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Original paper

Biological characteristics of *Punctularia atropurpurascens* through morphological and molecular analyses during development

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Abstract
In this study, further evidences of the presence of the (sub)tropical *Punctularia atropurpurascens* in Rome are reported. Besides its generally described saprotrophic behaviour, some of the new-found specimens revealed a parasitic ability of the fungus. Moreover, although reputed a quite rare fungal species in Italy, *P. atropurpurascens* shows great capacity to develop in its places of manifestation under favourable climatic and environmental conditions. A comparison of morphological and molecular analyses of the fungus under various conditions gives suggestive information on the underlying biology. Furthermore, proton NMR analyses of the biochemical composition of the red drops secreted by the fungus showed the presence of lipids involved in cellular signal transduction pathways as well as phlebiarubrone-related molecules.

Key words: phlebiarubrone; lipids; NMR analysis, *Quercus* spp.; ITS region; parasitic fungi

Introduction

Punctularia atropurpurascens (Berk. & Broome) Petch forms the genus *Punctularia* together with the species *P. strigosozonata* (Schweinitz) P. H. B. Talbot, in the family Punctulariaceae and order Corticiales (Larsson, 2007). *Punctularia atropurpurascens* is a purplish coloured, saprotrophic fungus that grows on rotting wood, in prevalence deciduous. Its superior surface is fluffy, membranous, waxy, with a purplish-brownish coloured central zone. The lower surface is adherent to the growth substrate. An aqueous exudate in the form of reddish coloured droplets is often present. The hymenium is found on the upper surface and it is distributed in a non-uniform manner. The hyphal system is monomitic, made up of only generative hyphae, which appear branched, septate and with clamp connections. Basidia produce four sterigma. The conidia, produced through sporogenesis, are chained, subglobose or ellipsoid, with a violet brown colour (Martini, 2016).

Recently, we reported the presence of *P. atropurpurascens* in the Villa Ada urban Park in Rome, Latium (Knijn and Ferretti, 2018). This report referred to a specimen growing on a rotting stump of *Quercus suber* L., thus showing a saprotrophic behaviour. The stump was contemporarily colonised at its base by *Meripilus giganteus* (Pers.) P. Karst., *Ganoderma lucidum* (Curtis) P. Karst. and *Fomes fomentarius* (L.) Fr. Here, we report further evidences of the presence of *P. atropurpurascens* in Rome, with some specimens showing a parasitic capability, as well as findings of a longitudinal study of this fungus.

The morphological evolution of five specimens is described; also, microscopic analyses were performed at different stages of the fungus' life cycle as well as at different stages of the fungus' age. The biochemical content of red droplets secreted by *P. atropurpurascens* was investigated by NMR analysis.

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Materials and Methods

Samples

After the first basidioma described by Knijn and Ferretti (2018), two more specimens of *P. atropurpurascens* were found in the same area of the Villa Ada urban Park called "Sughereta" dominated by holm oaks and cork oaks. The hosts of these specimens were two living trees of *Quercus ilex* L. already colonised by other lignicolous fungi (Fig. 1a). A fourth specimen was found outside of the park at a distance of two kilometres on a trunk of *Platanus* × *acerifolia* (Aiton) Willd.

In order to test *P. atropurpurascens'* capacity of proliferation, a fifth specimen was obtained by inoculating conidia into the knot of a cut branch of *Q. cerris*. Conidia were taken from the fungus growing on the cork oak stump. The inoculated branch was then put in an external environment, far from the site where conidia were collected. After a year and a half, in autumn 2018, *P. atropurpurascens* had colonised the surface and the inside of the trunk and proliferated on some leaves of *Celtis australis* L. that lay underneath the trunk (Fig. 1b). The specimens are itemised in Table 1.

Table 1 - Description of the five samples of *Punctularia atropurpurascens* used in this study. The columns feature the specimen codes in University of Tartu herbarium (Estonia) and ITS sequence codes in the UNITE database, the species and status of their host, the dates of first observation (asterisks * indicate sites that had been frequently inspected also before this date without observing the fungus) and of collection

Sample	Herbarium/UNITE codes	Host		Date of first observation	Collection date
PA1	TU111531/UDB034596	*Quercus suber*	stump	19/10/2016	14/01/2018
PA2	TU114604/UDB035018	*Platanus* × *acerifolia*	stump	25/05/2018	29/05/2018
PA3	TU114603/UDB035019	*Quercus ilex*	living tree	19/05/2018*	30/06/2018
PA4	TU114791/UDB039800	*Quercus ilex*	living tree	17/10/2018*	01/11/2018
PA5	TU114792/UDB039785	*Quercus cerris*	stump	03/11/2018*	03/11/2018
		Celtis australis	leaves		

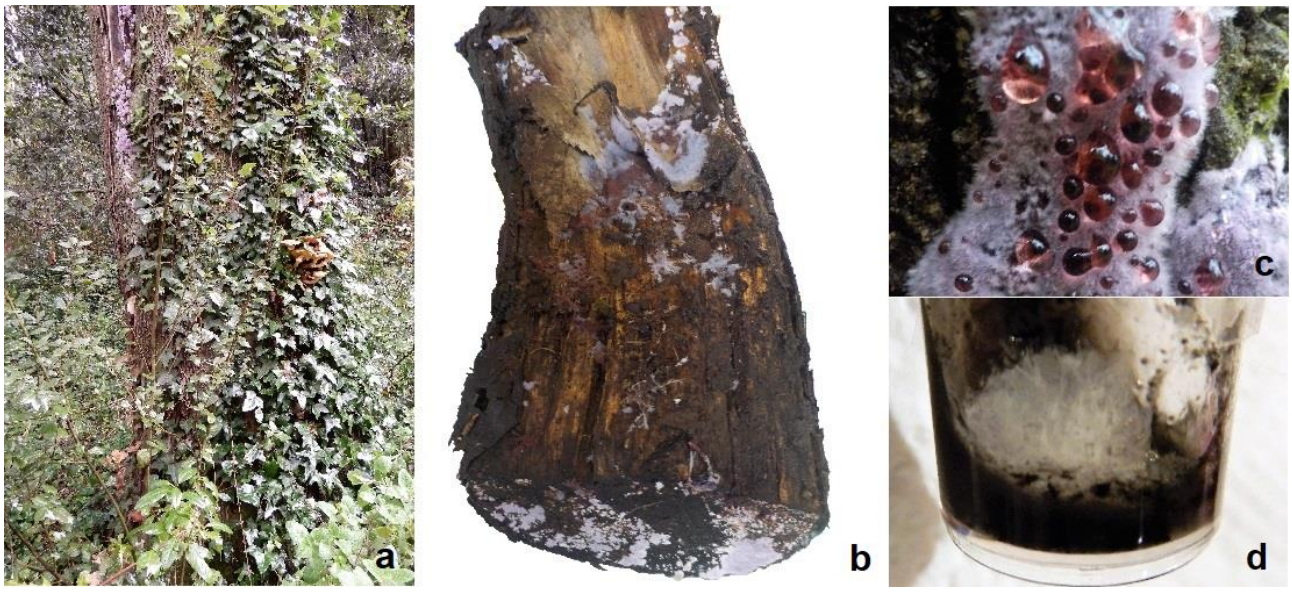

Fig. 1 - *Punctularia atropurpurascens* growing on a living tree of *Quercus ilex* (a), on a *Quercus cerris* cut branch (b); red droplets secreted by *P. atropurpurascens* (c)*;* hyphae layer formed in saline solution (d)

Moreover, several conidia were put in a suspension of sterilised saline solution (NaCl 0.9%, EDTA 0.002%) at room temperature. After more than a month, hyphae had formed on the surface of the suspension (Fig. 1d).

Morphological analysis

Microscope images of the fungi were obtained with a digital camera AmScope (Irvine CA, USA) MU500 mounted on an AmScope B490 microscope, operating at ×800 and ×2000 magnifications. Samples were analysed dry mount and wet mount in water.

Microscopical observations were made on fragments taken from the various specimens in the feathery-felted state as well as a part in the crusty state and in the latter case from both the almost dry centre and the still fluffy margins. Moreover, from the feathery-felted fungus, samples were taken from the inside of a pore left by the exuded red droplets as well as from the external contour of the pore. The sample in saline solution was analysed by microscopy at two intervals (after 20 days and 40 days).

Molecular analysis

Ribosomal DNA-based analysis was performed on all five specimens of the fungus in the frame of the UNITE project (Kõljalg et al., 2013; https://unite.ut.ee/). DNA extraction, PCR amplification of 18S partial, ITS1, 5.8S, ITS2, 28S partial regions and sequencing were performed as in Voitk et al. (2017). Sequences were aligned with the MUSCLE v3.8.425 algorithm (Edgar, 2004), in Aliview 1.25 software (Larsson, 2014) and fine-tuned manually. The Basic Local Alignment Search Tool (BLAST 2.8.1+; Camacho et al., 2009) was used to assess sequence identity. The BLAST Ring Image was generated with BRIG 0.95 software (Alikhan et al., 2011). For phylogenetic analyses, Bayesian inference was applied as before (Knijn and Ferretti, 2018); phylogenetic trees were edited using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/). The final mean standard deviation of the separated frequencies was less than 0.008.

Proton NMR analysis

The dried sample of red droplets secreted by the fungus at the stage shown in Fig. 1c and Fig. 3b was dissolved in $CDCl_3$ and proton 1D and 2D NMR spectra were collected. Proton NMR measurements applying a single-pulse sequence have been performed on a Bruker (Billerica MA, USA) Avance 400 MHz WB (9.4 T) spectrometer at 27 ºC. In total, 6000 repetitions of 8k points were acquired and FIDs were zero-filled to 32k points before transformation. A spectral width of 10 ppm was measured, corresponding to 4006 Hz at the magnetic field of 9.4 T.

Results

Specimen findings

During the period of investigation (October 2016–March 2019), the weather in Rome has been characterised by several dry periods, for instance from June to October 2017, as well as particularly humid ones, for instance February-March 2018, as can be seen from Fig. 2. These climate dynamics and the discovery of other specimens of *P. atropurpurascens* gave the opportunity to study this species' development in time under different climatic circumstances.

The fungus was detected again in the "Sughereta" area of the Villa Ada Park in May 2018, where a specimen was growing vertically on the trunk of a living holm oak tree (Fig. 1a) situated about 70 meters from the trunk of the cork oak that had been previously observed. Already in October 2016, this tree was observed to be colonised at its base by *M. giganteus* and by *Ganoderma adspersum* (Schulzer) Donk; moreover, in autumn 2018, groups of *Armillaria gallica* Marxm. & Romagn. were growing on its trunk.

A second observation of this species growing as a parasite occurred in October 2018 in the same area, on a trunk of a holm oak tree, all around its circumference. This tree is situated in a relatively open zone rather distant from the other colonized trees, a stream separated it from both the cork oak and the other holm oak. Again, this tree was colonised by groups of *A. gallica* and other lignicolous fungi at its base, causing the deterioration of the tree's wood: some branches were evidently dry,

others still vegetating. In March 2019, about five months after the first appearance of *P. atropurpurascens*, this tree had only dry branches.

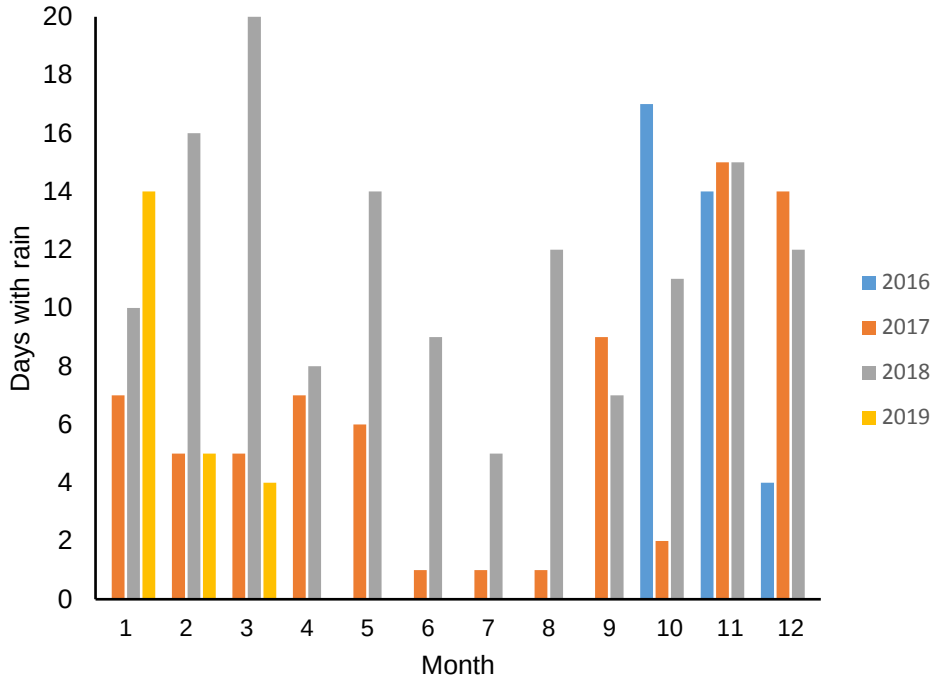

Fig. 2 - Number of rain days (> 0.2 mm) per month for the period of investigation (data obtained from http://www.lineameteo.it/)

Both observations can be considered as close to the first appearance of the fungus' basidiomata because the host trees are situated on a frequently inspected route. During the humid autumn of 2018, *P. atropurpurascens* extended on the bark and inside the trunks, with its characteristic feathery-felted morphology, exuding numerous reddish droplets (Fig. 1c).

Interestingly, *P. atropurpurascens* was also found to be present outside of the Villa Ada Park. Indeed, a specimen was observed on a trunk of *Platanus* × *acerifolia* (Aiton) Willd. along an avenue tree-lined with platans (Viale Regina Margherita), distant as the crow flies about two kilometres from the Park but only a few meters outside the large private Park of Villa Albani.

Maturation phases and morphological changes

The basidoma of *P. atropurpurascens* is stably present throughout the year, although its morphology undergoes evident transformations (Fig. 3). In the first stage of maturation (Fig. 3a), the fungus appears as sparse, light pink, fluffy tufts coming out of the clefts of the hosts bark. With time (Fig. 3b), the tufts spread and merge, becoming showy pink-coloured, while reddish droplets exude from pores. This stage generally occurs mid-autumn, but can also happen as late as mid-spring. Then, depending on humidity, the morphology of the fungus can transform in two different ways. If the weather is dry (Fig. 3g), the red droplets disappear and the fungus turns purple while its consistency becomes compact. When the weather is more humid stage 3b persists for a long time and with the temperatures rising, the centre of the fungus dries to a brownish crust and the colour of the droplets tend to rust (Fig. 3c). At the next stage (Fig. 3d) the fungus turns purple/blue at the centre while staying pink at the edges. Successively (Fig. 3e), the fungus darkens to deep purple and at the final stage (Fig. 3f) the fungus appears as black spots on the tree's bark. In dry seasons, the transition from stage 3g to 3f occurs directly in late winter or early spring.

Microscopical observations

Samples of all specimens were observed by microscopy, including those obtained from the evolution of the conidia in suspension (Fig. 4). All samples had similar microscopical as well as morphological appearances, in accordance with Martini (2016). The basidia seem to coincide in particular with the crust state of *P. atropurpurascens*. On the other hand, conidia are prevalent when the fungus has a feathery-felted morphology, that is, at the stadium of primary hyphae with the appearance of ramifications (dendrohyphidia).

Fig. 3 - Morphological changes undergone during the year by the basidiomata of *Punctularia atropurpurascens*. The long cycle follows stages a-b-c-d-e-f, however under dry circumstances the fungus can transform from stage b into g and directly into stage f

The fungus, at the initial phase of growth on each substrate (for example the *P. atropurpurascens* put in saline solution, *de novo* growth), is composed of hyphae without clamp connections, since these appear in the hyphae with the formation of the secondary mycelium (Stephenson, 2010). The clamp connections appear after several growth cycles, sometime after the fungus *P. atropurpurascens* has colonised its host.

Massive conidia were present inside the pores produced by the secreted reddish droplets, while they were scarce at the margins, essentially formed by primary filamentous hyphae. The conidia

appear with a reddish intracellular content, probably due to pigments like phlebiarubrone and its derivatives. Furthermore, conidia have hyaline as well as brownish violet cell walls, in particular with the growth of the fungus. Moreover, at this point violet-brownish-coloured granulations become noticeable on the hyphal filaments. At the first stages of growth, lipid droplets appear inside the conidia.

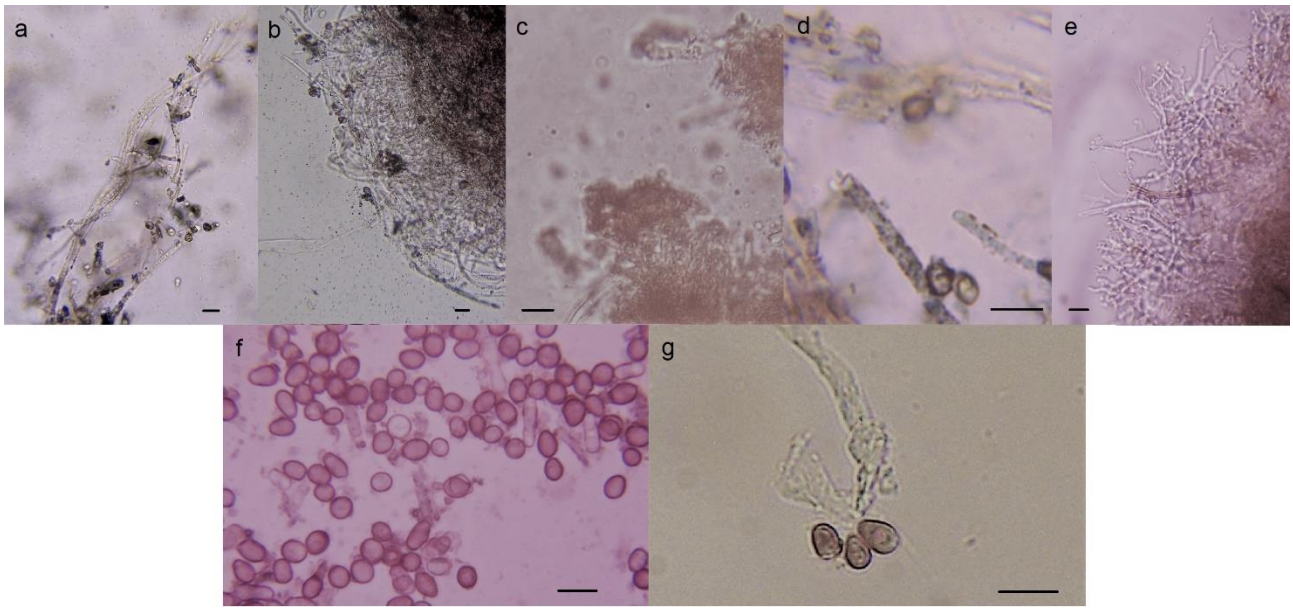

Fig. 4 - a) Hyphae from *Punctularia atropurpurascens* in saline solution for 20 days; no clamp connections were visible. b) Similar patterns were present in hyphae found the first time (17/10/2018) on *Quercus ilex*. c) Basidia in the fungus in the crust state, from the same tree. d) Hyphae in saline solution for 40 days; clamp connections were visible. e) *P. atropurpurascens* from *Quercus suber*; clamp connections were present. f, g) Conidia found in saline solution after 20 days (f) and 40 days, with red droplets present internally (g). Bar = 10 µm

Genetics

All five specimens were confirmed to be *P. atropurpurascens* by BLAST searches and phylogenetics (Fig. 5). After the alignment process, the dataset resulted in five sequences with a length of 938 bp. Nucleotide diversity was observed in the ITS1 (one SNP very close to the 5.8S region), ITS2 (three SNPs) and LSU (four insertions) regions (Table 2). The phylogram obtained through Bayesian inference grouped the five specimen in two clades (PA1, PA2 and PA3, PA4, PA5) with a node supported by a posterior probability of 0.997 (Fig. S1).

Table 2 - Single nucleotide polymorphisms (SNPs) in the genetic sequences of the *P. atropurpurascens* samples. Columns report obtained sequence length, BLAST identity and gaps between sample PA1 and the other four samples. "–" a gap existed in the reported locus, "·" no data

Region					**ITS1**	**ITS2**			**LSU**			
Position (bp)					204	477	516	529	842	894	900	928
Sample	**Accession No**	**Length (bp)**	**Ident**	**Gaps**								
PA1	UDB034596	915	1	0	A	A	T	C	–	–	–	·
PA2	UDB035018	937	0.995	0.003	G	A	C	C	G	A	A	G
PA3	UDB035019	930	0.992	0.003	G	G	C	T	G	A	A	–
PA4	UDB039800	600	0.993	0	G	G	C	T	·	·	·	·
PA5	UDB039785	938	0.993	0.003	A	G	C	T	G	A	A	G

Magnetic Resonance Spectroscopy

In order to confirm the presence of phlebiarubrone in the droplets secreted by the fungus, liquid was collected in November 2018 when fluffy tufts appeared (Fig. 3b) and ^{1}H NMR analysis was performed. The results show that signals from terphenylquinone (Anke et al., 1986), although of low intensity, are present in the aromatic region of the spectra (Fig. 6). Other resonances in the spectrum are due to acyl chains from lipids and to sterols; moreover, well visible signals arising from inositol can be observed, probably partly –O–phosphorylated (Gutierrez et al., 2007). Signals from double bonds are absent, indicating that the lipid acyl chains are saturated, as can be found in lipids forming the category of glycosyl inositol phosphoryl ceramides (GIPCs), while the sterol is related to ergosterol (Dupont et al., 2012).

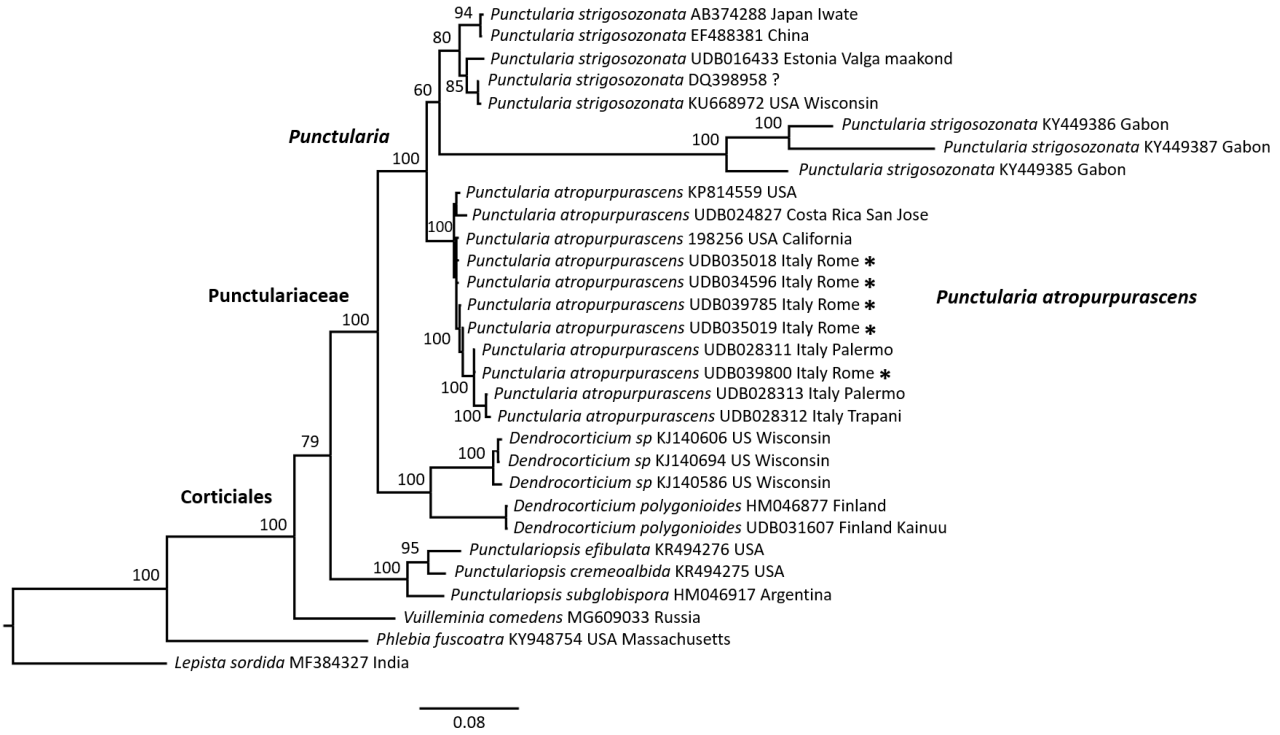

Fig. 5 - Phylogenetic tree with posterior probability values obtained through Bayesian inference based upon alignment of the nuclear ribosomal ITS sequences reported in Table S1, identified by the associated species, accession number and place of collection. The samples described in the article are indicated by an asterisk. Bayesian posterior probabilities are reported on each branch. The final mean standard deviation of the separated frequencies was 0.0075

Discussion

Although in Italy only few reports from Sicily are available on the (sub)tropical species *P. atropurpurascens* (Mondello, 2017), the results prove that, given favourable climatic and environmental conditions, this fungus has the ability to proliferate easily in its sites of appearance. In fact, *P. atropurpurascens* is present in the Villa Ada urban Park (Rome) and in a platan tree-lined avenue, two kilometres distant from the other specimens. Notably, the fungus showed parasitic as well as saprotrophic behaviour. In the "Sughereta" area of Villa Ada, both holm oak trees infected by *P. atropurpurascens* were alive but previously hit by the colonisation of other parasitic and saprotrophic fungi, while no healthy trees in the same area were affected. Evident presence of *P. atropurpurascens* basidiomata was found on a living holm oak where in previous years a flourishing cluster of *M. giganteus* had been noted at the base of its trunk. It is conceivable that when *P. atropurpurascens* behaves as a parasite, its development is successive to the aggression of the tree by other fungal parasites, thus previously weakened by its colonisation (Schwarze et al., 2000). No reports in literature were found on a possible synergism between different parasitic fungal genera with *P. atropurpurascens.*

Remarkably, it was found that the cyclic morphological transformations of the fungus can follow two distinct pathways in function of conditions of humidity, which seems an adapting strategy to withstand drought stress. It would be interesting to see if this duality also occurs in the (sub)tropical ambient where the fungus usually grows, however no data was found regarding this particular aspect. Petch (1916) did mention two quite different forms of *P. atropurpurascens* depending on humidity, describing the phases 3b and 3c in Fig. 3. It is striking that the morphological changes occurring in *P. atropurpurascens* during its life cycle are more significant than the macroscopic differences with the similar fungus *Hypochnella violacea* Auersw. Ex J. Schrot., alluding to a biological significance of these transformations.

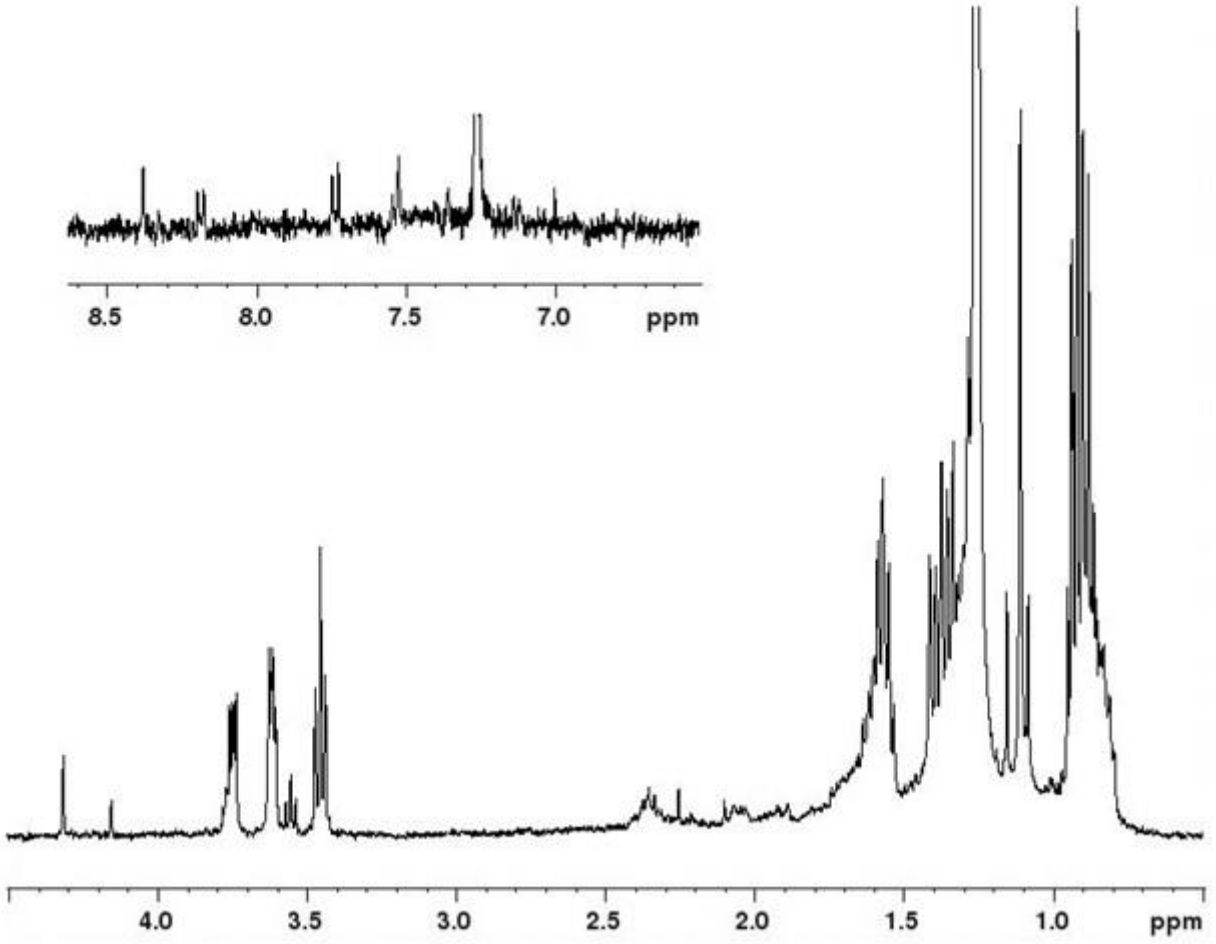

Fig. 6 - One-dimensional ^{1}H NMR spectrum in deuterated chloroform of a sample taken from dried red droplets exuded by *Punctularia atropurpurascens*. Signals arising from lipids' acyl chains are present (about 0.9 and 1.3 ppm), as well as from sterols (about 0.8, 1.1, 1.6 and 3.6 ppm) and inositol compounds (from 3.4 to 4.4 ppm). The aromatic region (high ppm) is shown as an insert, amplified by four to evidence signals from phlebiarubrone (7.5 and 7.7 ppm)

All samples of the various specimens showed a similar appearance at a morphological and microscopical level. Our observations put in evidence that the morphological variations occurring during growth cycle are not correlated with the host species, and neither with its saprotrophic or parasitic behaviour. In fact, the morphological transformations are induced by climate dynamics. On the other hand, the microscopical changes are related to growth cycle and age of the fungus.

Complex biochemical pathways underlie variations in fungal gene expression, also in relation with the specific nature and composition of its growth substrate (Skyba et al., 2016). It is reported that in *Fomitopsis pinicola* (Sw.) P. Karst. gene expression is regulated at different stages of growth in function of the genotype of the colonised substrate, regardless of its provenience (conifers or hardwoods) (Baojun et al., 2018). The genes that are regulated by substrate composition, encode for

enzymes with known or potential function in rot decay, some of them are involved in redox reactions with benzoquinones such as phlebiarubrone and its derivatives (Suzuki et al. 2006; Baojun et al., 2018).

Another question is what biochemical role phlebiarubrone and its derivatives play during fungus' development. It is reported that phlebiarubrone produced by *P. atropurpurascens* (Anke et al., 1986; Gill and Steglich, 1987), as well as by other macromycetes (Järvinen et al., 2016), has effects similar to inhibitors of DNA transcription, translation and replication in bacteria (Järvinen et al., 2016). Phlebiarubrone is essentially a diphenylbenzoquinone or terphenylquinone, like some DNA intercalating molecules (Di Marco et al., 1969; Hollstein, 1974; Weiss, 1992). Moreover, phlebiarubrone shows weak antibacterial and cytotoxic activity (Calì et al., 2003).

Proton NMR analyses performed on the reddish droplets secreted by *P. atropurpurascens* when basidiomata appeared as fluffy tufts (Fig. 3b), underline that the molecular content is not casual. In fact, the droplets are composed of derivatives of phlebiarubrone together with lipid molecules, most probably structured as micelles. The lipids are sterols related to ergosterol and inositol containing lipids, the major part with saturated acyl chains, as found in lipids forming the category of glycosyl inositol phosphoryl ceramides (GIPCs) (Guan et al., 2009; Burè et al., 2014). Both kinds of molecules are known to be involved in various signals transduction mechanisms through the cell membrane in fungi and plants (Rodrigues et al., 2000; Gutierrez et al., 2007; Alvarez-Vasquez et al., 2011; Gronnier et al., 2016) matching those in mammalian cells (Ferretti et al., 2003). Moreover, it was shown that the ergosterol biosynthetic pathway increases survival in fungi during the drying-wetting cycling periods (Dupont et al., 2012). The cycling periods are also undergone by *P. atropurpurascens*, being persistent throughout the year on the colonised host (Knijn and Ferretti, 2018). These processes could be related to the cyclic morphological transformations through two distinct pathways in function of the climatic conditions that have been described.

Overall, these findings suggest that the molecules secreted by *P. atropurpurascens* are not merely the guttation of an ensemble of aqueous substances, but indicate a biological role exists for the secretion of this kind of droplets and their components by the fungus. Therefore, *P. atropurpurascens* can be proposed as a model for signalling molecules' studies on how its compounds could modulate the growth and survival of fungi and conidia (Brown and London, 2000; Guan et al., 2009).

Acknowledgments

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