Characterization of the Italian population of *Ciborinia camelliae*

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ABSTRACT *Ciborinia camelliae* Kohn is the causal agent of camellia flower blight. The fungus infects only the flowers of camellias causing serious damage to the plant, particularly from an aesthetic point of view. The disease has been reported in almost all countries where camellia is grown for ornamental purposes, but there are not many studies on the variability of the population of this phytopathogen. The main objective of this study was to contribute to study the level of variability within the Italian population. More than 130 *C. camelliae* strains were collected from six localities distributed in five Italian regions and identified also based on molecular characterization of the ITS nucleotide sequences. The population variability was assessed by comparing the morphological characters. From a phenological point of view, 11 different colony morphotypes were identified, whose presence/absence and frequency are different in the various locations considered. The study of the taxonomically valid nucleotide sequences to differentiate fungi at the species level confirmed that the strains under study belong to a single species. To further investigate Italian population of *C. camelliae* we sequenced by a combination of long and short reads technologies the genome of a representative strain. This genome represents a worldwide reference for future *C. cameliae* diversity and pathogenicity studies.

KEY WORDS camellia flower blight, variability, morphotypes, genome, mitochondrial diversity

The ascomycete *Ciborinia camelliae* Kohn, inoperculate Discomycete of the Sclerotiniaceae, is the causal agent of camellia flower blight (CFB). It is a necrotrophic fungus that shows host and organ specificity infecting only flowers of species belonging to the genus *Camellia*.

The scientific literature from the first description of the problem up to the present day has been summarized in two reviews proposed by Taylor and Long (2000) and Saracchi and colleagues (2019).

The pathogen was first reported in Japan in 1919 (Kohn and Nagasawa 1984) and now is localized in several world countries where the camellia plants are cultivated. According to the European and Mediterranean Plant Protection Organization (EPPO) database, the fungus is recorded in Europe (France, Germany, Guernsey, Ireland, Italy, Netherlands, Portugal, Spain, Switzerland, and United Kingdom), Japan, New Zealand and North America (California, Florida, Georgia, Louisiana, South and North Carolina, Texas and Oregon).

In literature, information about *C. camelliae* is lacking especially those concerning pathogen populations and their variability. Diversity of the fungus has been investigated by exploring random-based genetic approaches (UP-PCR) as well as sequence-based approaches on a group of New Zealand and American strains demonstrating that the level of genetic variations within the two populations was relatively low (Butler et al. 2002; Taylor 2004; van Toor et al. 2005).

In order to improve the knowledge about the population of the pathogenic fungus *C. camelliae*, the phenotypic variability of numerous strains isolated from phytopathological samples collected in different Italian provinces is here described. Furthermore, for the purpose to contribute in improving the reference genome of the species a strain of the most common Italian morphotype was sequenced by a combination of long and short reads.

MATERIALS AND METHODS

The collection maintained in the laboratory of Plant Pathology at the Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Italy, includes more than 220 *C. camelliae* strains, isolated from symptomatic flowers and sclerotia collected from 13 localities distributed in seven Countries. Their identification at the species level was also confirmed using the molecular characterization of the ITS nucleotide sequences.

On the basis of the origin and on a first morphological description of the colonies, 47 strains were selected for the present study on the variability of the pathogen population.

The cultural and morphological characterization was performed following as described by Saracchi et al. (2022) on four different media: Czapek-Yeast Extract Agar medium (CYA), Malt-Extract Agar medium (MEA), Potato Dextrose Agar medium (PDA), and Malt Agar medium (MA). 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 color, (iv) mycelium texture, (v) pigment production, and (vi) morphology of the reverse. All isolates were classified into morphotypes based on their morphocultural characteristics after 21 days of incubation at 20 °C. For each strain, a morphotype pattern was then assessed by 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. The data relating to the identified morphotypes were analyzed not only on the basis of the frequency but also on the geographical origin of the strains.

The ITAC2 strain of *C. camelliae* was isolated from a sample collected in Oggebbio (Verbania, Italy). It was deposited in a public collection (DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) with the accession number DSM 112729. To obtain pure high-molecular-weight DNA, two types of materials were employed: mycelium and conidia, collected from a 14-day-old colony grown on Potato Dextrose Agar medium. DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). To sequence the ITAC2 genomic DNA, two different platforms were employed: Oxford Nanopore Technologies (ONT, Oxford, UK) and Illumina Hiseq, executed by Eurofins Genomics (Ebersberg, Germany). The MinION system (FLO-MIN-106 R9.4 flow-cell) was used to perform the ONT sequencing. The native barcoding protocol was carried out using both EXP-NBD104 and EXPNBD114 in conjunction with the SQK-LSK109 kit and also the SQK-RAD004 sequencing kit (Oxford Nanopore Technologies, Oxford, U.K.). The long Nanopore reads and short Illumina reads were analyzed using the European Galaxy web platform (https://usegalaxy.eu/) tools (Afgan et al. 2018) and Geneious Prime software, version 42 2021.1.1 (Biomatters, Auckland, New Zealand) as described in Valenti et al. (2022a) and Degradi et al. (2021). The genome sequence was compared with the NCBI reference assembly of *C. camelliae* ICMP 19812 (GCA_001247705.1) from New Zealand. After analyzing the nucleotide sequences, the mitochondrial genome was identified and assembled together with the aim to construct the complete and circular mitogenome. The results were compared also with data from the available Sclerotiniaceae mitogenomes (Valenti et al. 2022b) to understand the mitogenome arrangement and the evolution of this phytopathogenic fungus in comparison to other Sclerotiniaceae species and confirm the usefulness of mitochondrial analysis to define phylogenetic positioning of this sequenced species.

RESULTS AND DISCUSSIONS

Seventy-one *Ciborinia camelliae* strains from six Italian sites, grown on different culture media were categorized into different morphotypes shown in figure 1 and summarized in **table 1**.

An interesting result of this experimentation is the detailed demonstration that phenologically different colony morphologies exist within the context of a *C. camelliae* population. The frequency of these different morphotypes is not uniform geographically. Moreover different growth substrates contributed differently to the overall variability.

Colonies grown on MEA and PDA showed three different morphotypes each, while on CYA and MA the groups were only two. The frequency of the different morphotypes was not uniform: in the substrates CYA, MA, and MEA it was generally one morphotype that prevailed over the others and, in the specific case, the morphotypes A (88.7%), C (94.36%), and E (91.54%) were found to be predominant respectively. On PDA, about half of the colonies resulted belonged to the morphotype H, 17 colonies (23.94%) to the morphotype I, and only one colony to the L one which was the rarest.

Combining the data about the "morphotype" on each medium, for each strain we defined its "morphotype pattern", considering all the 71 strains eleven morphotype patterns were defined (**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. 2), and, considering the different regions, it was found with frequencies ranging from 69 to 100%.

A clear variability in morphological characteristics was demonstrated among strains isolated in the same locality. 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 the most frequent (69.49%).

By comparing the morphological profiles in relation to their origin, it emerges that none of them are typical of a single geographical area. Although the pathogen presents a wide morphological variability, differentiations only due to the geographical origin of the strains cannot, therefore, be hypothesized. The high variability of the strains could also be justified by the entry into a territory of genetically different strains of *C. camelliae*, through infected plants or vessels containing propagules of the pathogen, in particular its sclerotia. This could easily happen through the commercialization of plants, the exchange of specimens between camellia lovers, also, their uncontrolled importation.

This study summarizes the first description of the Italian morphotypes in *C. camelliae* and confirms the distribution of the pathogen in several Italian areas producing camellias. Van Toor and collaborators (2005) found differences in New Zealand and American strains, therefore, given the limited information in the literature on this pathogen, it would be interesting to extend the comparison also to strains of the pathogen from other areas where camellia is present.

To improve the study of variability and the comparison between strains, it would be further interesting to extend the research by adopting genetic approaches such as, for example, done by Van Toor (2005) first on the Italian population currently under study and then considering strains from different countries.

The analysis of the pathogenicity of the morphotype and the genetic determinants of the infection (Denton-Giles et al. 2020) could also help shed light on the biology of this fascinating pathogen and on potential control methods.

More than a century has passed since the first CFB description which was made in Japan by Hara in 1919. Still today there are many more unknown aspects than what is known about this pathogen. Also

from the genomic point of view, the information on *C. camelliae* is very limited. For the purpose to contribute in improving the reference genome of the species, a strain of the most common Italian morphotype was sequenced by a combination of long and short reads.

The ITAC2 strain of *C. camelliae*, isolated from a sample collected in Oggebbio (Verbania, Italy), was chosen as representative of the most frequent morphotype pattern.

The extraction of DNA - characterized by a high concentration of nucleic acid and with an excellent degree of purity, has not always been a simple operation. Frequently the release of mucilaginous substances has hindered the use of membranes designed to retain DNA in many commercial kits and reduced the yield when more traditional protocols were used. The best results were obtained by extracting DNA from plate-differentiated conidia rather than from the vegetative mycelium of the pathogen.

The long Nanopore reads and the short Illumina reads analysed using the European Galaxy web platform tools and Geneious Prime software produced 10,324,028 paired-end reads 2 x 151 b and 641,071 single-molecule long reads, with a mean length of 4,574 b. The mean quality score for Nanopore and Illumina data was 9.9 and 30.8, respectively. The hybrid assembly resulted in a coverage of 67x and 63x considering Illumina and Nanopore reads, respectively. The total length of the *C. camelliae* ITAC2 genome was 46.48 Mb. Comparing it with the NCBI reference assembly of *C. camelliae* ICMP 19812 (LGQK01001007) from New Zealand (**Table 2**), the genome improved in contiguity decreasing the contig number from 2,604 to 49. Instead, the N50 contig size increased from 31,803 to 2,726,972 bp and the completeness of assembly increased from 94.5% to 97.3% according to BUSCO analysis (Valenti et al. 2022a).

The obtained results clearly contribute to improving the existing reference genome of the species (Genbank: LGQK01001007). The qualitative-quantitative information relating to the genome of *C. camellie* further increases the possibilities of studying this pathogen in more depth, its relationships with the closest fungal species and, above all, it could help to better understand the relations with its host as well as the still obscure sides of the disease it induces on camellias.

Further interesting data emerged from this genomic study which allowed the first fully annotated mitochondrial DNA of *C. camelliae* which also contributed to the increasing the evolutionary knowledge of the Sclerotiniaceae pathogens (Valenti et al. 2022b).

The circular mitochondrial genome of *C. camelliae* strain ITAC2, submitted to the GenBank database with the accession number OK326902, has a total length of 114,660 bp (Fig. 3). The nucleotide composition is the following: 34.7% of A, 13.2% of C, 16.5% of G, and 35.7% of T with a GC content of 29.6%.

This mitogenome contains all 14 typical genes encoding the subunits of ATP synthase (*atp6, atp8, and atp9*), NADH dehydrogenase (*nad1, nad2, nad3, nad4, nad4L, nad5, and nad6*), apocytochrome b (*cob*), and cytochrome c oxidase (*cox1, cox2,* and *cox3*). Additionally, the conserved ribosomal protein-coding gene S3 (*rps3*), untranslated genes of the large and small ribosomal RNAs (*rnl* and *rns*), the ribonuclease P RNA (*rnpB*) gene, and 33 transfer RNAs (tRNA) genes were detected. Overall, the RNA region accounted for 9.26% of the whole mitochondrial genome. The length of individual tRNAs ranged from 69 to 85 bp and most of these genes were placed around the *rnl* region. The total tRNAs are related to 18 essential amino acids. In total, the mitogenome contains 62 putative genes, including 44 open reading frames (ORFs) for hypothetical proteins (15) and homing endonucleases of the LAGLIDADG (24) and GIY-YIG (5) families. BLASTn analyses indicate that 22 ORFs have high identities with mitochondrial ORFs of the genus *Monilinia*. A high similarity with the Sclerotiniaceae species was not

observed in 14 % of the ORFs. These exhibit the highest amino acid identity (ranging from 63.41 to 90.75%) with fungal species belonging to the family of Nectriaceae, Ceratocystidaceae, Cryphonectriaceae, Erysiphaceae, and Orbiliaceae. Evolutionary, the last two are respectively the closest and the most distant taxonomic group from the Sclerotiniaceae family.

The mitochondrial genome size is associated with a high content of introns (Deng et al. 2018). In *C. camelliae* mtDNA we detected 28 introns. Introns exhibit a variable length ranging from 1,015 bp to 4,750 bp.

The arrangements of the 15 protein-coding genes found in *C. camelliae* mitogenome were compared among some related species. Gene order in *C. camelliae* is identical to most of the Sclerotiniaceae species, except for minor differences due to replication events of *atp8*, *atp9*, and *cob* genes.

Phylogenetic analysis was also carried out using the amino acid sequences of 14 protein-coding mitochondrial genes considering the nine Sclerotiniaceae species with publicly available data.

To obtain a second mitochondrial set of genes from another *C. camelliae*, a partial mitochondrial genome composed of a contig list was obtained from the whole genome assembly of *C. camelliae* ICMP 19812 strain from New Zealand. *Glarea lozoyensis* was considered as an outgroup. The phylogenetic tree (Fig. 4), demonstrated a close connection between *C. camelliae* ITAC2 strain and the members of the Sclerotiniaceae family. The two *C. camelliae* strains group as an independent clade, distinct from the other Sclerotiniaceae species. They exhibit 95.4 and 98.3% of similarity in nucleotide and amino acid sequences, respectively.

The significant difference between the two *C. camelliae* strains may be due to a misassembly of the New Zealand strain genome or could represent the beginning of a speciation event. The Italian and New Zealand population are separated by geographic barriers and they may diverge in complete independence under different selection pressures (Dettman et al., 2008; Stukenbrock, 2013). In this study, the first mitochondrial genome of *C. camelliae* was described demonstrating the phylogenetic position of the species in the Sclerotiniaceae family using data obtained from high-throughput sequencing technologies.

In this work, the variability of *C. camelliae* in the context of the Italian population has been demonstrated. This variability could also affect the other populations of the pathogen present in the countries where CFB has been reported and it would be very interesting to extend the research to these aspects as well.

Considering the impact of globalization on the diffusion of the phytopathogens, the commercial exchange and collectors of camellia plants, it is very probable that the populations of the pathogen will still increase their variability. Subsequent differentiation of *C. camelliae* populations may one day also evolve into the formation of different species of the pathogen.

Studies on the *C. camelliae* genome and its variability will be able to provide further information useful for better understanding the pathogen and help study its relationship with the host, as well as protect it from the disease it induces.

ACKNOWLEDGEMENTS

The authors would like to thank the Italian Camellia Society for its help in retrieving plant pathological specimens and for partially funding the research.

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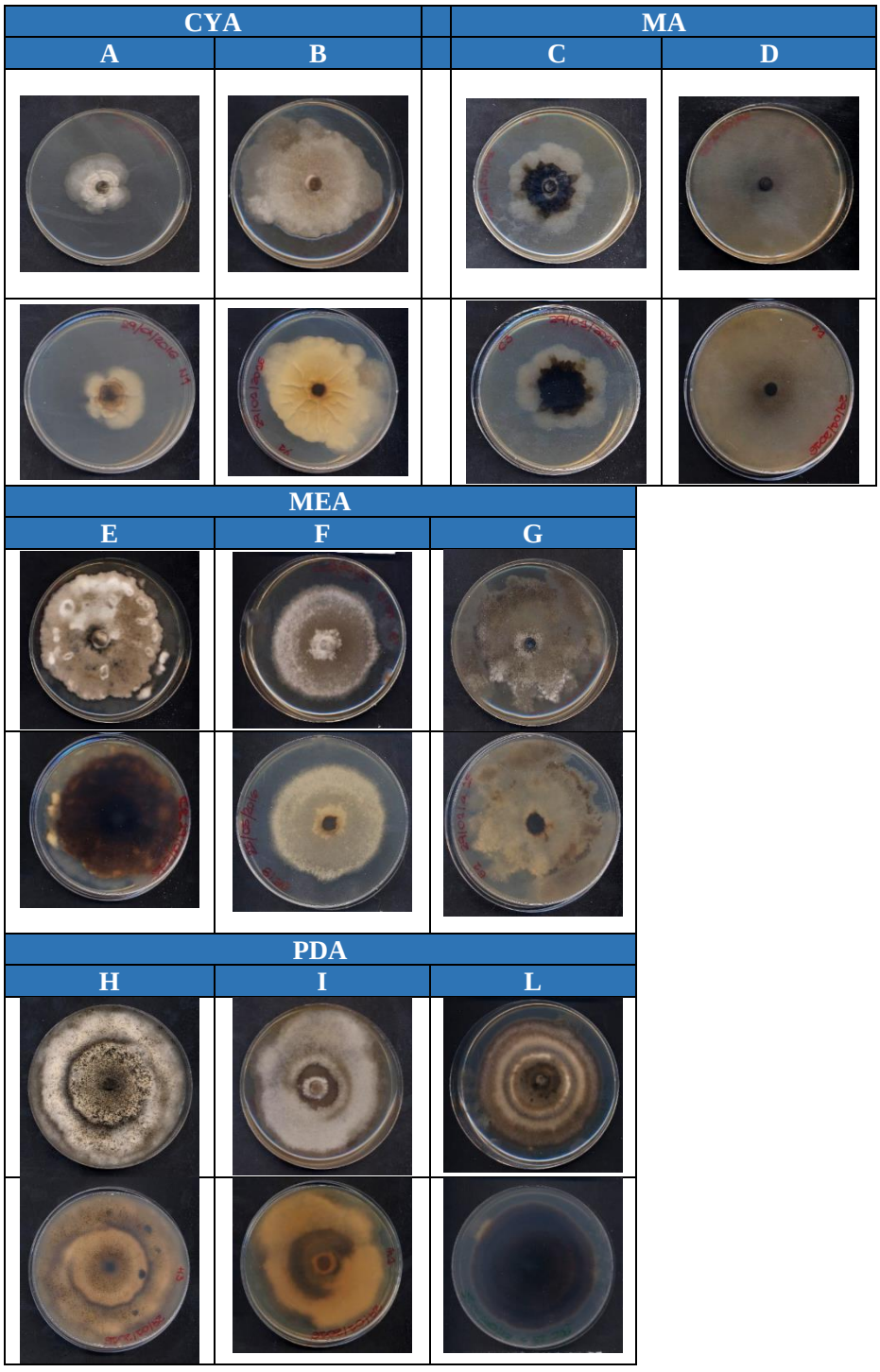
TABLES AND FIGURES

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.

Location	Geographic coordinates	Source	Strain	CYA	MA	MEA	PDA	Morphotype pattern
Tramezzina (CO)	45° 59´10.3186″ N 09° 13´51.6598″ E	Infected flower	CO1	B	C	E	I	8
			CO2	B	C	E	H	7
			CO5	A	C	E	H	1
			CO6	A	D	G	I	6
			CO7	A	C	E	I	2
			CO8	A	C	E	I	2
			CO9	A	C	E	I	2
			CO10	A	C	F	I	3
			CO11	B	C	E	I	8
			CO12	A	C	E	I	2
			CO13	A	C	E	I	2
			CO15	A	C	E	H	1
Genoa	44° 25´32.8807″ N 08° 49´09.0124″ E	Sclerotium	GE2	A	C	E	H	1
			GE6	A	C	E	H	1
			GE9	A	C	E	H	1
			GE13	A	C	E	H	1
			GE18	A	C	E	H	1
			GE19	A	C	F	I	3
			GE24	A	C	E	H	1
			GE27	A	C	E	H	1
			GE34	A	C	E	H	1
			GE40	A	C	E	I	2
Oggebbio (VB)	45° 59´47.5782″ N 08° 39´05.9659″ E	Sclerotium	ITAB2	A	C	E	I	2
			ITAB3	A	C	E	I	2
			ITAC1	A	C	E	H	1
			ITAC2	A	C	E	H	1
			ITAC3	A	C	E	H	1
			ITAD1	A	C	E	H	1
			ITAE1	A	C	G	H	4
			ITAE3	A	C	E	H	1
			ITAG1	A	C	E	H	1
			ITAG2	A	C	E	H	1
			ITAG3	A	C	E	H	1
			ITAH1	A	C	E	H	1
			ITAH2	A	C	E	H	1
			ITAH3	A	C	E	H	1
			ITAI1	A	C	E	H	1
			ITAI2	A	D	E	H	5
			ITAJ1	A	D	G	H	11
			ITAK	A	C	E	H	1
			ITAL	A	C	E	H	1
			ITAM	A	C	E	I	2

			ITAN	A	C	E	H	1
			ITAO	A	C	E	H	1
			ITAP	A	C	E	H	1
			ITAQ	A	C	E	H	1
			ITAR	A	C	E	H	1
			ITAS	A	C	E	H	1
			ITAT	A	C	E	H	1
			ITAU1	A	D	F	I	10
			ITAV1	B	C	E	H	7
			ITAX	A	C	E	H	1
			ITAY1	B	C	E	H	7
Capannori (LU)	43° 47´04.8685″ N 10° 33´47.5693″ E	Sclerotium	LU1	A	C	E	H	1
			LU2	A	C	E	H	1
			LU3	A	C	E	L	9
			LU4	A	C	E	H	1
Milan	45° 27´52.9056″ N 09° 13´27.0905″ E	Infected flower	MI01	B	C	E	I	8
			MI02	A	C	E	I	2
			MI03	B	C	E	H	7
			MI04	B	C	E	H	7
Portici (NA)	40° 48´45.8813″ N 14° 20´10.6897″ E	Infected flower	NA2	A	C	E	H	1
			NA5	A	C	E	H	1
			NA8	A	C	E	H	1
			NA10	A	C	E	H	1
			NA12	A	C	E	H	1
			NA15	A	C	E	H	1
			NA18	A	C	E	H	1
			NA19	A	C	E	H	1
			NA21	A	C	E	H	1
			NA23	A	C	E	H	1

Fig. 1 Myceliar morphotypes (**A-L**) obtained from the growth of the pathogen for 21 days at 20 °C on four different media (CYA, MA, MEA, and PDA).



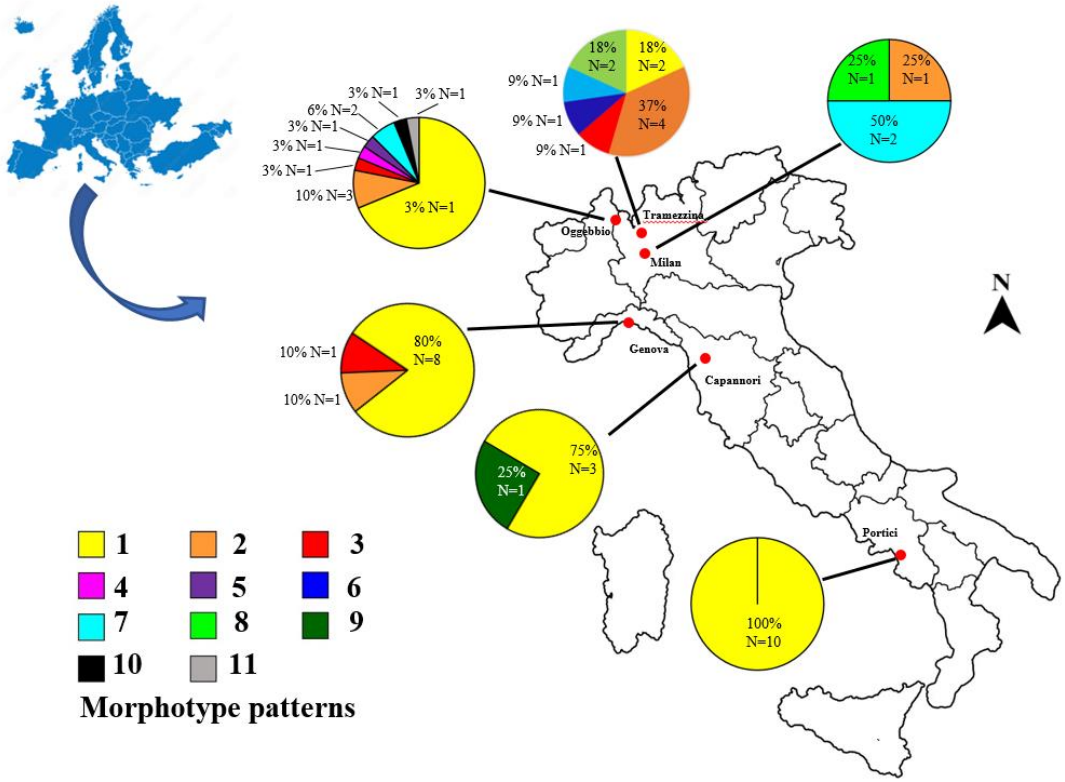


Fig.2 Italian distribution of Ciborinia camelliae morphotype patterns. Pie charts are proportional to morphotype patterns frequency in each area.

Table 2: Genome assembly comparison between Ciborinia camelliae ITAC2 and ICMP 19812 (GCA_001247705) strains.

Strain	*C. camelliae* ITAC2	*C. camelliae* ICMP 19812
Contigs	49	2,604
Largest contig (bp)	5,578,642	31,803
Genome size (Mb)	46.48	40.73
N_{50}	2,726,972	31,803
GC %	39.56	42.48
#N mismatches	9,280	90,878
BUSCO_Completeness%	97.3	94.5

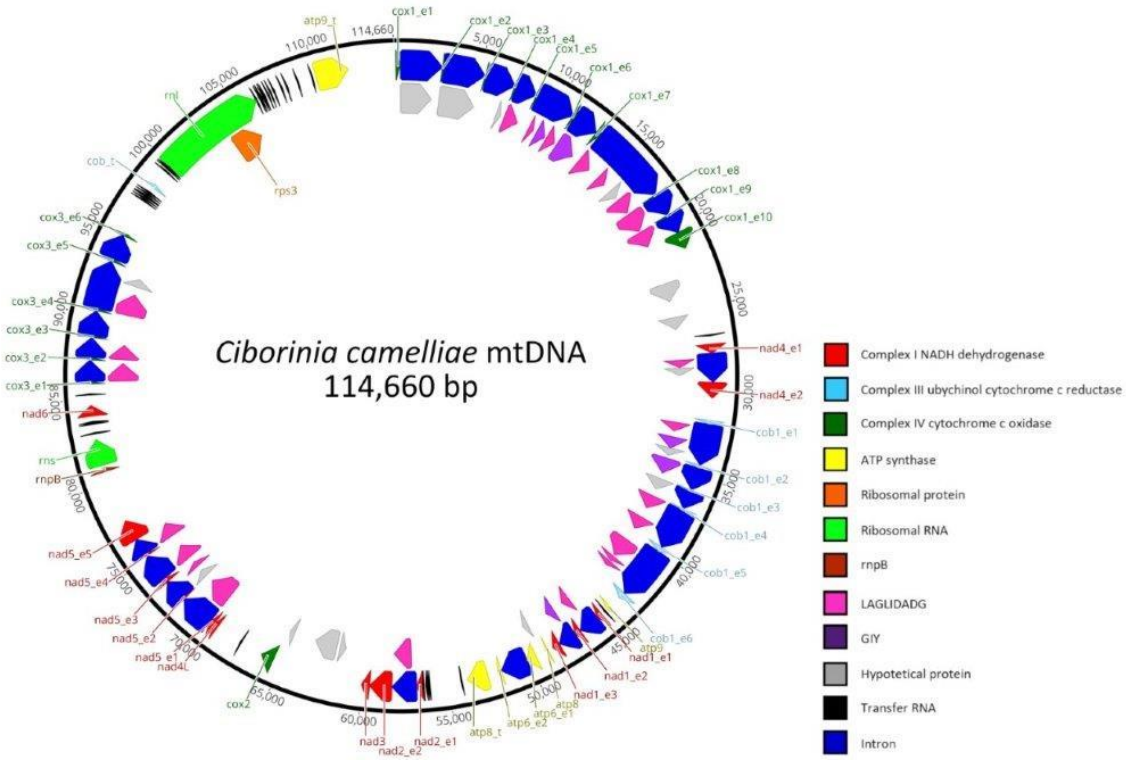


Fig.3 Genetic organization of Ciborinia camelliae mitogenome. Circular map is generated by Geneious Prime software, version 2021.1.1 (Biomatters, Auckland, New Zealand). Genes are represented by different colored blocks.

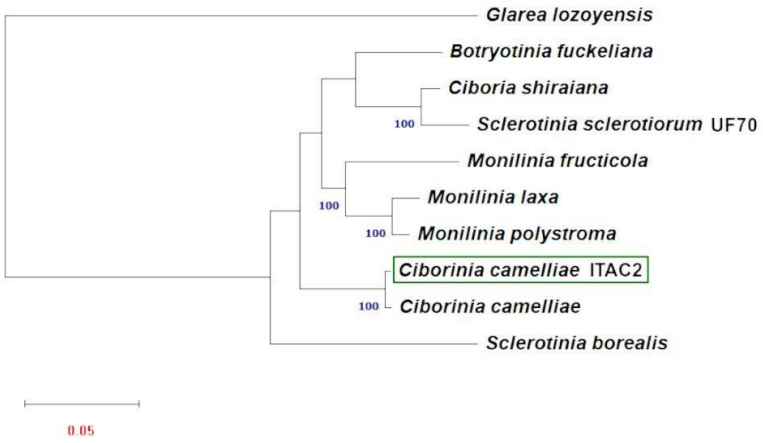


Fig. 4 Maximum Likelihood phylogenetic tree based on 14 econded mitochondrial genome. Blue numbers are bootstrap support values (> 90). Red number denote branch length. The GeneBank accession numbers are listed as follow: Botryotinia fuckeliana (KC 832409), Ciboria shiraiana (CM 017871.1), Ciborinia camelliae (GCA_00124770.5), Ciborinia camelliae strain ITAC2 (OK326902) Glarea lozoyensis (NC_031375.1), Monilinia fructicola (NC_056195.1), Monilinia laxa (NC_051483.1), Monilinia polystroma (PDEU00000000.1), Sclerotinia borealis (KJ434027), and Sclerotinia sclerotiorum (KT283062).