those in GenBank. The BLAST search showed high identity with D. amygdali, D. eres, D. foeniculina and D. phaseolorum accessions. For phylogenetic analyses, two representative isolates of closely related species, i.e., the ex-type together with one additional isolate when possible, were selected as references, and their corresponding sequences were retrieved from GenBank (Table 2). These sequences ($n = 38$), including Diaporthella corylina strain CBS 121124 which was used as outgroup, were added to those of the Spanish isolates ($n = 225$). The MP three-locus phylogeny showed that Spanish isolates of Diaporthe grouped into five distinct clades, four of them with known Diaporthe species (data not shown). The most abundant group, with 192 isolates, clustered with the ex-type isolate of D. amygdali (CBS 126679), the second ($n = 23$) with the ex-type of D. foeniculina (CBS 111553) and two single isolates each grouped with the ex-type of D. eres (CBS 109767), and with D. phaseolorum (CBS 116019). The remaining isolates ($n = 8$) clustered together, closely related to, but separated from, D. sterilis (CBS136969), suggesting that they could belong to a new species.

For accurate resolution of the species limits of our isolates, fragments of his and cal genes were sequenced in a set of 70 and 39 representative D. amygdali isolates, respectively, and for all isolates of the other groups (Table 1). The selection of the D. amygdali isolates was based on the province/region of origin and year of isolation. In addition, all GenBank sequences (ITS and tub) of two undescribed Diaporthe isolates (PMM 1657 and PMM 1660), which shared 100% identity with these loci of the potential new species, were included in the analyses (Table 2). Then, MP, ML and BI phylogenetic trees were constructed for the five-locus combined dataset, which included all taxa ($n = 265$) regardless of the level of completeness. A total of 2826 characters, including gaps (ITS: 1–564, tub: 565–1384, tef-1α: 1385–1814, his: 1815–2300 and cal: 2301–2826), were used in phylogenetic analyses, of which 1494 were
constant and 817 were parsimony informative. The MP analysis yielded a single most parsimonious tree (TL = 2387; CI = 0.647; RI = 0.955; RC = 0.618). The ML analysis resulted in a single best tree with the final ML optimization likelihood = −15029.27737. In the BI analysis, the ITS/tub/tef-1α/his/cal partitions had 158/341/310/210/308 unique site patterns, respectively, and the analysis read a total of 40,004 trees, sampling 30,004 of them. The topologies and branching order of the inferred trees were compared visually, and they were fully congruent among themselves and with the previous ITS/tub/tef-1α multilocus phylogeny. The ITS/tub/tef-1α/his/cal ML tree is presented with the support of all phylogenetic methods at the branches (Figure 2).

Figure 2. Randomized Accelerated Maximum Likelihood (RAxML) tree based on analysis of a combined dataset of ITS, tub, tef-1α, his and cal sequences. Bootstrap support values for Maximum Parsimony (MP) and ML higher than 70% and Bayesian posterior probabilities (PP) higher than 0.90 are shown at the branches (MP/ML/PP). Clades highlighted contain the isolates identified in the current study and the novel taxa is shown in red. Ex-type strains are indicated in bold. The tree is rooted using Diaporthella corylina (CBS121124). The scale bar represents the expected number of nucleotide substitutions per site.
Diaporthe amygdali represented 85.3% of the studied isolates and they were obtained from all sampled regions. The second most frequent species was D. foeniculina, with 23 isolates (10.2% of total), and it was recovered in all sampled regions except in La Rioja. Diaporthe eres and D. phaseolorum, each with only one isolate, were recovered from the Andalucía region. The remaining isolates (n = 8, 3.6% of total) were grouped together with 92% and 98% bootstrap support for MP and ML, respectively, and with 1 of BI posterior probability, but not with any known Diaporthe species. Therefore, they were putatively identified as belonging to a novel species described here and named D. mediterranea. This new species was obtained from the Islas Baleares and Comunidad Valenciana regions.

3.2. Taxonomy

Based on both the results of the phylogenetic inference and morphological characters, one new species of Diaporthe is described below (Figure 3).

**Diaporthe mediterranea** M. León, J. M. Rodríguez-Reina and J. Armengol, *sp. nov.*—MycoBank MB 836048; Figure 3.

***Typification***: Alcalá, Alicante province (Comunidad Valenciana), Spain. From *Prunus dulcis* twig canker, 2017, J. Armengol, DAL-34 (holotype; CBS H-24368—ex-type culture CBS 146754).

***Etymology***: Named after the Mediterranean Sea, because this species was found on almond trees from orchards located in the Alicante province (Comunidad Valenciana) and Mallorca (Islas Baleares) in Mediterranean coastal areas of Spain.

***Known distribution***: Spain.

***Description***: Conidiomata pycnidial, globose or irregular, solitary on PNA but also aggregated on MEA, PDA and OA, erumpent, dark brown to black, (mean diameter ± SD = 527 ± 104.8 µm, n = 30), whitish translucent to creamy conidial drops exuded from the ostioles. Conidiophores densely aggregated lining the inner cavity, smooth and hyaline, cylindrical, straight, reduced to conidiogenous cells (mean ± SD = 15.5 ± 2.7 × 2.2 ± 0.4 µm, n = 30). Paraphyses not observed. Alpha conidia produced in all the tested media, aseptate, fusiform, hyaline, multi-guttulate and acute at both ends, (mean ± SD = 6.6 ± 0.5 × 2.4 ± 0.2 µm, n = 30). Beta and gamma conidia not observed.

***Culture characteristics***: Colonies covering the medium within 7 d at 25 °C, with moderate aerial mycelium. Colonies on MEA, PDA and OA white at first, becoming light cream, mycelium flat on MEA and OA, denser and more felted on PDA. Reverse pale brown with light to dark grayish dots with age, with visible solitary and aggregate conidiomata at maturity on MEA, PDA and OA. Optimum growth temperature on PDA was 25.4 °C. Growth rates of colonies on PDA at 5, 10, 15, 20, 25, 30 and...
35 °C were 0.02, 0.11, 0.36, 0.44, 0.67, 0.57 and 0.01 mm per day, respectively. No growth was observed at 40 °C.

Additional materials examined: DAL24 Sant Llorenç d’Escardassar, Mallorca, Islas Baleares, Spain, 2014 and DAL174 Altea la Vella, Alicante, Comunidad Valenciana, Spain, 2018.

Notes: Diaporthe mediterranea was collected from P. dulcis in Spain. The BLASTn search showed 100% identity with the available sequences (ITS and tub) of two isolates named Phomopsis sp. 5 (PMM 1657 and PMM 1660), collected from Vitis vinifera in South Africa [41,42], which were not described as new species by any of the authors. Nevertheless, other loci are needed to better resolve the identity of these isolates. Phylogenetic analysis combining five gene loci showed that all the isolates of D. mediterranea clustered together in a highly supported clade (92/98/1) and displayed a close relationship but they were clearly differentiated from D. sterilis. Based on alignments of the separate loci, D. mediterranea differs from D. sterilis [43] in seven positions (6 nt and one indel of 1 nt) of 426 bp in tub (p-distance = 1.4%), 20 positions (4 nt and one indel of 16 nt) of 342 bp in tef1-α (p-distance = 1.5%), 21 nt of 434 bp in his (p-distance = 4.8%), and 3 nt of 469 bp in cal (p-distance = 0.6%). The ITS sequences of both species showed 100% identity. Morphologically, D. mediterranea mainly differs from D. sterilis in its capacity to produce alpha conidia, because all isolates representing D. sterilis could not be induced to sporulate on any of the culture media used by Lombard et al. [43], when this new Diaporthe species collected from Vaccinium corymbosum was described.

3.3. Pathogenicity Tests

All Diaporthe isolates inoculated on one-year-old twigs of almond cv. Vayro caused necrotic lesions of variable length (Figure 4). There was no effect of the experiment on the lesion length (p = 0.5032). Mean lesion length in canes inoculated with different Diaporthe isolates (n = 12 per inoculated isolate) ranged from 1.4 to 13.7 cm and control twigs treated with uncolonized PDA plugs showed a mean lesion length of 0.6 cm (Figure 5). Statistical analysis revealed significant differences in lesion length between the control and twigs inoculated with all isolates, except those of D. foeniculina, namely DAL-27 and DAL-61 (p = 0.7224 and p = 0.0117, respectively) and D. phaseolorum DAL-222 (p = 0.0239).

![Figure 4](image-url) NECROTIC LESIONS INDUCED BY THE DIAPORTHE SPP. INOCULATED ON ALMOND DETACHED CANES.
which were used to elucidate the diversity of phenotypical data and DNA sequence analyses.

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In five different regions of Spain from 2005 to 2019 resulted in a collection of 225 Diaporthe isolates, except for D. phaseolorum.

When isolates of the different Diaporthe species were grouped, significant differences in mean lesion length (cm) were also observed (p < 0.01). Twigs inoculated with D. mediterranea showed significantly longer mean lesions (11.3 cm) compared with D. amygdali. (Figure 6). There were no statistical differences among mean lesion length values caused by D. amygdali (7.7 cm), D. eres (8.4 cm) or D. phaseolorum (6.2 cm). However, twigs inoculated with D. foeniculina showed significantly shorter lesions (2.6 cm) compared with the other Diaporthe spp., except for D. phaseolorum.

Figure 6. Box plot of lesion length (cm) caused by Diaporthe spp. on almond detached twigs inoculated (n = 12 per isolate) with isolates of D. amygdali (seven isolates), D. eres (one isolate), D. foeniculina (two isolates), D. phaseolorum (one isolate) and D. mediterranea (three isolates). Black lines in the boxes show medians. Asterisks (*) indicate that values are significantly different than D. amygdali according to the Wilcoxon rank sum test (p < 0.01).

4. Discussion

The survey conducted on almond orchards showing twig cankers and shoot blight symptoms in five different regions of Spain from 2005 to 2019 resulted in a collection of 225 Diaporthe isolates, which were used to elucidate the diversity of Diaporthe species associated with this host using both phenotypical data and DNA sequence analyses.
This is the first study in which a collection of *Diaporthe* isolates from almond has been characterized using multilocus DNA sequence analysis with five loci (ITS, tub, tef-1a, cal and his), which has been recommended in previous phylogenetic studies of the genus *Diaporthe* for species identification and separation [14,17,44]. This analysis allowed the identification of four known *Diaporthe* species, namely: *D. amygdali*, *D. eres*, *D. foeniculina* and *D. phaseolorum*. Moreover, it also confirmed that eight isolates represented a novel phylogenetic species, newly described here as *D. mediterranea*.

*Diaporthe amygdali* was the most prevalent species, due to the largest number of isolates collected from widely separated almond growing regions in Spain. This fungus has been described on this crop in other Mediterranean countries, such as France [11], Greece [45], Hungary [46], Italy [47], Portugal [8,48] and Tunisia [49], where it is considered the main pathogen associated with twig cankers and shoot blight symptoms. In Mediterranean areas, *D. amygdali* has also been reported as a damaging agent in other fruit and nut crops, such as apricot [50], peach [9,51] and English walnut [32]. *Diaporthe amygdali* is also present in other continents, affecting diverse hosts: on almond and peach in the USA [53,54]; grapevine in South Africa [55]; peach in Japan [56]; peach and nectarine in Uruguay [57,58]; and peach, pear and walnut in China [59–61].

Regarding the other *Diaporthe* species found in our study: *D. eres* was previously reported on *P. dulcis* in Portugal [8], and *D. foeniculina* is present on almond in Italy, with one isolate (CBS 171.78) deposited at the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands) [62]. To our knowledge, our study represents the first report of *D. phaseolorum* on almond.

The isolates described in our work as belonging to the new taxon, *D. mediterranea*, were found only in two almond-growing regions in Spain: coastal areas of Alicante province (Comunidad Valenciana) and Mallorca (Islas Baleares). It is interesting to note that the ITS and tub sequences of two *Diaporthe* isolates, namely *Phomopsis* sp. 5 (PMM 1657 and PMM 1660), which were collected from *V. vinifera* in South Africa [41,42], showed 100% identity with the ITS and tub sequences of *D. mediterranea*. Further studies including other loci would be needed to resolve the identity of the South African isolates (PMM 1657 and PMM 1660).

Pathogenicity tests were performed using one-year-old almond twigs, as described by [8], who determined the capacity of *Diaporthe* spp. isolates from Portugal to cause lesions on this crop. Except for *D. foeniculina* and *D. phaseolorum*, all *Diaporthe* species inoculated to almond twigs cv. Vayro were able to cause lesions significantly different from those developed on the uninoculated controls. The most severe symptoms were detected on almond twigs inoculated with *D. mediterranea*. Therefore, this study provides novel information about the ability of this species to cause disease on *P. dulcis*, being more aggressive than the well-known pathogen *D. amygdali*. *Diaporthe eres* was also pathogenic to almond, but the incidence of this species and *D. phaseolorum* in the survey conducted in this study was extremely low, with only one isolate found in each species.

The present study is the first comprehensive attempt to characterize *Diaporthe* species associated with *P. dulcis* in Spain, combining morphology and multilocus DNA sequence analysis. Our results confirm *D. amygdali* as a key pathogen of almonds in Spain. Moreover, the new species *D. mediterranea* should also be considered as a potentially important causal agent of twig cankers and shoot blight on this crop, according to the high virulence shown in the pathogenicity tests. In Spain, the lack of information regarding the identity of *Diaporthe* species on almond and their pathogenicity hinders the development of efficient control strategies and the development of resistant varieties. These aspects have been addressed for the first time in this work and will contribute to the development of improved integrated disease management programs against twig canker and shoot blight disease.

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