Identification of *Arthrinium marii* as Causal Agent of Olive Tree Dieback in Apulia (Southern Italy)

Donato Gerin,¹ Franco Nigro,^{1,2} Francesco Faretra,^{1,2,†} and Stefania Pollastro^{1,2}

¹ Department of Soil, Plant and Food Sciences, University of Bari Aldo Moro, Bari 70126, Italy

² Selge Network, University of Bari Aldo Moro, Bari 70126, Italy

Abstract

Olive (*Olea europaea* L. var. *sativa*) is one of the most economically important tree crops grown in the Mediterranean basin. *Arthrinium* Kunze ex Fr. (teleomorph: *Apiospora* Sacc.) is a widespread fungal genus, and *Arthrinium marii* Larrondo & Calvo is a ubiquitous species, found in algae, soil, plants, and agricultural communities. *A. marii* was isolated from olive trees showing dieback from orchards located in Andria and in Fasano, Brindisi (Apulia, southern Italy) and identified based on morphological features and molecular analysis of four genomic regions (ITS, *TUB2*, *TEF1*, and

LSU). Two-year-old olive plants artificially inoculated with three representative *A. marii* isolates showed complete dieback within 6 months, and the fungus was reisolated, satisfying Koch's postulates. This is the first report of *A. marii* causing dieback on olive trees that could represent an important threat for olive cultivation.

Keywords: Arbosana, foliar chlorosis, ITS, *LSU* sequence, *TUB2* and *TEF1* sequences, withering and twig defoliations

Olive (*Olea europaea* L. var. *sativa*) is an economically important tree crop of Mediterranean countries, and the European Union is the leading world producer of olive oil and drupes (Food and Agriculture Organization of the United Nations 2017).

Although olive trees are very resistant to environmental stresses, several biotic entities can compromise their health. Fungal species as those belonging to the genus *Verticillium* are well known as vascular pathogens causing tree wilt, and fungi such as *Botryosphaeriaceae* spp. (*Diplodia* spp., *Dothiorella iberica*, *Lasiodiplodia theobromae*, *Neofusicoccum mediterraneum*), *Cytospora oleina*, *Eutypa lata*, *Phoma incompta*, *Phaeomoniella chlamydospora*, and *Phaeoacremonium* spp. are associated with olive tree decline, dieback, and cankers and more recently have acquired great importance worldwide (Carlucci et al. 2013, 2015; Ivic et al. 2010; Kaliterna et al. 2012; Moral et al. 2010; Nigro et al. 2013, 2014; Rhouma et al. 2010; Romero et al. 2005; Tosi and Natalini 2009; Úrbez-Torres et al. 2013).

A new olive cropping system consisting of intensive or superintensive plantings is expanding worldwide in order to increase productivity and profits (Tous et al. 2010). The plant density of 140 trees per hectare in traditional growing systems has become up to 400 and 2,500 trees per hectare, respectively, in intensive and superintensive growing systems (Russo et al. 2016).

These systems are characterized by different microclimatic conditions because they need more intensive use of irrigation and fertilization than traditional growing systems and by mechanical pruning and harvesting; consequently, the trees are more susceptible to diseases and pests (Graniti et al. 2011). In fact, in intensive and superintensive growing systems *Pseudomonas savastanoi* pv. *savastanoi* increases

on branches and twigs because of the injuries caused by crop management machinery (Tous et al. 2010). Additionally, an increase in *Verticillium* wilt has been seen (Jiménez-Díaz et al. 2011; López-Escudero and Mercado-Blanco 2011).

In 2018, dieback of about 30% of trees was observed in three 2-year-old olive orchards of cultivar Arbosana located in Andria and in Fasano, Brindisi (Apulia, southern Italy), and this study explored the causes of the dieback.

Materials and Methods

Sampling and fungal isolation. About 30% of olive trees (Arbosana) showing dieback were observed in three 2-year-old orchards located in Andria and in Fasano, Brindisi (Apulia, southern Italy). Ten representative symptomatic trees per orchard were randomly sampled and analyzed for wood symptoms in comparison with the healthy ones. Discolored wood tissues were surface-disinfested by submerging them for 2 min in 2% sodium hypochlorite solution, washed twice with sterile water, air dried, and then placed onto potato dextrose agar (PDA), infusion from 200 g of peeled and sliced potatoes kept at 60°C for 1 h, 20 g of dextrose, adjusted to pH 6.5, 20 g of agar, per liter) plates. Plates were incubated at 24 ± 1°C in the dark, and after 5 days *Arthrinium* sp. pure cultures were obtained. Three representative monoconidial isolates (DiSSPA_A1, DiSSPA_A2, and DiSSPA_A3) were obtained from pure cultures and used for the subsequent analyses.

Morphology and pathogenicity assay. Morphometric characters were assessed on 10-day-old colonies grown on PDA and malt extract agar (MEA; 20 g of malt extract and 20 g of agar, per liter) at 25 ± 1°C according to the synoptic keys proposed for *Arthrinium* species identification (Crous and Groenewald 2013), and conidia were quantified on the same plates, according to the method described by Crespo-Sempere et al. (2013). For each medium and isolate three replicated plates were used, and data were expressed as number of conidia × 10⁴/mm² of colony. Three hundred globose to elongate ellipsoid conidia were measured for each of the three monoconidial isolates DiSSPA_A1 to A3 with an optical microscope equipped with an ocular micrometer.

The pathogenicity of the DiSSPA_A1 to A3 isolates was investigated by artificial inoculation assays of 2-year-old olive tree cultivars Arbosana and Cellina di Nardò. Two different inoculation methods based on root (method 1) and wood inoculation (method 2) were compared. Briefly, for the method 1, isolates were cultured for 12 days on PDA at 25 ± 1°C in the dark, and conidia were scraped from the colony surface, suspended in sterile water with 0.05% Tween 20, and filtered on rayon-polyester filtration material to remove mycelium fragments. Roots of 2-year-old olive trees were

[†]Corresponding author: F. Faretra; francesco.faretra@uniba.it

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opportunistically cut at 20 cm in length, fully immersed in 2.5 liters of a suspension containing 5×10^4 conidia/ml, and maintained overnight at $25 \pm 1^\circ\text{C}$ before planting in 10- × 10- × 17-cm plastic pots containing a sand–lime–peat soil mixture. For the method 2, mycelial plugs (4 mm diameter) from the edge of 15-day-old colonies on PDA were placed in artificial wounds (5 mm long and 3 mm deep) under stem bark of olive trees potted as previously described, at 10 cm height from the crown and then protected with a layer of sealing film. Sterile water (method 1) and sterile PDA medium plugs (method 2) were used as the mock-inoculated controls, and six replicated plants were used for each treatment. Plants were maintained in the greenhouse ($25 \pm 2^\circ\text{C}$; 16-h daylight photoperiod) and at 6 months after inoculation (MAI), wood and roots of both the cultivars were inspected for internal symptoms with a destructive assay by dissecting the plants in three portions (about 10 cm each). The reisolation assay was performed by collecting from each portion five pieces (moving for each about 2 cm from bottom to top) of woody tissue surface disinfested as reported above and placed on PDA plates. Additionally, at 2, 4, and 6 MAI, the top length (budding) was measured, and canopy symptoms were assessed via an empirical scale of four classes (0 = healthy tree, 1 = $\leq 25\%$ of the tree showed symptoms [foliar chlorosis and necrosis], 2 = 26 to 50% of the tree showed symptoms, 3 = 51 to 75% of the tree showed symptoms, and 4 = 76 to 100% of the tree showed symptoms). Data were used to calculate the McKinney index (MKI) according to the following formula (McKinney 1923):

$$\text{MKI} = [\sum(f \times v)] / (n \times N) \times 100$$

where f is the number of symptomatic trees, v is the value of each class, N is the total number of examined trees, and n is the highest value of the classes occurring in the empirical scale.

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference test in CoStat software at the significance level $P \leq 0.05$. In terms of MKI, no differences were observed for inoculated trees among cultivars and isolates in preliminary two-way ANOVAs, so all data were used as biological replicates in comparing inoculated with mock-inoculated trees.

Molecular and phylogenetic analysis. Genomic DNA of the three monoconidial isolates was extracted according to the cetyl trimethylammonium bromide (CTAB)-based protocol described by De Miccolis Angelini et al. (2010). Briefly, 2-day-old fungal mycelium was collected from sterile cellophane overlapped to PDA plates. Mycelium, powdered under liquid nitrogen, was mixed with 600 μl of CTAB buffer (100 mM of Tris-Cl, pH 8.0; 1.4 M of NaCl; 20 mM of EDTA, pH 8.0; 2% cetyltrimethylammonium bromide [wt/vol]; 0.2% β -mercaptoethanol [vol/vol]). Samples were frozen and thawed three times with liquid nitrogen and a water bath at 75°C , and then incubated at 75°C for 1 h. After chloroform extraction, the clear supernatant was transferred to a new tube and precipitated with isopropanol. After 30 min at -80°C the pellet, collected by centrifugation at 14,000 rpm for 15 min, was washed with cold 70% ethanol, air dried, dissolved in TE (10 mM of Tris-Cl; 1 mM of EDTA, pH 8), treated with 0.1 $\mu\text{g}/\mu\text{l}$ of DNAase-free pancreatic RNAase for 2 h at 37°C , and finally precipitated by the addition of 0.6 volumes of 5 M of ammonium acetate and 2 volumes of cold absolute ethanol. The final DNA pellet, washed with 70% ethanol and air dried, was dissolved in ultrapure water and stored at -80°C until use. DNA was quantified with a spectrophotometer and stored at -20°C until use.

The ITS1–5.8S–ITS2 rDNA internal transcribed spacer (ITS) region, translation elongation factor 1- α (*TEF1*), β -tubulin (*TUB2*), and large subunit (*LSU*) of rDNA were amplified with the primers listed in Table 1. Amplifications were performed in 50 μl of reaction volume containing 1 $\times$ buffer, 2.5 mM of MgCl_2 , 100 nM of each dNTP, 100 nM of each primer, 1 U of Taq polymerase, and 100 ng of DNA template. PCR program consisted of an initial denaturation (94°C , 3 min), 35 cycles made up by denaturation (94°C , 1 min), annealing (58°C , 30 s), and extension (72°C , 1 min), followed by a final extension (72°C , 7 min).

PCR products were visualized on a 1.5% agarose gel (110 V for 110 min) and custom sequenced. Sequences were singularly analyzed for high sequence similarity through BLASTn by searching in the entire nucleotide collection database of GenBank (<https://www.ncbi.nlm.nih.gov>) and also limiting the search to the sequences from type material.

All ITS, *TEF1*, and *TUB2* sequences of *Arthrinium* sp. (GenBank accession numbers in Table 2) were previously aligned separately in SeqMan Pro software (alignment parameters: match size = 5, minimum match percentage = 10, maximum gaps per kilobase in sequence = 130), and only the sequences of 33 isolates (including 9 type specimens) that showed $\geq 80\%$ identity with those of isolates DiSSPA_A1 to A3 were trimmed with SeqMan Pro Software to obtain the concatenated ITS–*TEF1*–*TUB2* sequences. The *LSU* sequences were excluded because only a few *Arthrinium* sp. sequences were available. Phylogenetic analyses were conducted in MEGA version 6 (Tamura et al. 2013) using the maximum parsimony (MP) and maximum likelihood (ML) methods. The MP tree was obtained via the Subtree Pruning and Regrafting algorithm with search level 1, in which the initial trees were obtained by the random addition of sequences (10 replicates). For ML analysis, MEGA was used to infer the best model of nucleotide substitution for the dataset via the Tamura-Nei model and the nearest neighbor interchange heuristic search method. For both MP and ML, the branch stability was determined by 1,000 bootstrap replicates. *Nigrospora zimmermanii* ITS–*TEF1*–*TUB2* concatenated sequence was used as the outgroup.

Results

Symptom description, fungal isolation, and pathogenicity.

Symptomatic trees exhibited foliar chlorosis and twig defoliations. Wood discolorations were observed on transversal and longitudinal wood sections of branches and twigs, and colonies morphologically referable to *Arthrinium* sp. were predominantly obtained on PDA (Fig. 1).

Olive trees (Arbosana and Cellina di Nardò) were artificially inoculated with the three *Arthrinium marii* isolates DiSSPA_A1 to A3. Exclusively on *A. marii*-inoculated trees, no budding was observed up to 6 MAI, when complete dieback occurred. Conversely, noninoculated control trees grew about 10 cm per month (Fig. 2). At all times, no statistically significant differences between isolates and cultivar were ascertained (F and P always ≤ 1.6 and ≥ 0.2 , respectively) by analyses of inoculated trees. On the contrary, inoculated and mock-inoculated trees differed statistically in both root and wood inoculation assays (Table 3). The MKI was 0.0% on noninoculated control plants; 33.3, 63.9, and 86.1% on root-inoculated trees; and 19.4, 38.9, and 63.9% on wood-inoculated ones at 2, 4, and 6 MAI, respectively.

Table 1. Primers used in this study

Target gene or region	Primer name	Primer sequence (5'-3')	References
ITS1–5.8S rDNA–ITS2 region	ITS5	GGAAGTAAAAGTCGTAACAAGG	White et al. 1989
	ITS4	TCCTCCGCTTATTGATATGC	
Translation elongation factor 1-a	EF1-728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn 1999
	EF1-986R	TACTTGAAGGAACCCCTTACC	
β -tubulin	T1	AACATGCGTGAGATTGTAAGT	Glass and Donaldson 1995
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	
Large subunit rDNA region	LROR	ACCCGCTGAACCTTAAGC	O'Donnell and Cigelnik 1997
	LR5	TCCTGAGGGAACTTCG	

Table 2. Details of *Arthrinium* spp. strains used in the BLASTn and phylogenetic analysis

Species	Strain [*]	Host	Country	GenBank accession numbers ^y		
				ITS	TUB2	TEF1
<i>Arthrinium arundinis</i>	CBS 464.83	<i>Phragmites australis</i>	Netherlands	KF144888.1	KF144979.1	KF145021.1
<i>A. arundinis</i>	LC7277	<i>Bambusa</i> sp.	China	KY494750.1	KY705218.1	KY705146.1
<i>A. arundinis</i>	EGG3	<i>Lasiotera donacis</i>	France	MF627422.1	MF627424.1	MF627423.1
<i>Arthrinium bambusae</i>	LC7106	<i>Bambusa</i> sp.	China	KY494719.1	KY705187.1	KY705117.1
<i>A. bambusae</i>	LC7113	<i>Bambusa</i> sp.	China	KY494720.1	KY705188.1	KY806205.1
<i>A. bambusae</i>	LC7124	<i>Bambusa</i> sp.	China	KY494727.1	KY705195.1	KY806206.1
<i>Arthrinium camelliae-sinensis</i>	LC8181	<i>Brassica rapa</i> subsp. <i>oleifera</i>	China	KY494761.1	KY705229.1	KY705157.1
<i>A. camelliae-sinensis</i>	LC5007	<i>Camellia sinensis</i>	China	KY494704.1	KY705173.1	KY705103.1
<i>Arthrinium dichotomanthi</i>	LC8175	<i>Dichotomanthes tristaniicarpa</i>	China	KY494755.1	KY705223.1	KY705151.1
<i>A. dichotomanthi</i>	LC8176	<i>Dichotomanthes tristaniicarpa</i>	China	KY494756.1	KY705224.1	KY705152.1
<i>A. dichotomanthi</i>	LC4950	<i>Dichotomanthes tristaniicarpa</i>	China	KY494697.1	KY705167.1	KY705096.1
<i>Arthrinium gaoyouense</i>	CFCC52301	<i>Phragmites australis</i>	China	MH197124	MH236789.1	MH236793.1
<i>A. gaoyouense</i>	CFCC52302	<i>Phragmites australis</i>	China	MH197125	MH236790.1	MH236794.1
<i>Arthrinium guizhouense</i>	LC5322	Air	China	KY494709.1	KY705178.1	KY705108.1
<i>A. guizhouense</i>	LC5318	Air	China	KY494708.1	KY705177.1	KY705107.1
<i>Arthrinium hydei</i>	LC7103	<i>Bambusa</i> sp.	China	KY494715.1	KY705183.1	KY705114.1
<i>A. hydei</i>	CBS 114990	<i>Bambusa tuldoidea</i>	China	KF144890.1	KF144982.1	KF145024.1
<i>Arthrinium jiangxiense</i>	LC2831	<i>Bambusa</i> sp.	China	KY494686.1	KY806201.1	KY705085.1
<i>A. jiangxiense</i>	LC4577	<i>Maesa</i> sp.	China	KY494693.1	KY705163.1	KY705092.1
<i>A. jiangxiense</i>	LC5394	Soil	China	KY494711.1	KY705180.1	KY705110.1
<i>Arthrinium kogelbergense</i>	CBS 117206	Unknown algae	Croatia	KF144895.1	KF144987.1	KF145029.1
<i>A. kogelbergense</i>	CBS 113333	<i>Restionaceae</i>	South Africa	KF144893.1	KF144985.1	KF145027.1
<i>A. kogelbergense</i>	CBS 114734	<i>Uncus gerardii</i>	Sweden	KF144894.1	KF144986.1	KF145028.1
<i>Arthrinium malaysianum</i>	CBS 102053	<i>Macaranga hullettii</i>	Malaysia	KF144896.1	KF144988.1	KF145030.1
<i>A. malaysianum</i>	CBS 251.29	<i>Cinnamomum camphora</i>	Unknown	KF144897.1	KF144989.1	KF145031.1
<i>Arthrinium marii</i>	CPC 18904	<i>Phragmites australis</i>	Italy	KF144902.1	KF144994.1	KF145036.1
<i>A. marii</i>	CBS 497.90	—	Spain	MH862232.1	KF144993.1	KF145035.1
<i>A. marii</i>	CBS 200.57	<i>Beta vulgaris</i>	Netherlands	KF144900.1	KF144992.1	KF145034.1
<i>A. marii</i>	CBS 114803	<i>Pseudosasa hindsii</i>	China	KF144899.1	KF144991.1	KF145033.1
<i>A. marii</i> ^z	DiSSPA_A1	<i>Olea europaea</i>	Italy	MK602320.1	MK614695.1	MK645472.1
<i>A. marii</i> ^z	DiSSPA_A1	<i>Olea europaea</i>	Italy	MK602321.1	MK614696.1	MK645473.1
<i>A. marii</i> ^z	DiSSPA_A1	<i>Olea europaea</i>	Italy	MK602322.1	MK614697.1	MK645474.1
<i>Arthrinium obovatum</i>	LC8177	<i>Lithocarpus</i> sp.	China	KY494757.1	KY705225.1	KY705153.1
<i>A. obovatum</i>	LC8178	<i>Lithocarpus</i> sp.	China	KY494758.1	KY705226.1	KY705154.1
<i>A. obovatum</i>	LC4940	<i>Lithocarpus</i> sp.	China	KY494696.1	KY705166.1	KY705095.1
<i>Arthrinium ovatum</i>	CBS 115042	<i>Arundinaria hindsii</i>	China	KF144903.1	KF144995.1	KF145037.1
<i>Arthrinium phaeospermum</i>	CBS 142.55	Soil	Japan	KF144908.1	KF145000.1	KF145042.1
<i>A. phaeospermum</i>	CBS 114318	<i>Hordeum vulgare</i>	Iran	KF144907.1	KF144999.1	KF145041.1
<i>A. phaeospermum</i>	CBS 114317	<i>Hordeum vulgare</i>	Iran	KF144906.1	KF144998.1	KF145040.1
<i>Arthrinium phragmites</i>	CPC:18900	<i>Phragmites australis</i>	Italy	KF144909.1	KF145001.1	KF145043.1
<i>Arthrinium pseudoparenchymaticum</i>	LC8173	<i>Bambusa</i> sp.	China	KY494753.1	KY705221.1	KY705149.1
<i>A. pseudoparenchymaticum</i>	LC8174	<i>Bambusa</i> sp.	China	KY494754.1	KY705222.1	KY705150.1
<i>A. pseudoparenchymaticum</i>	LC7234	<i>Bambusa</i> sp.	China	KY494743.1	KY705211.1	KY705139.1
<i>Arthrinium pseudospegazzinii</i>	CBS 102052	<i>Macaranga hullettii</i>	Malaysia	KF144911.1	KF145002.1	KF145045.1
<i>Arthrinium pterospermum</i>	CPC:20193	<i>Lepidosperma gladiatum</i>	Australia	KF144913.1	KF145004.1	KF145046.1
<i>Arthrinium rasikravindrae</i>	LC5449	Unknown	China	KY494713.1	KY705182.1	KY705112.1
<i>A. rasikravindrae</i>	LC7115	<i>Bambusa</i> sp.	China	KY494721.1	KY705189.1	KY705118.1
<i>A. rasikravindrae</i>	LC7117	<i>Bambusa</i> sp.	China	KY494722.1	KY705190.1	KY705119.1
<i>Arthrinium sacchari</i>	CBS 372.67	Air	Unknown	KF144918.1	KF145007.1	KF145049.1
<i>A. sacchari</i>	CBS 664.74	Soil	Netherlands	KF144919.1	KF145008.1	KF145050.1
<i>A. sacchari</i>	CBS 301.49	<i>Bambusa</i> sp.	Indonesia	KF144917.1	KF145006.1	KF145048.1
<i>Arthrinium saccharicola</i>	CBS 831.71	Unknown	Netherlands	KF144922.1	KF145012.1	KF145054.1
<i>A. saccharicola</i>	CBS 463.83	<i>Phragmites australis</i>	Netherlands	KF144921.1	KF145011.1	KF145053.1
<i>A. saccharicola</i>	CBS 191.73	Unknown	Netherlands	KF144920.1	KF145009.1	KF145051.1
<i>Arthrinium subroseum</i>	LC7215	<i>Bambusa</i> sp.	China	KY494740.1	KY705208.1	KY705136.1
<i>A. subroseum</i>	LC7291	<i>Bambusa</i> sp.	China	KY494751.1	KY705219.1	KY705147.1
<i>Arthrinium thailandicum</i>	LC5630	Unknown	China	KY494714.1	KY806200.1	KY705113.1
<i>Arthrinium xenocordella</i>	CBS 478.86	Soil	Zimbabwe	KF145013.1	KF144925.1	KF145055.1
<i>A. xenocordella</i>	LC3486	<i>Camellia sinensis</i>	China	KY494687.1	KY705158.1	KY705086.1

^{*} CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CPC, Culture collection of Pedro Crous, housed at Westerdijk Fungal Biodiversity Institute; LC, working collection of Lei Cai, housed at Institute of Microbiology, Chinese Academy of Sciences, Beijing, China. Bold indicates type specimens.

^y ITS, partial ITS1–5.8S rDNA–ITS2; *TEF1*, translation elongation factor 1- α ; *TUB2*, β -tubulin.

^z For isolates DiSSPA_A1, DiSSPA_A2, and DiSSPA_A3 the accession numbers of the rDNA large subunit regions (LSU) sequences, used in the BLASTn analysis, were MK604516, MK604517, and MK604518, respectively.

At the end of the experiment (6 MAI), arthrinium-like colonies were always reisolated from discolored woody tissues, 25 to 30 cm away from the inoculation point (stem or roots) of the inoculated plants (Arbosana and Cellina di Nardò). Moreover, about 30% of roots showed discolorations only in the root-inoculated trees with *A. marii*. No arthrinium-like colonies were obtained from noninoculated control trees. Other fungi morphologically characterized, such

as *Trichoderma*, *Alternaria*, and *Cladosporium*, were isolated from all the plants, irrespective of artificial inoculation method.

Morphology of *A. marii* isolates. The morphology of *A. marii* isolates was observed on PDA and MEA after 10 days of incubation at 25°C in the dark. On both media, colonies were flat, spreading with sparse aerial mycelium. On PDA, cultures of the three isolates showed an olivaceous gray surface and olivaceous gray patches on the reverse surface. On MEA, the cultures appeared with a white-gray surface and gray patches on the reverse surface (Fig. 3A).

Mycelium consisted of smooth, hyaline, branched, and septate hyphae measuring 2 to 4 µm in diameter. Fifteen-day-old PDA cultures exhibited conidiophores in form of conidiogenous cells that were aggregated in clusters on brown (Fig. 3B), smooth, and ampulliform hyphae, producing conidia (Fig. 3C, D). Conidia were brown, smooth, granular, globose to elongate ellipsoid, and measuring 6 to 13 µm in diameter. Brown, elongated, sterile cells (average 22 × 5 µm), typical of some *Arthrinium* species (Samuels et al. 1981), were also observed (Fig. 3E).

Finally, for all isolates, the conidia production was higher on MEA (3.2 to 5.0×10^4 conidia per square millimeter) with respect to PDA (5.5 to 8.4×10^4 conidia per square millimeter) (Table 4).

Molecular and phylogenetic analysis. Sequences of 567, 833, 262, and 727 were obtained by sequencing the partial ITS, *LSU*, *TEF1*, and *TUB2* regions, respectively, of the three *A. marii* isolates DiSSPA_A1 to A3 (Table 2).

BLASTn analysis against the entire nucleotide collection database of GenBank revealed that all sequences showed 99 to 100% identity (coverage = 99 to 100%, e-value = 0.0) with different *Arthrinium* species. In more detail, for the three isolates the ITS region showed 100% similarity with *A. marii*, *Arthrinium* sp., and *Arthrinium phaeospermum* (e.g., KF144900.1, MH355544.1, and KY081461.1), the *LSU* showed 99% similarity with *A. marii*, *Arthrinium* sp., and *Arthrinium arundinis* (e.g., KF144946.1, MH109530.1, and KF993394.1), the *TEF1* showed 99% similarity with *A. marii* (e.g., KF145034.1), and the *TUB2* showed 100% similarity with *A. marii* and *Arthrinium hispanicum* (KF144992.1 and AB220289.1). Although none of them was a type specimen, *A. marii* was the only species showing the maximum identity for all four analyzed sequences.

Additionally, with the BLASTn analysis only against sequences from type material, all sequences (ITS, *LSU*, *TEF1*, and *TUB2*) of the three isolates showed 99 to 100% identity (coverage = 99 to 100%, e-value = 0.0) with *A. marii* type specimen (CBS 497.90, accession numbers in Table 2). The ITS and *LSU* regions of the three isolates showed identities >99%, although less than *A. marii* CBS 497.90, also with *Arthrinium guizhouense*, *Arthrinium longistromum*, and *Arthrinium pseudospegazzinii* type specimens (LC5322, MFLU 15-1184, and CBS 102052 strains, respectively). Finally, identities <96% with different *Arthrinium* type specimens were observed for *TEF1* and *TUB2* regions.

After the first alignment of ITS, *TUB2*, and *TEF1* sequences, 33 of 56 *Arthrinium* spp. showed identity >80% with the DiSSPA_A1 to A3 isolates. The most parsimonious tree obtained by MP analysis of concatenated ITS-*TEF1*-*TUB2* sequences (tree length = 545, consistency index = 0.608, retention index = 0.812, composite index = 0.621) is shown in Figure 4. The analysis using the ML and

Fig. 1. Apical shoots of A, healthy and B, symptomatic 2-year-old olive plants along with wood discoloration from which the 7-day-old *Arthrinium marii* colonies were obtained on potato dextrose agar (PDA).

Fig. 2. Olive plants inoculated or not with *Arthrinium marii*. a, Detail of budding in *A. marii* inoculated and noninoculated plants at 2 months after inoculation (MAI). b, General status of leaves at 2, 4, and 6 MAI of *A. marii* inoculated and noninoculated trees. Arrows indicate the absence of budding in the inoculated tree, and bracket marks indicate budding of noninoculated tree.

Table 3. McKinney index for olive trees artificially inoculated with *Arthrinium marii*^z

Inoculation	2 MAI	4 MAI	6 MAI
Wood			
<i>A. marii</i>	19.4 ± 2.8 a	38.9 ± 4.8 a	63.9 ± 2.8 a
Control	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
Root			
<i>A. marii</i>	33.3 ± 0.0 a	63.9 ± 2.8 a	86.1 ± 2.8 a
Control	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b

^z Data represent the mean values ± standard error. MAI, months after inoculation. For each MAI and inoculation assay, data followed by different letters were statistically significant at probability level $P \leq 0.05$ according to the Tukey test.

Tamura-Nei model resulted in a tree similar to that obtained with MP analysis, and with the highest log likelihood of -7344.64. Both analyses showed that the concatenated ITS-*TEF1*-*TUB2* sequences of all *Arthrinium* isolates obtained with this study clustered with *A. marii* type specimen, albeit with support $\leq 91\%$ (Fig. 4).

Discussion

In this study we identified *A. marii* as the causal agent of a severe dieback of olive trees observed in 2018 in three 2-year-old olive orchards, Arbosana located in Apulia (southern Italy). The fungus is ubiquitous in nature, and it has been reported in soils, plants, and agricultural communities (Agut and Calvo 2004; Crous and Groenewald 2013; Dai et al. 2016; Gnavi et al. 2017; Oliveira et al. 2012; Senanayake et al. 2015; Sharma et al. 2014; Wang et al. 2018) as well as in green algae (Gnavi et al. 2017). *Arthrinium* Kunze ex Fr. is an anamorph genus, which has been traditionally linked to the teleomorph genus *Apiospora* Sacc.

Arthrinium currently comprises 80 species (<http://www.indexfungorum.org>), and several of them have been documented as plant pathogens and endophytes (Mavragani et al. 2007; Sharma et al. 2014; Wang et al. 2018), although information on fungal biology and disease epidemiology is generally scanty. Reports on *Arthrinium* diseases are quite recent, but different species are known to be pathogens in different host plants, probably because of changes in environmental conditions, cultural practices, or the introduction of new cultivars (Crous and Groenewald 2013; Lo Piccolo et al. 2014; Mavragani et al. 2007). For example, *A. arundinis* causes brown culm streak of *Phyllostachys praecox*, is additionally found on different plant hosts in China (e.g., *Bambusa* sp., *Bothrocaryum controversum*, *Dichotomanthus tristaniaecarpa*, *Phyllostachys* sp., and *Osmanthus* sp.) (Chen et al. 2014; Wang et al. 2018), and has been isolated from the leaf of *Hordeum vulgare*, living leaves of *Fagus sylvatica* in Iran and Switzerland, and rosemary in Iran (Bagherabadi

et al. 2014; Crous and Groenewald 2013). *Arthrinium sacchari* causes damping-off of durum wheat in the semiarid Saskatchewan fields (Mavragani et al. 2007). *Arthrinium saccharicola* has been isolated from living and dead culms of *Phragmites australis* and even from the air in the Netherlands and France and from seawater in mangrove habitats in Hong Kong (Crous and Groenewald 2013; Miao et al. 2006). *Arthrinium xenocordella* is reported as causing fruit blight on *Pistacia vera* in Italy (Aiello et al. 2018). *A. phaeospermum*, responsible for culm rot on *Phyllostachys viridis* (Li et al. 2016), was recently demonstrated to be also associated with necrosis of olive leaves, and according to Koch's postulates the pathogenicity was proved only on leaves and not on twigs (Lo Piccolo et al. 2014). In this study, *A. marii* isolates were collected from branches and twigs of olive trees showing dieback, and they reproduced the disease when artificially inoculated on 2-year-old olive plants, satisfying Koch's postulates. The fungus was shown to be highly virulent; in fact, at 6 MAI it was reisolated 25 to 30 cm away from the inoculation point. A similar behavior was reported for *Botryosphaeria dothidea* and *Neofusicoccum mediterraneum*, causing ≤ 16 -cm lesions on olive branches within 1 MAI (Moral et al. 2010, 2017) and for *Neofabrea kienholzii* and *Phlyctema vagabunda*, both isolated from shoot

Table 4. Conidia production by *Arthrinium marii* isolates^z

Isolate	Conidia (No. $\times 10^4/\text{mm}^2$)	
	PDA	MEA
DiSSPA_A1	3.2 ± 0.4	6.6 ± 0.9
DiSSPA_A2	5.0 ± 0.7	8.4 ± 0.6
DiSSPA_A3	3.2 ± 0.5	5.5 ± 0.7

^z PDA, potato dextrose agar; MEA, malt extract agar. Data represent the mean values $\pm$ standard error.

Fig. 3. Morphology of the *Arthrinium marii* DiSSPA_A1 isolated from olive trees. **A**, Top and bottom of potato dextrose agar (PDA) and malt extract agar (MEA) cultures. **B**, details of PDA colony after 10 and 15 days after inoculation (DAI). **C**, Conidia and conidiogenous cell. **D**, Conidia. **E**, Sterile cells. In **C**, **D**, and **E**, bars represent 10 μm , and observations refer to 15-day-old PDA cultures.

lesions on olive in California and producing lesions up to 4 cm in length at 3 MAI, when inoculated into 2-year-old detached shoots (Trouillas et al. 2019).

The morphological features of the three isolates DiSSPA_A1 to A3 agreed with those described for *A. marii* by Crous and Groenewald (2013). In addition to the conidia, produced in higher amounts on MEA than on PDA, all isolates (DiSSPA_A1 to A3) also produced sterile

cells; these features are well known for some *Arthrinium* species, including *A. marii* (Crous and Groenewald 2013; Samuels et al. 1981).

Examination of morphological features and molecular analysis were both necessary for the appropriate identification of *A. marii*. Recently, a reassessment of *Arthrinium* sp. was proposed based on the phylogeny of *LSU* and *ITS* regions as well as concatenated *TUB2-TEF1* sequences (Crous and Groenewald 2013). According to the nucleotide

Fig. 4. Phylogenetic tree of concatenated *ITS-TEF1-TUB2* sequences of *Arthrinium* spp. generated by both maximum parsimony (MP) and maximum likelihood (ML) analyses. In the figure the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown on the nodes (MP/ML). *Nigrospora zimmermanii* was used as the outgroup. *Type specimens.

BLASTn analysis of ITS, *TUB2*, *TEF1*, and *LSU* gene sequences, *A. marii* (type specimen and other isolates) was the only species matching with a high identity (99 to 100%) for all four analyzed sequences. This result was also confirmed by phylogenetic analysis using the concatenated ITS–*TEF1*–*TUB2* sequences. In fact, all isolates (DiSSPA_A1 to A3) clustered with the type specimen of *A. marii* (CBS 497.90), although with a support of $\leq 91\%$ of the bootstrap test, and they represented a distinct clade in respect to the other species, including the recently described *Arthrinium gaoyouense* (Jiang et al. 2018). Additionally, our results confirm that strain CBS 114803, identified as *A. marii*, seems to represent an independent lineage, as recently reported by Pintos et al. (2019).

In conclusion, to our knowledge this is the first report of *A. marii*, a fungal species that seems ubiquitous in nature, as an agent of severe dieback of olive trees in southern Italy.

The spread of the fungus could represent a serious threat for olive cultivation, because of the challenges of the new planting systems (intensive and superintensive) and because of the introduction into agricultural practice of new varieties more adapted to the new growing system. Generally, the number of fungal species reported as causal agents of trees' decline and dieback is increasing, and the interactions established by different fungal species inhabiting olive trees are unknown (Graniti et al. 2011; Nigro et al. 2013, 2014). Additionally, fertilizers and biostimulants obtained from biomasses of different origin, including algae, are available on the market and commonly applied in agriculture because they are considered eco-friendly, but information on the effect of the algae mycobiota on cultivated plants are scanty. For instance, *A. marii* is a component of the mycobiota of the Mediterranean green alga *Flabellia petiolata* (Gnavi et al. 2017) and the calcareous brown alga *Padina pavonica* (Garzoli et al. 2018). It is crucial to improve basic knowledge on the microbial community interacting in the complex olive pathosystem in order to implement adequate protection strategies.

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