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 (nLSU), 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.

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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 commensal (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 Hysteriales, 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 Arthonia-ceae, 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 Arthonia-ceae 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 Arthoniaceae (Artho-niaceae, 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, ectocaricate 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 phylogenetic 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 Chiosdecton, Schismatoma, 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 Schismatoma periculum), 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 homoplasies in the morphological data (e.g. placement of Schismatoma, Lecanactis). Tehler & Irestedt (2007) investigated the phylogenetic relationships within the family Roccellaceae s. str. based on LSU and RP2 sequences from 48 taxa including mainly members of the genera Roccella and Rocellina. The results of these phylogenetic analyses also suggest that the fruticose/crustose habits have evolved multiple times in the family Roc-cellaceae 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 (Sparrarius 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 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 (multispor 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

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Voucher</th>
<th>Substrate</th>
<th>nuLSU</th>
<th>mtSSU</th>
<th>RPB2</th>
<th>Specimen in culture</th>
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<td>EU704009</td>
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<td>EU704012</td>
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<td>EU704050</td>
<td>EU704013</td>
<td>+</td>
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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 ciricins (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 bovine serum albumin (Ambion # 2616), 1 μl of 25 μM MgCl₂, 1.25 μ l U Taq DNA polymerase (Roche) and 1 μ l template genomic DNA. PCR was performed on Peltier Thermal Cyclers FTC-100 or FTC-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 RP2 protein-coding gene was amplified and sequenced using primers RPFB2-7cF and RPFB2-11aR (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 RP2 were sequenced directly using BigDye terminators (Applied Biosystems, Foster City, CA) and the amplification primers. For nuLSU, additional primers for sequencing were used: LR3R, 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 RP2 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 RP2 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 RP2 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 Erythrodocton 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 (Swoford 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 RP2 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, RP2/1st, 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 invariant 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.
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 = 100, 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 unidentifed tropical species), as well as Opegrapha atrata 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. nivacea, O. vermicellifera and O. vulgata form a strongly supported monophyletic group (ML BS = 100, PP = 100). This

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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. fuciformis 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

**Enterographa zonata** (Körb.) Källsten ex Torrente & Egea, **com. nov.**
MycoBank No.: MB 512317
Syn.: *Enterographa zonata* (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)


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 asci 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 asci 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, Erythrodercton, Lecanactis, Opegrapha, and Schismatoma.
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*, *Schizomattoma*, *Enterographa*, and *Erythrodercaton* 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. varià* 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. lithyriga*, *O. niveoatra*, and *O. vermicellifera*). These four species represent the core of the genus *Opegrapha* (Fig 2). All these species are corticulous, with the exception of *O. lithyriga*, which is saxicolous. The two folliculiferous *Opegrapha* species included in our study, *O. filicina* and *O. viridi-stellata*, 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 *Schizomattoma graphidioides*. Based on morphology, and because *O. rufescens* is more closely related to the type species of *Schizomattoma* (*S. periculum*, 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 *Schlerophyton* 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. anguina* (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 *Arthropeltion*, a genus without a proper excipulum. Future molecular data might show that *Paradoxomyces* is nested within *Arthropeltion* or even *Arthonia*.
Fig 2 – Three-locus (nuLSU + mSSU + 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 $\geq 70\%$ and a PP $\geq 95\%$ 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.
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 variabilis (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 Enterotheca hutchinsiae–Erythrocten 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 is 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 & Solitis 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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