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Morphotypes of Ciborinia camelliae Kohn infecting camellias in Italy

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Abstract:	Ciborinia camelliae Kohn is the causal agent of Camellia flower blight. The pathogen infects only flowers of camellias, causing serious damage to the plant. Seventy-one strains were collected from six Italian regions and were characterized at the phenotypic and genetic level. Morphotypes were identified based on their phenotypic differences in culture media. The Italian population consisted of eleven morphotypes. Internal transcribed spacer (ITS) regions of twenty-two isolates belonging to the different morphotypes were sequenced. All morphotypes have identical ITS sequences. The closest match of the ITS with the strain ICMP 19812 of Ciborinia camelliae from New Zealand (Massey University Arboretum, Palmerston North, 100 % sequence identity), and the morphological features confirmed the presence of a variable population of C. camelliae in six different Italian regions.
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Author Comments:	Dear Editor We performed the changes as suggested by the reviewer Morever find below a point by point reply to reviewers comments. We thank for the suggestions and we hope that the manuscript can be now accepted in JPP. We would appreciate a fast processing of the manuscript. Kind regards Marco Saracchi
Response to Reviewers:	We modified the text to show the current taxonomic definition of the species. No strains

	available in the mycobank directory are fully identical to the Italian strains. We specified the closest ID in mycobank and we specify that ICMP strain is the reference strain for C. camelliae genome project. For this reason the 100% ITS identity of the strain together with peculiar morphological characters of sclerotia formed on camellia petals and micromorphological characters of reproductive structures (completely different from those shown by Botrytis and Sclerotinia species) confirms species identity of Italian strains. The new sentence is now like this: Based on the results of ITS sequencing all strains representing the 11 morphotype patterns detected in Italy had an identical ITS region. Using www.mycobank.org platform the closest match was with different Botrytis cinerea strains (99.07% identity). All strains shared 100% identity with strain ICMP19812 type strain of Ciborinia camelliae from New Zealand (Massey University Arboretum, Palmerston North) used for the genome project of Ciborinia camellieae, with sequence accession number LGKQ00000000.1. The complete identity with a C. camelliae strain together with peculiar morphological characters of sclerotia formed on camelia petals and micromorphological description of reproductive structures (Kohn and Nagasawa 1984; Taylor and Long 2000; Saracchi et al. 2019) confirmed species identity of the examined strains.

Morphotypes of *Ciborinia camelliae* Kohn infecting camellias in Italy

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Summary: *Ciborinia camelliae* Kohn is the causal agent of Camellia flower blight. The pathogen infects only flowers of camellias, causing serious damage to the plant. Seventy-one strains were collected from six Italian regions and were characterized at the phenotypic and genetic level. Morphotypes were identified based on their phenotypic differences in culture media. The Italian population consisted of eleven morphotypes. Internal transcribed spacer (ITS) regions of twenty-two isolates belonging to the different morphotypes were sequenced. All morphotypes have identical ITS sequences. The closest match of the ITS with the strain ICMP 19812 of *Ciborinia camelliae* from New Zealand (Massey University Arboretum, Palmerston North, 100 % sequence identity), and the morphological features confirmed the presence of a variable population of *C. camelliae* in six different Italian regions.

Keywords: Camellia flower blight; morphological traits; ITS sequences, Sclerotiniaceae.

Ciborinia camelliae Kohn is a pathogenic fungus that affects plants of the genus *Camellia*. The pathogen infects only flowers causing a disease called camellia flower blight (CFB) (Taylor and Long 2000; Saracchi et al. 2019). The infection occurs when the spores land on camellia flowers, where they germinate, penetrate petal tissues and cause extensive damage to flowers (Taylor 2004). The symptoms first appear as small, irregular brown spots on the petals (Vingnanasingam 2002). Gradually, the infection spreads to the entire flower, destined to fall prematurely (Kohn and Nagasawa 1984) (Fig. 1a). A white ring of mycelium at the base of the flower, under the calyx, is a distinctive feature of the disease (Vingnanasingam 2002) (Fig. 1b). *Ciborinia camelliae* biological cycle is strongly related to sclerotia, developing from senescent petals. The mature sclerotium is dark due to accumulation of melanin and discoid (12 x 10 x 2 mm) (Kohn and Nagasawa 1984) (Fig. 1c). According to Whetzel (1945), species belonging to genus *Ciborinia* incorporate residue petal tissue into the sclerotial cortex and medulla. Cup-shaped apothecia (5-18 mm in diameter) (Fig. 1d), developed from overwintering sclerotia (Kohn and Nagasawa 1984), contain ascospores that can be spread by the wind for several kilometers (Saracchi et al. 2019). According to Hansen and Thomas (1940), ascospores are hyaline, one-celled, ovate, and guttulate (7.5-12.5 x 4.0-5.0 µm) (Fig. 1e). Globose conidia, visible as black spots, are produced in chains on senescent flowers and in *in vitro* culture (Taylor 2004) (Fig. 1f). Although CFB is widespread in almost all regions where camellia is grown (Hara 1919; Hansen and Thomas 1940; Stewart and Neilson 1993; Cook 1999; Peper 1999; Mansilla et al. 1999; Garibaldi et al. 2001 and Colombo et al. 2016), data on the pathogen are limited. The first severe damage of camellia flowers by *C. camelliae* has been reported in Northern Italy in 2000 and 2001 (Garibaldi et al. 2001; Gullino et al. 2001). The presence of the pathogen in different Italian regions was then reported (Colombo et al. 2016, Saracchi et al. 2019), but studies on the distribution of the pathogen and its variability are lacking, also due to the difficulties of its isolation from infected tissues, *in vitro* cultivation and maintaining the collections vital. The main objective of this study was to investigate the variability among strains collected at different sites in Italy by analyzing morphological characters and by ITS sequence verification.

Symptomatic flowers and sclerotia (also producing apothecia and ascospores) showing *C. camelliae* features were collected from public or private gardens located in six different Italian sites (Table 1). Pathogen strains were isolated into a pure culture by placing small pieces cut from the base of infected petals and mycelium from the flower receptacle (Fig. 1a and 1b) or sclerotium (1c), onto Potato Dextrose Agar (PDA: 800 ml/L of potato extract; 20 g/L glucose, BioFROXX, Germany; 15 g/L agar, Applichem, Germany) and PDA added with antibiotics (nalidixic acid 25 ppm, Sigma-Aldrich, Germany; tetracycline 25 ppm, Applichem, Germany; novobiocin 25 ppm, Sigma-Aldrich, Germany) to inhibit bacterial growth. All isolates listed in Table 1 are maintained in the laboratory of Plant Pathology at the Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Italy. The cultural and morphological characterization was performed for all 71 pathogenic strains. Colonies were grown on four different agar-media: 1) Czapek-Yeast Extract Agar medium (CYA: 35 g/L Czapek Dox broth, Difco Laboratories, USA; 2 g/L yeast extract, Difco Laboratories, USA; 15 g/L agar, Applichem, Germany), 2) Malt-Extract Agar medium (MEA: 30 g/L malt extract, VWR chemicals, Belgium; 15 g/L agar, Applichem, Germany; 20 g/L glucose, BioFROXX, Germany; 1g/L peptone,

Difco Laboratories, USA), 3) PDA2 medium (39 g/L Potato Dextrose Agar, Applichem, Germany) and 4) Malt Agar medium (MA: 30 g/L malt extract, VWR chemicals, Belgium; 15 g/L agar, Applichem, Germany). A disc of pathogen agar-mycelium (6 mm of diameter), taken from the edge of an actively growing colony on PDA medium, was inoculated upside down in the center of the Petri plate. Mycelial colonies were observed after 7, 14, and 21 days of incubation at 20°C in the dark. Three replicate plates were prepared for each strain and medium. For each colony, a set of parameters were recorded: (*i*) shape, (*ii*) size, (*iii*) colony's colour, (*iv*) mycelium texture, (*v*) pigment production, and (*vi*) morphology of reverse. All isolates were initially classified into ten morphotypes (A-L, Figure 2) based on their morphocultural characteristics after 21 days of incubation at 20°C. For each strain, a morphotype pattern was then assessed combining the resulting morphotypes on the four culture media. We defined the morphotype pattern as the whole morphological features obtained by the pathogen grown on four different substrates. Strains showing comparable cultural traits were grouped into the same morphotype pattern. To confirm the identification of the investigated strains as *Ciborinia camelliae*, ITS1 and ITS2 sequences were obtained from 22 isolates representing all the morphotype patterns and all the geographical areas investigated. Briefly 6 days potato-dextrose broth cultures were harvested, liophylized and extracted according to vanToor et al 2005. potato-dextrose broth. The ITS region was amplified with the universal primers ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) as described in Kamel et al. (2016). The PCR products were sequenced by Eurofins Genomics (Ebersberg, Germany) and all sequences were aligned using Geneious software (Biomatters, Auckland, New Zealand), version R11.1.4. Sequences were compared with the ITS sequences deposited in GenBank.

Seventy-one *Ciborinia camelliae* strains from six Italian sites were categorized into eleven morphotype patterns (Table 1). Among all investigated strains, morphotype pattern 1 was predominant (62%), while patterns 4, 5, 6, 9, 10, and 11 can be considered sporadic (1.3%). The highest percentage of morphotype pattern 1 was found in Portici strains, where all isolates exhibited the same morphotype. The presence of pattern 1 was identifiable in all sites, except in Milan (Fig. 3). Four out of six different regions were strongly dominated by morphotype 1 with a frequency ranging from 69% to 100%. Some strains, which belong to the same locality showed a clear variability in morphological characteristics. For example, among the 13 isolates from Tramezzina, 6 morphotype patterns were found. Within the Oggebbio population, the greatest morphological variability was found (specifically 8 morphotype patterns), although morphotype pattern 1 was observed in 22 out of 32 strains. Nevertheless, considering each group of strains belonging to the same geographical area, none exhibited a completely different morphological profile on all agar-media. Although the pathogen exhibits a wide morphological variability, we can not suppose differentiations due to the geographical origin of the strains.

Based on the results of ITS sequencing all strains representing the 11 morphotype patterns detected in Italy had an identical ITS region. Using www.mycobank.org platform the closest match was with different *Botrytis cinerea* strains (99.07% identity). All strains shared 100% identity with strain ICMP19812 type strain of *Ciborinia camelliae* from New Zealand (Massey University Arboretum, Palmerston North) used for the genome project of *Ciborinia camellieae*, with sequence accession number LGKQ00000000.1. The complete identity with *a C. camelliae* strain together with peculiar morphological characters of sclerotia formed on camelia petals and micromorphological description of reproductive structures (Kohn and Nagasawa 1984; Taylor and Long 2000; Saracchi et al. 2019) confirmed species identity of the examined strains.

This study represents the first description of the Italian morphotypes in *C. camelliae* and confirms the distribution of the pathogen in different Italian camellia-producing areas. Van Toor and coworkers (2005) found diversity in New Zealand's and American strains, therefore, given the restricted information in the literature on this pathogen, a genetic analysis of a worldwide collection of *C. camelliae*, comparing Italian collection and worldwide diversity is warranted in order to better investigate the pathogen evolution. The analysis of morphotype's pathogenicity and genetic determinants of infection (Denton-Giles et al 2020) may shed light on the biology of this fascinating pathogen.

Compliance with ethical standards

Conflicts of interest: Authors declare no conflict of interests.

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Legend

Table 1 Strain, origin (location with geographic coordinates, and source of isolation), colony morphotypes based on the description of fungal grown on four different media (CYA, MA, MEA, and PDA) for 21 days at 20°C. ITS GenBank accession numbers are reported for the sequenced strains

Fig. 1 *Ciborinia camelliae* symptomatic flower (a), mycelium developed on the receptacle (b), sclerotium (c), apothecium (d), ascospores (e), and conidia (f)

Fig. 2 Myceliar morphotypes obtained from the growth of the pathogen for 21 days at 20°C on four different media: CYA (A, B), MA (C, D), MEA (E-G), and PDA (H-I-L)

Fig. 3 Italian distribution of *Ciborinia camelliae* morphotype patterns investigated in the present study. Pie charts are proportional to morphotype patterns frequency in each area

Table 1 Strain, origin (location with geographic coordinates, and source of isolation), colony morphotypes based on the description of fungal grown on four different media (CYA, MA, MEA, and PDA) for 21 days at 20°C. ITS GenBank accession numbers are reported for the sequenced strains

	Strain origin			Morphotypes					
Strain	**Location**	**Geographic coordinates**	**Source**	**CYA**	**MA**	**MEA**	**PDA**	**Morphotype pattern**	**GenBank accession No.**
CO1	Tramezzina (CO)	45°59´10.3186" N 09°13´51.6598" E	Infected Flower	B	C	E	I	8	MW624701
CO2	Tramezzina (CO)	45°59´10.3186" N 09°13´51.6598" E	Infected Flower	B	C	E	H	7	
CO5	Tramezzina (CO)	45°59´10.3186" N 09°13´51.6598" E	Infected Flower	A	C	E	H	1	MW624702
CO6	Tramezzina (CO)	45°59´10.3186" N 09°13´51.6598" E	Infected Flower	A	D	G	I	6	MW624703
CO7	Tramezzina (CO)	45°59´10.3186" N 09°13´51.6598" E	Infected Flower	A	C	E	I	2	
CO8	Tramezzina (CO)	45°59´10.3186" N 09°13´51.6598" E	Infected Flower	A	C	E	I	2	
CO9	Tramezzina (CO)	45°59´10.3186" N 09°13´51.6598" E	Infected Flower	A	C	E	I	2	
CO10	Tramezzina (CO)	45°59´10.3186" N 09°13´51.6598" E	Infected Flower	A	C	F	I	3	
CO11	Tramezzina (CO)	45°59´10.3186" N 09°13´51.6598" E	Infected Flower	B	C	E	I	8	
CO12	Tramezzina (CO)	45°59´10.3186" N 09°13´51.6598" E	Infected Flower	A	C	E	I	2	
CO13	Tramezzina (CO)	45°59´10.3186" N 09°13´51.6598" E	Infected Flower	A	C	E	I	2	
CO15	Tramezzina (CO)	45°59´10.3186" N 09°13´51.6598" E	Infected Flower	A	C	E	H	1	MW624704
GE2	Genoa	44°25´32.8807" N 08°49´09.0124" E	Sclerotium	A	C	E	H	1	MW624705
GE6	Genoa	44°25´32.8807" N 08°49´09.0124" E	Sclerotium	A	C	E	H	1	
GE9	Genoa	44°25´32.8807" N 08°49´09.0124" E	Sclerotium	A	C	E	H	1	
GE13	Genoa	44°25´32.8807" N 08°49´09.0124" E	Sclerotium	A	C	E	H	1	
GE18	Genoa	44°25´32.8807" N 08°49´09.0124" E	Sclerotium	A	C	E	H	1	
GE19	Genoa	44°25´32.8807" N 08°49´09.0124" E	Sclerotium	A	C	F	I	3	MW624706
GE24	Genoa	44°25´32.8807" N 08°49´09.0124" E	Sclerotium	A	C	E	H	1	
GE27	Genoa	44°25´32.8807" N 08°49´09.0124" E	Sclerotium	A	C	E	H	1	
GE34	Genoa	44°25´32.8807" N 08°49´09.0124" E	Sclerotium	A	C	E	H	1	
GE40	Genoa	44°25´32.8807" N 08°49´09.0124" E	Sclerotium	A	C	E	I	2	MW624707
ITAB2	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	I	2	MW624708
ITAB3	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	I	2	
ITAC1	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAC2	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	MW624709
ITAC3	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAD1	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	

ITAE1	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	G	H	4	MW624710
ITAE3	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	MW624711
ITAG1	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAG2	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	MW624712
ITAG3	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAH1	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAH2	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAH3	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	MW624713
ITAI1	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAI2	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	D	E	H	5	MW624714
ITAJ1	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	D	G	H	11	MW624715
ITAK	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAL	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAM	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	I	2	
ITAN1	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	MW624716
ITAO	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAP	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAQ	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAR	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAS	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAT	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAU1	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	D	F	I	10	MW624717
ITAV1	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	B	C	E	H	7	MW624718
ITAX	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	A	C	E	H	1	
ITAY1	Oggebbio (VB)	45°59´47.5782" N 08°39´05.9659" E	Sclerotium	B	C	E	H	7	
LU1	Capannori (LU)	43°47´04.8685" N 10°33´47.5693" E	Sclerotium	A	C	E	H	1	
LU2	Capannori (LU)	43°47´04.8685" N 10°33´47.5693" E	Sclerotium	A	C	E	H	1	MW624719
LU3	Capannori (LU)	43°47´04.8685" N 10°33´47.5693" E	Sclerotium	A	C	E	L	9	MW624720
LU4	Capannori (LU)	43°47´04.8685" N 10°33´47.5693" E	Sclerotium	A	C	E	H	1	
MI1	Milan	45°27´52.9056" N 09°13´27.0905" E	Infected Flower	B	C	E	I	8	

MI2	Milan	45°27´52.9056" N 09°13´27.0905" E	Infected Flower	A	C	E	I	2	MW624721
MI3	Milan	45°27´52.9056" N 09°13´27.0905" E	Infected Flower	B	C	E	H	7	
MI4	Milan	45°27´52.9056" N 09°13´27.0905" E	Infected Flower	B	C	E	H	7	
NA2	Portici (NA)	40°48´45.8813" N 14°20´10.6897" E	Infected Flower	A	C	E	H	1	
NA5	Portici (NA)	40°48´45.8813" N 14°20´10.6897" E	Infected Flower	A	C	E	H	1	MW624722
NA8	Portici (NA)	40°48´45.8813" N 14°20´10.6897" E	Infected Flower	A	C	E	H	1	
NA10	Portici (NA)	40°48´45.8813" N 14°20´10.6897" E	Infected Flower	A	C	E	H	1	
NA12	Portici (NA)	40°48´45.8813" N 14°20´10.6897" E	Infected Flower	A	C	E	H	1	
NA15	Portici (NA)	40°48´45.8813" N 14°20´10.6897" E	Infected Flower	A	C	E	H	1	
NA18	Portici (NA)	40°48´45.8813" N 14°20´10.6897" E	Infected Flower	A	C	E	H	1	
NA19	Portici (NA)	40°48´45.8813" N 14°20´10.6897" E	Infected Flower	A	C	E	H	1	
NA21	Portici (NA)	40°48´45.8813" N 14°20´10.6897" E	Infected Flower	A	C	E	H	1	
NA23	Portici (NA)	40°48´45.8813" N 14°20´10.6897" E	Infected Flower	A	C	E	H	1	

Figure 1

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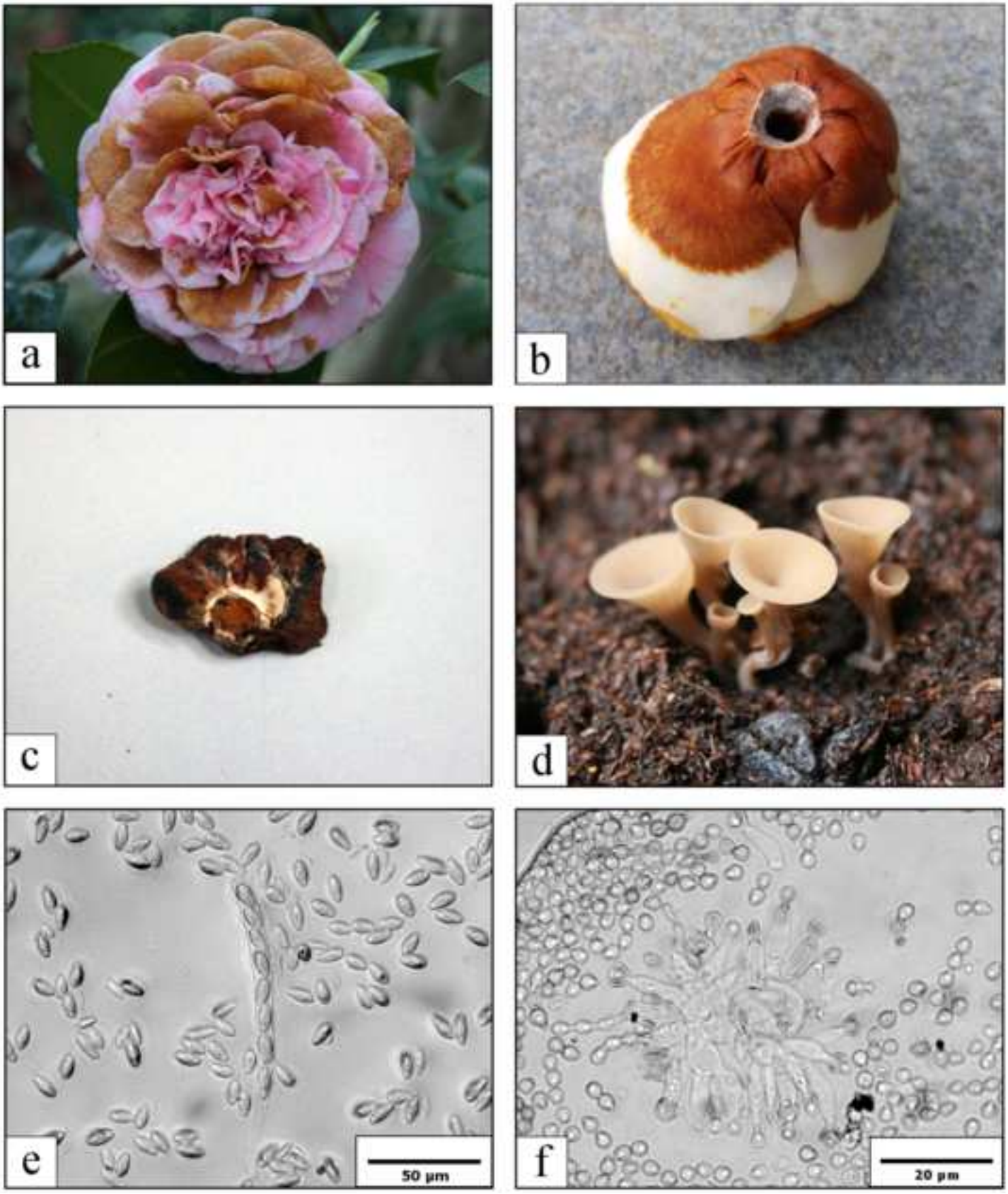

Figure 2

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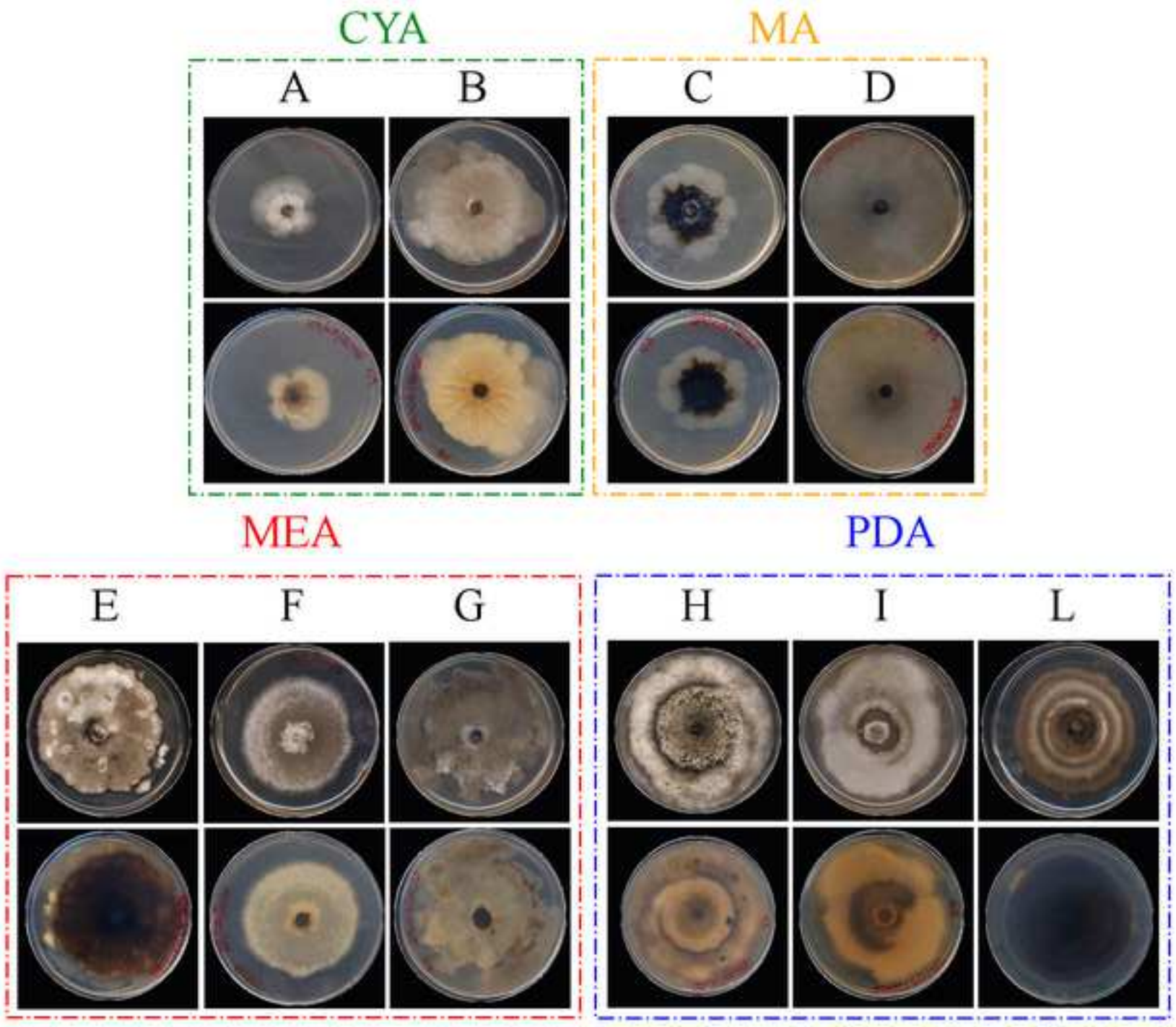

Figure 3

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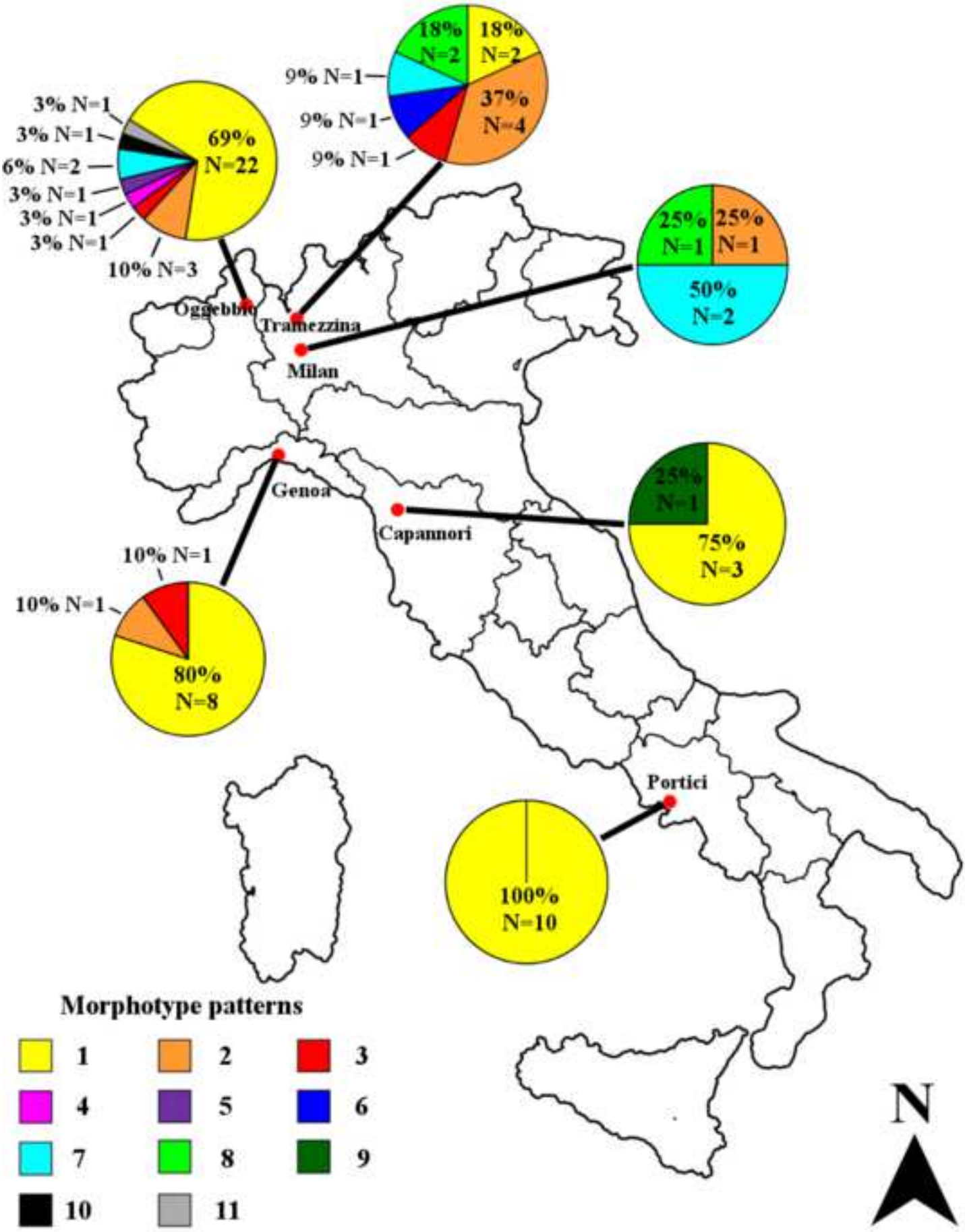

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