

Three species of *Colletotrichum* are associated with anthracnose of pomegranate in Brazil

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Abstract

Pomegranate is a fruit rich in vitamins and secondary metabolites used in traditional medicine and industry. However, production losses have been associated the anthracnose, disease caused by *Colletotrichum* species. This is an important disease of the pomegranate, as it affects the yield and the quality of the fruits. The present study aimed to investigate which species of *Colletotrichum* are associated with anthracnose disease in pomegranate in the Northeast region of Brazil, using multi-locus phylogenetic analysis and morpho-cultural characteristics. The total DNA extracted was amplified with *GAPDH*, *TUB2*, *CAL*, *ACT* genes and the ITS-rDNA region. The sequences obtained were used for the construction of phylogenetic trees of Bayesian inference. The mycelial growth rate, size and shape of the conidia and appressories were evaluated for the morpho-cultural characterization of the species. Six isolates were analysis and three species belonging to the *C. gloeosporioides* complex were identified in this study. This is the first report of *C. theobromicola* (2) in pomegranate fruit in Brazil and *C. siamense* (2) and *C. fructicola* (2) in the world.

Keywords: *Colletotrichum fructicola*, *Colletotrichum siamense*, *Colletotrichum theobromicola*, *Punica granatum*, multi-locus phylogeny

Pomegranate (*Punica granatum* L.) is a tree native to the Middle East but it is well adapted to the climatic conditions of Brazil. Commercial production of pomegranate has expanded internationally as well as within Brazil due to increasing interest from farmers. The fruit is rich in nutrients and active principles that can be used in the cosmetic, pharmaceutical and food industry (Kanetis et al., 2015; Hmid et al., 2017).

However, fungi of the genus *Colletotrichum* Corda have harmed the yield of this culture, causing the anthracnose disease. Anthracnose fruit rot is considered one of the most important fungal diseases of the pomegranate, both in pre-harvest and post-harvest, because it leads to significant financial losses, mainly due to the rot symptoms that depreciate the fruit and lead to rapid deterioration (Munhuweyi et al., 2016).

Although different species of the genus *Colletotrichum* cause anthracnose in several hosts, only

the species *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc had been reported in pomegranate, because its identification was based exclusively on morphological studies, host range and analysis of the internal ribosomal transcribed spacer region (ITS-rDNA) (Lakshmi et al., 2011; Thomidis & Exadaktylou, 2011; Rahimlou et al., 2014).

Nevertheless, the current taxonomy of the *Colletotrichum* genus has been based on multi-locus phylogenetic studies combined with phenotypic characteristics. Studies using these approaches concluded that *C. gloeosporioides* is a complex of species (Weir et al., 2012; Jayawardena et al., 2016). In addition, the species *C. theobromicola* Delacr. Bull and *C. tropicale* E.I. Rojas, S.A. Rehner & Samuels were reported on pomegranate fruits in India and Brazil, respectively, and *C. nymphaeae* (Pass.) Aa, *C. fioriniae* (Marcelino & Gouli) R.G. Shivas & Y.P. Tan, *C. simmondsii* R.G. Shivas & Y.P. Tan, *C. theobromicola*, *C. siamense* Prihastuti L. Cai

& K.D. Hyde, and *C. gloeosporioides* on pomegranate in United States, all using multi-locus analysis (Sharma et al., 2015; Silva-Cabral et al., 2019; Xavier et al., 2019). Therefore, the present study aimed to investigate which *Colletotrichum* species are associated with anthracnose disease on pomegranate in the northeastern region in Brazil, based in multi-locus phylogeny and morpho-cultural characteristics' approaches.

Pomegranate fruits with circular necrotic lesions were collected from domestic growers and food fairs from August 2012 until March 2013, in the states of Alagoas, Pernambuco and Bahia in Brazil. The isolates were obtained from symptomatic pomegranates and grown on potato-dextrose-agar (PDA). The colonies were stored in the Culture Collection of Phytopathogenic Fungi at the Universidade Federal de Alagoas (COUFAL).

Healthy pomegranate fruits (one fruit by isolate) were submitted to pathogenicity. Each fruits wounded by a needle sterile were inoculated with two 5 mm mycelial plugs (seven days old) placed separated equidistant from each other and kept in humid chamber. Control fruits were inoculated with agar plugs.

The total DNA was obtained from single-spore colonies growing for 7 days using the extraction protocol described by Doyle & Doyle (1987) and submitted to PCR reactions for amplification of the ITS-rDNA region, and partial sequences of the β -tubulin (*TUB2*) genes, glyceraldehyde-3-phosphate (*GAPDH*), calmodulin (*CAL*) and actin (*ACT*). The PCR conditions were the same as described by Weir et al. (2012). The products of the PCR reaction were purified and sequenced in both directions with the same primers used for amplification.

Sequences of the amplified regions were assembled using the Staden Package software and compared against the GenBank database for preliminary identification of the species. Next, the alignment of the consensus sequences was manually adjusted using the MUSCLE algorithm in MEGA6. The Bayesian Inference analysis (BI) was performed employing the method of the Markov chain Monte Carlo (MCMC) at the CIPRES web portal using MrBayes v. 3.2.3 to construct phylogenetic trees. The best nucleotide substitution model was determined for data from each genomic region using MrModeltest according to the Akaike Information Criterion (AIC). An initial BI analysis using the *GAPDH* data set was performed for the seven isolates obtained in this study. A second BI analysis was carried out to confirm the identification of the isolates based on the concatenated data set (*ACT*, *CAL*, *GAPDH*, ITS and *TUB2*).

Sequences of the species from *C. gloeosporioides*

complex obtained from GenBank were included in the analysis (Table supplementary 1). The partial sequences obtained in this study were deposited in GenBank. The alignment and tree were deposited in TreeBASE (<http://www.treebase.org>; accession number: 23044).

For morpho-cultural characterization, colonies were grown on synthetic PDA incubated at 25°C for 7 days and measured daily. The colour of the colonies was evaluated in the 7th day. Format and size of fifty conidia and appressories were also measured. Data were submitted to analysis of variance (ANOVA) ($p < 0.05$) by Tukey's test.

Six isolates were obtained from Alagoas (COUFAL0042, COUFAL0043, COUFAL0044), Bahia (COUFAL0040 and COUFAL0041) and Pernambuco (COUFAL0049). All isolates tested caused symptoms at 7 to 10 days after inoculation. The control fruits showed no symptoms. Sequences of the *GAPDH* gene showed high similarity with sequences of three species from *C. gloeosporioides* complex deposited in the GenBank: *C. siamense*, *C. theobromicola* and *C. fructicola* Prihastuti L. Cai & K.D. Hyde. The results of both phylogenetic BI analysis confirmed the prior identification of these three species (Figure 1).

The isolates produced hyaline, straight, cylindrical to clavate conidia with often broadly rounded ends and brown appressoria of irregular format. Colonies varied between grey to white with mean growth of 5.8 mm/day (Table 1). All morpho-cultural features were similar to the species from *C. gloeosporioides* complex (Rojas et al., 2010; Weir et al., 2012).

These species are widespread in different world locations causing anthracnose on several hosts. However, *C. fructicola* was not previously reported on pomegranate anywhere in the world, while *C. siamense* and *C. theobromicola* were reported only in the USA and the latter also in India, probably because of the lack of studies using multi-locus phylogeny for the identification of species from *Colletotrichum* in this host (Alaniz et al., 2015; Meetum et al., 2015; Sharma et al., 2015; Liu et al., 2016; Costa et al., 2019). Moreover, the species *C. gloeosporioides* was not reported in the present study.

Studies carried out in Brazil with other hosts using multi-locus phylogenetic, in which anthracnose had also been attributed exclusively *C. gloeosporioides* based in morphological characteristics, corroborate with this result, reporting *C. fructicola* on cashew, pine cone, soursop and mango; *C. siamense* on cashew, pine cone, soursop and mango, *C. tropicale* on cashew, pomegranate, pine cone, soursop and mango, *C. asianum* on mango, *C.*

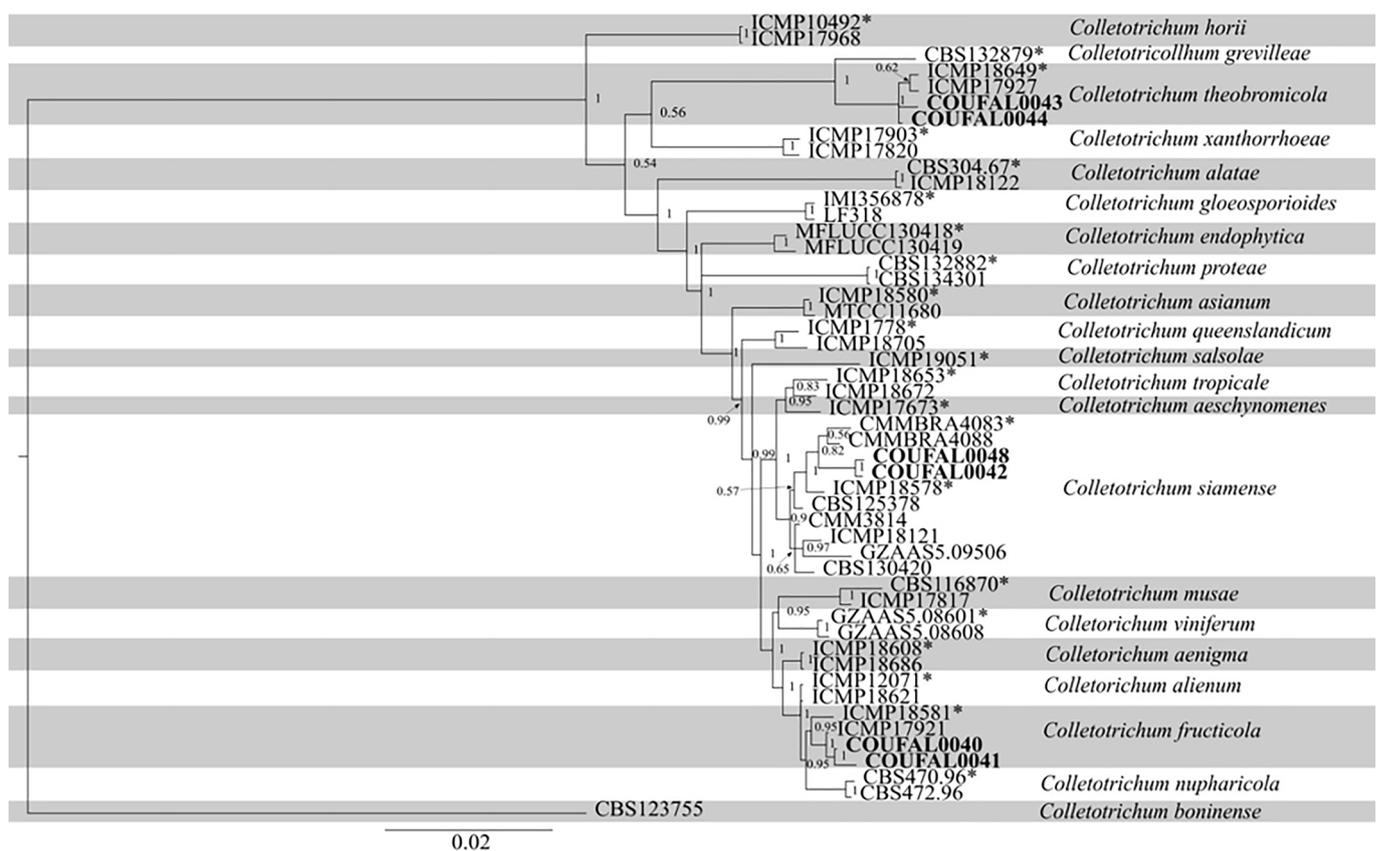


Figure 1. Multi-locus phylogenetic tree inferred from the Bayesian analysis using *GAPDH*, *TUB2*, *ACT* and *CAL* genes and ITS region for *C. gloeosporioides* complex. The combined data set consisted of 2614 nucleotides. The SYM+I model of evolution was used for all gene. The values of posterior probability > 0.5 are indicated above knots. Ex-type cultures are marked with an asterisk. The strains used in this study are emphasized in bold. The tree was rooted with *Colletotrichum boninense*.

Table 1. Summary of morphological and cultural characteristic of *Colletotrichum* strains.

Species	Conidia(µm)			Appressoria(µm)		Growth rate (mm/day)	Colonies
	Length	Width	Shape	Length	Width		
<i>C. fruticola</i> COUFAL0040 COUFAL0041	13.2c (7.72-18.12)	4.3b (2.31-6.04)	Cylindrical	9.7a (7.07-14.17)	6.5b (4.43-9.19)	4.0c	Greyish, greendarkreverse
<i>C. theobromicola</i> COUFAL0043 COUFAL0044	16.7a (13.32-20.95)	4.6a (3.18-5.68)	Cylindricalto clavate	8.5b (6.6-11.79)	6.6b (4.13-9.28)	6.2a	Gray with white edges, dark gray reverse
<i>C. siamense</i> COUFAL0042 COUFAL0048	14.2b (12.59-17.62)	4.6a (3.68-5.37)	Cylindrical	9.7a (5.8-15.19)	7.3a (5.1-10.43)	5.3b	White, greenishreverse

Means followed by the same letter in the column do not differ statistically by the Tukey test at 5% probability.

theobromicola on pine cone and sour sop, *C. chrysophilum*, *C. fragariae* and *C. queenslandicum* on cashew (Lima et al., 2013; Veloso et al., 2018; Costa et al., 2019; Silva-Cabral et al., 2019). *Colletotrichum gloeosporioides* was reported only in the studies of Costa et al. (2019) and Veloso et al. (2018), but with low incidence, one isolate obtained from pine cone leaf and two isolates obtained from cashew inflorescence, respectively.

These results raise an intriguing question about this species that needs to be better clarified: Does this species behave as an endophytic pathogen, as indicated by the study of Waculicz-Andrade et al. (2017) or is it not a common tropic pathogen as concluded by Phoulivong et al. (2010)?

This is the first report of the species *C. fruticola* causing anthracnose on pomegranate fruits in the world and of *C. siamense* and *C. theobromicola* in Brazil. This knowledge is critical to the development of more efficient control measures of anthracnose disease of pomegranate, inasmuch as, little was known about the *Colletotrichum* species associated with this crop.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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