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Alternaria muriformis sp. nov., a New Species in Section *Chalastospora* Isolated from Herbivore Dung in Spain

Isabel Iturrieta-González [1,2] and Josepa Gené [3,*]

[1] Department of Preclinic Sciences, Medicine Faculty, Laboratory of Infectology and Clinical Immunology, Center of Excellence in Translational Medicine-Scientific and Technological Nucleus (CEMT-BIOREN), Universidad de La Frontera, Temuco 4810296, Chile; isabel.iturrieta@ufrontera.cl
[2] Jeffrey Modell Center of Diagnosis and Research in Primary Immunodeficiencies, Center of Excellence in Translational Medicine, Medicine Faculty, Universidad de La Frontera, Temuco 4810296, Chile
[3] Unitat de Micologia i Microbiologia Ambiental, Facultat de Medicina i Ciències de la Salut and IISPV, Universitat Rovira i Virgili, 43201 Reus, Spain
* Correspondence: josepa.gene@urv.cat

Abstract: In a survey of the mycobiota from the dung of herbivorous animals collected in natural areas in Spain, an *Alternaria* isolate was found. Morphological data and a multi-locus phylogenetic approach carried out through Maximum Likelihood and Bayesian Inference analyses with three gene markers (i.e., the internal transcribed spacer of rDNA, glyceraldehyde-3-phosphate dehydrogenase, and plasma membrane ATPase) revealed that it represents a novel *Alternaria* species in *Chalastospora*. *Alternaria muriformis* sp. nov. is described and illustrated here. It is closely related to *Alternaria abundans*, *Alternaria armoraciae*, and *Alternaria breviramosa*, but can be easily differentiated by its production of muriform conidia. Key morphological features of the members of the *Chalastospora* section are provided.

Keywords: *Ascomycota*; *Alternaria*; *Chalastospora*; new species; herbivore dung; dematiaceous hyphomycetes; phylogeny; *Pleosporaceae*; taxonomy

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1. Introduction

Alternaria, erected in 1816 [1], is currently one of the richest-species genera in the order *Pleosporales* (*Dothideomycetes*), with a wide environmental distribution and adaptation to diverse ecological lifestyles. It includes saprophytic species mainly inhabiting decaying plant material but also species associated with living plants, such as endophytes or phytopathogens [2–4]. Phytopathogenic species cause disease in a wide variety of important agronomic host plants, including ornamentals, fruits, vegetables, and other crops, affecting both pre- and post-harvested stages [5–8]. Several species are also able to cause animal and human infections, such as *Alternaria alternata*, *Alternaria infectoria*, *Alternaria triticina*, and the recently described pathogenic species *Alternaria anthropophila*, *Alternaria atrobrunnea*, and *Alternaria guarroi* [9–12]. Furthermore, *Alternaria* spp. are notable for their ability to produce secondary metabolites with phytotoxic, cytotoxic, antifungal, and antimicrobial effects, some of which have beneficial applications in the biotechnological and chemical industries [8,13,14].

In the last decade, the genus *Alternaria* has been taxonomically reevaluated based on several multi-gene phylogenetic analyses using a combination of various gene markers like the internal transcribed spacer of rDNA (ITS) and protein-coding genes, such as glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), plasma membrane ATPase (*ATPase*), RNA polymerase second largest subunit (*rpb2*), and translation elongation factor 1-alpha (*tef1*) [2,12,15]. As a result, the genus currently contains more than 380 species, which are distributed in 29 sections and seven monotypic lineages [4,7,15–18]. The section *Chalastospora*, which was erected by Woudenberg et al. [15] and typified by *Alternaria cetera*

(E.G. Simmons), was introduced to accommodate species previously included in the hyphomycetous genera *Chalastospora* [19] and *Embellisia* [20]. The section was morphologically characterized as producing simple or branched primary conidiophores from which pale to medium brown conidia originated singly or in chains. Conidia are usually narrowly ellipsoid to ellipsoid or ovoid, beakless, with no to multiple transverse eusepta, and rarely longitudinal or oblique septa [15]. The species in the section are consistently separated in terms of their genetic differences based on multilocus sequence typing (MLST) of three concatenated loci (i.e., ITS, *gapdh,* and *ATPase*) [2]. Currently, the *Chalastospora* section comprises seven species, most of which have been found on plant material, i.e., *Alternaria abundans* (E.G. Simmons), *Alternaria armoraciae* (E.G. Simmons and C.F. Hill), *Alternaria breviramosa* (Woudenberg and Crous), and *A. cetera*. The *Alternaria obclavata* (Crous and U. Braun) was described from air, and *Alternaria malorum* (Rühle, U. Braun, and Crous and Dugan) and *Alternaria pobletensis* (Iturrieta-González and Dania García and Gené) from herbivore dung, with *A. malorum* having also been reported as an opportunistic pathogen in humans [2,15,21].

The aim of the present study was to characterize, using a polyphasic approach combining phenotypic and sequence data, a putative novel species of *Alternaria* in the section *Chalastospora* isolated from herbivore dung collected in a natural area of Catalonia (Spain).

2. Materials and Methods

2.1. Sampling and Isolation of Fungi

Dung samples collected from different geographical regions of Spain were incubated in moist chambers at room temperature (ca. 24 °C) in darkness and examined periodically for about two months. Interesting fungi were isolated on potato dextrose agar (PDA; Pronadisa, Madrid, Spain) and preserved at the culture collection of the Medical School of Rovira i Virgili University (FMR; Reus, Spain) for further studies. The *Alternaria* isolate FMR 17518 was revived for morphological and molecular analysis.

Taxonomic information and nomenclature for the new species were deposited in MycoBank (https://www.mycobank.org/). Ex-type culture and holotype (as a dry colony) were deposited at the Westerdijk Fungal Biodiversity Institute (CBS, Utrecht, The Netherlands).

2.2. DNA Extraction, PCR, Sequencing, and Phylogenetic Analysis

Genomic DNA was extracted from colonies growing on PDA for 7 to 14 days at 25 °C in darkness, according to Müller et al. [22]. For a preliminary identification and later for establishing phylogenetic relationships, we amplified and sequenced the ITS region, *ATPase,* and *gapdh* gene markers according to the loci used in previous studies [2]. Amplification of the ITS barcode was performed using the primer pairs ITS5/ITS4 [23], ATPDF1/ATPDR1 for *ATPase* [24], and gpd1/gpd2 for *gapdh* [25] (Table 1).

Table 1. List of primer pair sets used for PCR and sequencing.

Locus	Primer	Sequence (5′–3′)	References
Internal transcribed spacer (ITS)	ITS5	GGAAGTAAAAGTCGTAACAAGG	[23]
	ITS4	TCCTCCGCTTATTGATATGC	
Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*)	gpd1	CAACGGCTTCGGTCGCATTG	[25]
	gpd2	GCCAAGCAGTTGGTTGTGC	
Plasma membrane ATPase (*ATPase*)	ATPDF1	ATCGTCTCCATGACCGAGTTCG	[24]
	ATPDR1	TCCGATGGAGTTCATGATAGCC	

PCR products were purified with a Qiagen PCR Purification Kit (Qiagen, Inc., Valencia, CA, USA) and stored at −20 °C until sequencing. The same pairs of primers used for the amplification were used in sequencing the products, which were processed at Macrogen

Europe (Macrogen Inc., Madrid, Spain). The sequences of each isolate were edited using SeqMan v.7.0.0 (DNAStar Lasergene, Madison, WI, USA) to obtain the consensus sequences.

The sequences obtained were compared with those in the National Center for Biotechnology Information (NCBI) database, and those of the species related to our unidentified isolate were retrieved from GenBank for phylogenetic analysis (Table 2).

Multiple sequence alignments of the individual loci and combined analysis were performed using the MEGA (Molecular Evolutionary Genetics Analysis) software v.6.0 [26], through the ClustalW algorithm [27], refined with MUSCLE [28] in the same platform, and manually adjusted as necessary. Phylogenetic reconstructions were made using Maximum Likelihood (ML) and Bayesian Inference (BI) under MEGA software v.6.0 and MrBayes v.3.2.6 [29], respectively. The combined analysis of these phylogenetic markers was tested through the incongruence length difference (ILD) implemented in the Winclada program [30]. ML bootstrap values (bs) $\geq$ 70% were considered significant. For the BI phylogenetic analysis, the best nucleotide substitution model was determined using jModelTest [31]. The parameter settings used were two simultaneous runs of five M generations and four Markov chains, sampled every 1000 generations. The 50% majority-rule consensus tree and posterior probability values (pp) were calculated after discarding the first 25% of the samples. A pp value of $\geq$0.95 was considered significant. Sequence data generated in the present study were deposited in GenBank (Table 2).

Table 2. *Alternaria* species included in the phylogenetic analysis and their GenBank accession number.

Species	Section	Isolates [1]	Sources	GenBank Accession Numbers [2]			References
				ITS	*gapdh*	*ATPase*	
A. abundans	*Chalastospora*	CBS 534.83 [T]	*Fragaria* sp. and stolon	JN383485	KC584154	JQ671802	[15,32]
A. armoraciae	*Chalastospora*	CBS 118702 [T]	*Armoracia rusticana*	KC584182	KC584099	LR134098	[2,15]
A. breviramosa	*Chalastospora*	CBS 121331 [T]	*Triticum* sp.	FJ839608	KC584148	LR134099	[2,15]
A. cetera	*Chalastospora*	CBS 121340 [T]	*Elymus scabrus*	JN383482	AY562398	LR134101	[15,32]
A. malorum	*Chalastospora*	CBS 135.31	*Malus sylvestris* and fruit	JQ693638	JQ646278	JQ671800	[33]
	Chalastospora	FMR 17369	Rabbit dung	LR134074	LR134077	LR134029	[2]
A. obclavata	*Chalastospora*	CBS 124120 [T]	Air	KC584225	KC584149	LR134100	[2,15]
A. pobletensis	*Chalastospora*	FMR 16448 [T]	Herbivore dung	LR133896	LR133897	LR133903	[2]
A. muriformis	***Chalastospora***	**FMR 17518 [T]**	**Herbivore dung**	**OQ421258**	**OQ425406**	**OQ425407**	**Present study**
A. caricis	*Nimbya*	CBS 480.90 [T]	*Carex hoodii*	AY278839	AY278826	JQ671780	[15,32]
A. scirpicola	*Nimbya*	CBS 481.90	*Scirpus* sp.	KC584237	KC584163	JQ671781	[15,32]

[1] CBS: culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; FMR: Facultat de Medicina, Universitat Rovira i Virgili, Reus, Spain; [T] indicates ex-type strains. [2] ITS: internal transcribed spacers and intervening 5.8S nrDNA; *ATPase*: plasma membrane ATPase gene; *gapdh*: glyceraldehyde-3-phosphate dehydrogenase. The novel species described in this study is indicated in bold.

2.3. Phenotypic Study

Macroscopic characterization of the colonies was performed on PDA, potato carrot agar (PCA; potato 20 g, carrot 20 g, agar 13 g, and distilled water 1 L), and oatmeal agar (OA; oatmeal 30 g, agar 13 g, and distilled water 1 L) for 7 days at 25 °C in darkness. The colors of the colonies in descriptions were based on Kornerup and Wanscher [34]. Cardinal temperatures for growth were tested in duplicates on PDA after 7 days in darkness, at 5 °C intervals from 5 °C to 40 °C, as well as at 37 °C.

The microscopic characterization was carried out after 7 days at 25 °C in darkness, following the recommendations of Simmons [19]. Measurements and descriptions of the microscopic structures were taken from the specimens mounted in Shear's solution growing on the media described above. Photomicrographs were obtained using a Zeiss Axio-Imager M1 light microscope (Zeiss, Oberkochen, Germany) with a DeltaPix Infinity X digital camera.

3. Results

The preliminary comparison of the ITS sequence in our isolate with those in the NCBI confirmed its taxonomic position in the genus *Alternaria* section *Chalastospora*, showing 98.66% of sequence identity with two species in this section, i.e., *A. abundans* CBS 535.83 and *A. armoraciae* CBS 118702. Based on this preliminary result, the phylogenetic reconstruction for each locus was performed through ML analysis. The best nucleotide substitution model determined with the MEGA program was Kimura two-parameter (K2 + G) for ITS and *gapdh*, and Tamura Nei with gamma distribution (T93 + G) for *ATPase* (Supplementary Material).

Multi-locus reconstruction of the section *Chalastospora* was performed using the three recommended loci for these sections, and through ML and BI analyses. The alignment comprised a total of 2216 bp (i.e., ITS 536 bp, *gapdh* 485 bp, and *ATPase* 1195 bp), including 310 variable sites (i.e., ITS 62 bp, *gapdh* 88 bp, and *ATPase* 160 bp) and 195 being phylogenetically informative (i.e., ITS 31 bp, *gapdh* 43 bp, and *ATPase* 121 bp). The best nucleotide substitution model for the ML using the combined analysis of these three loci was Tamura-Nei with gamma distribution (T93 + G) and for BI was Kimura two-parameter with gamma distribution and invariant sites (K80 + G + I) for the ITS region, and Hasegawa-Kishino-Yano with invariant sites (HKY + I) for *ATPase* and *gapdh*. The phylogenetic tree obtained showed that the isolate FMR 17518 formed a single distant branch, which was placed in a supported clade (89% bs/0.99 pp), along with the three well-supported species of *A. abundans*, *A. armoraciae*, and *A. breviramosa* (Figure 1). The phylogenetic distance, high support values of the lineages, and the morphological differences with the related species allow us to propose a new species in the genus *Alternaria*, which is described in the taxonomy section.

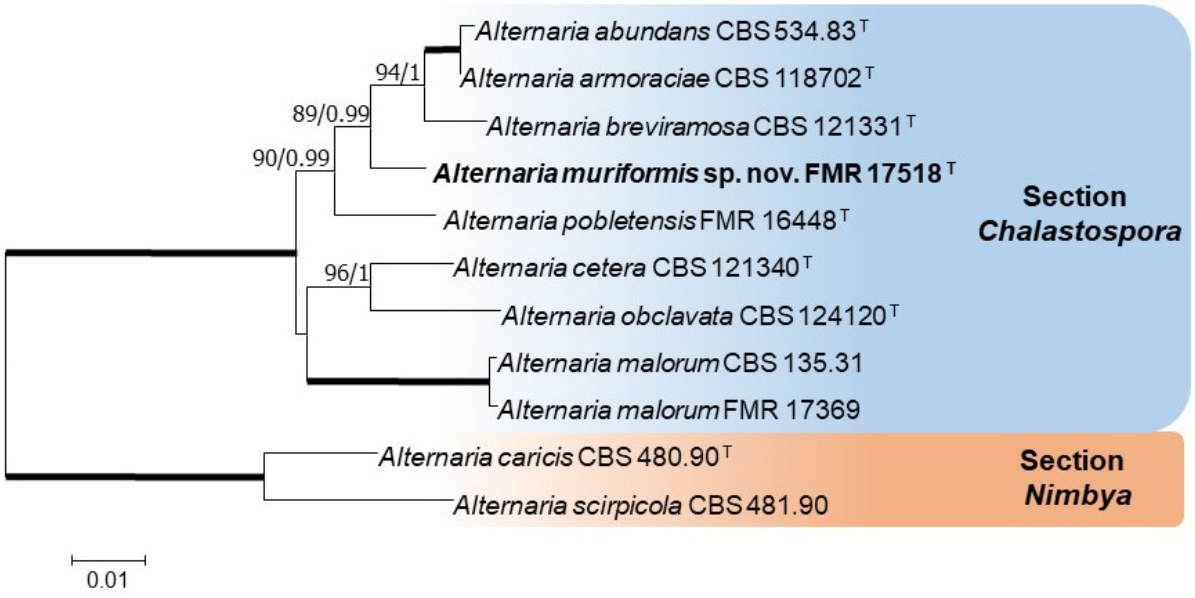

Figure 1. Phylogenetic tree constructed with ITS (536 bp), *gapdh* (485 bp), and *ATPase* (1195 bp) and sequences of ex-type strains of *Alternaria* species in the section *Chalastospora* and rooted with *Alternaria caricis* CBS 480.90 and *Alternaria scirpicola* CBS 481.90 (section *Nimbya*). Bootstrap support (bs) values greater than 70% and Bayesian posterior probabilities (pp) greater than 0.95 are given at the nodes (bs/pp). Bold branches indicate a bs/pp of 100/1. The novel species described in this study is indicated in bold. ^{T} indicates an ex-type of strain.

Taxonomy

Alternaria muriformis (Iturrieta-González and Gené) sp. nov.—MycoBank MB847820 (Figure 2).

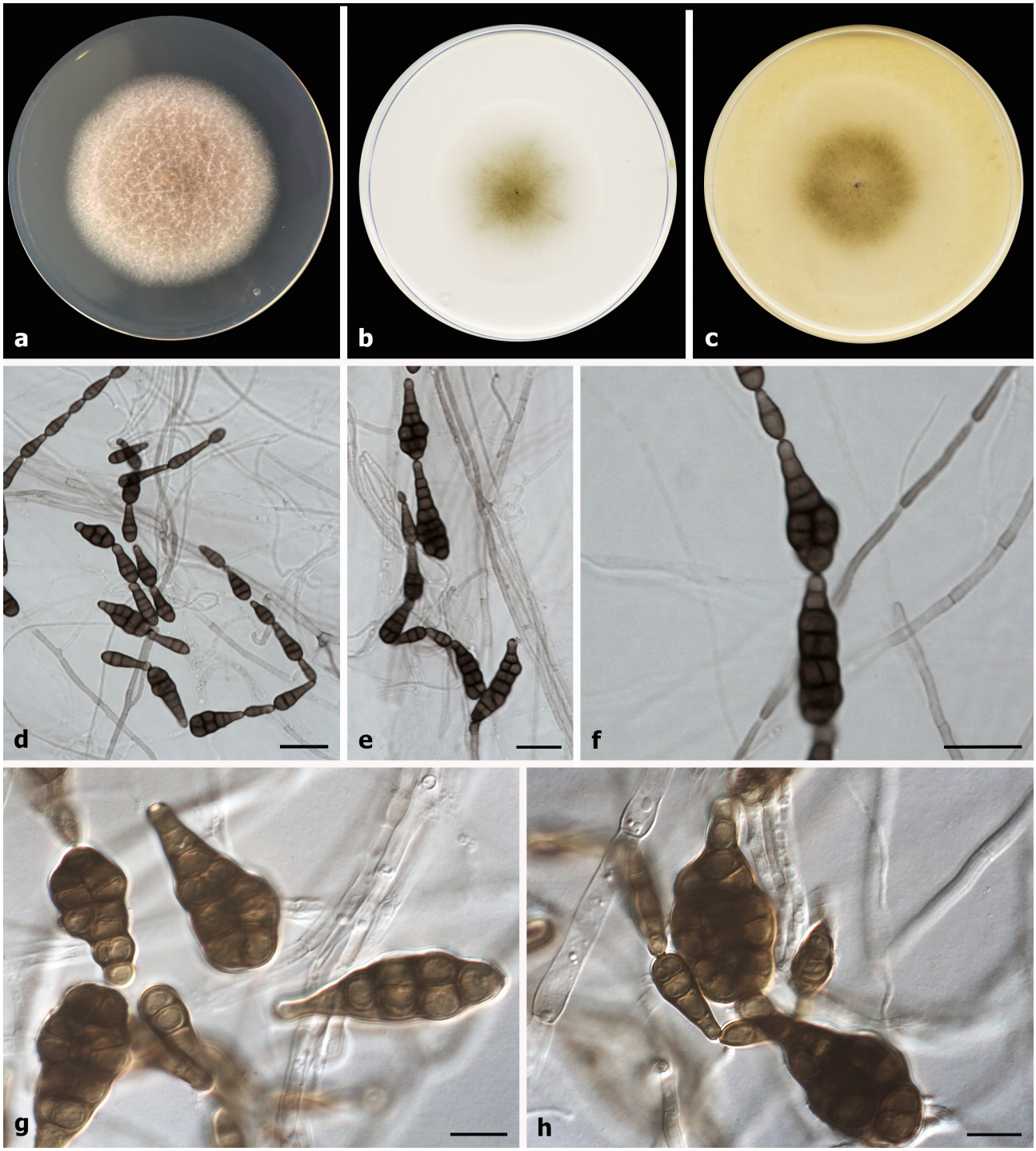

Figure 2. *Alternaria muriformis* (ex-type FMR 17518). (**a**) Colonies on PDA; (**b**) colonies on PCA; (**c**) colonies on OA; (**d**–**h**) Conidia. Scale bars (**d**–**f**) = 20 µm and (**g**,**h**) = 10 µm.

Etymology: The epithet refers to the production of muriform conidia in OA culture.

Culture characteristics (at 25 °C for 7 days): Colonies on PDA reaching 58–61 mm diam, blond-white to yellowish-white (4C4/4A2), cottony, abundant aerial mycelium, margins regular; reverse yellowish-brown (5F8/5D5), yellowish-white final edge (4A2). On PCA attaining 49–50 mm diam, flat, slightly velvety, scarce aerial mycelium, margins regular; reverse grey (1D4) to colorless towards the periphery. On OA reaching 55–56 mm diam, flat, slightly floccose, scarce aerial mycelium, margins regular; surface and reverse olive (4E3) to colorless.

Cardinal temperature for growth: minimum of 15 °C; optimum of 20 °C; and maximum of 30 °C.

Morphological description of the asexual morph (on OA at 25 °C for 7 days): Mycelium is superficial and immersed. Hyphae 1–4 µm wide, septate, branched, subhyaline to pale olivaceous, smooth-walled to verruculose. Conidiophores micronematous to semi-macronematous, arising laterally or terminally from aerial hyphae, erect to slightly flexuous, unbranched, 10.5–73 × 3–4.5 µm, pale olivaceous to yellowish brown, smooth-walled, with 1–2 terminal conidiogenous loci. Conidia forming unbranched or slightly branched chains, with up to 15 conidia in the unbranched part, commonly ellipsoidal or obclavate, 10–40 × 4–14 µm, with darkened middle transverse septa, some constricted, (1–)3–5(–7) transverse septa, and 0–1(–2) longitudinal or oblique septa per transverse segment, yellowish-brown to brown, smooth-walled; conidia with muriform conidial bodies are present, 37–45 × 16–33 µm. Secondary conidiophores can be formed as lateral conidiogenous loci from the conidial body. Furthermore, sexual morphology was not observed.

Known distribution: In Spain (as seen in this article: lifestyle-saprobic on herbivorous dung).

Specimen examined: Spain, Catalonia, Barcelona province, Pontons (N 41.40590° E 1.50918°), dung of an unidentified herbivorous animal, June 2018, J. Gené and I. Iturrieta-González (holotype FMR H-17518, culture ex-type FMR 17518).

Notes: *Alternaria muriformis* is placed in a supported clade in *Alternaria* section *Chalastospora* (Figure 1), and it is phylogenetically related to *A. armoraciae*, *A. abundans*, and *A. breviramosa*. However, the new species differs morphologically from its relatives in the production of muriform conidia [19,35,36] (Table 3). Despite following the recommendations of Simmons [19] for morphological characterization of the new fungus, we observed sporulation exclusively on OA at 25 °C.

Table 3. Comparison of the conidial morphology among *Alternaria* species in section *Chalastospora*.

Species	Conidia					References
	Shape	Size (µm)	Transverse Septa Numbers	Longitudinal or Oblique Septa Numbers (*)	Ornamentation	
A. abundans	Ovoidal Obclavate	20–30 × 10–12 40–50 × 8–12	3–6(–8)	0–1	Usually smooth	[36]
A. armoraciae	Ovoidal to ellipsoidal	15–35 × 8–12	3–5	0–1	Smooth	[19]
A. breviramosa	Ellipsoidal to fusiform	(8–)10–15(–17) × 3(–3.5)	0–1(–2)	Absent	Smooth	[35]
A. cetera	Ellipsoidal to narrow-ovoid	18–22 × 3–4(–5)	1–3	Absent	Smooth	[19,37]
A. malorum	Ellipsoidal-ovoidal, cylindrical, or fusiform	6–14(17) × 2–4	Absent	Absent	Smooth	[38]
A. obclavata	Obclavate	(23–)26–30(–35) × (3.5–)4	0–3	Absent	Smooth	[35]
A. pobletensis	Obpyriform or obclavate, and some ellipsoidal or subcylindrical	8–50 × 5–20	(1–)3–7(–9)	0–1(–2)	Smooth or verruculose	[2]
A. muriformis	**Ellipsoidal or obclavate**	**10–40 × 4–14**	**(1–)3–5(–7)**	**0–1(–2)**	**Smooth**	**Present study**
	Muriform	**37–45 × 16–33**				

* per transverse segment. The novel species described in this study is indicated in bold.

4. Discussion

Morphological traits have long been the basis for species identification in the genus *Alternaria* [19]. However, due to the limited number of taxonomically informative features, especially to distinguish closely related species, the use of DNA sequence data and multigene analysis is now required, not only for identification purposes but also for delineating novel species. The molecular markers recommended for this purpose have been defined for various *Alternaria* sections over the course of several studies [2,15,33,39,40]. Therefore, based on those molecular approaches, numerous new species, mainly in the sections *Alternaria*, *Infectoriae*, *Porri*, and *Radicina*, have been described in recent years [2,12,18,41], but only a few in the section *Chalastospora* [2]. This section comprises a small group of eight *Alternaria* species, including the new species *A. muriformis* (Table 3), which are well delineated by using the ITS, *gapdh,* and *ATPase* gene markers. Of note is that despite the ITS barcode being considered a gene marker only able to classify *Alternaria* species at the section level [2,15,33,40], in section *Chalastospora,* each locus is able to discriminate each species (see Supplementary Material). However, significant statistical support to establish phylogenetic relationships among species can only be achieved with the combination of the three markers. In our multi-locus analysis, the isolate investigated here formed a highly supported branch with both ML and BI analysis (89/0.99) that reinforces the proposal of *A. muriformis*. With the exception of *A. obclavata*, which has been exclusively reported from air samples [35], most species in *Chalastospora* have been isolated from vegetal substrates, as mentioned before. However, *A. malorum* [21,42], *A. pobletensis* [2], and now *A. muriformis* have also been recovered from the dung of herbivorous animals, suggesting that this is a good source to find taxonomically interesting *Alternaria* species, not only for this section but also for other *Alternaria* groups, as previously reported by Marin-Felix et al. [2]. Animal dung, and specifically herbivore dung, contains hemicellulose, cellulose, lignin, high nitrogen content, minerals, and high moisture content, which constitute a good substrate for fungal growth [43]. This is how, in recent years, a significant number of new species and new records have been described from this type of substrate [2,44–48].

The genus *Alternaria* contains species with an important role as producers of metabolites [14,49]. Although some metabolites have been shown to be toxic in plants and animals, they have also been shown to have biotechnological applications with excellent antioxidative, herbicidal, antibacterial, antiparasitic, antitumor, and enzyme inhibitory properties, which reinforces the need to study potential new metabolites produced by new species in the genus [49–52].

This study introduces a new *Alternaria* species for the section *Chalastospora* based on a polyphasic approach combining morphological and molecular characterization. Future research is required in order to elucidate the ecology of *A. muriformis,* its role as a possible pathogenic species in toxin production, and its potential biotechnological applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15050606/s1, Figure S1: Maximum Likelihood tree of the section *Chalastospora* constructed with ITS region; Figure S2: Maximum Likelihood tree of the section *Chalastospora* constructed with *gapdh*; Figure S3: Maximum Likelihood tree of the section *Chalastospora* constructed with *ATPase*.

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