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Research Article

Diversity of *Biscogniauxia mediterranea* within Single Stromata on Cork Oak

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Charcoal canker, caused by the fungus *Biscogniauxia mediterranea*, is one of the most frequent diseases of cork oak in Portugal. The pathogen has been considered a secondary invader that attacks only stressed hosts; however, in recent years, an increasing number of young trees exhibiting the disease symptoms have been recorded. A collection of monoascosporic cultures isolated from single stromata of *B. mediterranea* in cork oak from different locations was analyzed by means of microsatellite—Primed Polymerase Chain Reaction—using three microsatellite primers, in order to detect the genetic variation of the population thus discussing its plasticity and ability to adapt to different conditions. The results showed a high level of genetic variability among isolates obtained from the same stroma, being impossible to distinguish isolates from individual stromata neither from different geographical location.

1. Introduction

Quercus suber L., cork oak, is the most emblematic tree of Portugal due to its high environmental, social, and economical value. *Biscogniauxia mediterranea* (De Not.) O. Kuntze (Xylariaceae, Xylariales) is well known as the causal agent of charcoal canker in cork oak [1]. This fungus can live as an endophyte in all of the aerial organs of the oak plants and can act as an opportunistic pathogen when the hosts suffer prolonged periods of stress. In those conditions, *B. mediterranea* is able to rapidly colonize the xylem and bark tissues, induce necrosis and canker formation, and accelerate tree decline and eventually death [2–4]. The great abundance of inoculum produced on colonized parts of the tree and the dispersal of fungal ascospores, airborne and by insects, is important factors accounting for fungal spread in the forests [5–7].

Recent observations on Portuguese cork oak stands revealed the increased incidence of charcoal canker and the presence of atypical symptoms, especially in young trees, which questioned whether some alteration occurred on the disease epidemiology [8, 9]. The evidence of high genetic variability and the heterothallic mating system can support the adaptive strategy of the fungus and its epidemiology [10], particularly facing actual conditions of climate change which appear to favor the impact of charcoal disease in *Q. suber* forests [11].

With this work we intended to evaluate the diversity of *B. mediterranea* within individual hosts in Portugal, through the analysis of Microsatellite—Primed Polymerase Chain Reaction (MSP-PCR) profiles of monoascosporic cultures isolated from single stromas in cork oak.

2. Material and Methods

A collection of 16 isolates of *B. mediterranea* was analyzed, eight monoascosporic isolates obtained from two stromata from adult declined *Q. suber* sampled in Comporta (A) and Grândola (B) (Alentejo, Portugal). Monoascosporic cultures

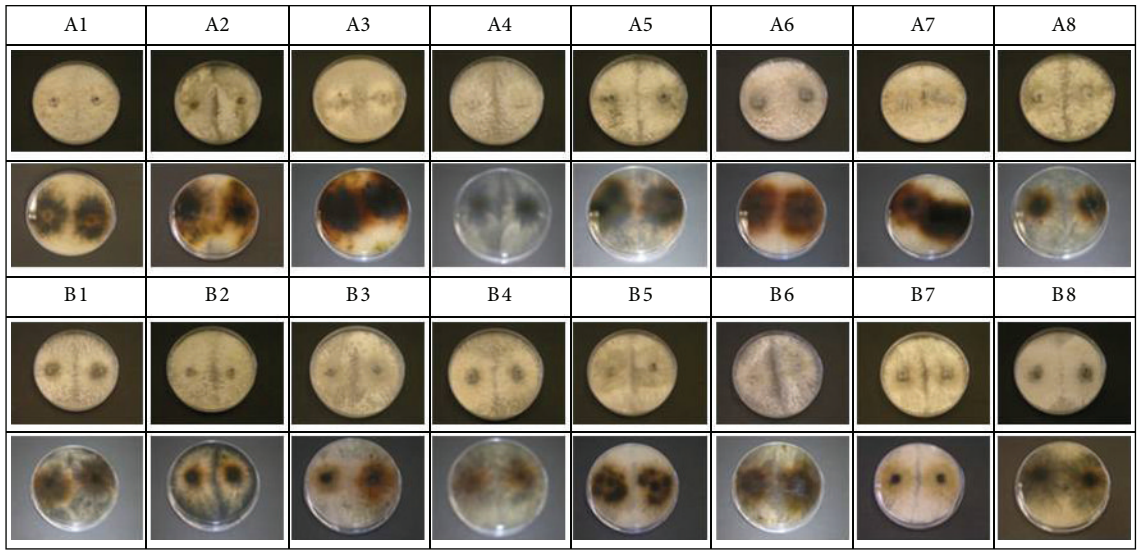

Figure 1: Seven days colonies on PDA of monoascospores isolates of *Biscogniauxia mediterranea* from the stromas A and B (surface and reverse).

were started by incubating stromata in Petri dishes at 22°C and 100% relative humidity. After 12 h the ascospores discharges on the dishes were collected, and single ascospores were individualized with a needle and plated on Potato Dextrose Agar (PDA, Difco, USA) acidified with lactic acid (1 mL lactic acid 85%/L PDA, PDAA) and incubated at 25 ± 1°C in darkness [12]. Seven-day growth cultures on PDA were evaluated for the general aspect of the colony, density, and surface and reverse colors (according to Rayner color chart [13]). Voucher specimens of each isolate were deposited in the fungal collection at Micoteca da Estação Agronómica Nacional (MEAN) at INIAV (Oeiras, Portugal).

DNA was isolated from mycelia scraped from the surface of a PDA plate and extracted with the DNeasy Plant Mini Kit (Qiagen, USA), following the manufacturer's instructions. Instead of using ground lyophilized mycelia, fresh mycelium was used and disrupted by adding approximately 50 μL of glass beads (425–600 μm diameter) to the extraction buffer and vortexing for 2 min before and after RNase A incubation [14].

The collection of 16 isolates was analyzed by means of MSP-PCR. The profiles were generated following the protocol of Uddin and Stevenson [15] using the primers (CAG)5, (GACA)4, and (GTG)5. PCR reactions were carried out in a total reaction volume of 25 μL containing approximately 10 ng of genomic DNA (quantified by Nanodrop 2000 Spectrophotometer, Thermo Scientific, USA), 1 μM of either oligonucleotide primer, 1x Dream Taq buffer (DreamTaq PCR Master Mix, Fermentas, Germany) which includes Taq polymerase (unknown concentration), 0.2 mM dNTPs, and 3 mM $MgCl_2$. Thermal cycling was performed on a Tgradient Thermocycler (Biometra, Germany) using the following parameters: an initial incubation at 94°C for 2 min, followed by 40 cycles of 30 s at 93°C, 1 min at 53°C, 30 s at 72°C, and a final 72°C extension period of 10 min. Amplicons were separated by electrophoresis at 7 V cm^{-1} in agarose gel (1.5%) containing 0.5 μg/mL ethidium bromide and 1x TBE running buffer. Data analysis was visualized by Versa Doc Gel Imaging System (BioRad, USA). The isolates were clustered on the basis of their profiles in consensus dendrogram built with NTSYSpc2 (Numerical Taxonomy and Multivariate Analysis System, version 2.1) using DICE coefficient and UPGMA.

3. Results

The isolates of *B. mediterranea* presented high variability in culture, especially in pigmentation and presence of aerial mycelium. Monoascosporic isolates from the same stroma showed evident differences among cultures (Figure 1). Cultural aspects of the seven days colonies from both stromas varied from velvety to wholly with mycelial tufts dispersed in the culture to velvety with sectors (according to the density) and tufts, density media to high. Colors differ from white with vinaceous buff aerial mycelium, grayish sepia to smoke grey, or butt margin with olivaceous center. In some colonies dark brown exudates are frequent. The reverse of the colonies varies from buff margin with umber to olivaceous center, buff to honey margin, saffron to sienna center, or pale mouse grey to mouse grey, with darker spots dispersed in the culture or strong diffusible pigment.

The molecular analysis of monoascosporic isolates by MSP-PCR resolved distinct amplification banding patterns between 0,25 and 1,2 kb for the primer (CAG)5, between 0,25 kb and 0,7 kb for (GACA)4, and between 0,5 and 1,3 kb for (GTG)5, resulting in a total of 20 different band positions. The three primers generated different amplification patterns among isolates even from the same stroma. One consensus dendrogram was obtained from combined analysis of the profiles generated by the three primers for isolates from a single stroma (Figure 2) and for the all set of isolates (Figure 3).

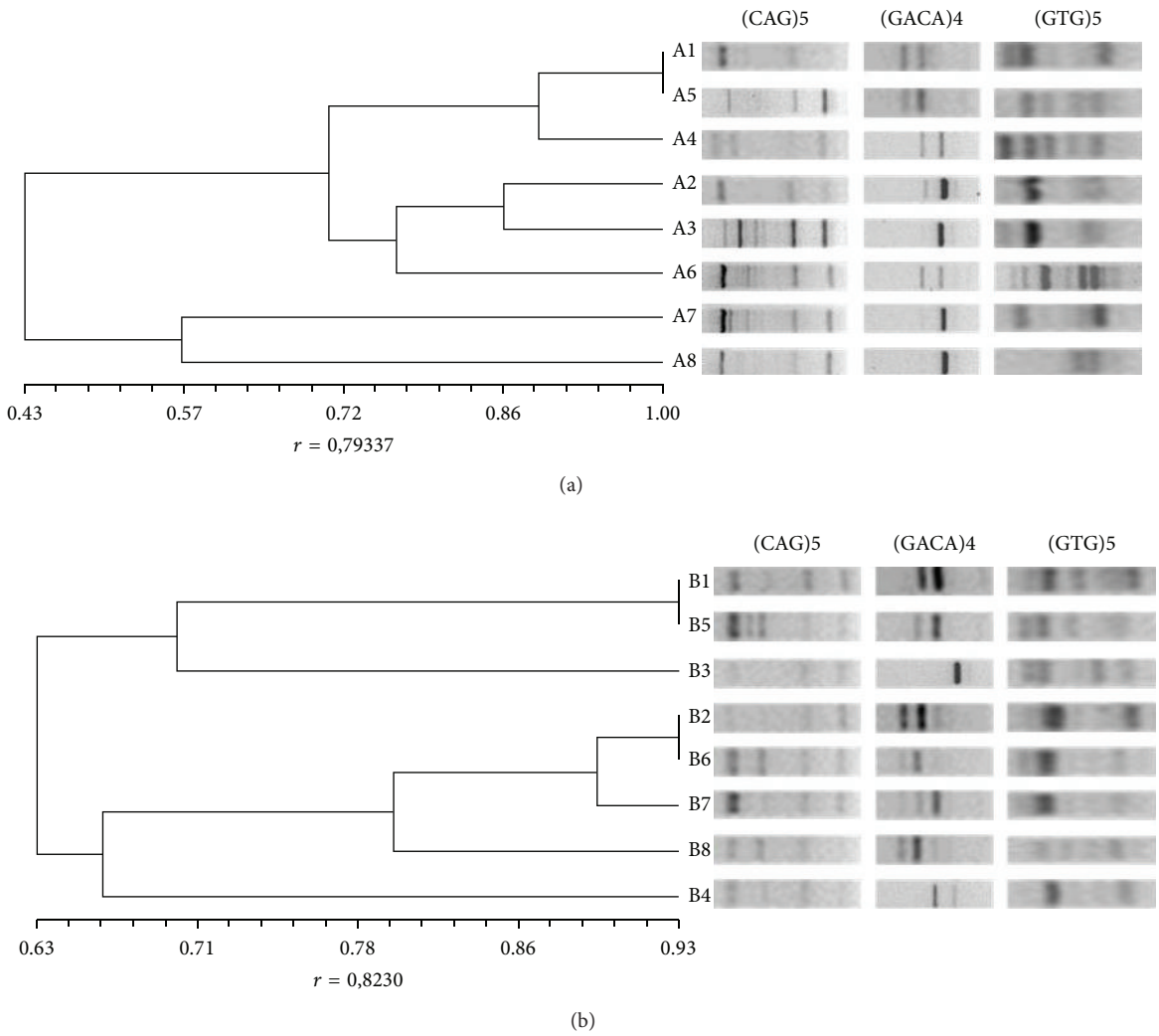

Figure 2: Consensus dendrograms from (CAG)5, (GACA)4, and (GTG)5 MSP-PCR profiles performed in NTSYSpc2 using DICE's correlation coefficient and UPGMA. Scale bar represents percentage of similarity. (a) Analysis of *Biscogniauxia mediterranea* isolates obtained from stroma A (Comporta); (b) analysis of *B. mediterranea* isolates obtained from stroma B (Grândola).

Among the monoascosporic cultures originated from the stroma collected both in Comporta and in Grândola, the isolates exhibited a high level of variability within each stroma, clustering in two main groups. In Comporta, a cluster with two isolates and another with six isolates were formed with 43% similarity. In the first group the two isolates were 57% similar and in the second group the isolates clustered at different levels higher than 70% similarity, with only two 100% similar isolates (Figure 2). In Grândola, the two main groups were 63% similar, presenting three isolates in a cluster in which one is segregated with 70% similarity and two were up to 90% similar, and the other five isolates clustered at different levels of similarity higher than 65%, with also two isolates up to 90% similar (Figure 2). The joint analysis of isolates from the two sites showed a high variability among all, and the isolates were grouped in increasing levels of similarity above 46%, with no distinction between the isolates of each local (Figure 3).

4. Discussion

In the present study, a high genetic variability of *B. mediterranea* was detected within populations of monoascosporic cultures isolated from single stromata and from different hosts/localities. Individually, the MSP-PCR primers profiles showed a high degree of diversity among isolates from each sampled stroma, and a joint analysis of all isolates did not reveal clustering according to the different stroma. The use of combined MSP-PCR profiles has strengthened these observations, highlighting the vast genetic diversity among isolates. The occurrence of high genetic variability of *B. mediterranea* in a single stroma is in line with the results presented by Vannini et al. [10] in which the variability of the fungus was assessed by random amplified polymorphic DNA (RAPD); however, this approach allowed the discrimination of monoascosporic isolates within a single ascus, a single stroma, and among stromata. Also Schiaffino et al. [16], using

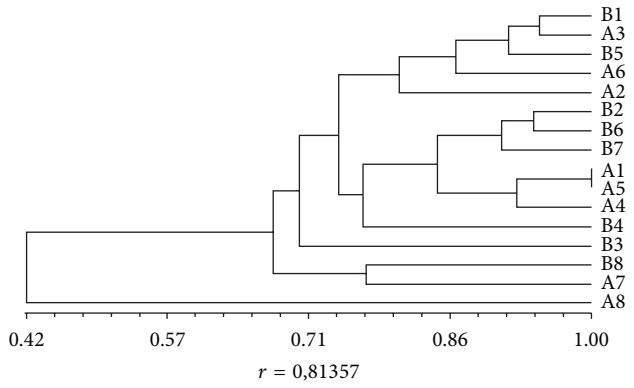

Figure 3: Analysis of *Biscogniauxia mediterranea* total isolates. Similarity consensus dendrogram from (CAG)5, (GACA)4, and (GTG)5 MSP-PCR profiles performed in NTSYSpc2 using DICE's correlation coefficient and UPGMA.

the same technique, showed high level of genetic variability among isolates from a restricted area of Sardinia as well as among isolates from different localities.

The application of neutral genetic markers to plant pathogens has enabled the finding that a lesion is often colonized by several genetically distinct individuals, suggesting that host coinfection is relatively common. Examples include *Phaeosphaeria nodorum* [17] and *Mycosphaerella graminicola* on wheat [18], *Alternaria* sp. on pear leaves [19], *Ascochyta rabiei* on chickpea [20], *Aspergillus flavus* on cotton [21], *Rhynchosporium secalis* on barley [22], and *Leptosphaeria maculans* on oilseed rape [23].

The presence of high genetic variability also in small populations could be partially explained by the consideration that ascospores are the most important dispersal and inoculums units in *B. mediterranea*, as in other Xylariaceae, and those new genotypes can spread over long distances [24]. The high rate of sexual reproduction and the heterothallic mating system of this fungus represent an important internal source of genetic variability of the population [10]. Being a heterothallic fungus, the occurrence of multiple genotypes in the same lesion allows isolates of opposite mating types to come together and reproduce sexually. Frequent sexual reproduction in turn will ensure frequent recombination and increased evolutionary adaptability [18].

In addition, infection by different genotypes can occur at different times of the host life cycle, keeping the fungus as endophyte. However, colonization of the tissues and reproduction occur at the same time from all the infection points, when the host is subjected to stress. Such behavior could explain how this fungus, though being considered a weakness parasite, is able to kill large trees in a short period [10].

Large variability of *B. mediterranea* population is extremely important for its epidemiology since it provides the fungus with genetic flexibility for long-term survival and adaptation to the environment. Coexistence of pathogen clones within the same host plant has manifold biological implications beyond the increased opportunities for sexual reproduction. For example, coexistence can affect host health or infectiousness and affect the transmission success of individual clones, thus shaping the evolution of traits such as virulence/aggressiveness or fungicide resistance [18].

It is important to consider this high adaptive capacity of *B. mediterranea*, notably in the scenario of climate change. The predictions that comprise fungus' physiological features already indicate that the impact of charcoal disease in *Q. suber* forests will be favored under the aggravated climate conditions for the Mediterranean basin [2, 25].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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