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Towards a new classification of the *Arthoniales* (*Ascomycota*) based on a three-gene phylogeny focussing on the genus *Opegrapha*

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ABSTRACT

A multi-locus phylogenetic study of the order *Arthoniales* is presented here using the nuclear ribosomal large subunit (nuLSU), the second largest subunit of RNA polymerase II (*RPB2*) and the mitochondrial ribosomal small subunit (mtSSU). These genes were sequenced from 43 specimens or culture isolates representing 33 species from this order, 16 of which were from the second largest genus, *Opegrapha*. With the inclusion of sequences from GenBank, ten genera and 35 species are included in this study, representing about 18 % of the genera and *ca* 3 % of the species of this order. Our study revealed the homoplastic nature of morphological characters traditionally used to circumscribe genera within the *Arthoniales*, such as exciple carbonization and ascomatal structure. The genus *Opegrapha* appears polyphyletic, species of that genus being nested in all the major clades identified within *Arthoniales*. The transfer of *O. atra* and *O. calcarea* to the genus *Arthonia* will allow this genus and family *Arthoniaceae* to be recognized as monophyletic. The genus *Enterographa* was also found to be polyphyletic. Therefore, the following new combinations are needed: *Arthonia calcarea* (basionym: *O. calcarea*), and *O. anguinella* (basionym: *Stigmatidium anguinellum*); and the use of the names *A. atra* and *Enterographa zonata* are proposed here. The simultaneous use of a mitochondrial gene and two nuclear genes led to the detection of what seems to be a case of introgression of a mitochondrion from one species to another (mitochondrion capture; cytoplasmic gene flow) resulting from hybridization.

Introduction

The *Arthoniales* are one of the main lichenized groups of the *Pezizomycotina* and are currently classified in the *Arthoniomycetes* (Hibbett *et al.* 2007; Spatafora *et al.* 2006). Their ascomata are usually apothecial in contrast to their closest relatives, the *Dothideomycetes* (Spatafora *et al.* 2006). Most species form lichen symbioses with trentepohlioid algae. The order currently

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includes three families (*Arthoniaceae*, *Chrysothricaceae*, and *Roccellaceae*), *ca* 55 genera and *ca* 1200 species. More than half of the species are included in the genera *Arthonia* and *Opegrapha* with *ca* 400 and 300 species, respectively (Kirk *et al.* 2001). The order is a major component of the lichen flora of many forest types, especially in the tropics where many corticolous and foliicolous species occur. It is also well represented in saxicolous habitats, especially in subtropical coastal habitats with a Mediterranean or desert type climate (Mediterranean area, Socotra island, southern California, the central Chilean coast and southern Africa) (Follmann & Werner 2003; Tehler 1983, 1990). Over 100 species belonging to the *Arthoniaceae* and *Roccellaceae* are known to grow as lichenicolous fungi on diverse hosts. Most of them are highly host-specific and commensalic (Lawrey & Diederich 2003).

The family concept within the *Arthoniales* has changed considerably during the past decades. Luttrell (1973) classified the *Arthoniaceae*, *Opegraphaceae* (including the *Roccellaceae*) and *Lecanactidaceae* in the order *Hysteriales* on the basis of their ascomata being somewhat similar to those of the *Hysteriaceae*, with boat-shaped to linear carbonaceous pseudothecia opening by a longitudinal slit. He suggested that the *Arthoniaceae* could be related to the *Myriangiales* owing to the structure of the ascomata being a tangled mass of hyphae in which the globoid asci are embedded. Henssen & Jahns (1974) distinguished the families *Arthoniaceae*, *Opegraphaceae*, *Lecanactidaceae,* and *Roccellaceae* in the *Arthoniales* assuming that the latter three families are more closely related than all to the *Arthoniaceae*. Earlier, Poelt (1973) suggested that the *Lecanactidaceae* should not be segregated from the *Opegraphaceae*. Arx & Müller (1975) placed the *Arthoniaceae* in the order *Dothideales*, omitting *Lecanactidaceae*, *Opegraphaceae,* and *Roccellaceae* from their classification. Barr (1979) placed the *Opegraphaceae* and *Roccellaceae* in the *Hysteriales*, and the *Arthoniaceae* in the *Myriangiales*. The *Arthoniales* (*Arthoniaceae*, *Chrysothricaceae,* and tentatively the *Seuratiaceae*) and *Opegraphales* (*Opegraphaceae* and *Roccellaceae*) were accepted as separate orders by Hawksworth & Eriksson (1986) who published both names validly. Within the *Opegraphales*, the species with a crustose, ecorticate thallus and lecideine ascomata were included in the *Opegraphaceae*, whilst the *Roccellaceae* (*sensu* Tehler 1990, 1993) included species with a crustose or fruticose, usually corticate thallus and ascomata with a well-developed thalline margin. Hafellner (1988) suggested a close relationship between the *Opegraphales* and *Arthoniales,* which were later merged in the class *Arthoniomycetes* (Eriksson & Winka 1997).

Tehler's (1990) first phylogenic hypothesis of the *Arthoniales*, focusing mostly on the *Roccellaceae* and based on morphological, chemical, and anatomical data, confirmed *Arthoniales* and *Opegraphales* together as a monophyletic group. He suggested including the *Opegraphales* in the *Arthoniales*. Hawksworth *et al.* (1995) and Grube (1998) expanded the *Roccellaceae* to include the *Opegraphaceae* and other genera, such as *Chiodecton*, *Schismatomma,* and *Syncesia*, considered of uncertain family affiliation by Tehler (1993). Current generic concepts are mainly based on characters such as thallus structure, chemistry, and ascomatal anatomy, including the degree of ascomatal carbonization, internal ascomatal structure, ascus types, and ascospore septation.

So far, only few representatives of *Arthoniales* have been included in molecular phylogenetic studies, and almost no molecular data have been published for the crustose taxa, including the important genera *Arthonia* and *Opegrapha,* and very few taxa had more than one locus in GenBank. Tehler (1995a,b), who published the first *Arthoniales* sequences (nuSSU), found incongruence between molecular and morphological datasets. In Tehler (1995a), *Lecanactis abietina* did not cluster with other members of the *Arthoniales* (*Arthonia radiata*, *Dendrographa leucophaea,* and *Schismatomma pericleum*), but strangely was found to be closely related with *Porpidia crustulata* (sub. *Lecidea crustulata*) of the *Lecanorales*. When the same sequences were included in a broader phylogenetic context, including representative species from the *Ascomycota* and *Basidiomycota*, the monophyly of the *Arthoniales* was found to be well-supported (Gargas *et al.* 1995). Based on multilocus phylogenetic analyses, the *Arthoniomycetes* have been reported to be sister to the *Dothidiomycetes* by Lutzoni *et al.* (2004) but with low support. Spatafora *et al.* (2006) confirmed this result using a more extensive taxon and locus sampling.

Myllys *et al.* (1998) used partial sequences from the nuSSU rDNA of 18 taxa to investigate the phylogenetic relationships in the order *Arthoniales* focusing on the family *Roccellaceae*. Because this locus was too conservative for solving phylogenetic relationships among closely related genera, ITS data were added to an extended dataset including 33 taxa to provide more resolution (Myllys *et al.* 1999). Significant incongruence between the molecular and morphological datasets were shown and assumed to be due to a high level of homoplasy in the morphological data (e.g. placement of *Schismatomma*, *Lecanactis*). Tehler & Irestedt (2007) investigated the phylogenetic relationships within the family *Roccellaceae s. str.* based on LSU and *RPB2* sequences from 48 taxa including mainly members of the genera *Roccella* and *Roccellina*. The results of these phylogenetic analyses also suggest that the fruticose/crustose habits have evolved multiple times in the family *Roccellaceae s. str.* and that character states, such as fruticose and crustose, may have been overemphasized in morphologically based classifications.

The order *Arthoniales* was never subjected to a broad and exhaustive molecular phylogenetic study. The two main genera of this order, *Arthonia* and *Opegrapha*, are considered as heterogeneous assemblages (Grube *et al.* 1995; Matzer 1996; Pentecost & Coppins 1983) based on morphology. Some allied genera, including the recently monographed genus *Enterographa* (Sparrius 2004), can also be considered as heterogeneous. No sequences from these crustose genera have ever been included in analyses focusing on the *Arthoniales*. The aim of this paper is to confront the current morphology–anatomy-based classification with a multi-locus phylogeny of the *Arthoniales* and to discuss the taxonomic value of diagnostic characters used to define genera and families within this order.

Material and methods

Contaminations with co-occurring fungi are frequent when using standard DNA isolation protocols on lichen thalli (see Hofstetter *et al.* 2007). This is especially the case with taxa having inconspicuous thalli and collected in the tropics (see Arnold *et al.* in press), such as most *Opegrapha* species. DNA amplifications have been particularly difficult for

members of the *Arthoniaceae*. Therefore, cultures of the mycobionts were necessary to ensure the reliability of sequences obtained from such taxa. Furthermore, many genera from the *Arthoniales* are very rare and, therefore, fresh specimens for molecular studies are difficult to obtain. Tropical taxa are often poorly known and in need of a taxonomic revision, especially in large genera such as *Arthonia* and *Opegrapha*, and therefore identifications of many species are often problematic.

Taxon sampling and cultures

Thirty-one mycobionts were cultured for the purpose of this study (Table 1). Cultures were isolated from ascospores (multispore cultures) of freshly collected material on malt–yeast-extract medium as described by Yoshimura *et al.* (2002). When cultures were not available, well-preserved and freshly collected lichen specimens lacking any visible symptoms of fungal infection were used for DNA isolation. The DNA of 12

Table 1 – Specimens and DNA sequences used in this study, with their respective voucher information

Name	Voucher	Substrate	nuLSU	mtSSU	*RPB2*	Specimen in culture
Arthonia cinnabarina	Rwanda, *D. Ertz* 8730 (BR)	Bark	–	**EU704046**	**EU704009**	+
A. didyma	Belgium, *D. Ertz* 7587 (BR)	Bark	**EU704083**	**EU704047**	**EU704010**	+
A. radiata	Belgium, *D. Ertz* s. n. (BR)	Bark	–	**EU704048**	**EU704011**	+
Arthonia sp. 1	Rwanda, *D. Ertz* 7775 (BR)	Bark	**EU704084**	**EU704049**	**EU704012**	+
Arthonia sp. 2	Florida, *D. Ertz* 9090 (BR)	Bark	–	**EU704050**	**EU704013**	+
Chiodecton natalense	Zambia, *D. Ertz* 6576 (BR)	Bark	**EU704085**	**EU704051**	**EU704014**	–
Cryptothecia candida	Gabon, *D. Ertz* 9260 (BR)	Leaf	–	**EU704052**	**EU704015**	+
Cryptothecia sp.	Rwanda, *D. Ertz* 8472 (BR)	Bark	–	**EU704053**	**EU704016**	+
Cudonia circinans	JP232; AFTOL-ID353	–	12025062	46411455	52699795	–
Curvularia brachyspora	ATCC58872; ATCC12330	–	12025063	46411456	6606124	–
Dendrographa leucophaea	California, *L. Sparrius* 7999 (DUKE)	Bark	47499217	47499218	**EU704017**	–
Enterographa anguinella	Gabon, *D. Ertz* 10027 (BR)	Bark	**EU704086**	**EU704054**	**EU704018**	+
Enterographa sp. 1	Gagon, *D. Ertz* 9770 (BR)	Leaf	**EU704087**	**EU704055**	**EU704019**	+
E. crassa	France, *D. Ertz* 5041 (BR)	Bark	**EU704088**	**EU704056**	**EU704020**	–
E. crassa	France, *D. Ertz* 7554 (BR)	Bark	–	**EU747080**	–	+
E. crassa	Belgium, *D. Ertz* 7561 (BR)	Bark	–	**EU747081**	–	+
E. crassa	Luxembourg, *D. Ertz* 7621 (BR)	Bark	–	**EU747082**	–	+
E. hutchinsiae	Belgium, *D. Ertz* 10066 (BR)	Bark	**EU704089**	**EU704057**	**EU704021**	–
E. hutchinsiae	Belgium, *D. Ertz* 10064 (BR)	Rock	–	**EU747083**	–	–
Erythrodecton granulatum	Gabon, *D. Ertz* 9908 (BR)	Bark	**EU704090**	**EU704058**	**EU704022**	–
Lecanactis abietina	Belgium, *D. Ertz* 5068 (DUKE)	Bark	47499219	47499220	49175462	–
Lecanactis sp. 1	Rwanda, *D. Ertz* 7995 (BR)	Bark	**EU704091**	**EU704059**	**EU704023**	+
Lecanactis sp. 2	La Réunion, *D. Ertz* 4780 (BR)	Bark	**EU704092**	**EU704060**	**EU704024**	–
Opegrapha atra	France, *D. Ertz* 8911 (BR)	Bark	–	**EU704061**	**EU704025**	+
O. bicolor	Rwanda, *D. Ertz* 8731 (BR)	Bark	**EU704093**	**EU704062**	**EU704026**	+
O. calcarea 1	France, *D. Ertz* 7545 (BR)	Rock	–	**EU704063**	**EU704027**	+
O. calcarea 2	France, *D. Ertz* 7539 (BR)	Rock	–	**EU704064**	**EU704028**	+
O. calcarea 3	France, *D. Ertz* 7540 (BR)	Rock	–	**EU704065**	**EU704029**	+
O. celtidicola	Portugal, *P. Diederich* 16053 (BR)	Bark	**EU704094**	**EU704066**	**EU704030**	+
O. filicina	Rwanda, *D. Ertz* 7994 (BR)	Leaf	**EU704095**	**EU704067**	**EU704031**	+
O. lithyrga	Belgium, *D. Ertz* 8784 (BR)	Rock	**EU704096**	**EU704068**	**EU704032**	+
O. longissima	Florida, *D. Ertz* 9155 (BR)	Bark	**EU704097**	**EU704069**	**EU704033**	+
O. niveoatra	Belgium, *D. Ertz* 7529 (BR)	Bark	**EU704098**	**EU704070**	**EU704034**	+
O. ochrocheila s. lat.	Rwanda, *D. Ertz* 8624 (BR)	Bark	**EU704099**	**EU704071**	**EU704035**	+
O. ochrocheila 1	Luxembourg, *D. Ertz* 7519 (BR)	Bark	**EU704100**	**EU704072**	**EU704036**	+
O. ochrocheila 2	Belgium, *D. Ertz* 7500 (BR)	Rock	**EU704101**	**EU704073**	**EU704037**	+
O. rufescens	Belgium, *N. Vigneron* 75 (BR)	Bark	**EU704102**	**EU704074**	**EU704038**	–
O. varia	France, *D. Ertz* 7570 (BR)	Bark	**EU704103**	**EU704075**	**EU704039**	+
O. vermicellifera	Belgium, *D. Ertz* 7562 (BR)	Bark	**EU704105**	**EU704077**	**EU704041**	+
O. viridis	Luxembourg, *D. Ertz* 7619 (BR)	Bark	**EU704106**	**EU704078**	**EU704042**	+
O. cf. *viridis*	Rwanda, *D. Ertz* 7807 (BR)	Bark	**EU704107**	**EU704079**	**EU704043**	+
O. viridistellata	La Réunion, *D. Ertz* 4795 (BR)	Bamboo stem	**EU704104**	**EU704076**	**EU704040**	–
O. vulgata	Belgium, *D. Ertz* 7564 (BR)	Bark	**EU704108**	**EU704080**	**EU704044**	+
O. zonata	Belgium, *N. Vigneron* 104 (BR)	Bark	**EU704109**	**EU704081**	**EU704045**	–
O. zonata	Belgium, *D. Ertz* 9230 (BR)	Rock	–	**EU747084**	–	–
Roccella fuciformis	Tenerife, *P. Diederich* 15572 (DUKE)	Rock	46411443	**EU704082**	110669591	–
Schismatomma pericleum	*A. Tehler* 7701 (S)	Bark	12025091	50429157	–	–
Seynesia erumpens	SMH1291 (F)	–	12025093	46411476	52699873	–

GenBank accession numbers (in bold) refer to sequences (106) generated by this project. All other sequences (18 GenBank identification numbers) were obtained directly from GenBank.

additional taxa was sequenced directly from specimens. We obtained 106 new sequences from 43 specimens belonging to 33 taxa from continental Africa (Gabon, Rwanda, Zambia), Europe (Belgium, France, Luxembourg, Portugal), La Réunion, and North America (California, Florida). Eighteen sequences were added from GenBank. The three outgroup species were chosen based on Lutzoni *et al.* (2004): *Curvularia brachyspora* (*Dothideomycetes*), *Seynesia erumpens* (*Sordariomycetes*), and *Cudonia circinans* (*Leotiomycetes*). In total, the dataset for the multi-locus phylogenetic tree presented here includes 43 specimens representing 38 species.

Molecular data

Genomic DNA was isolated from mycobiont cultures or from lichen specimens using the Puregene Genomic DNA Purification Kit (GENTRA Systems, Minnesota) following the manufacturer's Plant Tissue extraction protocol. Amplification reactions were prepared for a 50 µl final volume containing 5 µl 10 × Taq Buffer (Roche, Basel), 2.5 µl of each of the 20 µM primers, 1 µl of 10 mg ml^{-1} bovin serum albumin (Ambion # 2616), 1 µl of 25 mM $MgCl_2$, 1.25 U Taq DNA polymerase (Roche) and 1 µl template genomic DNA. PCR was performed on Peltier Thermal Cyclers PTC-100 or PTC-150 (MJ Research-Biorad, Hercules, CA). A targeted fragment of about 1.4 kb at the 5' end of the nuLSU rDNA was amplified using primers LR0R (Rehner & Samuels 1994), LIC15R (Miadlikowska *et al.* 2002), or LIC24R (Miadlikowska & Lutzoni 2000) with LR7 (Vilgalys & Hester 1990). A fragment of about 1 kb of the *RPB2* protein-coding gene was amplified and sequenced using primers fRPB2-7cF and fRPB2-11aR (Liu *et al.* 1999). Primers for amplification and sequencing of the mtSSU rDNA were mrSSU1 and mrSSU3R (Zoller *et al.* 1999). Cloning, when required, was performed with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hiklen). The yield of the PCRs was verified by running the products on a 1 % agarose gel using ethidium bromide. Both strands of nuLSU, mtSSU, and *RPB2* were sequenced directly using BigDye terminators (Applied Biosystems, Foster City, CA) and the amplification primers. For nuLSU, additional primers for sequencing were used: LR3R, LR3, LR5, and LR5R (Vilgalys & Hester 1990; Vilgalys' website, http://www.botany.duke.edu/fungi/mycolab). Sequence fragments were assembled with Sequencher version 4.6 (Gene Codes Corporation, Ann Arbor, MI). Sequences were subjected to BLAST searches to verify their closest relatives and to detect potential contaminations.

Phylogenetic analyses

NuLSU, mtSSU, and *RPB2* sequences for taxa listed in Table 1 were aligned using MacClade 4.05 (Maddison & Maddison 2002). The alignment of nuLSU sequences was improved using the secondary structure of *Saccharomyces cerevisiae* (http://www.rna.icmb.utexas.edu) following Kjer (1995).

Because it was not possible to complete the nuLSU, mtSSU, and *RPB2* sequences for the same set of 43 samples, analyses for incongruence among loci were carried out on datasets with 33 samples for which all three genes were sequenced (Fig 1), in addition to using the most complete datasets for each gene (i.e., 34 nuLSU, 43 mtSSU, and 42 *RPB2* sequences; Tables 1 and 2). To detect significant conflicts among datasets, each single-locus alignment was analysed separately using maximum likelihood (ML) with RAxML-VI-HPC (Stamatakis *et al.* 2005). Bootstrap (BS) proportions were calculated with 1 K BS replicates implementing the GTRMIX model with gamma distribution, approximated with four categories. A conflict among single-locus datasets was considered significant if a well-supported monophyletic group, e.g. ML BS ≥ 70 % (Mason-Gamer & Kellogg 1996) was found to be well-supported as non-monophyletic using a different locus. Because we detected a significant topological conflict between the mitochondrial gene tree and the two nuclear gene trees within one clade with four taxa, we sequenced the mtSSU of additional specimens of three species of this clade to verify whether the conflict could be due to contaminations (Table 1: samples *Enterographa crassa* Ertz 7554, 7561, 7621; *E. hutchinsiae* Ertz 10064, and *Opegrapha zonata* Ertz 9230). These additional sequences were all identical to those used in the analyses (samples *E. crassa* Ertz 5041, *E. hutchinsiae* Ertz 10066, and *O. zonata* Ertz 9230). We were not able to verify the mtSSU of *Erythrodecton granulatum* because we had only one specimen of this species. We also tested each gene separately to determine whether nucleotide base composition heterogeneity could explain this result. A chi-square test of homogeneity of base frequencies across taxa was performed with PAUP 4.0b10 (Swofford 2002).

Two combined three-locus datasets were assembled: a 33-taxon combined dataset with no missing sequences and a 43-taxon dataset (supermatrix approach) with one missing sequence of the *RPB2* gene and nine missing sequences of the nuLSU gene. ML search for the most likely tree on the three-locus datasets for 33 and 43 taxa was conducted with 1 K replicates using RAxML with the same settings as applied in the BS analyses on single genes, but recognizing five data partitions (nuLSU, mtSSU, *RPB2*/1st, 2nd and 3rd codon positions). ML BS values were derived from 1 K BS replicates using RAxML with the same settings as applied on the original concatenated datasets. In addition, Bayesian analyses using Bayesian Metropolis coupled MCMC (B-MCMCMC) as implemented in MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) were conducted on the three-locus 43-taxon supermatrix dataset with the same five data partitions as in the ML analysis. Models of evolution for the Bayesian analysis were estimated using the Akaike Information Criterion (AIC) as implemented in Modeltest v3.7 (Posada & Crandall 1998). Bayesian analyses were implemented with four independent chains, with every 500th trees sampled for 20 M generations, using a GTR model of nucleotide substitution (Rodríguez *et al.* 1990) including proportion of invariable sites and a gamma distribution of four categories. To ensure that the runs reached stationarity and converged on the same log-likelihood level, chains were examined by eye and using AWTY (http://ceb.csit.fsu.edu/awty). Posterior probabilities (PP) and 50 % majority-rule consensus tree were generated from the last 30 K of the 40 K trees sampled. PP ≥ 95 % and ML BS ≥ 70 % were considered to be significant.

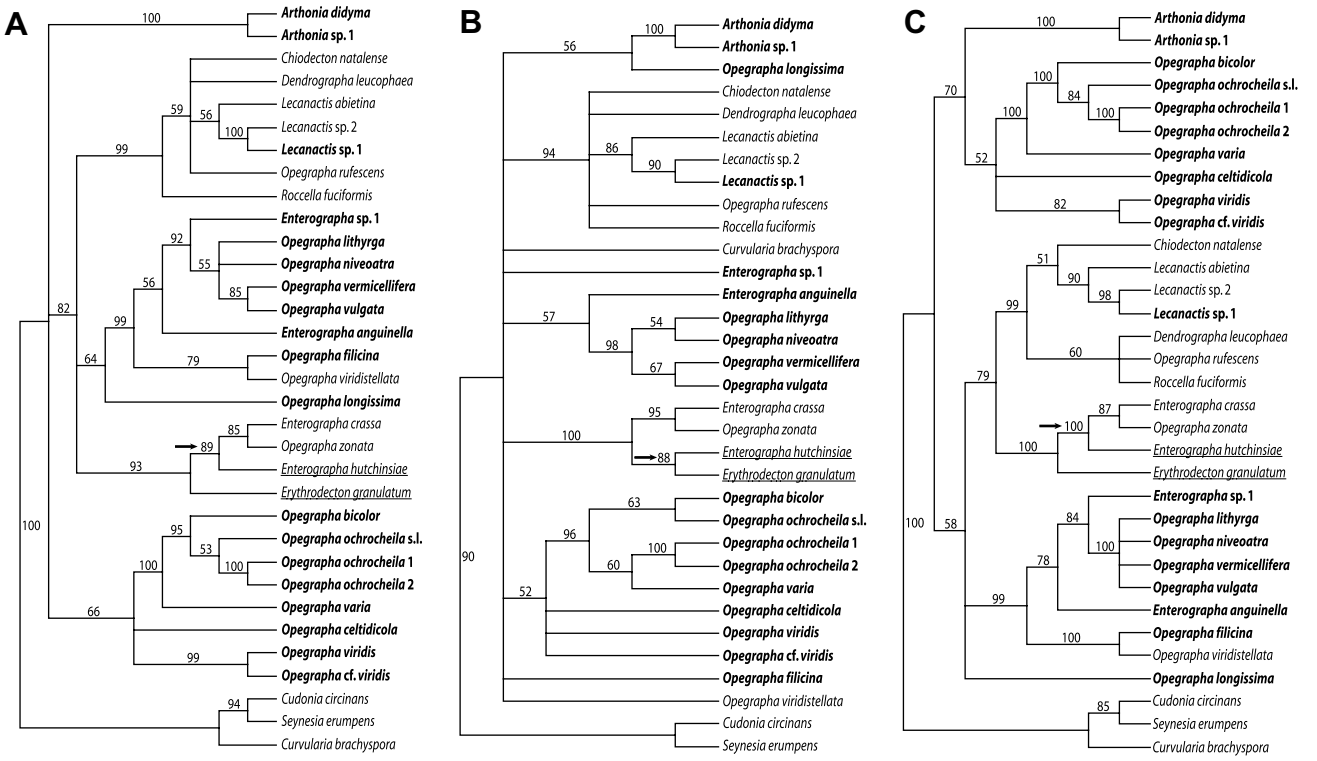

Fig 1 – Detection of incongruence among single-gene phylogenies based on ML BS analyses of all samples (33 samples representing 31 species) for which we were successful in obtaining the three genes targeted for this study. (A) nuLSU, (B) mtSSU, and (C) *RPB2*. Arrows point to the conflicting relationship, considered to be significant using a 70 % BS support threshold value. Taxa which are part of this topological conflict are underlined. Taxa for which DNA was isolated from axenic cultures, rather than directly frowhich are m thalli, are shown in bold.

Results

Single-locus phylogenetic analyses

The congruence analyses revealed one significant conflict between the mitochondrial and the nuclear genes (Fig 1). The phylogeny based on mtSSU shows *Enterographa hutchinsiae* as being sister to *Erythrodecton granulatum* (Fig 1B), whereas the nuclear gene phylogenies (nuLSU and *RPB2*) show independently the relationships of these two taxa as being paraphyletic (Fig 1A, C). All other inconsistencies among single-gene topologies were non-significant, i.e. resulting from the expected lack of accuracy associated with datasets containing few characters. No conflicts were detected between the two nuclear gene phylogenies.

Multi-locus phylogenetic analyses

Because the inclusion or exclusion of the mtSSU dataset, and of the taxon causing these topological conflicts (*Erythrodecton granulatum*), in this multi-gene study of the *Arthoniales* did not alter our conclusions, these three datasets were concatenated without removing *E. granulatum*. Three main, well-supported (ML BS $\geq$ 97 and PP = 100), monophyletic groups were recovered within the *Arthoniales*, corresponding to the *Arthoniaceae*, *Opegrapha varia* group, and *Roccellaceae s. str.* (Fig 2). Two well-supported sister groups were revealed within the *Arthoniaceae*. One group comprises the five species of *Arthonia* (*A. cinnabarina*, *A. didyma*, the generic type *A. radiata*, and two unidentified tropical species), as well as *Opegrapha atra* and three specimens of *O. calcarea* (ML BS = 87; PP = 100). The relationships between these taxa are well resolved and supported. The second monophyletic group (ML BS = 89; PP = 94) is represented by two *Cryptothecia* species, *C. candida* and *C.* sp.

The phylogenetic placement of the *Opegrapha varia* group remains uncertain (Fig 2). If the addition of data reveals this group of species to be sister to the *Roccellaceae s. str.*, these species would continue to be classified within the *Roccellaceae*. *Roccellaceae s. str.* include here two well-supported main groups and *Opegrapha longissima* with a poorly supported phylogenetic placement. The largest of these groups includes two distinct monophyletic groups: the *Roccella* and *Enterographa* groups. The *Roccella* group includes eight species representing six genera (Fig 2). Relationships within this group are poorly supported, except for the three *Lecanactis* species that form a well-structured and strongly supported monophyletic group (ML BS = 100, PP = 100). The *Enterographa* group includes four species from three different genera (*Enterographa*, *Erythrodecton*, and *Opegrapha*). The second main group within the *Roccellaceae s. str.* (*Opegrapha vulgata* group) includes eight species from two genera (*Enterographa* and *Opegrapha*). *O. lithyrga*, *O. niveoatra*, *O. vermicellifera* and *O. vulgata* form a strongly supported monophyletic group (ML BS = 100, PP = 100). This

Table 2 – Summary of alignment lengths and number of included and excluded characters for each dataset

Datasets	Total no. of characters	No. of excluded characters	No. of included characters	Percentage of included characters	No. of variable characters
34 nuLSU sequences	2801	1491	1310	46.8	435
43 mtSSU sequences	2185	1675	510	23.3	235
42 RPB2 sequences	921	96	825	89.6	497
43 Concatenated sequences	5907	3262	2645	44.8	1167

group, together with *Enterographa anguinella* and *E.* sp. 1 form another well-supported group that is sister to a clade including the foliicolous species *O. filicina* and *O. viridistellata*.

Taxonomy

Based on our multi-gene phylogenetic study, the following new combinations are proposed:

Arthonia calcarea (Turner ex Sm.) Ertz & Diederich, **comb. nov.**
MycoBank No.: MB 512316
Basionym: *Opegrapha calcarea* Turner ex Sm., *Engl. Botan.* **25**: tab. 1790 (1807).

Opegrapha anguinella (Nyl.) Ertz & Diederich, **comb. nov**.
MycoBank No.: MB 512317
Basionym: *Stigmatidium anguinellum* Nyl., *Ann. Sci. nat. Bot.*, sér. 4, **19**: 381 (1863).
Syn.: *Enterographa anguinella* (Nyl.) Redinger, *Feddes Repertorium* **43**: 62 (1938).

Furthermore, we find more appropriate to use the following two combinations in the future:

Arthonia atra (Pers.) A. Schneid., *Guide Study Lich.*: 131 (1898), instead of *Opegrapha atra* Pers., *Bot. Mag.* (Roemer & Usteri) **7**: 30 (1794)
Enterographa zonata (Körb.) Källsten ex Torrente & Egea, *Bibl. Lich.* 32: 198 (1989), instead of *Opegrapha zonata* Körb., *Syst. Lich. Germ. (Breslau)*: 279 (1855).

Discussion

The family *Arthoniaceae* is characterized by globose to clavate asci with a strongly thickened tholus belonging to the *Cryptothecia*, *Arthothelium*, or *Arthonia* type, as illustrated in Grube (1998). The most striking result of the present study is the phylogenetic position of *Opegrapha atra* and *O. calcarea* (Fig 3K–L) nested within the genus *Arthonia* (Fig 2), which implies that the *Arthoniaceae* are only monophyletic when these species are included. An important morphological character that can support the inclusion of these two *Opegrapha* species within *Arthonia* is the ascus type [shortly clavate with an apically thickened wall, as in the type species *A. radiata* (Fig 3M–N)]. In his identification key to the genera of the *Arthoniales*, Grube (1998) used the ascus type to separate the *Arthoniaceae* from the other *Arthoniales* and, interestingly, noticed that the asci of the '*Opegrapha calcarea* group' are similar to those of *Arthonia*. Moreover, the type of ascomatal amorphous cell wall pigment in *O. atra* and *O. calcarea* is very similar, if not the same, to this type of pigment in *Arthonia radiata*, which differs from the pigment in other more or less well-delimited groups in *Arthonia* (Grube pers. comm.).

The genus *Cryptothecia* differs from *Arthonia* by the lack of well-developed ascomata and by globose asci loosely scattered in the thallus. Sometimes asci are aggregated in distinct thallus patches and are then always separated by hydrophobic plectenchyma (Grube 1998). Both *Cryptothecia* species included in our study, the foliicolous *Cryptothecia candida* and an unidentified corticolous *Cryptothecia* species, form a monophyletic group sister to *Arthonia* (Fig 2).

Additional species and genera will have to be added in future studies in order to confirm the monophyly of the *Arthoniaceae*. The most interesting results are expected in the large (*ca* 400 species) and heterogeneous genus *Arthonia* (Grube *et al.* 1995; Matzer 1996).

The family *Roccellaceae* in its current delimitation (Eriksson 2006; Kirk *et al.* 2001) is polyphyletic in our tree (Fig 2). If we accept that *O. atra* and *O. calcarea* belong to the *Arthoniaceae* (see above), two distinct well-supported clades can be distinguished within the remaining paraphyletic *Roccellaceae*.

The *O. varia* group is strongly supported and might represent a distinct family (Fig 2). According to Torrente & Egea (1989), all species of that group have an ascus of the '*Varia* type', with the exception of *O. viridis s. lat.* that has an ascus of the '*Vulgata* type'. We do not have any diagnostic character state to support this group. Species included in this group need to be included in another genus, or other genera, given that the type species of *Opegrapha* is *O. vulgata* (Figs 2 and 3A–B). Current synonyms of the genus *Opegrapha* exist to accommodate such species. However, more taxa need to be included in future phylogenetic studies before attempting to circumscribe such putative genera.

Within the *Roccellaceae s. str.*, the *Roccella* group comprises the generic type, *R. fuciformis* (Fig 2). This group can be considered as the core of the family *Roccellaceae*. The three *Lecanactis* species, including the generic type *L. abietina* (Fig 3O–P), form a well-supported monophyletic group. This result agrees with the phylogenetic analyses performed on 24 species of the genus using morphological, anatomical, and chemical data that supported *Lecanactis* as monophyletic (Tehler & Egea 1997).

The family *Opegraphaceae* was accepted by many authors before being included in the *Roccellaceae* by Hawksworth *et al.* (1995). Amongst the genera present in our tree, the *Opegraphaceae* comprised the genera *Chiodecton*, *Enterographa*, *Erythrodecton*, *Lecanactis*, *Opegrapha,* and *Schismatomma*,

whereas the *Roccellaceae* included *Dendrographa* and *Roccella* (e.g. Eriksson & Hawksworth 1993). Interestingly, the *Roccella* group (Figs 2, 3O–T) includes members of crustose and fruticose genera (*Opegraphaceae* and *Roccellaceae*, respectively; in their traditional sense). The fruticose growth form was used to define the *Roccellaceae sensu* Tehler (1990), but this feature is probably homoplasious as indicated by Myllys *et al.* (1999). Because the *Roccella* group is strongly supported and comprises typical members of both families, we confirm that the family *Opegraphaceae* in its traditional sense is not monophyletic. In the phylogenetic study by Tehler (1990) based on morphological, chemical and anatomical data from the *Arthoniales*, *Lecanactis* (represented by the generic type, *L. abietina*) was sister to *Opegrapha* (represented by the generic type, *O. vulgata*). Our results clearly show an incongruence between the morphological and the molecular data as the genus *Lecanactis* is more closely related to members of the genera *Chiodecton*, *Dendrographa*, *Roccella*, *Schismatomma*, *Enterographa*, and *Erythrodecton*, than to the *O. vulgata* group.

Traditionally, the genus *Opegrapha* included species with lirelliform ascomata having a distinct carbonized excipulum (Fig 3). Our study revealed that species previously recognized as *Opegrapha* are found in all main monophyletic groups across the *Arthoniales* (Fig 2). The polyphyly of the genus can only be partly explained by morphological characters. The carbonization of the excipulum cannot be used alone to characterize the genus *Opegrapha*. However, it is the only morphological character state used to distinguish *Opegrapha* from *Enterographa* and other genera. These results led to the problem of choosing phenotypic character states reflecting monophyletic groups (genera in this case). So far, the *O. varia* group includes only *Opegrapha* species for which we do not have any morphological synapomorphies. However, the placement of this group is uncertain. The available data cannot exclude a putative sister relationship to the *Roccellaceae s. str.* (Fig 2).

The *O. vulgata* group includes the type species of the genus *Opegrapha*, *O. vulgata* (Fig 3A–B), together with three very closely related species (*O. lithyrga*, *O. niveoatra,* and *O. vermicellifera*). These four species represent the core of the genus *Opegrapha* (Fig 2). All these species are corticolous, with the exception of *O. lithyrga*, which is saxicolous. The two foliicolous *Opegrapha* species included in our study, *O. filicina* and *O. viridistellata*, form a monophyletic group sister to the rest of the taxa part of the *O. vulgata* group. They share a common photobiont genus, *Phycopeltis*, whereas all other *Opegrapha* species included in our study are in symbiosis with *Trentepohlia*.

The position of *Opegrapha rufescens* (Fig 3Q–R) within the *Roccella* group demonstrates that it does not belong to *Opegrapha s. str.* Pentecost & Coppins (1983) already noticed that a high similarity exists between some forms of *O. rufescens* and *Schismatomma graphidioides*. Based on morphology, and because *O. rufescens* is more closely related to the type species of *Schismatomma* (*S. pericleum*, Fig 3S) than to members of *Opegrapha s. str.*, it would be convenient to subsume *O. rufescens* within this genus. However, both species do not form a monophyletic group in our tree and relationships among these taxa and other most closely related taxa are poorly supported (Fig 2). More, fast-evolving, molecular characters are required to resolve the relationships between the different taxa of the *Roccella* group with high confidence.

The *Enterographa* group includes the type species of the genus *Enterographa*, *E. crassa* (Figs 2, 3E–F). As the excipulum of *Opegrapha zonata* is only carbonized in the upper half, being hyaline below (Fig 3G–H), the generic position of that species was a matter of debate. This species was described as an *Opegrapha* by Körber (1855), transferred to *Enterographa* by Torrente & Egea (1989), but maintained in *Opegrapha* by other authors (e.g., Pentecost & James 1992). *Enterographa* and *Sclerophyton* were recently monographed by Sparrius (2004) who accepted 35 species in the former and 14 in the latter genus. Both genera were distinguished from *Opegrapha* by a poorly developed, non-carbonized excipulum. In that monograph, *O. zonata* was included in *Opegrapha* and excluded from *Enterographa,* despite the lower parts of the excipulum being not fully carbonized. In our analyses, *O. zonata* is nested within *Enterographa s. str.*, i.e. sharing a more recent common ancestor with the type species of the genus (*E. crassa*) than with *E. hutchinsiae*, a morphologically more similar species to *E. crassa*. Therefore, we should refer to this species as *Enterographa zonata* as concluded by Torrente & Egea (1989).

E. anguinella (Fig 3C–D) and *E.* sp. 1 form a paraphyletic assemblage within the *O. vulgata* group (Fig 2). Therefore, these two *Enterographa* species are not part of *Enterographa s. str.* and should be considered as belonging to the genus *Opegrapha*. Morphologically, they are distinguished from *Enterographa s. str.* by more or less prominent, elongate lirellae, whilst typical *Enterographa* species have immersed, frequently grouped, punctiform to lirelliform ascomata. The inclusion of more species in future molecular studies will be needed to better understand which phenotypic characters can be used to delimit the genus *Enterographa*.

Usage of morphological key characters obscured classification of natural groups within the Arthoniales

Our molecular study clearly shows that many of the morphological features traditionally used to define genera within the *Arthoniales* were homoplastic. The development and carbonization of the excipulum were interpreted as important diacritical traits at the generic level within the *Arthoniales*. Most *Arthonia* species, including the generic type species *A. radiata*, have a rudimentary excipulum (Fig 3M–N), whereas *Opegrapha* species are characterized by the formation of a well-developed, usually thick excipulum (e.g. Fig 3A–B). As demonstrated with the position of *O. atra* and *O. calcarea* within *Arthonia* (Fig 2), the development of the excipulum cannot be used to define the genus *Arthonia* (Fig 3K–N). Similarly, Matzer (1996) described two lichenicolous species, *A. intermedia* and *A. pseudopegraphina*, with a well-developed, lateral excipuloid tissue. Coppins (1989) also mentioned the presence of a quite distinct excipuloid tissue for *A. excipienda*. Both authors considered that this character state is not sufficient to exclude such species from *Arthonia*. We predict that more taxonomic changes in other genera not included in our analyses will prove necessary. For instance, Matzer (1996) described the new genus *Paradoxomyces* characterized by a well-developed and carbonized exciple similar to that of *Opegrapha*, and by asci and muriform ascospores similar to those of *Arthothelium*, a genus without a proper excipulum. Future molecular data might show that *Paradoxomyces* is nested within *Arthothelium* or even *Arthonia*

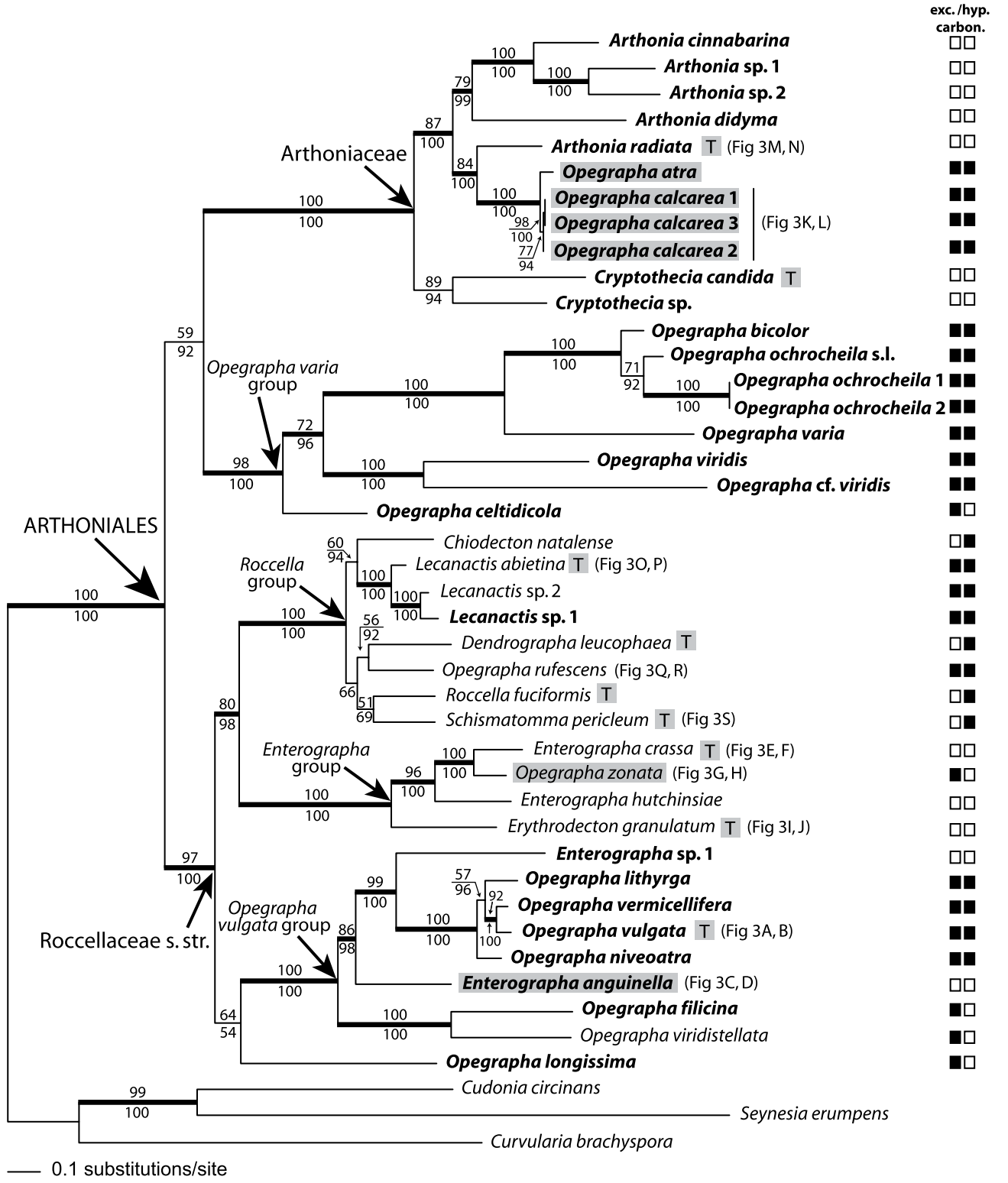

Fig 2 – Three-locus (nuLSU + mtSSU + *RPB2*) ML tree representing phylogenetic relationships among 40 members of the *Arthoniales*. ML BS values are shown above, and PPs are shown below, internal branches. Internal branches with a BS value ≥ 70 % and a PP ≥ 95 % are considered strongly supported and represented by thicker lines. Taxa sequenced from cultures are shown in bold. Generic types included in this tree are highlighted with a 'T' following the species name. Taxa for which a nomenclatural change is proposed here have their names highlighted with a pale grey box. The distribution of two character states is indicated at the right of the tree. The left squares give information about the carbonization of the excipulum: a white square refers to a hyaline or very reduced excipulum, whereas a black square refers to a carbonized excipulum. The right column of squares similarly gives information about the carbonization of the hypothecium.

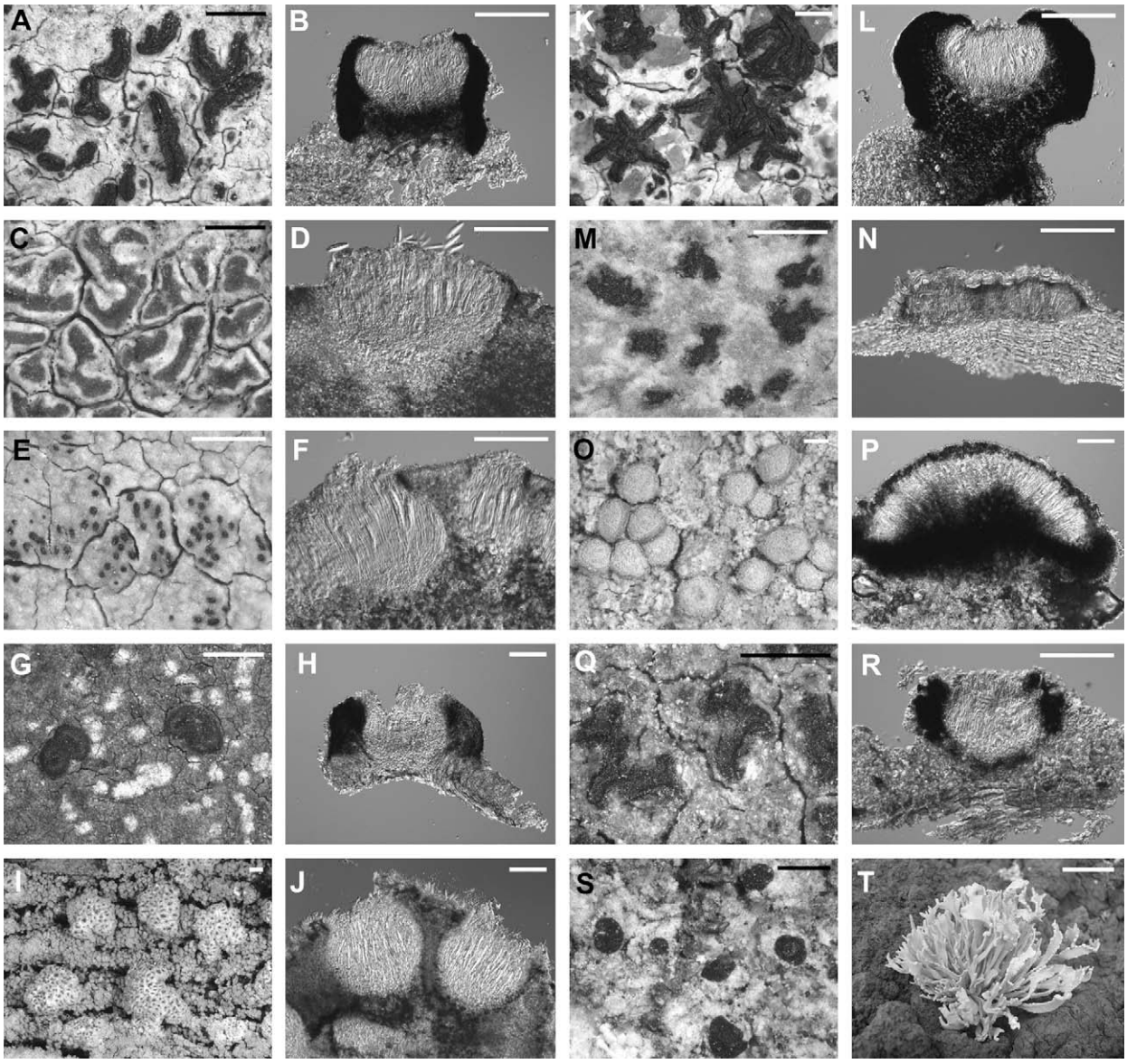

Fig 3 – Overall view of the morphological diversity of the *Arthoniales*. (A) Thallus of *Opegrapha vulgata* with lirellae (*Ertz* 7303). (B) Cross-section through a lirella of *O. vulgata* (*Ertz* 7303). (C) Thallus of *Enterographa anguinella* with lirellae (*Ertz* 10027). (D) Cross-section through a lirella of *E. anguinella* (*Ertz* 10027). (E) Thallus of *E. crassa* with ascomata (*Ertz* 5041). (F) Cross-section through an ascoma of *E. crassa* (*Ertz* 5041). (G) Thallus of *O. zonata* with ascomata (*Vigneron* 104). (H) Cross-section through an ascoma of *O. zonata* (*Vigneron* 104). (I) Thallus of *Erythrodecton granulatum* with perithecioid ascocarps aggregated into stroma-like structures (*Ertz* 9908). (J) Cross-section through ascocarps of *E. granulatum* (*Ertz* 9908). (K) Thallus of *O. calcarea* with lirellae (*Ertz* 7545). (L) Cross-section through a lirella of *O. calcarea* (*Ertz* 7545). (M) Thallus of *Arthonia radiata* with ascomata (*Ertz* 10096). (N) Cross-section through an ascoma of *A. radiata* (*Ertz* 10096). (O) Thallus of *Lecanactis abietina* with apothecia (*Ertz* 5068). (P) Cross-section through an apothecium of *L. abietina* (*Ertz* 5068). (Q) Thallus of *O. rufescens* with lirellae (*Vigneron* 75). (R) Cross-section through a lirella of *O. rufescens* (*Vigneron* 75). (S) Thallus of *Schismatomma pericleum* with ascomata (*Diederich* 14942). (T) Thallus of *Roccella* sp. on rock. Bars = (A, C, E, G, I, K, M, O, Q, S) 500 μm; (B, D, F, H, J, L, N, P, R) 100 μm; (T) 1 cm.

owing to a similar ascus type and despite the well-developed excipulum. Grube & Giralt (1996) have shown that, apart from the muriform ascospores, several *Arthothelium* species are so similar to *Arthonia* that they might belong to this genus.

The polyphyly of the genera *Enterographa* and *Opegrapha* suggests that rapid evolutionary transitions between a carbonized and non-carbonized state seems most likely (Fig 2). Observations by Diederich on a specimen of *Opegrapha varia* (*Diederich* 12656) in which part of the lirellae are de-carbonized and yellowish pink supports this evolutionary scenario. Similar observations have been done on specimens of *Graphis* (*Graphidaceae*, *Ostropales*) (Staiger *et al.* 2006).

Differential evolutionary history between mitochondrial and nuclear genes and the detection of hybridization

Phylogenetic conflicts among closely related and recently diverged species have been reported as a signature of hybridization (Mallet 2005). The mitochondrial tree (Fig 1B) *versus* nuclear trees (Fig 1A, C) showing strongly supported sister versus paraphyletic relationships for the *Enterographa hutchinsiae–Erythrodecton granulatum* pair represent one manifestation of this pattern. This pattern detected here is not due to a nucleotide base frequency bias of the mtDNA. We could reject homogeneity of base frequencies across taxa only for the *RPB2* gene ($P < 0.0001$), yet both nuLSU and *RPB2* strongly supported *E. hutchinsiae* and *E. granulatum* as paraphyletic (Fig 1A, C). Therefore, the models of evolution used for our ML BS analyses were robust to the variation in base frequency across taxa for the three genes used in this study. Based on simulation studies (Alfaro *et al.* 2003), the use of ML to estimate BS support values is the most accurate method currently available to estimate phylogenetic confidence. Therefore, this significant discrepancy between the mitochondrial tree and our two nuclear trees are unlikely to be the result of inaccurate BS support estimation.

As mitochondria are maternally inherited in most ascomycetes studied so far (Lee & Taylor 1993; Reich & Luck 1966; Röhr *et al.* 1999), this type of evolutionary discrepancy between both genomes reported here match the expectation of cytoplasmic gene flow, where the mitochondrion from one species introgresses another, analogous to chloroplast capture in plants (Rieseberg & Soltis 1991; Tsitrone *et al.* 2003). Concerted evolution might be rather different in mt-rDNA because recombination is often severely limited by uniparental inheritance or failure of organelles to fuse and exchange genomes (Birky 2001). Therefore, comparing phylogenies derived from mitochondrial and nuclear genes could be useful in detecting gene flow among *Arthoniales* species, and fungi in general. However, this might be the sole utility of the mtSSU within the *Arthoniales*, as its resolving power is weak for inferring deeper relationships within this order (Fig 1B). Because of the tremendous diversity of endolichenic fungi found in lichen thalli (Arnold *et al.* in press), detection of hybridization through a comparison of mitochondrial and nuclear trees is best implemented using cultures of lichen mycobionts derived from ascospores.

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