doi:10.5598/imafungus.2011.02.01.13 IMA FUNGUS · VOLUME 2 · NO 1: 97–103

ARTICLE

Molecular phylogenetic studies on the lichenicolous *Xanthoriicola physciae* reveal Antarctic rock-inhabiting fungi and *Piedraia* species among closest relatives in the *Teratosphaeriaceae*

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Abstract: The phylogenetic placement of the monotypic dematiaceous hyphomycete genus *Xanthoriicola* was investigated. Sequences of the nLSU region were obtained from 11 specimens of *X. physciae,* which formed a single clade supported both by parsimony (91 %), and maximum likelihood (100 %) bootstraps, and Bayesian Posterior Probabilities (1.0). The closest relatives in the parsimony analysis were species of *Piedraria*, while in the Bayesian analysis they were those of *Friedmanniomyces*. These three genera, along with species of *Elasticomyces*, *Recurvomyces*, *Teratosphaeria*, and sequences from unnamed rock-inhabiting fungi (RIF), were all members of the same major clade within *Capnodiales* with strong support in both analyses, and for which the family name *Teratosphaeriaceae* can be used pending further studies on additional taxa.

Key words:
Ascomycota
Capnodiales
Friedmanniomyces
hyphomycetes
lichenicolous fungi
Piedrariaceae
rock inhabiting fungi

Article info: Submitted 10 May 2011; Accepted 26 May 2011; Published 7 June 2011.

INTRODUCTION

The generic name *Xanthoriicola* was introduced for the species *X. physciae* (Hawksworth & Punithalingam 1973). This fungus appears to be obligately lichenicolous on *Xanthoria parietina* in Europe, and is also reported from Africa and Asia (Siefert *et al*. 2011). The fungus primarily occurs in the apothecia, growing through the hymenium, with broad cupulate enteroblastic conidiogenous cells generating conidia at the surface (Fig. 1). The conidia are dark brown, spherical, single-celled, and have a coarse, warted surface ornamentation. This fungus was illustrated by line drawings in Hawksworth & Punithalingam (1973), and photomicrographs and scanning electron micrographs are presented in Hawksworth (1979). The surfaces of infected apothecia become sooty black and so are easily seen in the field. Whole swards of the host lichen are rarely affected, so while deleterious to the host it does not destroy their populations. It has not been reported as growing in isolated culture, and experiments to inoculate fresh specimens of the host have proven unsuccessful (T.F. Preece, unpubl. data). Furthermore, no sexual state has been discovered or postulated by association with other fungi that occur on the same host lichen.

The fungus is particularly unusual in that the conidia are formed "semi-endogenously", that is within the lower part of the collarette of the conidiogenous cells. This is a rare situation in hyphomycetous conidial fungi, and is otherwise seen only in *Craspedodidymum, Cystodendron, Lambinonia, Metacapnodium,* and some groups of *Phialophora s. lat*. (Ellis 1976, Seifert *et al*. 2011). In addition, some studies by Raman spectroscopy suggested that *Xanthoriicola physciae* might form scytonemin, a protective pigment only otherwise known in cyanobacteria (Preece 2009) – although that report now seems likely to have been a result of contamination from cyanobacteria growing on the surface of the hymenium.

Xanthoriicola physciae consequently appeared, on morphological grounds to occupy an isolated position amongst the conidial fungi. This investigation was initiated in order to determine its phlyogenetic relationships.

MATERIALS AND METHODS

Choice of additional taxa and outgroup

In addition to the *Xanthoriicola* specimens sequenced, 48 specimens of *Dothideomycetes* were included in the molecular study (Figs 2–3). The sampling was selected to include taxa that were close to our new sequences in GenBank, i.e. mainly conidial members of the *Teratosphaeriaceae*, together with other representatives of

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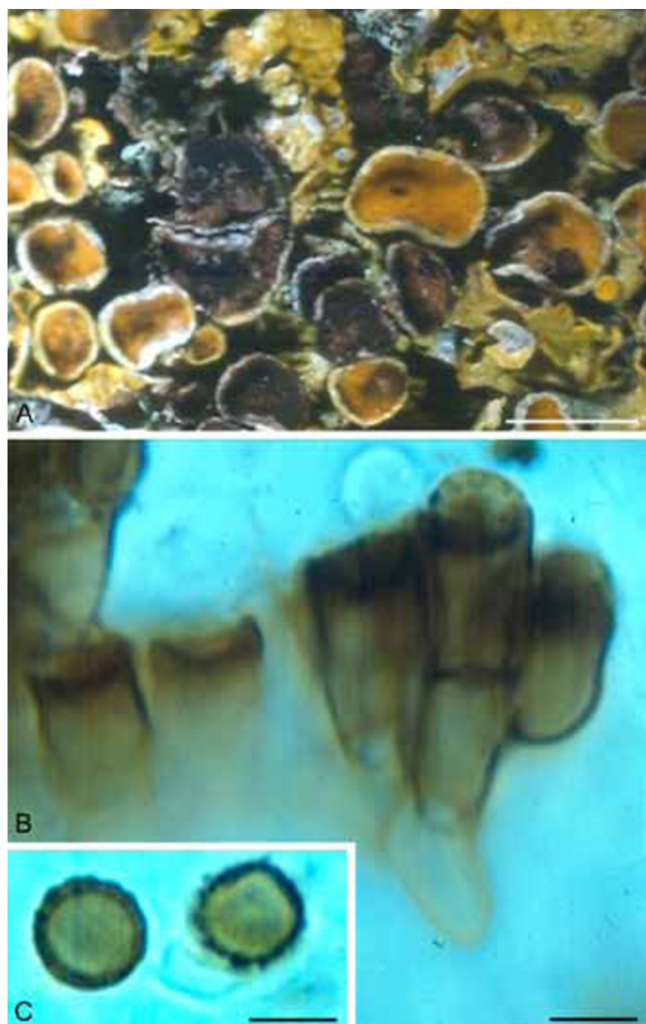

Fig. 1. *Xanthoriicola physciae* (IMI 164974). **A.** Apothecia of *Xanthoria parietina* infected by the fungus. **B.** Conidiogenous cells in the upper part of the hymenium. **C.** Conidia. Bars A = 5 mm, B–C = 5 µm.

Capnodiales and *Dothideales*. *Dothidea insculpta* was used as outgroup.

Voucher information, and GenBank accession numbers of newly sequenced taxa are provided in Table 1.

DNA extraction

DNA was extracted directly from dried specimens. Fungi growing in the host apothecia were carefully excised with the point of a sterile scalpel blade to minimize as much as possible the obtaining of host tissue. Total DNA was extracted using the Qiagen DNeasy Plant MiniKit, according to the manufacturer's instructions.

Amplification and sequencing

A fragment of *ca*. 1000 bp in the nLSU was amplified using the primers LR0R (R Vilgalys, www.biology.duke.edu/fungi/mycolab/primers.htm), LR5 (Vilgalys & Hester 1990), and also ones specifically designed in our laboratory to selectively amplify the DNA of *Xanthoriicola physciae*, avoiding that of the host. The primers we designed were: X158F (5'-GAGAGGATGCTTCTGGGCA-3') and X756R (5'-CCGAAGCTCCCACCTCCGTT-3'). Primer combinations used were LR0R/LR5, LR0R/ X756R, and X158F/LR5PCR.

PCR amplifications were performed using Illustra™ Hot Start PCR beads, according to the manufacturer's instructions, and using the settings in Hawksworth *et al*. (2010).

Before sequencing, the PCR products were purified using the Viogene PCR-M Clean-up System or the enzymatic method Exo-sap-IT©.

Sequence alignment and phylogenetic analysis

Sequences were aligned using MAFFT v. 6.611 (Katoh *et al*. 2002, Katoh & Toh 2008) using the procedures described in Wedin *et al*. (2009). The ambiguous regions in the alignment were identified and eliminated using Gblocks v. 0.91b (Castresana 2000).

Maximum parsimony and parsimony bootstrap analyses were performed using PAUP v. 4.0b10 (Swofford 2003) with the following settings: gaps were treated as "missing data", 1 000 random addition sequence replicates, TBR branch swapping, steepest descent off, collapse branches if minimum length is 0, MulTrees on, and with 1000 trees allowed to be saved in each replicate. For the bootstrap analyses (Felsenstein 1985) we used: heuristic search settings identical with the above analysis, but with ten random addition replicates, 1000 bootstrap replicates, a full heuristic search, and retained groups with a frequency > 50 %. Parsimony-uninformative characters were excluded from these analyses.

Maximum likelihood analyses (ML) were achieved using the program Garli v. 0.951 (Zwickl 2006). Runs were terminated after 10 000 generations with no significant improvement in – lnL. Improvement values were set to 0.01 with a total improvement lower than 0.05 compared to the last topology recovered. Bootstrap support was assessed using 1 000 tree replicates under the same parameters as above.

We used the Bayesian method of Huelsenbeck *et al*. (2001) to analyse the data by Markov Chain Monte Carlo (MCMC) sampling as implemented in the software MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001). Likelihood models were selected for each of the three gene regions using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) as implemented in jModeltest (Posada 2008). We used full likelihood optimization and selected from among only the 24 models implemented in MrBayes. Following this scheme, a GTR+I+G model was chosen for the nuclear LSU rDNA data using both criteria. The number of discrete gamma categories was kept at default four. Bayesian prior distributions included treating all tree topologies as equally likely, a uniform (0, 50) distribution for the gamma shape parameter, a uniform (0, 1) distribution for the proportion of invariable sites, and a flat (1, 1, 1, 1, 1, 1) Dirichlet for the rate matrix. Two parallel runs were performed, each with five chains, four of which were incrementally heated with a temperature of 0.15. The analysis was diagnosed for convergence every 100 000 generations, measured as the average standard deviation of splits across

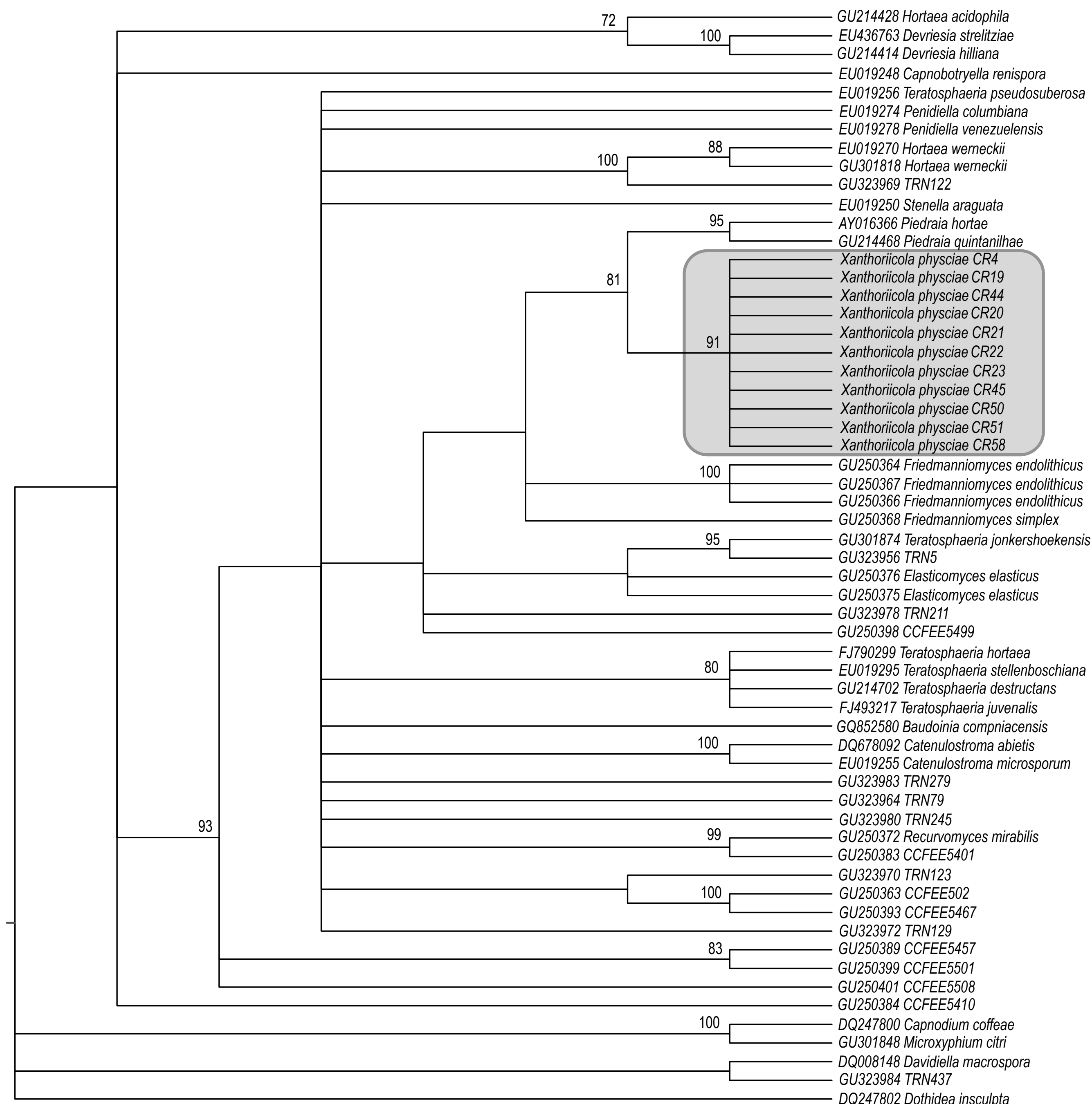

Fig. 2. Consensus tree of 725 000 equally most parsimonious trees from the analysis of the nLSU dataset. Bootstrap values ≥ 70 % are indicated over branches. Species name and GenBank accession number are given for each terminal. Sequences from cultures which have not been named are referred to by culture reference numbers. The tinted box includes *Xanthoriicola physciae*.

runs in the last half of the analysis. Every 100th tree was saved, and the first half of the run was discarded as burn-in.

RESULTS

We generated 11 new nLSU rDNA sequences (Table 1), which were aligned together with sequences already available in GenBank.

The matrix contained 775 characters from which 167 unambiguously aligned parsimony informative sites were used in the parsimony analysis. Our maximum parsimony analysis resulted in 725 000 most parsimonious trees of 644 steps, with CI = 0.393 and RI = 0.692.

The Bayesian analysis halted after 4 800 000 generations, when the average standard deviation of split frequencies across runs was then lower than 0.01 (= 0.0096). We considered the two runs to have converged and a majority rule consensus tree was constructed from the 48 000 trees of the stationary tree sample. Bayesian and ML analyses produced congruent phylogenies.

The 11 specimens of *Xanthoriicola physciae* formed a single clade supported both by parsimony bootstrap (91 %),

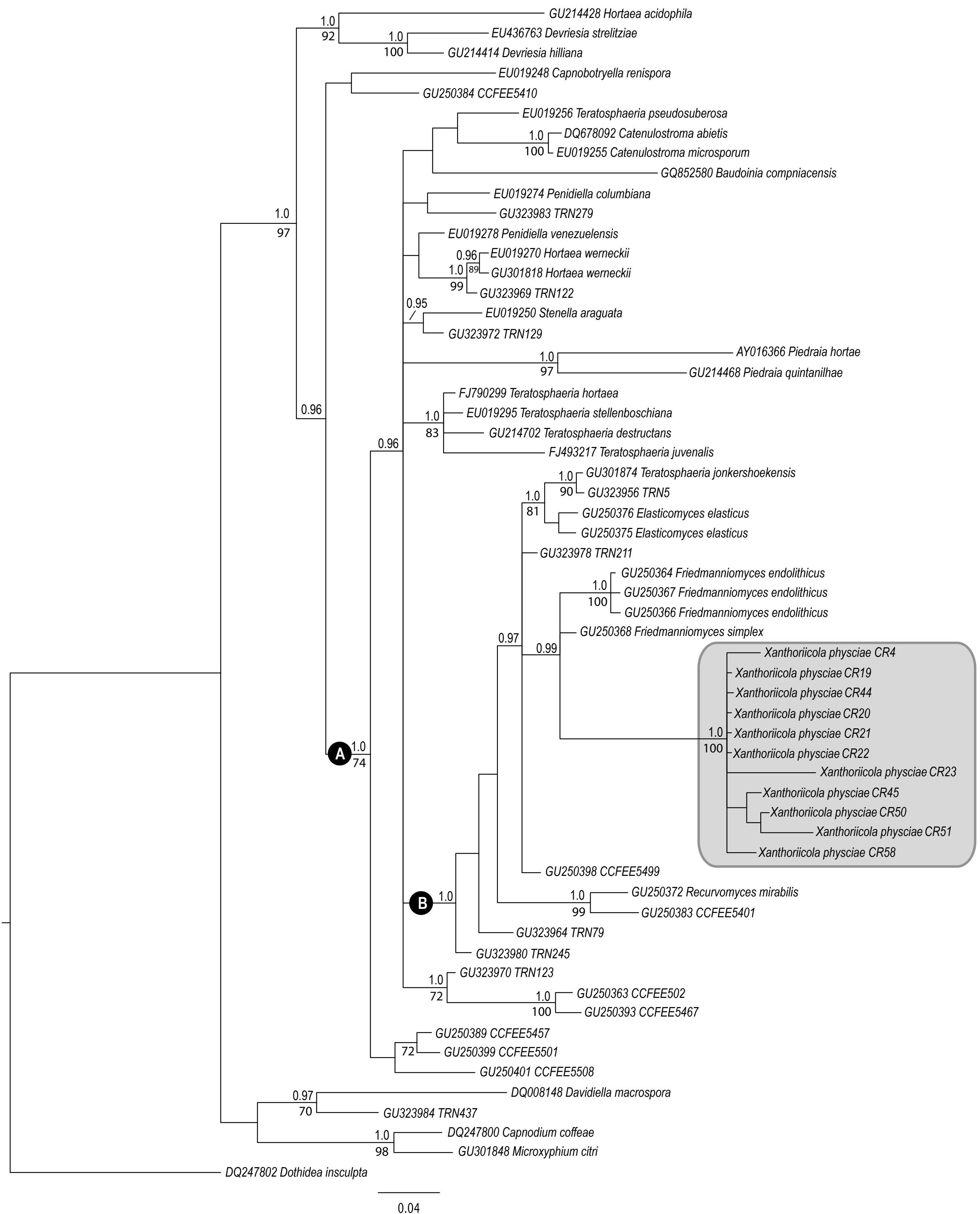

Fig. 3. 50 % majority rule Bayesian consensus tree with average branch lengths from the analysis of the nLSU dataset. Bayesian posterior probability values ≥ 0.95 are indicated over branches and ML bootstrap values ≥ 70 %, below branches. Branch lengths are scaled to the expected number of substitutions per site. A tinted box includes *Xanthoriicola physciae*. See the text for discussion of the clades distinguished as "A" and "B".

Table 1. Specimens of *Xanthoriicola physciae* from which sequence data were obtained, with details of reference collections where they are held and GenBank accession numbers.

Specimen no.	Reference collection no.	GenBank accession no.	Country	Locality
CR4	MAF-LICH 16882	JN040487	UK, England	St. Martins, Shropshire
CR19	MAF-LICH 16883	JN040488	UK, England	Kinton, Oswestry, Shropshire
CR20	MAF-LICH 16884	JN040489	UK, Wales	Kidwelly Quay, Carmarthenshire
CR21	MAF-LICH 16885	JN040490	UK, England	Ashtead, Surrey
CR22	MAF-LICH 16886	JN040491	UK, England	Headly Heath, Surrey
CR23	MAF-LICH 16887	JN040492	UK, England	Ashtead, Surrey
CR44	IMI 402504	JN040493	Germany	Regierungsbezirk Oberbayern, Bayern
CR45	K(M)116894	JN040494	UK, Wales	Aferedw, Powys
CR50	MAF-LICH 16888	JN040495	UK, England	Richmond upon Thames, Surrey
CR51	MAF-LICH 16889	JN040496	UK, England	Tickhill, S. Yorkshire
CR58	MAF-LICH 16890	JN040497	UK, England	Slapton Ley NNR, S. Devon

ML bootstrap (100 %), and Bayesian Posterior Probabilities (1.0) (Figs 2–3).

Some incongruence was found between the results of parsimony analysis and the two other analysis methods used, and therefore two topologies are shown. The maximum parsimony reconstruction is shown in Fig. 2. Since no conflicts were found between the Bayesian and the ML topologies, only the Bayesian reconstruction is shown in Fig. 3, with ML bootstrap values added. When the maximum parsimony method was used, *Piedraia* species appeared as the closest relatives of *Xathoriicola*, together forming a clade with 81 % Bootstrap support (Fig. 2). However, the Bayesian and maximum likelihood methods both recovered *Friedmanniomyces* as the sister group of *Xanthoriicola*, although only with strong phylogenetic support by the Bayesian method (Bayesian posterior probability = 0.99; Fig. 3). In this analysis, *Xanthoriicola* and *Friedmannimyces* are included in a more inclusive monophyletic group together with *Elasticomyces elasticus*, *Recurvomyces mirabilis*, *Teratosphaeria jonkershekensis*, and diverse unnamed rock inhabiting fungi (Bayesian posterior probability = 1.0).

DISCUSSION

The systematic placement of the monotypic lichenicolous genus *Xanthoriicola* has remained obscure in the absence of any known sexual stage. Both the analyses we undertook place it in the same general area of the fungal phylogenetic tree, but with some differences as to the taxa revealed as the closest known relatives.

In the maximum parsimony tree (Fig. 2), the genus *Piedraia* forms the sister group. *Piedraia* comprises two known species both of which form minute ascomata on hair; *P. hortae* on human hair ("black piedra") and *P. quintanilhae* on that of chimpanzees. The ascomata occur as black nodules on the hair, and have vermiform single-celled ascospores; the ascospores have whip-like extensions at both ends in *P. hortae*, but such extensions are absent in those of *P. quintanilhae*. No asexual state is known, and reports of one in *Trichosporon* are attributable to mixed infections. Excellent illustrations of *P. hortae*, and references to key literature, are provided by de Hoog *et al*. (2000). Sister to the *Piedraia*/*Xanthoriicola* clade in this analysis, but without bootstrap support, are two species of the hyphomycete genus *Friedmanniomyces,* which has no known sexual state.

Interestingly, in the Bayesian analysis (Fig. 3) it is the *Friedmanniomyces* species that appear as the sister group, with high support for the clade (0.99), the relationship to the *Piedraia* species being unresolved. This leads us to suspect that the relationship between *Xanthoriicola* and *Piedraia*, observed in the parsimony analyses, could be due to a long-branch attraction effect. Further analyses using additional molecular markers will be needed to ascertain this relationship. *Friedmanniomyces endolithicus* is found growing intermixed with cryptoendolithic lichen hyphae in the surface layers of sandstones in Antarctica. It has pale brown hyphae which give rise to chains of schizolytically produced doliiform conidia which are brown, 0(–3)-septate, and have truncated ends, and also forms multicellular balls of thick-walled brown cells (Seifert *et al*. 2011: pl 2D). In *F. simplex,* which occurs in similar situations, the conidia are darker brown, 1(–2)-celled, generally more elongated, with peculiar and often terminal chlamydospore-like cells, but no multicellular conidial balls. Both species grow in pure culture, and are described in detail by Selbmann *et al*. (2005).

Fungi in the clade labelled "B" in the Bayesian tree, with 1.0 support (Fig. 3) include two monotypic genera. *Recurvomyces mirabilis* which was also isolated from sandstone in Antarctica (Selbmann *et al*. 2008), but unlike the *Friedmanniomyces* species produces 0–1-septate, subhyaline to yellowish brown, thin-walled, elongate-ellipsoid conidia forming enteroblastically from the eponymous conidiophores which are characteristically often bent back towards the hyphae on which they arise. *Elasticomyces elasticus* was originally isolated from thalli of *Usnea antarctica* in Antarctica (Selbmann *et al*. 2008), but has since been found obtained from rock surfaces in the Alps, Andes, and Himalayas (Selbmann, pers. comm.). The conidium production in *Elasticomyces* is particularly distinctive. The pale to dark brown conidia are produced as arthrospores from long hyphae, the apices of which can continue to grow

while the distal regions break up into 1-septate to multiseptate fragments; the conidium walls can be smooth or somewhat rough. Superb illustrations of this fungus and of *Recurvomyces mirabilis* are provided by Selbmann *et al.* (2008).

Also present in clade "B" (Fig. 3) are *Teratosphaeria jonkershoekensis*, in which no conidial state is known, and numerous unnamed sequences, apparently all derived from cultures or sequences obtained from rocks; rock-inhabiting fungi (RIF). Of especial interest because of its sister group position to the *Friedmanniomyces*/*Xanthoriicola* clade is CCFEE5499. That fungus was isolated from rock surfaces in the Alps and presents as a non-sporulating, dark, felty on the surface, crustose mycelium, with a morphology characteristic of RIF; ITS analyses show it to be distinct from *F. endolithicus* but it does fall in a separate group of fungi all isolated from rock surfaces (Selbmann, pers. comm.).

The clade labelled as "A" (with 1.0 bootstrap support), which includes *Piedraia* and the *Friedmanniomyces*/*Xanthoriicola* clade, also comprises the clinically important black yeast *Hortaea werneckii* (responsible for "tinea nigra" on human hands or more rarely feet; de Hoog *et al*. 2000) and several other fungi of which species of *Teratosphaeria* predominate (Fig. 3). *Teratosphaeria* had been synonymized with *Mycosphaerella,* but, was resurrected by Crous *et al.* (2007) and placed in the new family *Teratosphaeriaceae*, a sister family to *Mycosphaerellaceae*. The type species, *T. fibrillosa,* has a superficial stroma linking the ascomata (not seen in most other species), ascospores becoming brown and verruculose while in the ascus and with a mucous sheath, a multilayered endotunica in the ascus, pseudoparaphysoidal remnants disappearing with age, and ostiolar periphyses present in some other species. Around 60 species are now recognised, but some have no known anamorphs, and some no known teleomorph. The anamorphs have been referred to 12 different genera, including *Hortaea* and *Pseudotaeniolina*.

We note that two sterile filamentous lichens, *Cystocoleus* and *Racodium*, also proved to have affinities with *Hortaea werneckii* (Muggia *et al.* 2008). Indeed, Crous *et al.* (2009) found that *Cystocoleus* fell into the *Teratosphaeriaceae* clade, while *Racodium* was more basal and incertae sedis. LSU sequences for these genera were included in the preliminary analyses to look for a possible relation to *Xanthoriicola*, but both grouped with sequences of the *Capnodiales* acting as an outgroup and so were not included in the final analyses. However, it is nevertheless interesting to note that these lichenized fungi have some phylogenetic relationship to fungi able to colonise lichen and plant tissues, and rock surfaces. All these are under extreme environmental conditions of dryness, large temperature fluctuations, solar radiation, and nutrient shortage. The adaptation of these different fungi to such diverse ecological strategies merits further exploration.

We conclude that *Xanthoriicola* should, on the basis of the LSU analyses carried out here, be most appropriately referred to the same family as *Piedraia* and *Teratosphaeria* in *Capnodiales.* This clade has strong support in both the maximum parsimony (93 %) and Bayesian (1.0) analyses. We note that *Piedrariaceae* (Barr 1979) is an earlier family name than *Teratosphaeriaceae* (Crous *et al.* 2007), but we do not take that up here as we consider that the affinity with *Piedraria* may be accentuated by long-branch attraction, and also that much more taxon-sampling in this part of the fungal tree of life is necessary to be confident about familial circumscriptions.

This study shows that technical advances can facilitate progress in the resolution of systematic placements for lichenicolous fungi with no sexual state and currently of uncertain position, and also that an understanding of the relationships of these taxa has implications for phylogenetic reconstructions in the *Dothideomycetes*. Further, molecular phylogenetics, using DNA from specimens, has the power to resolve problematic generic and species concepts in lichenicolous fungi.

ACKNOWLEDGEMENTS

We are grateful to Tom F. Preece for access to the results of his unpublished experiments with this fungus, to Mark R.D. Seaward for comments on the scytonemin report, and to Laura Selbmann for her new information of *Elasticomyces* and the origin and morphology of isolate CCFEE5499. Tom F. Preece, R. Nigel Stringer, and Heidi Döring are thanked for collecting additional material for use in our study. This investigation was undertaken as a part of a research grant to DLH from the Ministerio de Educación y Ciencia of Spain (Proyectos I+D CGL 2008-01600).

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