Neopestalotiopsis australis Causing Scab Disease on Byrsonima crassifolia in Mexico.

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Abstract

Nanche (*Byrsonima crassifolia*) has great economic importance in the dry and humid tropics in Mexico, where is used for the elaboration of different drinks, foods, and candies; also in the traditional medicine to treat fever and stomach disorders. Since 2021, a high incidence of disease-causing scabby fruit in young nanche fruits has been detected in Las Choapas, Veracruz, Mexico. Disease symptoms were visible on the skin of fruits which progressed as fruits matured. Therefore, the objectives of this study were to identify the causal agent of this scab disease based on morphology and molecular techniques and to determine their pathogenicity. Fifteen isolates were recovered from diseased fruits. Because the isolates presented similar morphology, two of them were selected to determine their identity and pathogenicity. On the basis of colony characteristics, conidial morphology, and phylogenetic analyses of the internal transcribed spacers, partial translation elongation factor 1-α gene, and partial β-tubulin gene sequence the isolates were identified as *Neopestalotiopsis australis*. Koch's postulates demonstrated the pathogenicity of *N. australis* on nanche. This is the first report implicating *N. australis* as a causal agent of Scab Disease on *B. crassifolia* in Mexico and in the world.

Introduction

Nanche or nance [*Byrsonima crassifolia* (L.) Kunth] is a seasonal species native to the dry tropical forests of southern Mexico, the Antilles, Central America and the Amazon basin in South America. It belongs to the Malpighiaceae family (Duarte, 2011); it grows as a shrub in the dry tropics and as a tree in the humid tropics (Maldonado-Peralta *et al*. 2020). In its areas of production, fruit is consumed fresh and sold by roadside vendors. Also, the fruit is used in the industry for the elaboration of ates, bottled soft drinks, jams, syrups, ice creams, liqueurs, creams, jellies, or cakes, among others (Medina-Torres *et al*. 2004). The leaves and the trunk bark are rich in tannins, as well as the unripe fruits. The tannins are used for tanning and a decoction of this bark is used to cure diarrhea, to lower fever and as an anti-inflammatory. The nanche is a good source of energy, lipids, food fiber, calcium, and vitamin C. Studies shows that the nanche also has powerful antioxidant compounds, like phenolic compounds and carotenoids (Silva *et al*. 2008; Irías-Mata *et al*., 2018). However, its use is limited to the recollection of fruits in the production season, despite the fact that it has a highly profitable fruit potential. This species has a regional and national market where market demand is not yet met and can play an important role as a source of economic income (Bayuelo-Jiménez *et al*. 2006; Duarte, 2011). In 2019, 1,491.85 ha of nanche were harvested, with an average yield of 5.38 ton ha^{-1}. The state of Veracruz ranks fourth in terms of area planted with 141 ha (SIAP, 2021).

Fruit diseases caused by *Penicillium* sp., *Phytium* sp., and *Sphaceloma* sp., species have been reported to cause losses in nanche plantations in Nayarit, Mexico (Castañeda-Salinas, 2007). Since 2021, a disease causing scabby fruit in young nanche fruits has been detected in Las Choapas, Veracruz, Mexico. Disease symptoms were visible on the skin of young fruits which progressed as fruits mature. The symptoms were widespread with 50 to 70% symptomatic fruits. As a consequence, the fruit production was reduced. Thus, the objective of this work was to determine the etiology of the disease.

Materials And Methods

Sample collection.— In May 2021, diseased fruit samples of nanche were collected in Las Choapas, Veracruz (17°40'53.32"N, 94°00'08.64"W; altitude 29 m), the collection sites were surrounded by natural vegetation; management practices in nanche plants included occasional mechanical weed control and sometimes formation

pruning. The samples were transported to the Laboratory for observations and disease diagnosis. Symptomatic fruits were examined under a stereomicroscope for the presence of signs.

Isolation and Purification. — To isolate the pathogen, pieces of fruit (5 mm^2) were excised from the margin between healthy and diseased tissue of symptomatic samples, sterilized with 1.5% sodium hypochlorite for 2 min, rinsed three times with sterilized distilled water for 1 min, then dried on sterilized paper towels, plated onto potato dextrose agar (PDA), and incubated at 22°C with a 12 h photoperiod for 3 days. Mycelial plugs from the edge of fungal hyphae developing from the tissues were aseptically transferred to fresh PDA and were incubated at 22°C with a 12 h photoperiod for 7 days. Monoconidial cultures of 15 homogenous isolates of *Neopestalotiopsis* were obtained using the single-spore method as described by Akinsanmi et al. (2004). Single-spore isolates were stored in tubes with PDA covered with sterile mineral oil at 15°C for subsequent study. Because the fifteen isolates were morphologically similar, two representative isolates were randomly selected for morphological and molecular characterization.

Morphological characterization.— Mycelial growth and the fungal structures morphology of the CPO 27.130 and CPO 27.136 isolates was determined on PDA (ten Petri plates for each isolate). Monoconidial cultures on PDA were incubated at 22°C with a 12 h photoperiod for 12 days. The diameter of cultures was measured daily and the average growth rate was obtained. Fungal structures were examined in lactophenol cotton blue on slides (Parija and Prabhakar, 1995), and over 50 measurements were obtained from conidia and other fungal structures using a compound microscope, and digital images were recorded. Mean, standard deviation (SD) and 95% confidence intervals were calculated.

DNA extraction, PCR amplification, sequencing and phylogenetic analyses.— Total genomic DNA was extracted from fresh fungal mycelia, scraped from the margin of a colony grown on a PDA plate, incubated at 25°C using the 2% cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990), with the addition of 3 M sodium acetate. The ITS, TEF and TUB partial genes of two representative isolates (CPO 27.130 and CPO 27.136) were amplified using the following pairs of primers: ITS1/ITS4 (White et al. 1990), EF1-728F/EF1-986R (Carbone *et al*., 1999) and T1/Bt2b (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997). Amplification conditions for ITS and TEF followed Crous et al. (2013) and for TUB, Lee et al. (2004). The PCR products were verified by staining with Ethidium Bromide after separation on 1.2% agarose electrophoresis gels. All PCR products were cleaned using ExoSAP-IT (Affymetrix, Thermo Fisher Scientific) and sequencing was carried out by the Macrogen (Rockville, MD, USA).

Partial sequences for ITS, TEF and TUB obtained in this study were deposited in NCBI's GenBank database (Table 1) and were compared in the GenBank database to refence sequences using the BLAST search tool (Basic Local Alignment Search Tool). Reference strings available on GenBank were selected and aligned was performed by using BioEdit v7.2.5 (Hall, 1999) using ClustalW Multiple alignment method (Thompson et al. 1994), and the concatenated alignment (ITS, TUB, TEF) was generated. The trees were produced using MEGA V7 (Molecular Evolutionary Genetics Analysis, Version 7) (Kumar et al. 2016). The evolutionary history was inferred using the Maximum Likelihood (ML)method based on the Tamura-Nei model (Tamura and Nei, 1993), with 1000 bootstrap iterations. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The isolate codes, hosts, location and Genbank numbers of the strains used in the

phylogenetic analyses can be viewed in Table 1. As outgroup sequence for this analysis, we used *Nothoseiridium podocarpi*.

Table 1
Strains used in phylogenetic analyses and their GenBank accession numbers.

Species	Strain	Host/Substrate	Location	GenBank accession		
				ITS	TUB	TEF
Neopestalotiopsis australis	**CPO 27.130**	Byrsonima crassifolia	**Mexico: Veracruz**	**ON312539**	**ON316845**	**ON316847**
Neopestalotiopsis australis	**CPO 27.136**	Byrsonima crassifolia	**Mexico: Veracruz**	**ON312570**	**ON316846**	**ON316848**
Neopestalotiopsis australis	CBS 114159	*Telopea* sp.	Australia: New South Wales	KM199348	KM199432.1	KM199537.1
N. clavispora	CBS 447.73	Decaying wood	Sri Lanka	KM199374	KM199443	KM199539
N. piceana	CBS 225.30	*Mangifera indica*	–	KM199371	KM199451	KM199535
N. piceana	CBS 254.32	*Cocos nucifera*	Indonesia: Sulawesi	KM199372	KM199452	KM199529
Neopestalotiopsis javaensis	CBS 257.31	*Cocos nucifera*	Indonesia: Java	KM199357	KM199437	KM199543
N. formicarum	CBS 115.83	Plant debris	Cuba	KM199344	KM199444	KM199519
N. formicarum	CBS 362.72	Dead Formicidae (ant)	Ghana	KM199358	KM199455	KM199517
N. saprophytica	CBS 115452	*Litsea rotundifolia*	Hong Kong	KM199345	KM199433	KM199538
N. surinamensis	CBS 450.74	Soil under *Elaeis guineensis*	Suriname	KM199351	KM199465	KM199518
Neopestalotiopsis cubana	CBS 600.96	Leaf litter	Cuba	KM199347	KM199438.1	KM199521.1
N. honoluluana	CBS 111535	*Telopea* sp.	USA: Hawaii	KM199363	KM199461	KM199546
N. honoluluana	CBS 114495	*Telopea* sp.	USA: Hawaii	KM199364	KM199457	KM199548
N. mesopotamica	CBS 299.74	*Eucalyptus* sp.	Turkey	KM199361	KM199435	KM199541
N. mesopotamica	CBS 336.86	*Pinus brutia*	Iraq	KM199362	KM199441	KM199555
N. rosae	CBS 101057	*Rosa* sp.	New Zealand	KM199359	KM199429	KM199523

ITS: internal transcribed spacers; *TUB*: partial beta-tubulin gene; *TEF*: partial translation elongation factor 1-alpha gene. Sequences generated in this study are emphasised in bold face.

Species	Strain	Host/Substrate	Location	GenBank accession		
				ITS	TUB	TEF
N. rosae	CBS 124745	*Paeonia suffruticosa*	USA	KM199360	KM199430	KM199524
N. surinamensis	CBS 111494	*Protea eximia*	Zimbabwe	JX556232	KM199462	KM199530
Pestalotiopsis australis	CBS 114193	*Grevillea* sp.	Australia: New South Wales	KM199332	KM199383	KM199475
P. australis	CBS 114474	*Protea neriifolia × susannae*	South Africa	KM199334	KM199385	KM199477
P. oryzae	CBS 111522	*Telopea* sp.	USA: Hawaii	KM199294	KM199394	KM199493
P. parva	CBS 278.35	*Leucothoe fontanesiana*	–	KM199313	KM199405	KM199509
N. eucalypticola	CBS 264.37	*Eucalyptus globulus*	–	KM199376.1	KM199431.1	KM199551.1
Pestalotiopsis arceuthobii	CBS 434.65	*Arceuthobium campylopodum*	USA	KM199341.1	KM199427.1	KM199516.1
P. arengae	CBS 331.92	*Arenga undulatifolia*	Singapore	KM199340.1	KM199426.1	KM199515.1
P. australasiae	CBS 114126	*Knightia* sp.	New Zealand	KM199297.1	KM199409.1	KM199499.1
P. biciliata	CBS 124463	*Platanus × hispanica*	Slovakia	KM199308.1	KM199399.1	KM199505.1
P. chamaeropis	CBS 186.71	*Chamaerops humilis*	Italy	KM199326.1	KM199391.1	KM199473.1
P. colombiensis	CBS 118553	*Eucalyptus eurograndis*	Colombia	KM199307.1	KM199421.1	KM199488.1
P. diploclisiae	CBS 115587	*Diploclisia glaucescens*	Hong Kong	KM199320.1	KM199419.1	KM199486.1
P. grevilleae	CBS 114127	*Grevillea* sp.	Australia	KM199300.1	KM199407.1	KM199504.1
P. hawaiiensis	CBS 114491	*Leucospermum* sp. cv. 'Coral'	USA: Hawaii	KM199339.1	KM199428.1	KM199514.1
P. hollandica	CBS 265.33	*Sciadopitys verticillata*	Netherlands	KM199328.1	KM199388.1	KM199481.1

ITS: internal transcribed spacers; *TUB*: partial beta-tubulin gene; *TEF*: partial translation elongation factor 1-alpha gene. Sequences generated in this study are emphasised in bold face.

Pathogenicity test.— The pathogenicity test was carried out with the two representative isolates (CPO 27.130 and CPO 27.136), tested on detached unripe nanche fruits (45 days after anthesis). Detached healthy fruits were rinsed with tap water, sterilized with 1% NaOCl solution for 2 min and rinsed twice with sterile distilled water. Then the fruits were dry and placed on a sterilized paper in clean boxes and were wounded in a single site at the ecuatorial zone, wound was made by removing a piece of fruit epidermis (1.5 mm). A mycelial plug (5 mm in diam.) was removed from the margin of 5-days-old growing colonies on PDA at 22°C and was placed on the wounded site of each fruit. Each *Neopestalotiopsis* isolate was inoculated on fifthy fruits. Nanche fruits inoculated with PDA plug without fungal mycelium were used as the control. Boxes were incubated at 22 ± 2°C with 12 h of light. The inoculated fruits were monitored daily for development of symptoms. Re-isolations were performed as described above to fulfill Koch's postulates. Pathogenicity testing was performed twice.

Results

Symptoms.— The main diagnostic symptoms were irregular, brown, corky lesions on the exocarp of fruit, and characteristically delineated by a narrow light-brown margin. Lesions were initially reddish-brown and became brown when mature. In the later stages of the disease black acervuli were developed on the surface of the lesions that produced abundant masses of conidia (Fig. 1).

Morphological characterization.— Colonies on PDA attaining 90 mm diam after 12 d at 22°C, with lobate edge, pale white-coloured, with dense aerial mycelium on the surface with black conidiomata; reverse similar in colour. *Conidiomata* sporodochial in culture on PDA, solitary or aggregated in clusters, semi-immersed to superficial, brown to black, scattered or clustered, shining, punctiform, pulvinate, ellipsoidal, elongate or irregular in outline, sometimes confluent; exuding black conidia in a slimy, globose mass. *Conidiophores* indistinct, often reduced to conidiogenous cells. *Conidiogenous cells* discrete, ampulliform to lageniform, hyaline, simple, 8.5–10.4 × 2.4–3 µm, apex 1–2 µm diam. *Conidia* fusoid, ellipsoid, straight to slightly curved, 4-septate, (18.6–)21–22.5(–23.7) × (6.7–)7.2–7.6(–8.2) µm, x ± SD = 21.69 ± 1.7 × 7.4 ± 0.4 µm; basal cell conic with a truncate base, hyaline, thin-walled, 10.5–3 µm long; three median cells doliiform, (13–)14.3–15(–16.5) µm long, x ± SD = 14.6 ± 0.79 µm, versicoloured, septa darker than the rest of the cell (second cell from the base pale brown, 4.2–5.5 µm long; third cell darker brown, 4–6 µm long; fourth cell darker brown, 3–5.5 µm long); apical cell 2–3.5 µm long, hyaline, subcylindrical to obconic, thin-walled; with 3 tubular apical appendages, arising from the apical crest, unbranched, filiform, flexuous, (16–)20–22(–23.5) µm long, x ± SD = 21.3 ± 2.4 µm; basal appendage single, tubular, unbranched, centric, 2.5–4.7 µm long.

Pathogenicity tests.— The two tested isolates were pathogenic on nanche fruits. Initial symptoms occurred eight days after inoculation (dai), all inoculated fruits developed irregular, brown lesions (the same symptoms of fruits observed in the field); after 11 dai the lesions increased in size, and acervuli were observed; whereas control fruits remained healthy (Fig. 3). To fulfill Koch's postulates, pieces of fruit (5 mm^2) were excised from the margin between healthy and diseased tissue of symptomatic fruits, and sterilized, plated, and incubated as described above; the pathogen was reisolated, the colony characteristics and morphometric measurements were similar to the isolates CPO 27.130 and CPO 27.136. Thus, *Neopestalotiopsis australis* fulfilled the criteria stipulated by the Koch's postulates and was identified as the causal agent of the scab disease of nanche.

The ML analyses of the combined sequence data (ITS + TUB + TEF) of the Mexican isolates of *Neopestalotiopsis* from nanche formed a separate, well-supported clade (Fig. 4). Phylogenetic inference, colony characteristics, and conidial morphometric indicated that these isolates correspond to the specie *Neopestalotiopsis australis*.

Discussion

In recent years, *Neopestalotiopsis* is a relatively important plant pathogenic genera able to infect wide host ranges (Keith et al. 2006; Maharachchikumbura et al. 2011). In the case of fruit crops, *Neopestalotiopsis* has been reported to cause significant diseases including leaf spots of guarana plant (Gualberto et al. 2021), and mango (Gerardo-Lugo et al. 2020); root and crown rot on strawberry (Obregón et al. 2018; Machín et al. 2019; Sun et al. 2021;); scab of guava (Solarte et al. 2018); fruit canker of guava (Haq *et al*. 2021); dry flower disease of macadamia (Akinsanmi et al. 2017; Prasannath et al. 2021). In this investigation, *Neopestalotiopsis australis* was determined to infect nanche fruits in Las Choapas, Veracruz; the pathogenicity test confirmed that the inoculated isolates were pathogenic on nanche fruits, inducing brown lesions with conidia exuding a cirrus from black acervuli. According to the scientific literature this fungi has been only reported infecting eucalyptus stems in Brazil (Santos et al. 2020), associated with cladode brown spot on *Nopalea cochenillifera* in Brazil (Conforto et al. 2019) so this is the first report of *N. australis* causing scab disease on *Byrsonima crassifolia* in Mexico and in the world.

The colony characteristics and morphometric parameters are consistent with those described by Maharachchikumbura et al. (2014) and Santos et al. (2020). Phylogenetic analysis using the maximum likelihood method based on the combined ITS, TEF and TUB sequences placed the isolates CPO 27.130 and CPO 27.136 in the same clade as the isolate CBS 114159 of *Neopestalotiopsis australis*. It is important to mention that the morphological characteristics of the colonies exhibited variability after being subcultured, especially in colony morphology like color, growth rate, and time of sporulation; however conidial characteristics (length, width) were more consistent in their mean, standard deviation, and 95% confidence intervals, also in color and number of appendages. Therefore, these variables were of greater relevance in the identification. These observations are according to those reported by Tejesvi et al. (2007), and Maharachchikumbura et al. (2011) who suggest that colony morphology characteristics are plastic, and variable.

In field, all developmental stages of nanche fruit may be affected, but principally unripe nanche fruits. When the infection occurs in young fruits, brown fissures are formed in the lesions; the fissures may occur because the damaged tissue cannot expand as the fruit grows, and splits, forming deep fissures. The more the fruit grows, the more these areas of original damage break up and the space in between is filled by corky tissue. These symptoms are similar in appearance to those caused by *Neopestalotipsis* sp., in guava fruits in Colombia (Solarte et al. 2018). On the other hand, when de infection occurs in mature fruits or detached fruits no fissures are formed because the fruits stops growing any larger and there are not tissue expansion. That is the reason for why in the inoculated fruits there is no presence of fissures.

At the moment, the disease has been observed in different backyards of homes, but represents a threat for the emerging nanche industry in México, for this reason is advisable to carry out studies to determine how widespread the inoculum of *N. australis* is in Mexico, in addition to knowing its ecology and if it is affecting other crops. Likewise, future monitoring of *N. australis* will be necessary to confirm seasonal patterns and assess its geographic distribution. Considering the economic and ecological impacts of this fungi in other regions, the evaluation of plant damage should be considered, as well as the study of its epidemiology, including the range of hosts and potential natural enemies, to understand its characteristics. Therefore, appropriate management strategies, proper surveillance, and prevention approaches should be investigated and applied.

Declarations

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Availability of data and material (data transparency): All data generated or analysed during this study are included in this article.

Code availability (software application or custom code): Not applicable.

Authors' contributions: All authors contributed to the study conception and design. Also, all authors read and approved the final manuscript. Specific contributions by author are: **Magnolia Moreno-Velázquez:** conceptualization, methodology, investigation, writing-original draft. **Sergio Hernández-Pablo:** methodology, investigation, interpretation of data. **Andres Quezada-Salinas:** investigation, project administration, supervision, writing-review and editing. **Dionicio Alvarado-Rosales:** morphological identification, field work. **Johan Rodríguez-Mendoza:** formal analysis (molecular analysis, phylogenetic analysis, molecular methodology). **Juan Manuel Tovar-Pedraza:** methodology, investigation. **Guillermo Márquez-Licona:** investigation, identification. **Luz de Lourdes Saavedra-Romero:** field characterization, survey.

Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals

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Figures

Figure 1

Symptoms and signs of scab disease on naturally infected nanche fruits. (a–b) Lesions on 50-days-old fruits; (c–d) Acervuli in the lesions; (e) Masses of spores emerging from an acervulus in form of black cirrus; (f) Conidia.

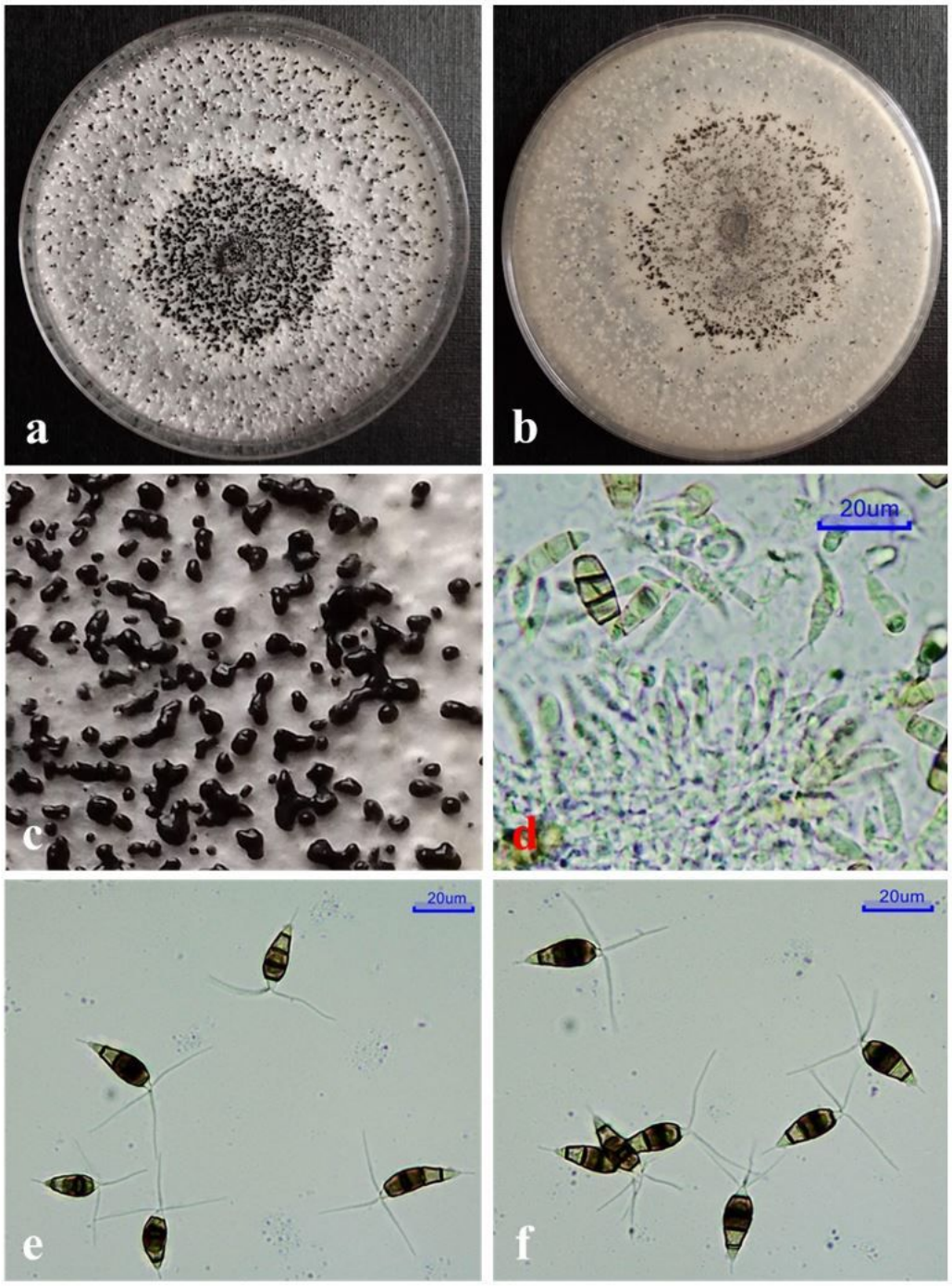

Figure 2

Morphological and cultural characteristics of *Neopestalotiopsis australis* isolated from scab present in the fruit of *Byrsonima crassifolia*. (a–b) Culture on PDA; (a) Upper surface view of the 12d old culture; (b) Reverse view of the culture 12d old culture; (c) Colony sporulating on PDA; (d) Conidiogenous cells giving rise to conidia; (e–f) Conidia.

Figure 3

Results of pathogenicity test of *Neopestalotiopsis australis* on nanche fruits. (a–b) Eight days after inoculation (a injured fruit control, b inoculation with a mycelial plug on injured fruit); (c–d) Eleven days after inoculation, acervuli formation; (e) acervulus; (f) Conidia.

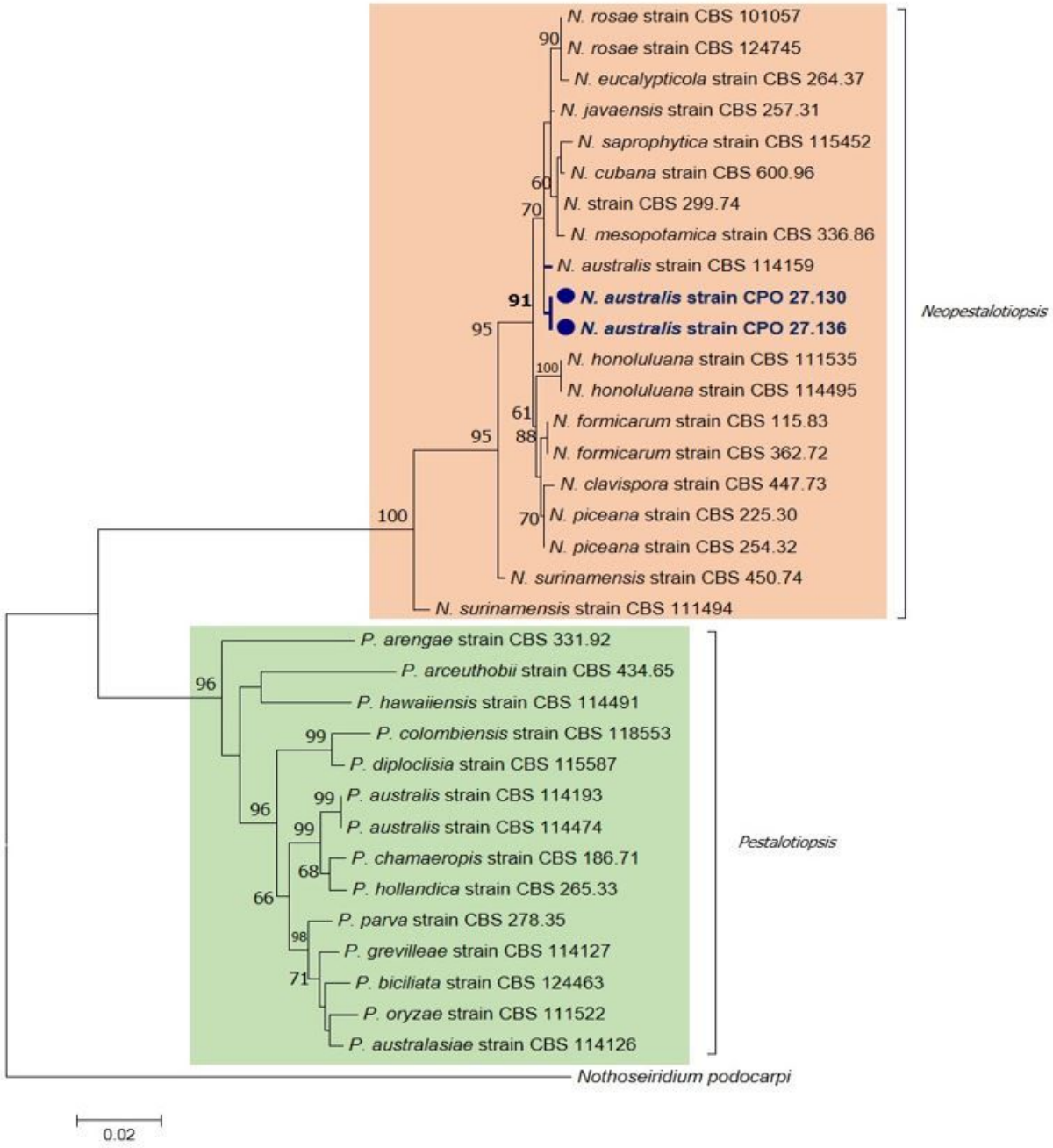

Figure 4

Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-4784.45) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 35 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1193 positions in the final dataset.