Ramophialophora, a new anamorphic genus of *Sordariales*

Misericordia Calduch, Josepa Gené*, Alberto M. Stchigel, José F. Cano and Josep Guarro

Unitat de Microbiologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, 43201-Reus, Spain

**Correspondence*: Josepa Gené, josepa.gene@urv.es

Abstract: *Ramophialophora vesiculosa* gen. et sp. nov., a dematiaceous hyphomycete isolated from a soil sample in Spain, is described and illustrated. It is characterised by erect and branched conidiophores with branches ending in sterile vesicles, mono- or polyphialidic, terminal and lateral conidiogenous cells with flared collarettes, and spherical, brown conidia with a protuberant basal hilum. ITS rDNA sequence analysis reveals its relationship with the *Sordariales*.

Taxonomic novelties: *Ramophialophora* Calduch, Stchigel, Gené & Guarro gen. nov., *Ramophialophora vesiculosa* Calduch, Stchigel, Gené & Guarro sp. nov.
Key words: hyphomycetes, *Phialophora*, *Ramophialophora*, soil, *Sordariales*, Spain.

INTRODUCTION

During a continued survey of soil-borne microfungi from different phytogeographical areas of Spain, we recorded several interesting fungi from the Muniellos Integral Biological Reserve, Asturias. A rare anamorphic fungus was isolated using a soil-baiting technique (Barnett *et al*. 1974, Davet & Rouxel 2000). It is a dematiaceous hyphomycete that shows some of the typical characteristics of *Phialophora* Medlar, i.e. more or less pigmented, phialidic conidiogenous cells with flaring collarettes, forming conidia in slimy masses. However, in addition, it has other relevant features such as the presence of repeatedly branched conidiophores with terminal, hyaline or subhyaline, sterile vesicles on the conidiophore axis and branches, a characteristic of the conidiophore never described in *Phialophora* or any other morphologically similar genera. This fungus is proposed here as a new species and genus. Sequence analysis of the ITS region (ITS1, ITS2 and the 5.8S gene) confirmed its close relationship with members of the *Sordariales*, and its genetic differences with representative species of *Phialophora*.

MATERIALS AND METHODS

Soil samples were collected in the Muniellos Integral Biological Reserve, Asturias, Spain. This reserve comprises a surface of 5542 ha at 650–1642 m altitude. The average annual temperature is 6–10 ºC and the annual average rainfall is 1400–2300 L/m^2. The vegetation is composed of forests of *Quercus petraea* (Matt.) Liebl., *Q. pyrenaica* Willd., *Betula celtiberica* Rothm & Vasc., *Fagus sylvatica* L., riparian forests and mixed forests of *Fraxinus excelsior* L. and *Acer pseudoplatanus* L. (Fernández & Bueno 1996).

Soil samples free of organic matter (A_o horizon) were placed in sterilized polyethylene bags, closed with rubber bands and labelled. In the laboratory, they were stored in a refrigerator at 4–7 ºC until processed. To stimulate fungal growth, the samples were placed in sterile Petri dishes, moistened with sterile distilled water, covered with small, thin pieces (approx. 2 cm^2) of sterile wood, and incubated at 22–25 ºC in 12 h of darkness alternating with 12 h of cool white fluorescent light. The whole plates were examined under the stereomicroscope every week 6 mo. To isolate fungi developing on the wood pieces, propagules were transferred, using a sterile dissection needle, to Petri dishes containing potato carrot agar (PCA; 20 g potatoes, 20 g carrots, 20 g agar, 1 L dist. water) and oatmeal agar (OA; 30 g filtered oat flakes, 20 g agar, 1 L dist. water). Cultures were incubated at room temperature. Photomicrographs were obtained with a Leitz Dialux 20 light microscope and scanning electron microscope Jeol JSM-6400.

The ITS regions (ITS-1 only partial, ITS-2 complete) and 5.8S rRNA gene (complete) of our isolate were sequenced using the ITS-5 and ITS-4 primers (White *et al*. 1990). DNA extraction, amplification and sequencing were performed as in Solé *et al*. (2002). A BLAST sequence homology search (Altschul *et al*. 1997) was performed to compare our sequence with others from the GenBank database. For the phylogenetic study, sequences from eight strains of *Sordariales* (*Cercophora appalachianensis* O. Hilber & R. Hilber AF177155, *Chaetomium nigricolor* L. M. Ames AJ458185, *Chaetosphaeria chloroconia* W. Gams & Hol.-Jech. AF178542, *Coronatomyces cubensis* D. García, Stchigel & Guarro AJ458187, *Phialophora phaeophora* W. Gams, the

anamorph of *Chaetosphaeria pygmaea* (P. Karst.) Constant., K. Holm & L. Holm AF083191, *Podospora anserina* (Rabenh.) Niessl AF388930, *P. austrohemisphaerica* N. Lundq. AY026939, *Thielavia hyrcaniae* Nicot AJ271581), and four strains of *Phialophora* spp. (*P. americana* (Nannf.) S. Hughes U31840, *P. richardsiae* (Nannf.) Conant U31844, *P. verrucosa* Medlar U31848 and *P. verrucosa* AF050282) obtained from the GenBank were aligned with Clustal W (version 1.5) of multiple sequence alignment computer programme (Thompson *et al*. 1994) and then adjusted visually where necessary. Cladistic analyses using the Neighbour-joining method (Saitou & Nei 1987) were performed with the MEGA 2.1 computer program (Kumar *et al*. 2001). The tree was constructed using the Kimura-2-parameter distance model (Kimura 1980) with pairwise deletion of gaps. The robustness of branches was assessed by bootstrap analysis with 1000 replicates.

RESULTS AND DISCUSSION

Taxonomy

Ramophialophora Calduch, Stchigel, Gené & Guarro, **gen. nov.** MycoBank MB500021.

Etymology: Latin *ramus* = branch, referring to the branched conidiophores, and *-phialophora* due to the *Phialophora*-like conidiogenous cells.

Ad hyphomycetes pertinens. Coloniae in ligno sterili effusae, pilosae, olivaceo-brunneae. Hyphae pallide brunneae, septatae, ramosae. Conidiophora macronematosa, mononematosa, erecta, septata, ramosa, brunnea, sursum vesicula sterili, subhyalina et subsphaerica vel clavata terminata. Cellulae conidiogenae monophialidicae vel polyphialidicae, discretae, terminales et laterales, plerumquam lageniformes, pallide olivaceae, collari patelliformi, paulo obscuriore praeditae. Conidia in massa mucosa aggregata, unicellularia, brunnea, sphaerica, cum hilo basilari protuberante. Teleomorphosis ignota.

Species typica: *Ramophialophora vesiculosa* Calduch, Stchigel, Gené & Guarro, sp. nov.

Hyphomycetes. *Colonies* on sterile wood effuse, hairy, olivaceous-brown. *Mycelium* superficial and immersed in the substrate. *Hyphae* pale brown, septate, branched. *Conidiophores* macronematous, mononematous, erect, cylindrical, septate, branched, brown to dark brown, becoming paler towards the apex, smooth- and rather thick-walled; conidiophore axis and branches usually ending in a sterile, subhyaline, subspherical to clavate vesicle. *Conidiogenous cells* monophialidic or polyphialidic, discrete, terminal and lateral, lageniform, pale olivaceous, smooth-walled, with conspicuous, slightly darker collarettes. *Conidia* in slimy masses, one-celled, brown, spherical, with a protuberant basal hilum. *Teleomorph* unknown.

Ramophialophora vesiculosa Calduch, Stchigel, Gené & Guarro, **sp. nov.** MycoBank MB500022. Figs 1–11.

Etymology: Latin *vesiculosus* = bearing vesicles, referring to the vesicles on the conidiophore.

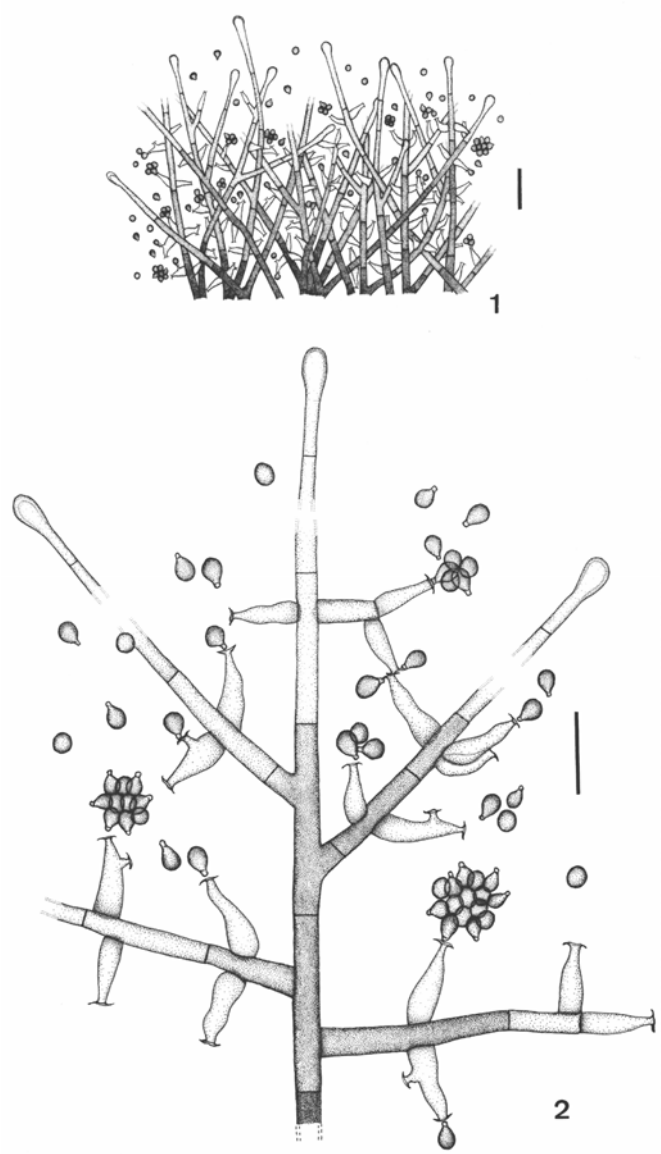
Coloniae in ligno sterili effusae, pilosae, olivaceo-brunneae. Mycelium superficiale et in substrato immersum. Hyphae 1–1.5 µm latae. Conidiophora cylindrica, septata, ramosa, brunnea vel atrobrunnea, pallidiora ad apicem, longitudine indeterminata, 2–3 µm lata, laevia, saepe crassitunicata, sursum vesicula sterili, subhyalina et subsphaerica vel clavata terminata, 3.5–6.5 µm lata in parte latissima. Cellulae conidiogenae discretae, terminales et laterales, plerumquam lageniformes, 5.5–12 µm longae, 2.5–4 µm latae in parte maxima, pallide olivaceae, laeves, collari patelliformi, 2–2.5 µm lato, paulo obscuriore praeditae. Conidia unicellularia, brunnea, laevia, crassitunicata, sphaerica, 2.5–3 µm diam, cum hilo basilari cylindrico, subhyalino, 0.5–1 × 1 µm protuberante. Teleomorphosis ignota.

Hyphomycetes. *Colonies* on sterile wood effuse, hairy, olivaceous-brown. *Mycelium* superficial and immersed in the substrate. *Hyphae* pale brown, septate, branched, 1–1.5 µm wide. *Conidiophores* macronematous, mononematous, erect, cylindrical, septate, branched, brown to dark brown, becoming paler towards the apex, length indeterminate, 2–3 µm wide, smooth- and rather thick-walled; conidiophore axis and branches usually ending in a sterile, subhyaline, subspherical to clavate vesicle 3.5–6.5 µm wide at the broadest part. *Conidiogenous cells* monophialidic or polyphialidic, discrete, terminal and lateral, lageniform, often waisted, sinuous, 5.5–12 µm long, 2.5–4 µm wide at the broadest part, pale olivaceous, smooth-walled, with conspicuous collarettes; collarettes widely flaring, often curved backwards, 2–2.5 µm wide, slightly darker. *Conidia* one-celled, brown, smooth- and thick-walled, spherical, 2.5–3 µm diam, with a subhyaline, cylindrical, 0.5–1 × 1 µm, basal hilum. *Teleomorph* unknown.

Cultural characteristics: Colonies on OA at 25 ºC growing slowly, attaining 21–25 mm diam in 21 d, velvety, brown at the centre, with dark brown to black radiations composed mainly of conidiophores and conidia; reverse brown to dark brown. Colonies on PCA at 25 ºC, attaining 27–32 mm diam in 21 d, with similar macroscopic characteristics to those observed on OA. On both media the microscopic features were very similar to those observed on the wood.

Specimen examined: **Spain**, Asturias Province, Muniellos Integral Biological Reserve, from forest soil, 26 June 1999,

col. A. M. Stchigel & M. Calduch, **holotype** IMI 389151, ex-type cultures CBS 110629 and FMR 7712.

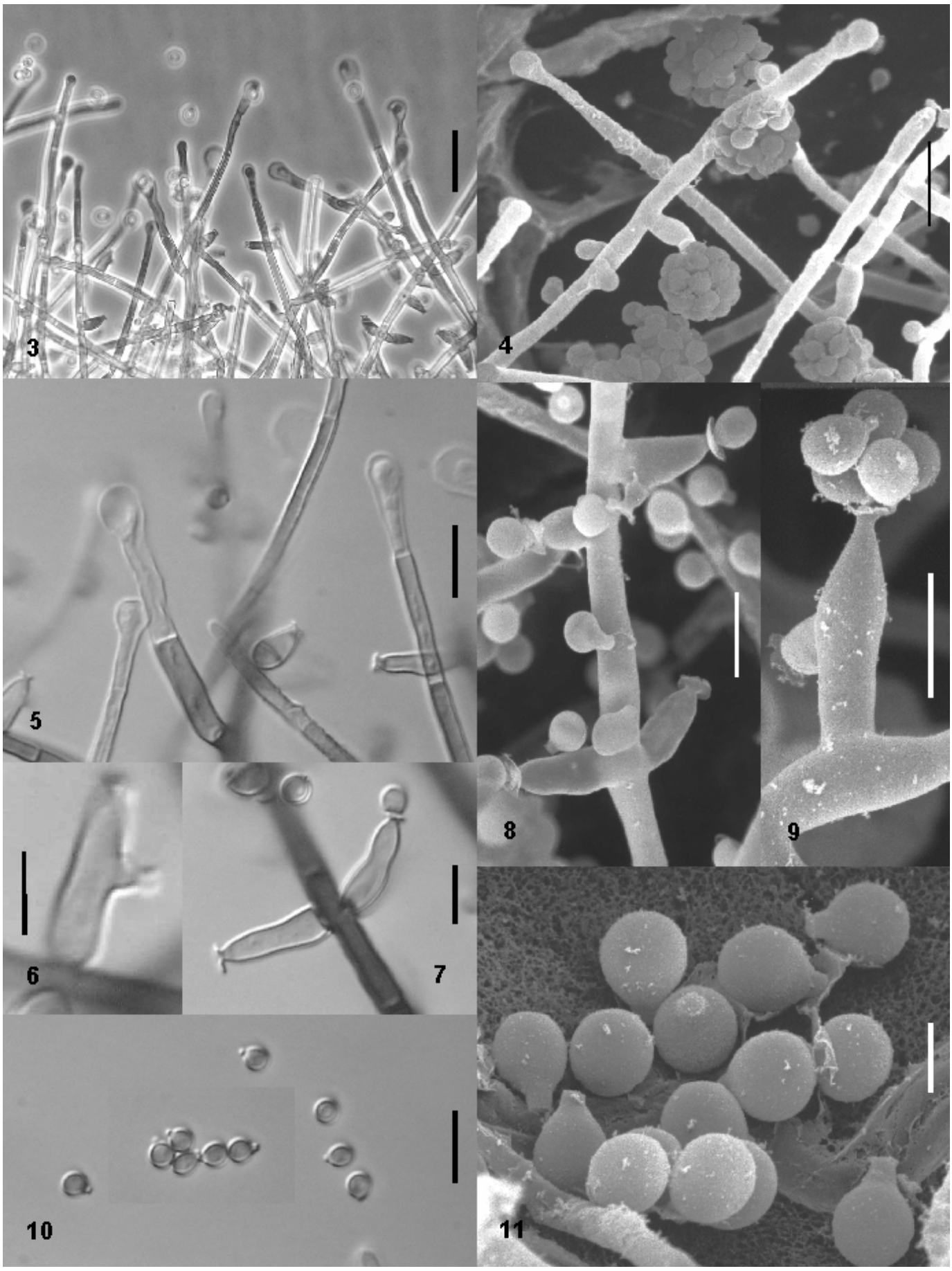


Figs 1–2. *Ramophialophora vesiculosa* (IMI 389151). 1. Habit sketch. 2. Detail of a conidiophore bearing conidiogenous cells and conidia. Scale bars: 1 = 20 µm, 2 = 10 µm.

Notes: *Ramophialophora vesiculosa* was initially identified as a species of *Phialophora* (Schol-Schwarz 1970) due to the presence of aseptate conidia emerging from terminal or intercalary phialidic conidiogenous cells with well-defined collarettes and forming slimy masses. However, its erect and branched conidiophores with hyaline to subhyaline, sterile vesicles have never been seen in *Phialophora*. In addition, *Phialophora* has been considered a poorly defined and highly polyphyletic genus (Gams 2000), and the inclusion of our specimen would increase its heterogeneity even further. Moreover, there is a current tendency to segregate *Phialophora* into more phenotypically and genetically coherent genera (Gams & McGinnis 1983, Crous *et al*. 1996, Gams 2000).

Similar conidiophores to those of *Ramophialophora* have been described in other dematiaceous hyphomycetes, such as *Gonytrichum* Nees & T. Nees (Gams & Holubová-Jechová 1976), *Dicyma* Boulanger (von Arx 1982), *Phaeostalagmus* W. Gams (Gams & Holubová-Jechová 1976) or *Surculiseries* Okane, Nakagiri & Tad. Ito (Okane *et al*. 2001). However, their conidiogenesis and the arrangement of the conidiogenous cells on the conidiophore differ considerably from those of our specimen. Conidiogenous cells of *Dicyma* and *Surculiseries* are always polyblastic, while those of *Gonytrichum* and *Phaeostalagmus* are monophialidic. In addition, *Gonytrichum* has conidiogenous cells arising from collar hyphae that encircle the conidiophore branches, and *Phaeostalagmus* has simple or branched conidiophores with verticillate conidiogenous cells which arise sub- or terminally from the conidiophore stipe or their branches. Furthermore, species of the latter genus never have globose conidia as those of *Ramophialophora vesiculosa*. Some *Phialophora* species have conidiogenous cells and conidia morphologically similar to those of *R. vesiculosa*. These are *P. cyclaminis* F. H. Beyma (Ellis 1976), *P. japonica* Iwatsu & Udawaga (Iwatsu & Udawaga 1985), *P. richardsiae* (Ellis 1976) and the *Phialophora* anamorph of *Podospora austrohemisphaerica* (Lundqvist *et al*. 1999). However, none of them shows a conidiophore differentiation as seen in our specimen. In addition, the conidia of *P. cyclaminis* are hyaline, those of *P. japonica* are pale olivaceous to olivaceous, and *P. richardsiae* produces two types of conidia, i.e., globose and darkly pigmented, and ellipsoidal and hyaline. The anamorph of *P. austrohemisphaerica* shows the most similar conidia to those of the new species, but they can be differentiated because in the former they are hyaline (brownish in mass) and emerging from phialides, which usually present an irregular or verticillate arrangement on short side branches of the vegetative mycelium. Moreover, the conidia of *P. austrohemisphaerica* never germinate *in vitro*, which suggests a spermatial function of these propagules (Lundqvist *et al*. 1999, Gams 2000).

To confirm our proposal and to know the phylogenetic relationships between *R. vesiculosa* and other members of *Phialophora*, we sequenced the ITS region of our isolate and compared the sequence with others deposited in GenBank. The BLAST search revealed that *R. vesiculosa* was genetically close to some members of the *Sordariales*, such as *Cercophora appalachianensis*, *Coronatomyces cubensis*, *Chaetomium nigricolor* and *Thielavia hyrcaniae*, with 95–96 % of homology mainly located in the 5.8S and ITS2 regions, and showing a very low sequence homology with members of the *Chaetothyriales*, where *Phialophora verrucosa*, the type species of the genus, *P. americana* and other related *Phialophora* species are included.



Figs 3–11. *Ramophialophora vesiculosa*, IMI 389151. 3. Conidiophores. 4, 5. Detail of the conidiophore branches ending in a sterile vesicle. 6–9. Conidiogenous cells showing collarettes and conidia. 10, 11. Conidia. 3. Phase contrast, 4, 8, 11 SEM, 5–7, 10 DIC. Scale bars: 3 = 20 µm, 4, 5 = 10 µm, 6–9 = 5 µm, 10 = 10 µm, 11 = 2 µm.

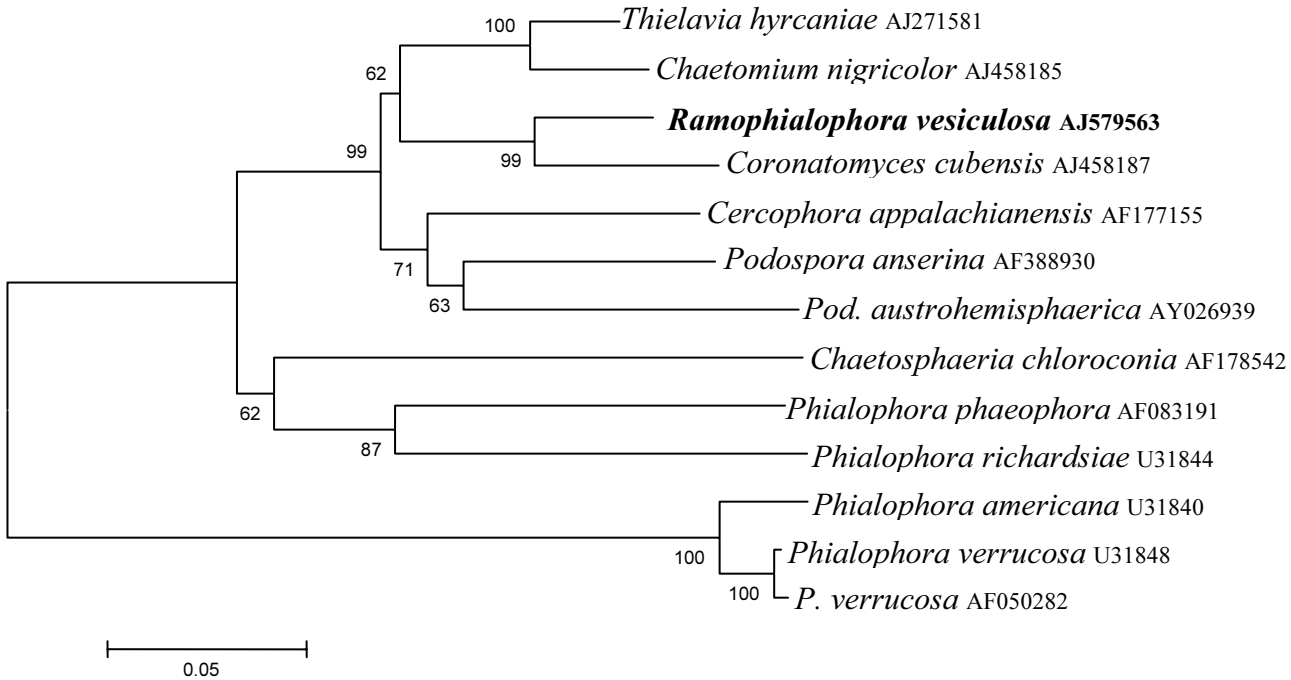


Fig. 12. Neighbour-joining tree based on nucleotide sequences of the 5.8S rRNA gene and ITS region (ITS1, partial sequence). Branch lengths are proportional to distance. Bootstrap replication frequencies (1000 replications) are indicated at the internodes (*Pod. = Podospora; P. = Phialophora*).

The phylogenetic tree shown in Fig. 12 is built up with the sequence of our isolate and from another twelve species obtained from GenBank. We included some members of *Sordariales* (*Chaetomium nigricolor*, *Coronatomyces cubensis*, *Podospora anserina*, *Thielavia hyrcaniae*), some of them with *Phialophora* or *Phialophora*-like anamorphs (*Cercophora appalachianensis*, *Chaetosphaeria pygmaea* and *Podospora austrohemisphaerica*). We also included some other anamorphic species morphologically similar to our taxon, such as *Gonytrichum chlamydosporium* G.L. Barron & G.C. Bhatt, the anamorph of *Chaetosphaeria chloroconia*, and some *Phialophora* species (*P. richardsiae*, *P. verrucosa* and *P. americana*). The tree showed two strongly supported monophyletic clades. The former contained all members of *Sordariales* and *P. richardsiae*. The latter species has been considered with an undefined taxonomic position (Yan *et al*. 1995, Gams 2000), and has now been transferred to the genus *Pleurostomophora* D. Vijaykrishna, L. Mostert, R. Jeewon, W. Gams, K.D. Hyde & Crous (this volume). The second clade comprised *P. americana* and *P. verrucosa* of the *Chaetothyriales*, which constituted a natural outgroup (100 % bootstrap index). *Ramophialophora vesiculosa* was included in the subcluster of the *Sordariales* and grouped with *Coronatomyces cubensis* with a bootstrap index of 99 %. This is a recently described ascomycetous species, with no anamorph, that was isolated from a Cuban soil sample (García *et al*., this volume). The morphological features of this taxon are typical of a member of the order *Sordariales*, although a more concrete taxonomic level has not been resolved. Our results seem to indicate that both fungi have a common sordariaceous ancestor, but to confirm this and to know its position at a higher taxonomical rank a more extensive molecular study is needed with more strains and the sequencing of different genes.

ACKNOWLEDGEMENTS

The authors are grateful to E. Descals (IMEDEA, CSIC-UIB, Esporles, Mallorca, Spain) for reviewing the manuscript prior to submission, to the Consejería de Agricultura of Principado de Asturias, Spain, for permission to collect the samples used in this study and to A. Moreno and F. Gilgado for their assistance in molecular techniques. This work was supported by CICYT (Comisión Interministerial de Ciencia y Tecnología) grant REN 2000-1521.

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