Charcoal Canker of Pear, Plum, and Quince Trees Caused by *Biscogniauxia rosacearum* sp. nov. in Southern Italy

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Abstract

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The genus *Biscogniauxia* is paraphyletic to members of the family Xylariaceae and includes at least 52 species to date that are mainly pathogens of dicotyledonous angiosperm trees. Most of these are forest trees, such as those in the genera *Acacia*, *Acer, Alnus*, *Eucalyptus*, *Fraxinus, Populus*, and *Quercus*, and other species of minor importance. *Biscogniauxia* spp. have been reported as endophytes or secondary invaders that attack only stressed plants. During a survey in rosaceous orchards in southern Italy, several charcoal cankers were observed and stroma samples were collected. A collection of 31 *Biscogniauxia* isolates was analyzed. Their phylogenetic relationships were determined through study of the internal transcribed spacer, β-tubulin, and actin gene sequences. Combining morphological, cultural, and molecular data, a new species of *Biscogniauxia* is described here as *Biscogniauxia rosacearum*. This new species was isolated for the first time from rosaceous hosts in Apulia. Pathogenicity tests showed that it causes symptoms on stems when artificially inoculated and produces stromata on the bark surface.

Biscogniauxia Kuntze in the family Xylariaceae (order Xylariales) had long been known as *Nummularia* Tul. & C. Tul., until Miller (1961) placed most of its members in the section *Applanata* of *Hypoxylon*. A few years later, Pouzar (1979, 1986) and Ju et al. (1998) defined the current concept of the genus *Biscogniauxia*. This genus has been recorded worldwide, including in the United States (Gonzàlez and Rogers 1993; Rogers et al. 2008), Africa (Linnakoski et al. 2012; Mugambi et al. 2009), Asia (Whalley et al. 2012), and Europe (Cannon et al. 1985; Ragazzi 2009; Ragazzi et al. 2012; Rappaz 1995). At present, it includes at least 52 species (Ju and Rogers 2001; Ju et al. 1998; Mugambi et al. 2009; Rappaz 1995; Rogers et al. 2000, 2008; Vasilyeva and Stephenson 2007, 2010; Vasilyeva et al. 2012; Whalley et al. 1990, 2000) that are exclusively parasites of dicotyledonous angiosperm trees such as species of *Acacia, Acer, Alnus, Artocarpus, Carya, Celtis, Coprosma, Eucalyptus, Fagus, Fraxinus, Gluta, Lithocarpus, Padus, Phyllirea, Pisonia, Populus, Psidium, Quercus, Rhamnus, Rubus*, and *Tilia* (Ju and Rogers 2001; Ju et al. 1998; Mugambi et al. 2009; Rappaz 1995; Rogers et al. 2000, 2008; Vasilyeva and Stephenson 2007; Vasilyeva et al. 2012; Whalley et al. 2000).

The genus *Biscogniauxia* appears to be paraphyletic (Peláez et al. 2008; Sánchez-Ballesteros et al. 2000), with species of the closely related genera *Camillea* Fr. and *Obolarina* Pouzar clustered inside it (Pažoutová et al. 2010).

Species of *Biscogniauxia* such as *Biscogniauxia mediterranea* are known as the causal agents of charcoal cankers on oak trees. In Italy, *B. mediterranea* is the main species associated with the decay of oak trees, especially *Quercus cerris*, *Q. robur*, *Q. frainetto*, and *Q. pubescens* (Ragazzi et al. 1989; Vannini and Scarascia Mugnozza 1991; Vannini et al. 1996).

This pathogen has always been considered a secondary fungal invader that attacks only stressed or old hosts. It is thought that it can live as an endophyte in all of the aerial organs of oak trees (rarely in the leaves), and has a latent phase that does not result in any symptoms (Wilson 1995). However, it can act as an opportunistic pathogen when the host is weakened by abiotic or biotic factors such as prolonged drought periods (Biocca and Motta 1995, Vannini and Scarascia Mugnozza 1991) or when attacked by other pathogens. Thus, these fungi are facultative saprophytes that spend most of their life cycle as parasites but can also persist for long periods on dead material (Nugent et al. 2005). *B. mediterranea* can rapidly colonize xylem and bark tissues to induce necrosis and canker formation, and to accelerate tree decline and eventually cause death (Desprez-Loustau et al. 2006; Linaldeddu et al. 2011). The great abundance of inoculum produced on colonized parts of a tree and the dispersal of ascospores either aerially or by insects is an important factor in the spread of this fungus in forests.

During a survey in fruit orchards throughout the Apulia region (southern Italy) from 2013 to 2014, cankers and unusual stromata were observed on the stems and branches surface of many fruit trees, including pear, plum, and quince, in Canosa di Puglia (Bari-Andria-Trani Province). A detailed phylogenetic study was performed to determine the correct taxonomic position of the fungi collected in this study. Therefore, the main objectives of the present study were to identify and characterize isolates of *Biscogniauxia* collected from pear, plum, and quince trees in southern Italy, as well as to demonstrate that they can cause disease symptoms on rosaceous hosts using pathogenicity assays.

Materials and Methods

Fungal isolates. During the summers of 2013 and 2014, several charcoal cankers were observed on the stems and branches of pear, plum, and quince trees of orchards in Canosa di Puglia (Bari-Andria-Trani) (41°13′21.77″N, 16°2′52.96″E) in Apulia, southern Italy. The stromata observed were applanate and black and resembled those of *Biscogniauxia* spp. (Fig. 1). Representative samples of these charcoal cankers were collected from 62 symptomatic trees (33 from quince, 21 from pear, and 8 from plum) during the growth stage. The ages of the fruit trees varied from 13 to 20 years old. The samples were transported to the laboratory for analysis. A portion of the stromata was removed with a sterilized scalpel and surface sterilized (Fisher et al. 1992). The stromatal contents were scooped out and placed into a tube containing sterile distilled water; then, a sterile wire loop was used to spread 300 μl of this suspension on petri dishes containing 15 ml of potato dextrose agar (PDA; 3.9% potato dextrose agar; Oxoid Ltd.), which were then incubated at 23°C (±2°C) in the dark. After 24 to 36 h of incubation, single germinating ascospores were transferred to fresh PDA plates. All fungal colonies that were morphologically similar to *Biscogniauxia* spp. were retained. Twenty-four strains of *Biscogniauxia* spp. are maintained in the culture collection

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of the Department of Science of Agriculture, Food and the Environment, of the University of Foggia (Italy), and the reference strains are in the collection of the Centraalbureau voor Schimmelcultures (CBS) Utrecht, The Netherlands. To compare the morphological and molecular differences, seven strains of *Biscogniauxia* spp. from *Q. pubescens* available from the collection of the abovementioned department, collected and maintained over the last 3 years from woods in two localities in the Apulia region that are in a national nature reserve: Incoronata (Foggia; 41°23′29″N, 15°39′35″E) and Accadia (Foggia; 41°10′00″N, 15°20′00″E), were also included in this study.

Morphology. The *Biscogniauxia* asexual morphs were morphologically characterized on malt extract agar (MEA; 2% malt extract [Oxoid Ltd.] and 1.5% agar [Difco]), oat meal agar (OA; 30 g of oat, 8 g of Oxoid agar, and 1,000 ml of water) (Crous et al. 2009), and PDA, with incubation at 25°C (±2°C) in the dark for 2 weeks. Colony morphology and color were defined on MEA, OA, and PDA at 25°C (±2°C) after 16 days, using the color charts of Rayner (1970). Cardinal temperatures for growth were determined by incubation of the PDA plates in the dark at temperatures of 5 to 40°C at 5°C intervals. Radial growth was measured on PDA plates after 8 days at 25°C (±2°C). The macroscopic features of the stromata were determined under a stereomicroscope (Nikon SMZ 645) and photographs were taken with a digital camera (Nikon Coolpix P900). The microscopic features of the sexual morphs were determined directly from fresh stromata by dissecting perithecia from stromata and placing the contents in distilled water, methylene blue solution, lactic acid, and Lugol's solution. The microscopic features of asexual morphs were determined using the slide culture technique of Carlucci et al. (2012). The microscopic examination was under Normaski differential interference contrast optics (Leica DM5500 microscope) and the images were recorded with a digital camera (Leica DFC420). The dimensions and structures of the asci, ascospores, ascal apical ring, conidiophores, and conidia were determined in 100% lactic acid or Lugol's solution from 30 measurements made with a Leica LAS measurement module (Leica Microsystem GmbH) from images recorded at ×40 or ×100 magnification. The 5th and 95th percentiles were defined for all of the measurements, with the extremes given in parentheses.

DNA isolation and polymerase chain reaction amplification. Genomic DNA of all of the isolates was extracted according to Carlucci et al. (2013). The *5.8S rDNA* gene and flanking internal

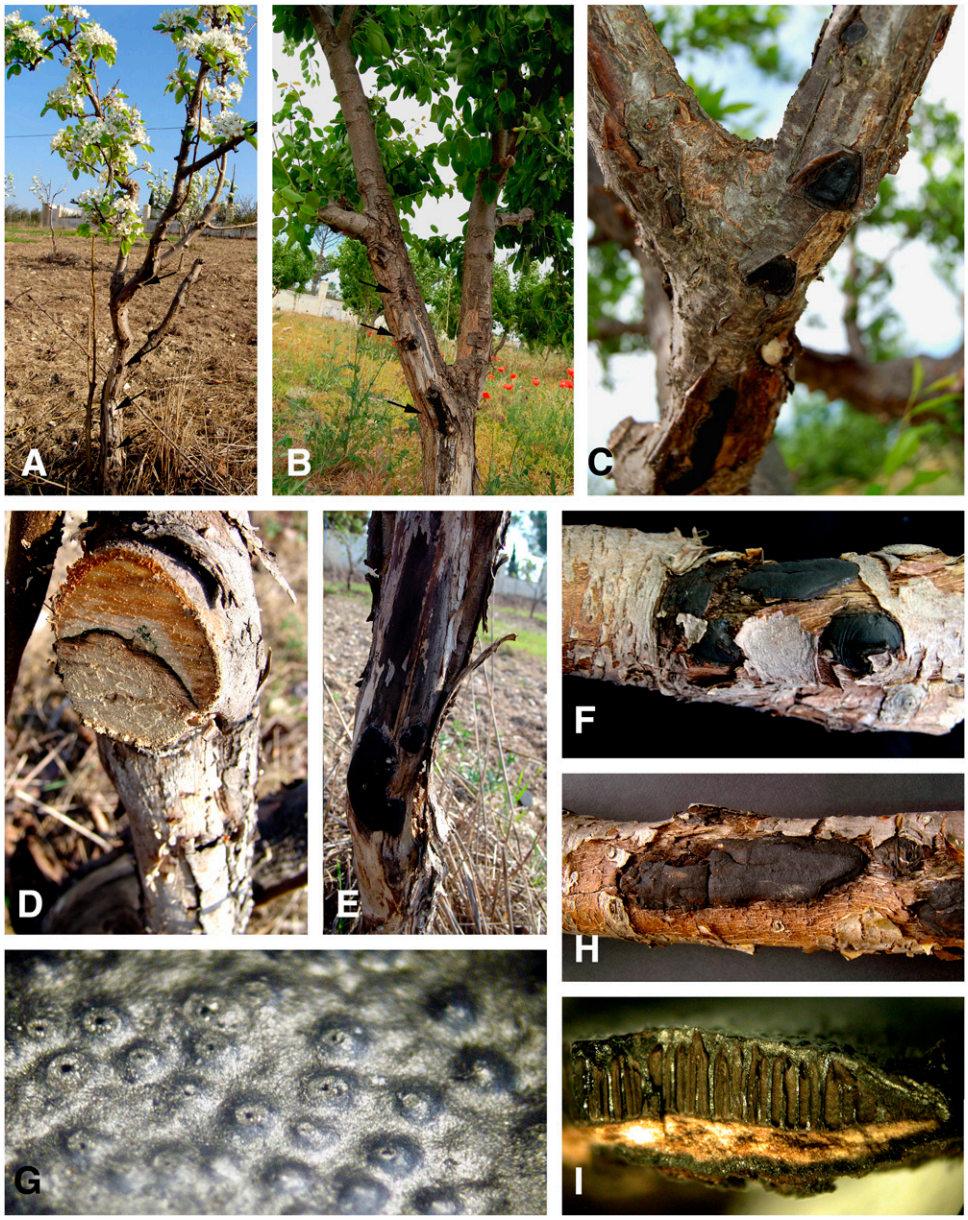

Fig. 1. A to **C,** Disease symptoms and charcoal stromata on quince, plum, and pear trees, respectively (black arrows). **D,** Dead wood in cross-section of pear branch corresponding to charcoal stromata. **E** to **G,** Stromata on trunk and branch bark of Rosaceae hosts. **H,** View of the stromatal surface. **I,** Section through the stroma, showing perithecia.

transcribed spacer (ITS) regions, the *partial* β-*tubulin* gene (TUB), and the *partial actin* gene (ACT) were amplified and sequenced using the primer pairs ITS1 and ITS4 (White et al. 1990), T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995), and ACT-512F and ACT-783R (Carbone and Kohn 1999), respectively, with all primer pairs supplied by Eurofins Italy service. Polymerase chain reactions (PCR) were performed in an S1000 thermal cycler (Bio-Rad). The ITS amplifications were according to Carlucci et al. (2012). The TUB and ACT amplifications were according to Raimondo et al. (2014). The amplification products were verified by agarose gel electrophoresis. The amplified PCR fragments were purified with NucleoSpin extract II purification kits (Macherey-Nagel) before DNA sequencing. The two strands of the PCR products were sequenced by the Eurofins Italy service.

Phylogenetic analysis. Nucleotide sequences were edited with BioEdit v.7.0.9 (http://www.mbio.ncsu.edu/BioEdit). The ITS and TUB/ACT datasets comprised sequences of closely related *Biscogniauxia* spp. selected in BLAST searches in GenBank. The ITS sequences were aligned with ClustalX v. 1.83 (Thompson et al. 1997). Alignment gaps were treated as fifth character, and all of the characters were unordered and of equal weight. Maximum-parsimony analysis was performed with PAUP v. 4.0b10 (Swofford 2003) using the heuristic search option, with 1,000 random taxa additions and tree bisection and reconstruction as the branch swapping algorithm. Branches of zero length were collapsed, and all multiple, equally parsimonious trees were saved. Bootstrap support values were calculated from 1,000 heuristic search replicates and 100 random taxon additions. The tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI), were calculated and the resulting trees were visualized with TreeView, v. 1.6.6 (Page 1996).

The TUB and ACT sequences were combined and aligned with ClustalX v. 1.83 (Thompson et al. 1997). A partition homogeneity test of the TUB/ACT alignment was conducted with PAUP v. 4.0b10 (Swofford 2003) to test pairwise congruence between the sequence datasets. Phylogenetic analysis was performed as described above but with treatment of the gaps as missing data. Newly generated sequences were deposited in GenBank (Table 1), the alignment and trees were deposited in TreeBase (www.treebase.org), and the taxonomic novelties were deposited in MycoBank (www.mycobank.org) (Crous et al. 2004). *Annulohypoxylon cohaerens* (strain BCRC34013; GenBank ITS = EF026140, TUB = AY951655, and ACT = AY951766) and *Hypoxylon rubiginosum* (strain BCRC34116; GenBank ITS = EF026143, TUB = AY951751, and ACT = AY951862) were used as outgroups in the phylogenetic analysis of ITS and TUB/ACT sequences.

Pathogenicity tests. Four isolates of *B. rosacearum* (Bx25, Bx26, Bx28, and Bx29 from *Q. pubescens*, *Prunus domestica*, *Cydonia oblonga*, and *Pyrus communis*, respectively) were used in the pathogenicity tests, which were carried out in September 2013 on wood stems (diameter = 3 to 5 cm) of about 15- to 20-year-old pear, plum, and quince trees in open fields in orchards in Canosa di Puglia (average temperature was 24°C and relative humidity was 66%). The bark of the wood stems was removed from a surface about 10 cm long and 3 to 4 cm wide, using a sterilized scalpel. A suspension of 1×10^6 ascospores/ml (5 ml) was sprayed on this wound. After inoculation, the wounds were wrapped with wet, sterile cotton wool and sealed with Parafilm for about 10 days. Controls were mock inoculated with sterile distilled water. Each experiment included 10 replicates per host and per isolate. The inoculated wood stems were examined every 4 months until May 2015. The leaf growth on the inoculated stems and stroma appearance and size on wood stems were assessed to fulfill Koch's postulates.

Statistical analyses were performed using Statistica (version 6; StatSoft). To test whether the data followed normal distributions, a Shapiro-Wilk test was used. The homogeneity of variance was assessed using Levene tests. Factorial analysis of variance (ANOVA) was performed to determine the significance of the *B. rosacearum* isolates on the plant hosts inoculated and to detect any interaction between these factors (plant host–isolate). One-way ANOVA was performed to evaluate the significant differences in percentages of stromata produced on stems inoculated by each *B. rosacearum* isolates and any difference due to plant host (pear, plum, and quince). Fischer's tests was used for the comparison of the treatment means at $P < 0.01$.

Results

Phylogenetic analysis. The ITS sequences generated for the 31 isolates studied (Table 1) were aligned with 36 sequences retrieved from GenBank, which represented closely related species of *Biscogniauxia*. The dataset consisted of 67 taxa, which included *A. cohaerens* (EF026140) and *H. rubiginosum* (EF026143) as outgroups. After alignment and exclusion of incomplete portions at either end, the ITS dataset consisted of 783 characters (including alignment gaps). Of the 783 characters, 219 were constant, whereas 124 were variable and parsimony uninformative. Maximum-parsimony analysis of the remaining 440 parsimony-informative characters resulted in one most-parsimonious tree (TL = 2,323, CI = 0.513, RI = 0.724, RC = 0.371, and HI = 0.487; TreeBase S17923). The phylogenetic tree showed two different clades of *B. mediterranea* with bootstrap values of 100% (Fig. 2). In particular, 26 isolates sequenced in this study matched 16 sequences of those available in the GenBank as *B. mediterranea* in clade 1, whereas the other 5 sequences matched the 5 sequences retrieved from the GenBank as *B. mediterranea* in clade 2. The last clade included the sequence of a specimen examined and described as *B. mediterranea* (Candoussau, F. 366) from Ju et al. (1998).

The partition homogeny test on the TUB/ACT alignments of *Biscogniauxia* produced *P* values of 0.120, which indicated that the datasets were congruent and could be combined. TUB/ACT sequences were generated for 31 isolates and aligned with 22 sequences retrieved from GenBank. The dataset consisted of 53 taxa, including the two outgroup taxa *A. cohaerens* (TUB = AY951655 and ACT = AY951766) and *H. rubiginosum* (TUB = AY951751 and ACT = AY951862). After alignment and exclusion of incomplete portions at either end, the dataset consisted of 1,371 characters (including alignment gaps). Of the 1,371 characters, 533 were constant, whereas 163 were variable and parsimony uninformative. Maximum-parsimony analysis of the remaining 675 parsimony-informative characters resulted in five most-parsimonious trees (TL = 2432, CI = 0.595, RI = 0.689, RC = 0.410, and HI = 0.405; TreeBase S17924). The phylogenetic tree shows a topology similar to that of the ITS tree (Fig. 3).

Taxonomy. The phylogenetic tree from the ITS analyses (Fig. 3) showed 25 species resolved, of which 2 species belong to the genus *Camillea* (*Camillea tinctor* and *C. obularia*), 2 belong to *Obolarina* (*Obolarina dryophila* and *O. persica*), 1 belongs to *Theissenia* (*Theissenia cinerea*), 1 belongs to *Durotheca* (*Durotheca rogersii*), and 19 belong to *Biscogniauxia*. Because both of the phylogenetic trees clearly showed two distinct clades that included sequences named as *B. mediterranea*, we considered that clade 2 is really related to *B. mediterranea* species, because the specimen examined as *B. mediterranea* (Candoussau, F. 366) (Ju et al. 1998) was in this clade. Instead, clade 1 included a novel species described here.

Biscogniauxia rosacearum *A. Carlucci & M. L. Raimondo, sp. nov.* MycoBank MB816045 (Fig. 4).

Etymology. Named after the hosts, which belong to the family Rosaceae, from which this species was isolated for the first time.

Stromata. Erumpent through bark, applanate, irregularly ovate in outline, 10 to 90-mm long, 10 to 20 mm wide, and 0.5 to 1.5 mm thick, with blackish mature surface. Carbonaceous tissues immediately beneath surface and between perithecia, grayish to blackish, coriaceous. The tissue below the perithecial layer of about 50-μm thickness, grayish to blackish, coriaceous, persistent.

Perithecia. Dark brown, tubular, monostichous, minute, 0.18 to 0.24 mm in diameter, 0.6 to 1 mm high, with carbonaceous stromatal material surrounding individual perithecia.

Ostioles. 40 μm in diameter, papillate, with punctate ostiolar openings, encircled with a disc of 0.2 to 0.3 mm in diameter formed by dehiscence of surrounding tissue.

Table 1. Fungal cultures and specimens used in the present phylogenetic study

Species, cultures[v]	Host	Location	Collector	GenBank accession numbers[u]		
				ITS	β-TUB	ACT
Annulohypoxylon cohaerens						
YMJ 310	*Fagus* sp.	France, Ariege	J. Fournier	EF026140	AY951655	AY951766
Biscogniauxia anceps						
YMJ 123	*Corylus avellana*	France, Landes	…	EF026132	AY951671	AY951783
B. arima						
YMJ 122[w]	Wood	Mexico, San Luis Potosi State	F. San Martin.	EF026150	AY951672	AY951784
B. atropunctata						
YMJ 128	Wood	United States, North Carolina	L. F. Grand	JX507799	AY951673	AY951785
B. atropunctata						
9201	*Juniperus virginiana*	United States	M. Hoffman	EF419906	…	…
B. atropunctata var. *intermedia*						
ATCC38987	…	United States	…	AF201705	…	…
B70M	*Quercus* sp.	Costa Rica	…	AJ390412	…	…
B. bartholomaei						
ATCC 38992	Wood	Russia	L. N. Vasilyeva	AF201719	…	…
B. capnodes						
YMJ 138	Corticated wood	Taiwan, Taipei	…	EF026131	AY951675	AY951787
B. citriformis						
YMJ 88113012	Wood	Taiwan	Y.-M. Ju and H. M. Hsieh	JX507800	AY951677	AY951790
YMJ 129	*Casuarina equisetifolia*	United States	J. D. Rogers	JX507801	AY951678	AY951789
B. cylindrispora						
YMJ 89092701[x]	Bark of *Cinnamomum*	Taiwan, Taipei	…	EF026133	AY951679	AY951791
B. formosana						
YMJ 8903220[x]	Bark	Taiwan, Taipei	Y.-M. Ju	JX507802	AY951680	AY951792
B. granmoi						
YMJ 135	Bark of *Prunus padus*	Austria, Steiermark	W. Maurer and C. Scheuer	JX507803	AY951681	AY951793
B. latirima						
YMJ 90080703	Bark	Taiwan, Tai-tung County	…	EF026135	AY951683	AY951795
B. marginata						
MFLUCC 12-0740	…	France	A. Gardiennet	KJ958407	…	…
ATCC 62608	*Quercus* sp.	Pennsylvania	…	AJ390417	…	…
B. mediterranea						
Candoussau, F. 366, YMJ 147[y]	Corticated wood of *Fagus* sp.	France, Oloron	F. Candoussau	EF026134	AY951684	AY951796
CBS 280.61	…	United States	…	AJ390413	…	…
Ohu 19B	Stem of *Opuntia humifusa*	United States	…	KF850388	…	…
EPU38CA	*Echinacea purpurea*	United States	C. R. Carvalho	KP127979	…	…
G410	*Silybum marianum*	United States	…	KM215652	…	…
Bx63	*Quercus pubescens*	Italy, Apulia, Accadia	F. Lops	KT253501	KT253535	KT253504
Bx69	*Q. pubescens*	Italy, Apulia, Accadia	F. Lops	…	…	…
Bx70	*Q. pubescens*	Italy, Apulia, Incoronata	A. Carlucci	KT253502	KT253536	KT253505
Bx83	*Q. pubescens*	Italy, Apulia, Accadia	M. L. Raimondo	…	…	…
Bx85	*Q. pubescens*	Italy, Apulia, Incoronata	A. Carlucci	KT253503	KT253537	KT253506
B. nummularia						
CBS969.70	*Fagus sylvatica*	England	…	AJ390415	…	…
B.NUMM3	…	Italy	A. Mazzaglia	AJ246231	…	…
H86	Inner bark of *Salix alba*	Slovakia, Jursky Sur	P. Srutka	GQ428318	GQ428324	GQ428312
MUCL 51395	*Fagus* sp.	France	…	JX658444	…	…
B. philippinensis var. *microspora*						
YMJ 89041101	Bark	Taiwan	…	EF026136	AY951685	AY951797
B. repanda						
ATCC 62606	…	…	…	AJ390418	…	…
B. rosacearum						
CFB993	*Q. ilex*	Spain	…	AF250624	…	…
JC55	*Q. ilex*	Spain	…	AF280625	…	…
JC809	*Q. ilex*	Spain	…	AF326479	…	…
JC2640	*Q. ilex*	Spain	…	AF326482	…	…
2C7	…	Italy	A. Mazzaglia	AJ246222	…	…

(*continued on next page*)

[u] ITS = internal transcribed spacer, TUB = β-tubulin, and ACT = actin.
[v] Isolates studied are indicated in bold.
[w] Isotype.
[x] Holotype.
[y] Specimen examined by Ju et al. (1998).
[z] Ex-type.

Table 1. (*continued from preceding page*)

Species, cultures[v]	Host	Location	Collector	GenBank accession numbers[u]		
				ITS	β-TUB	ACT
3961	*Holcus lanatus*	Spain	…	FN394711	…	…
PC05.008	*Platypus cylindrus*	Portugal, Alentejo	…	FR734186	…	…
BM07.003	*P. cylindrus*	Portugal, Alentejo	…	FR734187	…	…
CPC 18215	*Q. castaneifolia*	Iran	…	JF295127	…	…
CPC 18216	*Q. castaneifolia*	Iran	…	JF295128	…	…
CPC 18217	*Q. castaneifolia*	Iran	…	JF295129	…	…
A06A	Arthropods	Portugal	…	JQ781705	…	…
A06D	Arthropods	Portugal	…	JQ781708	…	…
A06E	Arthropods	Portugal	…	JQ781709	…	…
A60A	Arthropods	Portugal	…	JQ781799	…	…
ASR_H74_10A	*Pinus sylvestris*	Spain	…	JX421711	…	…
Bx1	*Pyrus communis*	Italy, Apulia, Canosa di Puglia	F. Lops	KT253495	KT253529	KT253515
Bx3	*Cydonia oblonga*	Italy, Apulia, Canosa di Puglia	F. Lops	KT253487	KT253521	KT253507
Bx4	*Prunus domestica*	Italy, Apulia, Canosa di Puglia	F. Lops	KT253491	KT253525	KT253511
Bx5	*Pyrus communis*	Italy, Apulia, Canosa di Puglia	M. L. Raimondo	…	…	…
Bx6	*C. oblonga*	Italy, Apulia, Canosa di Puglia	F. Lops	…	…	…
Bx9	*Prunus domestica*	Italy, Apulia, Canosa di Puglia	F. Lops	…	…	…
Bx10	*Pyrus communis*	Italy, Apulia, Canosa di Puglia	F. Lops	KT253496	KT253530	KT253516
Bx13	*P. communis*	Italy, Apulia, Canosa di Puglia	A. Carlucci	…	…	…
Bx14	*C. oblonga*	Italy, Apulia, Canosa di Puglia	M. L. Raimondo	KT253488	KT253522	KT253508
Bx15	*C. oblonga*	Italy, Apulia, Canosa di Puglia	A. Carlucci	…	…	…
Bx17	*C. oblonga*	Italy, Apulia, Canosa di Puglia	M. L. Raimondo	…	…	…
Bx18	*Prunus domestica*	Italy, Apulia, Canosa di Puglia	A. Carlucci	KT253492	KT253526	KT253512
Bx19	*C. oblonga*	Italy, Apulia, Canosa di Puglia	F. Lops	KT253489	KT253523	KT253509
Bx22	*P. domestica*	Italy, Apulia, Canosa di Puglia	F. Lops	…	…	…
Bx24	*Pyrus communis*	Italy, Apulia, Canosa di Puglia	A. Carlucci	…	…	…
Bx25	*Q. pubescens*	Italy, Apulia, Incoronata	F. Lops	KT253499	KT253533	KT253519
Bx26, CBS 141046[z]	*Prunus domestica*	Italy, Apulia, Canosa di Puglia	A. Carlucci	KT253493	KT253527	KT253513
Bx27	*Pyrus communis*	Italy, Apulia, Canosa di Puglia	A. Carlucci	…	…	…
Bx28, CBS 141002	*C. oblonga*	Italy, Apulia, Canosa di Puglia	A. Carlucci	KT253490	KT253524	KT253510
Bx29	*P. communis*	Italy, Apulia, Canosa di Puglia	A. Carlucci	KT253497	KT253531	KT253517
Bx30	*C. oblonga*	Italy, Apulia, Canosa di Puglia	F. Lops	…	…	…
Bx31	*Prunus domestica*	Italy, Apulia, Canosa di Puglia	F. Lops	…	…	…
Bx39	*P. domestica*	Italy, Apulia, Canosa di Puglia	F. Lops	…	…	…
Bx40	*Pyrus communis*	Italy, Apulia, Canosa di Puglia	F. Lops	KT253498	KT253532	KT253518
Bx52	*Prunus domestica*	Italy, Apulia, Canosa di Puglia	F. Lops	KT253494	KT253528	KT253514
Bx55	*Q. pubescens*	Italy, Apulia, Accadia	A. Carlucci	KT253500	KT253534	KT253520
B. simplicior						
YMJ 136	Wood of *Rhamnus cathartica*	France, Roquebrune Montseron	…	EF026130	AY951686	AY951798
Biscogniauxia sp.						
PP103	*Hevea brasiliensis*	Peru	…	FJ884075	…	…
VegaE4-39	*Coffea arabica*	United States, Hawaii	…	EU009960	…	…
SUT290	…	Thailand	…	DQ322095	…	…
B. uniapiculata						
YMJ 90080608	Bark	Taiwan, Tai-tung	Y.-M. Ju and H. M. Hsieh	JX507805	AY951687	AY951799
Camillea obularia						
ATCC 28093	Rotten wood	Puerto Rico	…	AJ390423	…	…
C. tinctor						
CBS 203.56	*Sassafras* sp.	United States	…	AJ390421	…	…
SUT260	…	Thailand	…	DQ322082	…	…
YMJ 363	Dead wood	Martinique	C. Lechat	JX507806	JX507795	JX507797
Durotheca rogersii						
YMJ 92031201[x]	Trunk of *Machilus zuihoensis*	Taiwan, Nan-Tou	Ju Y.-M. and H. M. Hsieh	EF026127	EF025612	EF025597
Hypoxylon rubiginosum						
YMJ 24	Wood of *Fraxinus*	United Kingdom, North Wales	…	EF026143	AY951751	AY951862
Obolarina dryophila						
H76	Inner bark of *Salix alba*	Slovakia, Jursky Sur	Petr Srutka	GQ428317	GQ428323	GQ428311
O. persica						
YMJ 1461[x]	Dying *Q. brantii*	Iran	M. Mirabolfathy	JX507807	JX507796	JX507798
Theissenia cinerea						
YMJ 90071615[x]	Wood stump	Taiwan	H. M. Hsieh and Y.-M. Ju	EF026128	EF025613	EF025598

Paraphyses. Filiform, tapering, sparsely septate, with oily contents, about 1.5 μm in diameter near the base and about 1.1 μm in diameter near the apex.

Asci. Eight-spored, cylindrical, arranged in uniseriate manner, short stipitates, with oily contents, (78.9–) 88.8 to 94.2 (–109.1) (mean, 91.5) μm long and (5.1–) 6.4 to 6.9 (–8.6) (mean, 6.7) μm wide, with spore-bearing parts 79.2 to 85.0 μm long, the stipes 7 to 12 μm long, with apical ring clearly bluing in Lugol's iodine reagent, discoid, 1.4 μm long and 2.6 μm wide.

Ascospores. Brown to dark brown, unicellular in both mature and immature ascospores, nearly equilateral, with broadly rounded ends, smooth, (8.0–) 9.2 to 9.8 (–11.2) (mean, 9.5) μm long and (4.0–) 5.0

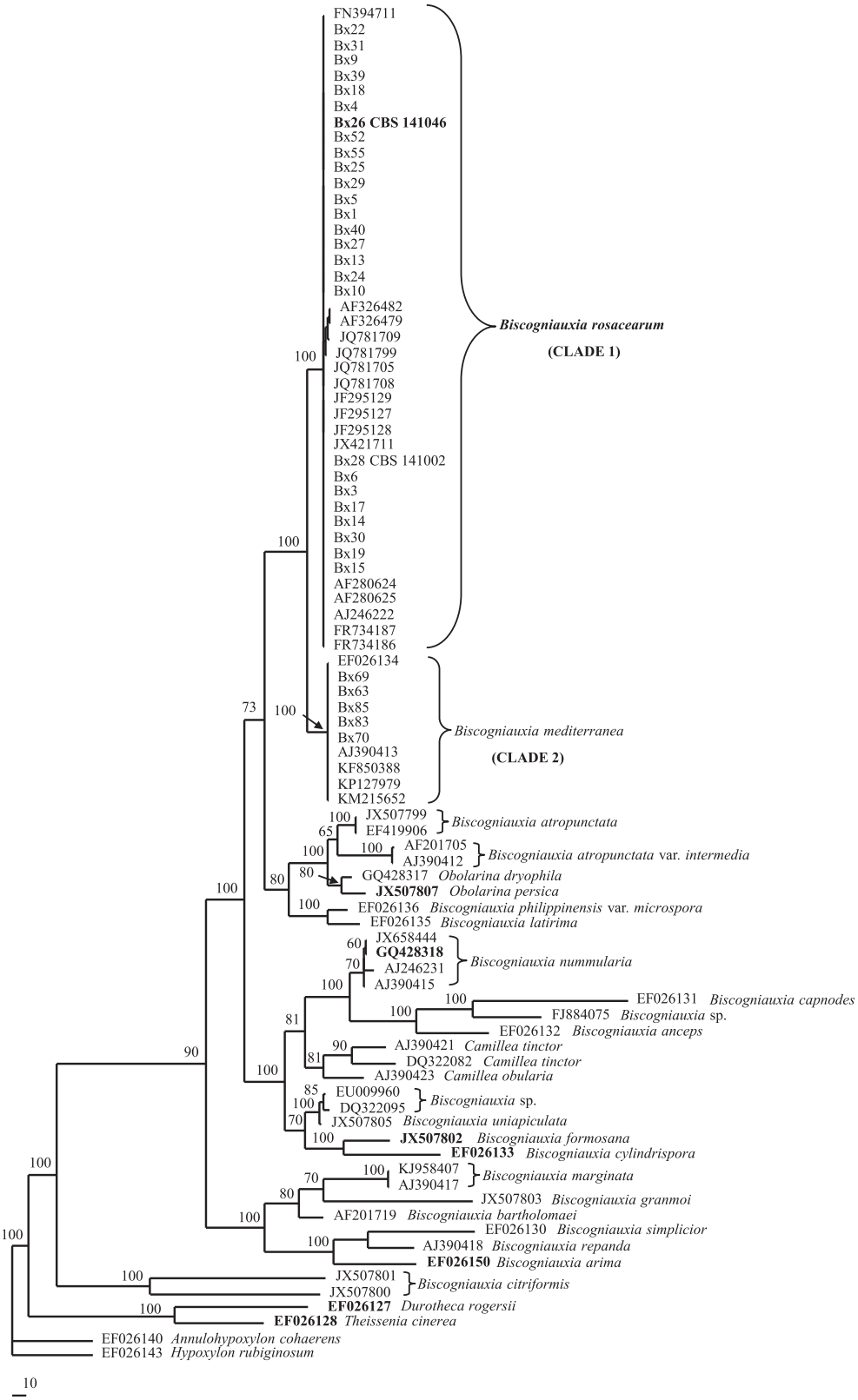

Fig. 2. Most parsimonious tree obtained from internal transcribed spacer alignment with bootstrap values from 1,000 replicates shown at the internodes. Ex-type sequences are highlighted in bold. *Annulohypoxylon cohaerens* and *Hypoxylon rubiginosum* were included as outgroups.

to 5.4 (–6.3) (mean, 5.2) µm wide, enveloped by a hyaline sheath. Germ slit straight, parallel to the long axis of the spore, almost full spore length.

Mycelia. Consisting of branched and septate hyphae, smooth to finely roughened, subhyaline to yellowish, occurring singly or in bundles of up to six, forming hyphal coils.

Conidiogenous structure. With *Nodulisporium*-like branching pattern, as defined by Ju and Rogers (1996).

Conidiophores. Hyaline to brownish, smooth to finely roughened, dichotomously branched, with two or three, and sometimes more, conidiogenous cells on each terminus.

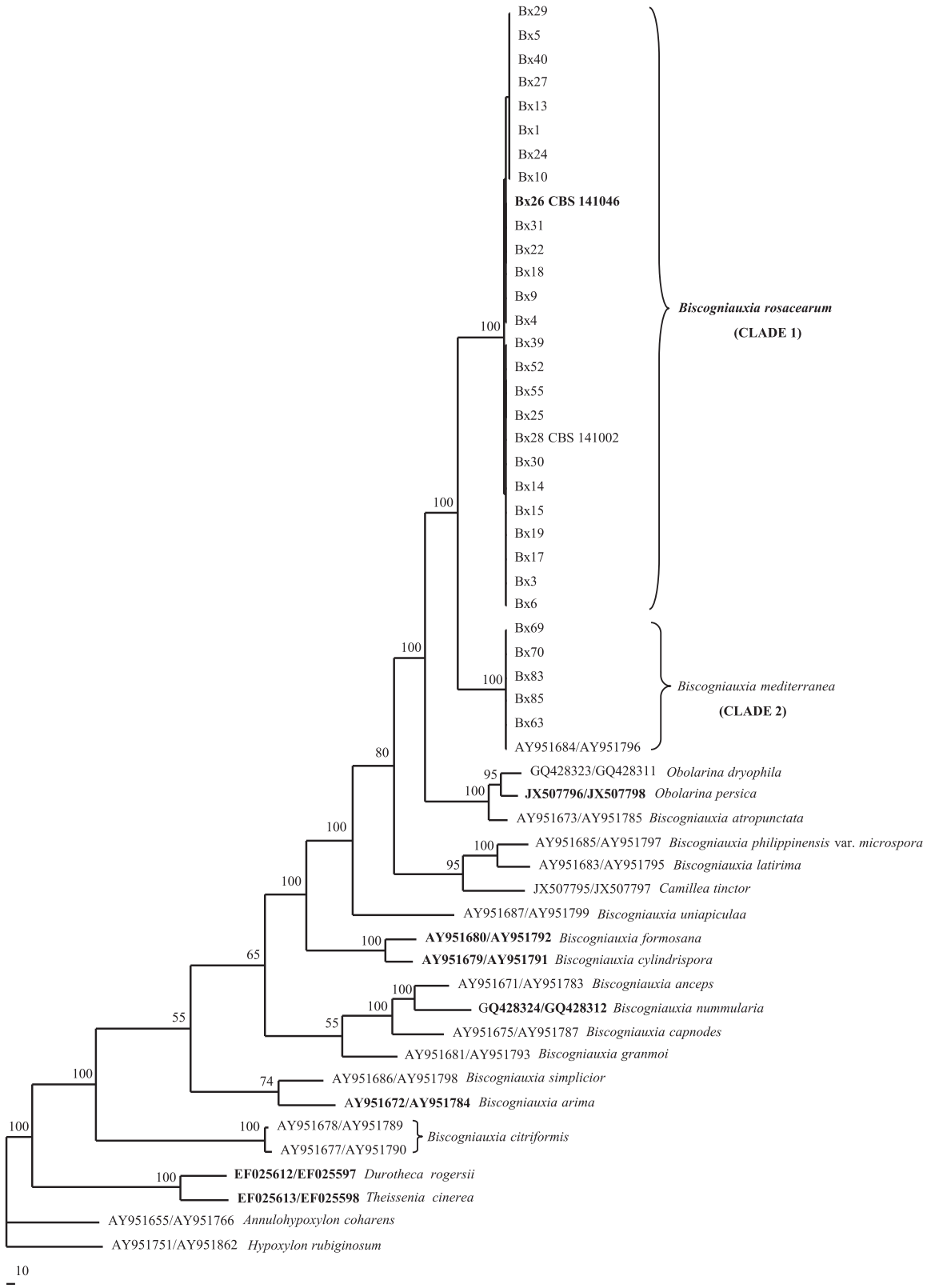

Fig. 3. One of the five most parsimonious trees obtained from the combined alignment of the *partial* β-*tubulin*/*partial actin* (*TUB*/*ACT*) gene sequence data, with bootstrap values from 1,000 replicates shown at the internodes. Accession numbers of the sequences obtained from the GenBank nucleotide database are indicated on the tree in the format *TUB*/*ACT*. Ex-type sequences are highlighted in bold. *Annulohypoxylon cohaerens* and *Hypoxylon rubiginosum* were included as outgroups.

Conidiogenous cells. Cylindrical, hyaline to brownish, smooth to finely roughened, (9.27–) 5.3 to 6.39 (–3.64) (mean, 5.85) µm long and (3.08–) 2.49 to 2.69 (–1.90) (mean, 2.59) µm wide, bearing denticulate conidial secession scars on apical region.

Conidia. Produced holoblastically in sympodial sequence, hyaline, smooth, obovoid to clavate, (3.31–) 5.72 to 6.26 (–8.23) (mean, 5.99) µm long and (1.66–) 2.17 to 2.32 (–2.89) (mean, 2.25) µm wide, with flattened base indicating former point of attachment to conidiogenous cells.

Cultural characteristics. Colonies reaching a radius of 90 mm after 8 days at 25°C (±2°C). Minimum temperature for growth, 7°C; optimum, 25°C; maximum, 39°C. Colonies on MEA aerial, with entire margin; after 21 days, pale smoke gray (21‴f) to tawny-olive (17″i) above, argus brown (13m) to verona brown (13″k) in reverse. Colonies on PDA aerial, with entire margin; after 21 days, deep olive buff (21‴b) to olive-buff (21‴d) above, buffy olive (21″k) to olive (21″m) in reverse. Colonies on OA aerial, with entire margin; after 21 days, avellaneous (17‴b) to olive (21″m) above, pale olive-buff (21‴f) to black (*1) in reverse.

Substrate. Different species of Rosaceae, including *Prunus domestica, Pyrus communis*, and *Cydonia oblonga.*

Known distribution. Apulia, Italy.

Specimens examined. Italy, Apulia, Canosa di Puglia, on wood of *Prunus domestica* (Rosaceae), August 2013, Carlucci A. Bx26 (HOLOTYPE CBS H-22145 dried PDA colony, culture ex-type Bx26 = CBS 141046; ITS sequence KT253493, TUB sequence KT253527, ACT sequence KT253513, MycoBank MB816045.

Other specimen examined. Italy, Apulia, Canosa di Puglia, on wood of *Cydonia oblonga* (Rosaceae), September 2013, Carlucci A. Bx28 = CBS 141002; ITS sequence KT253490, TUB sequence KT253524, ACT sequence KT253510.

Notes. According to DNA sequence analysis, *B. rosacearum* is most closely related to *B. mediterranea* and *B. atropunctata*. This species, however, differs in several aspects from *B. mediterranea* and *B. atropunctata*. Asci and ascospores of *B. rosacearum* are smaller than *B. mediterranea*, and the conidiogenus structure is *Nodulisporium*-like. It differs from *B. atropunctata* on account of the mature stromatal surface, which is colored rather than white.

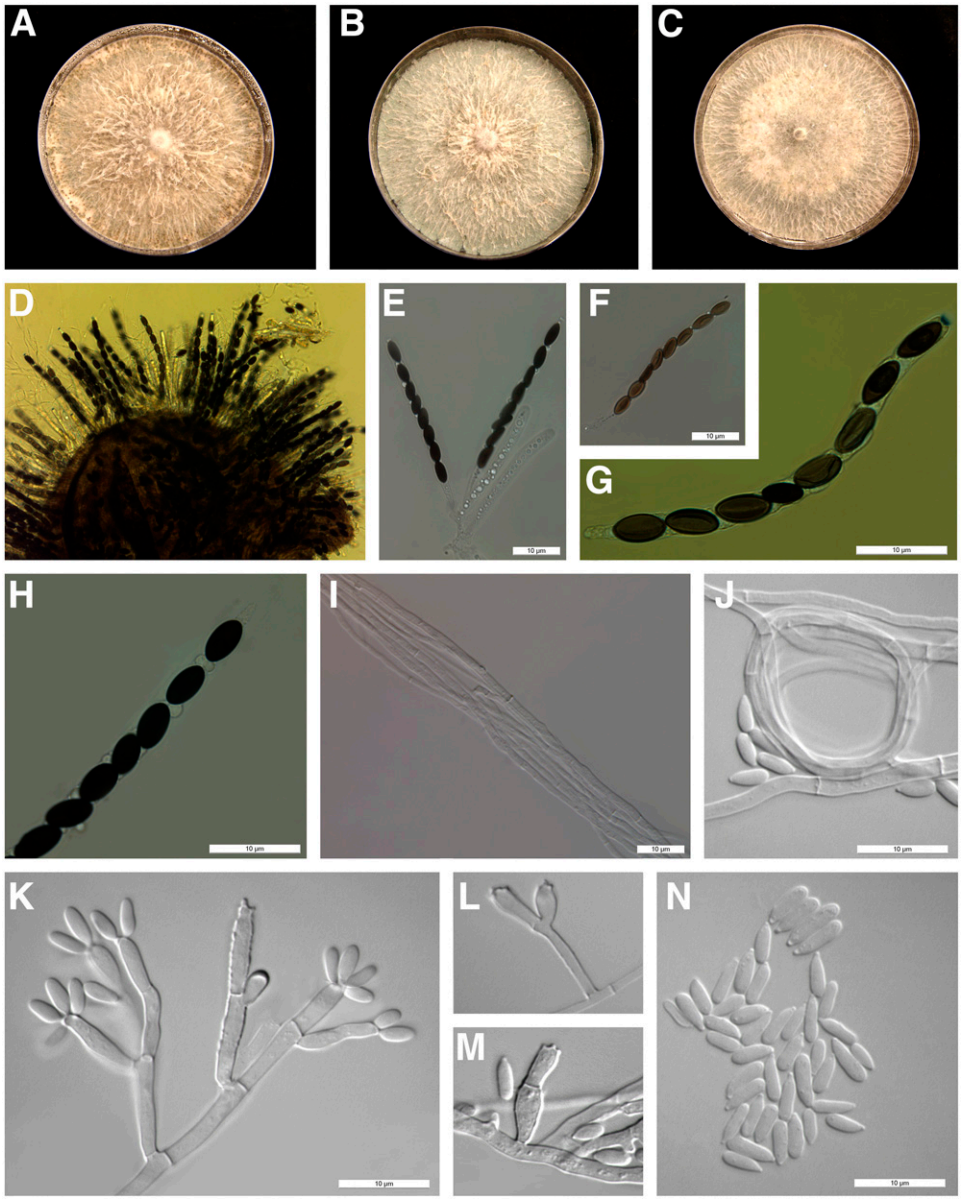

Fig. 4. *Biscogniauxia rosacearum* sp. nov. (Bx26 = CBS 141046). **A** to **C,** Sixteen-day-old colonies on **A,** malt extract agar; **B,** potato dextrose agar; and **C,** oat meal agar at 25°C. **D,** Asci in Lugol's reagent. **E** and **F,** Asci in 100% acid lactic. **G,** Ascus with apical ring in Lugol's reagent, showing ascospores with germ slit. **H,** Ascum in 100% acid lactic showing oil contents. **I,** Mycelia in bundles of up to six. **J,** Hyphal coils. **K** to **M,** Branched conidiophores and phialides. **N,** Conidia.

Pathogenicity tests. The results of pathogenicity tests were all determined at 20 months after inoculations. All *B. rosacearum* isolates tested in the inoculation experiment produced three to five charcoal stromata on inoculated stems on all three of the hosts inoculated. Stroma diameter varied from 0.8 to 2 cm. The inoculated stems produced poor vegetation, including flowers and fruit. According to the Shapiro-Wilk test, the data from the pathogenicity tests, on wood stems at 20 months after inoculation, followed a normal distribution ($W = 0.77$, $P < 0.00000$). The Levene test revealed that the homogeneity of variance was significant ($F = 7.58$, $P < 0.000000$). Factorial ANOVA demonstrated that there were significant differences among hosts inoculated with *B. rosacearum* isolates ($F = 40.5$, $P < 0.000000$). The average percentages and standard deviations of artificially inoculated pear, plum, and quince stems showing stromata (by one-way ANOVA) are reported in Table 2. All *B. rosacearum* isolates produced stromata on stems, although with different percentages. Of the inoculated hosts, the most susceptible to *B. rosacearum* isolates was quince, of which all inoculated stems showed stromata on bark (100%). Plum stems were less susceptible (ranging from 82 to 100%) whereas pear stems were the most tolerant, with 78 to 92% showing stromata. No significant differences in aggressiveness were observed among the isolates used in the artificial inoculation, whereas all of the controls did not produce stromata. The fungi were always reisolated from all wood samples inoculated, regardless of whether they had produced the stromata, thus fulfilling Koch's postulate.

Discussion

This phylogenetic reconstruction based on ITS and TUB/ACT gene sequences has allowed us to distinguish a new species within the *Biscogniauxia* genus, as the here-described *B. rosacearum*. This is different from *B. mediterranea* because it is very well supported, as indicated in both of the phylogenetic trees. Also, the morphological features of *B. rosacearum* confirm that these are two different species because, although according to the molecular analyses it is more closely related to *B. mediterranea*, *B. rosacearum* has asci and ascospores that are smaller and its conidiogenus structure is *Nodulisporium*-like, whereas *B. mediterranea* shows conidiogenus structures that are *Periconiella*-like, as defined by Ju and Rogers (1996).

Clade 1 of the ITS phylogenetic tree related to the new species here described for the first time, *B. rosacearum*, also includes 16 sequences named incorrectly as *B. mediterranea* from different hosts of European and Mediterranean Basin areas (Collado et al. 2001; Mirabolfathy et al. 2011; Sánchez Márquez et al. 2010; Trovão et al. 2013). Clade 2 appears to be correctly related to *B. mediterranea* species, because it includes five sequences from different hosts mainly from different states in the United States (Raja et al. 2015; Sánchez-Ballesteros et al. 2000; Silva-Hughes et al. 2015), one sequence (EF026134) from a European country (Hsieh et al. 2010), and five sequences from Italy collected in this study. Moreover, the sequence from France referred to a specimen examined by Ju et al. (1998) and given as *B. mediterranea* (Candoussau, F. 366). In particular, Collado et al. (2001) considered the fungal isolates indicated as two clades from their phylogenetic tree as sequences from two conspecific species, due to different geographic origins (i.e., Italy, Spain, Morocco, and the United States), although they claimed that the same clades were well distinguished and supported by high bootstrap values. Therefore, on the basis of morphological and phylogenetic features, we state that the two clades (1 and 2) do not relate to two conspecific species but, instead, to two different species of the genus *Biscogniauxia*.

The presence of charcoal stromata from fruit hosts such as pear, plum, and quince is very uncommon. In any case, on the basis of pathogenicity tests, it was possible to assess the ability of *B. rosacearum* isolates to infect pear, plum, and quince stems. On the other hand, in the older literature (Læssøe et al. 1999; Pouzar 1979; Vasilyeva 1988), some species of *Biscogniauxia* were related to some rosaceous hosts but no sequences of these are available from molecular data banks. *B. mediterranea* has never been isolated from rosaceous hosts, whereas *B. pruni* is reported from *Prunus padus* in Austria (Læssøe et al. 1999; Vasilyeva 1988), *B. marginata* from *Malus communis* (Pouzar 1979) in the Czech Republic, and *B. granmoi* from *P. padus* in Austria (Læssøe et al. 1999). No sequences reported in the phylogenetic tree of the present study are related to fungi from rosaceous hosts. Most *Biscogniauxia* spp. are associated with forest hosts, and mainly with *Quercus* spp. worldwide (De Sousa Santos 2003; Mirabolfathy et al. 2011; Ragazzi et al. 1989; Vannini and Scarascia Mugnozza 1991; Vannini et al. 1996). Moreover, we report that this fungus can cause severe damage to pear, plum, and quince trees, because it produced charcoal stromata when artificially inoculated under open-field conditions. Therefore, we strongly believe that this is a pathogen of rosaceous hosts, because this study is the first where *B. rosacearum* sp. nov. infects fruit hosts, although we also report for first time the occurrence of this novel *Biscogniauxia* spp. from *Q. pubescens* in southern Italy.

This putative adaptation or speciation of a species belonging to the *Biscogniauxia* genus to new plant hosts might have been influenced by unknown factors, which must be further investigated.

Table 2. Pathogenicity tests carried out with four *Biscogniauxia rosacearum* isolates inoculated on pear, plum, and quince stems

ID isolate	Host	Stem showing stromata (%)[x]	SD[y]	Min–max[z]	Reisolation (%)
Bx28	Pear	78 A	7.9	70–90	100
Bx26	Plum	82 A	6.3	70–90	100
Bx29	Pear	88 B	4.2	80–90	100
Bx25	Pear	89 B	5.7	80–100	100
Bx28	Plum	90 BC	6.7	80–100	100
Bx26	Pear	92 BC	6.3	80–100	100
Bx29	Plum	95 CD	5.3	90–100	100
Bx26	Quince	100 D	0	100–100	100
Bx29	Quince	100 D	0	100–100	100
Bx25	Plum	100 D	0	100–100	100
Bx25	Quince	100 D	0	100–100	100
Bx28	Quince	100 D	0	100–100	100

[x] Values followed by a different uppercase letter in each column are significantly different according to Fisher's test ($P < 0.01$).
[y] Standard deviation.
[z] Minimum and maximum values detected on the basis of 10 observations.

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