Research Article

High frequency of character transformations is phylogenetically structured within the lichenized fungal family Graphidaceae (Ascomycota: Ostropales)

H. THORSTEN LUMBSCH1, SITTIPORN PARNMEN1,2, EKAPHAN KRAICHAK1, KHWANRUAN BUTSATORN PAPONG3 & ROBERT LÜCKING1

1Science & Education, The Field Museum, Chicago, IL, 60605 USA
2Department of Medical Sciences, Ministry of Public Health, Tivanon Road, Nonthaburi 11000, Thailand
3Department of Biology and Natural Medicinal Mushroom Museum, Faculty of Science, Mahasarakham University, Kantarawichai, Maha Sarakham Province, 44150, Thailand

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Graphidaceae is a large family of over 2000 predominantly tropical, lichenized fungal species encompassing a remarkable range of morphological and chemical diversity. The majority of species belongs in subfamily Graphidoideae, which also exhibits the greatest amount of variation. Various phenotype characters have traditionally been used for classification at the genus and species levels, but their correlations with phylogenetic clades are poorly known. Using a multilocus approach, we reconstructed a phylogeny for 224 taxa, representing all main genera within subfamily Graphidoideae, and employed ancestral character reconstruction and character transformation analyses to understand the evolution of morphological, anatomical and chemical characters within this group. In addition, we examined the changes of habitat and photobiont types over the phylogeny. For this purpose, we focused on 10 characters, including thallus and ascoma features and chemistry. Since previous studies have shown that results may differ depending on the reconstruction method used, both Maximum-parsimony and Maximum-likelihood approaches were employed and multistate coding of characters was used. We reconstructed the ancestral states for 64 well-supported major clades in the family and found support for the ancestor of Graphidoideae being a tropical species with a trentepohlioid photobiont, apothecioid, solitary ascomata lacking both a columella and lateral paraphyses, and having non-amyloid ascospores. The frequency of transformations of morphological and chemical characters over the phylogeny of Graphidaceae was computed, resulting in a high frequency of reversible transformations for some characters, such as secondary chemistry, whereas other characters, such as photobiont, hymenial persistence or ascoma aggregation, exhibited low frequency of transformations. However, we found that even in the character with the highest number of transformations, secondary chemistry, the shifts were highly structured phylogenetically, suggesting that the evolution of the character, rather than the character state itself, can be used to predict phylogenetic relationships with certain accuracy.

Key words: Ancestral character reconstruction, character traits, lichens, molecular phylogeny, taxonomy

Introduction

An important aspect of evolutionary biology is the understanding of the historical process of phenotypic character changes over the tree of life. In groups with poor fossil record, such as fungi in the phylum Ascomycota, including lichenized groups (Taylor et al., 1995, 1997, 1999; Yuan et al., 2005; Berbee & Taylor, 2007; Krings et al., 2012), ancestral character reconstruction and stochastic mapping, inferred from patterns of traits in living organisms, provide an avenue for addressing character evolution (Pagel, 1999; Lutzoni et al., 2000; Huelsenbeck et al., 2003; Pagel et al., 2004). A growing body of studies employ these methods to better understand trait evolution in lichenized fungi (Lutzoni & Pagel, 1997; Lutzoni et al., 2001; Ihlen & Ekman, 2002; Blanco et al., 2006; Buschbom & Barker, 2006; Lumbsch et al., 2006; Crespo et al., 2007; Gueidan et al., 2007; Schmitt et al., 2009; Schmitt & Lumbsch, 2009; Schoch et al., 2009; Baloch et al., 2010; Prieto et al., 2012). While powerful in assessing character evolution, limitations of these methods have also been shown (Ekman et al., 2008) potentially yielding
contradicting results, depending on taxon sampling and the employed method of phylogenetic reconstruction.

Graphidaceae, which has recently been expanded to include the previously separated families Asterothyriaceae, Gomphillaceae, Solorinellaceae and Thelotremataceae (Mangold et al., 2008b; Baloch et al., 2010; Rivas Plata & Lumbsch, 2011; Rivas Plata et al., 2012a, 2012b), is one of the largest families of lichen-forming fungi with over 2000 accepted species (Lücking et al., 2013; Rivas Plata et al., 2013). The classification within this large fungal clade has dramatically changed over the last decade. The traditional circumscription of the family and genera was largely based on ascoma (rounded vs. lirellate or pseudostromatic) and ascospore types (septation and pigmentation) (Müller, 1887; Wirth & Hale, 1963, 1978; Hale, 1974, 1978). The use of these characters was long perceived as artificial and replaced by a classification among thelotremoid taxa based on excipular structures (Salisbury, 1972a, 1972b, 1978; Hale, 1980, 1981). However, major systematic revisions only started after seminal treatments on graphidoid and thelotremoid taxa respectively by the school of Klaus Kalb (Staiger, 2002; Frisch et al., 2006) with a more refined classification based on a combination of phenotypic characters. Molecular studies have further changed the classification with the identification of additional clades that are accommodated in new or resurrected genera and a revised concept at the family level (Staiger et al., 2006; Mangold et al., 2008a, 2008b; Nelsen et al., 2010; Rivas Plata et al., 2010a, 2012a, 2012b, 2013; Berger et al., 2011; Lücking et al., 2011, 2012, 2013; Rivas Plata & Lumbsch, 2011; Cáceres et al., 2012; Parmmen et al., 2012a, 2013).

Within Graphidaceae, up to four subfamilies are currently accepted (Hodkinson, 2012; Rivas Plata et al., 2012a; Lücking et al., 2013). Subfamily Graphidoideae constitutes by far the largest clade of this family of crustose, primarily tropical lichen-forming fungi (Rivas Plata et al., 2012a). The species currently placed in this subfamily represent the core of the former families Graphidaceae and Thelotremataceae. This apparent incongruence of traditional, morphology-based classifications and molecular phylogenies suggest that these fungi are extremely variable with regard to phenotype characters, with a high probability of characters evolving in parallel in unrelated clades, as has been shown for this family and for various other lineages in the Ascomycota (Blanco et al., 2004, 2006; Crespo et al., 2007; Tehler & Irestedt, 2007; Mugambi & Huhndorf, 2009; Schnitt et al., 2009; Lumbsch et al., 2010b; Parmmen et al., 2010; Muggia et al., 2011; Rivas Plata et al., 2011; Rivas Plata & Lumbsch, 2011).

With the availability of molecular data, we are now able to study the evolution of phenotypic characters previously used in the classification of Graphidaceae. While most species in the family are tropical crustose lichens, some species also occur in subtropical habitats, such as Redonographa (Lücking et al., 2013) or have their distribution centre in non-tropical areas, such as Diploschistes (Lumbsch, 1989). Two main types of photobionts are found in the family, chlorococcoid and trentepohlioid, correlating with ecological features of the involved lichens (Friedl & Gärtner, 1988; Nelsen et al., 2011). While numerous species have corticated thalli (Hale, 1981; Staiger, 2002; Frisch et al., 2006), others lack a cortex (Lumbsch, 1989). A cortex is a distinct layer of fungal hyphae covering the upper and/or lower side of the thallus. The ascomata can be either roundish (apothecoid) or elongate (lirellate), but there is no evidence regarding the evolutionary function of ascoma shape. A few lineages form mazaedioid ascomata which consist of spore masses that accumulate on top of the ascomata. These are highly adapted to wind dispersal of the ascospores (Lumbsch et al., 2004), while the majority of species in the family are characterized by ascomata with persistent hymenia. A sterile tissue within the hymenium, the so-called columnella, which is often partially or totally carbonized, occurs in many tropical species; it is assumed to protect the hymenium against fungivores (Rivas Plata & Lumbsch, 2011). Sterile hyphae that grow from the margin into the central cavity of the ascoma, so-called lateral paraphyses (Henssen, 1995) or peripherys (Hale, 1981) occur in several lineages, but their function is unknown. Ascospores in the family show different reaction to iodine, being either amyloid, hemiamyloid or non-amyloid (Baral, 1987; Rivas Plata & Lumbsch, 2011), depending on the presence and absence and chemical nature of internal wall substances.

We are now able to address questions on the possible functions of these characters in terms of ecology and adaptations of species to particular (micro-) habitats and niches. Generally, fast-evolving characters can be assumed to be ecologically modified, whereas highly conserved characters have low levels of correlation with ecological parameters, unless the clade in question is also ecologically uniform (Felsenstein, 1985; Harvey & Purvis, 1991; Coddington, 1994; Ackerly, 2003; Kraichak, 2012). In order to understand the patterns of the character evolution in this group of lichenized fungi, we assembled a dataset of four loci including 224 taxa representing all major clades of subfamily Graphidoideae, Fissurinoideae and Redonogaphideae and outgroups for phylogenetic analysis. We then performed ancestral character state reconstructions of seven representative morphological and anatomical characters, secondary chemistry, the type of photobiont and the vegetation type zone. The main objectives of this study were: (1) to characterize the hypothetical ancestor of the subfamily Graphidoideae, to better understand the evolution of phenotypic and ecological characters in the core group of the family Graphidaceae; (2) to identify the number and phylogenetic signal of
character state transformations of these characters along the reconstructed phylogeny; and (3) to understand the impact of ecology on character transformations.

Materials and methods

Taxon sampling and molecular methods

The taxon sampling included the major clades of subfamilies Graphidioideae, Redonographeeideae, and Fissurinoideae in Graphidaceae, plus five taxa of the genus Gyalecta as outgroup, based on previous molecular studies (Lumbsch et al., 2010a; Parmen et al., 2010). Two hundred and twenty-four species were included in the analyses (Table 1). We selected taxa for this study to represent the morphological and chemical diversity in the group and to include all major genera, except for the taxa in subfamily Gomphillioideae, from which a limited number of materials and DNA sequences were available for the current study. We included only species for which we obtained at least two of the four loci studied: nuclear LSU rDNA, mitochondrial SSU rDNA, and the protein-coding RPB1 and RPB2 genes. New sequences were generated for this study using the Sigma REDEXtract-N-Amp Plant PCR Kit (St. Louis, Missouri, USA) for DNA isolation following the manufacturer’s instructions, except that 40 µL of extraction buffer and 40 µL dilution buffer were used. DNA dilutions (5×) were used in PCR reactions of the genes coding for the nuLSU, mtSSU, RPB1 and RPB2, respectively. Primers and PCR amplification condition were the same as described previously (Parmen et al., 2012a, 2012b; Schmitt et al., 2012; Rivas Plata et al., 2013). One hundred and fifteen new sequences were generated for this study (21 mtSSU, 16 nuLSU, 54 RPB1 and 24 RPB2).

Sequences alignments and phylogenetic analyses

Alignments were done in Geneious Pro 5.5.2 (Drummond et al., 2012). Ambiguously aligned portions were removed manually. The single-locus and concatenated alignments were analysed by maximum likelihood (ML) and a Bayesian approach (B/MCMC). To test for potential conflict, ML bootstrap analyses (with 2000 pseudoreplicates) were performed on the individual datasets, and resulting single-tree trees were examined for conflict, i.e. incongruences with at least75% bootstrap support (Lutzoni et al., 2004).

The ML analysis of the concatenated alignment was performed with the program RAxML-HPC2 (version 7.3.1) on XSEDE (Stamatakis, 2006) using the default rapid hill-climbing algorithm. The model of nucleotide substitution chosen was GTRGAMMA, according to the results from the model selection by jModelTest2 (Guindon & Gascuel, 2003; Darriba et al., 2012). The dataset was partitioned into eight parts (mtSSU, nuLSU and each codon position of RPB1 and RPB2), and each gene partition was treated as independent. Introns in RPB1 and RPB2 sequences were removed from the analysis. Bootstrap estimates were carried out using 2000 pseudoreplicates (Stamatakis et al., 2008).

The B/MCMC analysis was conducted using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001), with the same substitution model as in the ML analysis. Two parallel runs with 10 000 000 generations each, starting with a random tree and employing four simultaneous chains, was executed. No molecular clock was assumed. Heating of chains was set to 0.2. Posterior probabilities were approximated by sampling trees using a variant of Markov Chain Monte Carlo (MCMC) method. To avoid autocorrelation, only every 1000th tree was sampled. The first 4000 trees were discarded as burn in. We used AWTY (Nylander et al., 2007) to compare splits frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was reached. A majority-rule consensus tree with average branch lengths was calculated from the remaining 6000 sampled trees using the sumt option of MrBayes. Posterior probabilities were obtained for each clade. Clades with bootstrap support above 70% under ML and Bayesian posterior probabilities above 0.95 were considered as strongly supported. Phylogenetic trees were visualized using the program TreeView (Page, 1996).

Ancestral character state reconstruction

Characters for the analysis including habitat, photobiont, cortex, six ascomatal characters, and secondary chemistry were analysed using multistate character coding with characters treated as unordered (Table 2 and Table S1, see online supplemental material, which is available from the article’s Taylor & Francis Online page at http://dx.doi.org/10.1080/14772000.2014.905506). The coding was done based on our own observations of the material and recently published studies (Lücking, 2009; Lücking et al., 2009, 2013; Mangold et al., 2009; Lumbsch et al., 2010a; Rivas Plata et al., 2010b, 2012a, 2012b, 2013; Berger et al., 2011; Rivas Plata & Lumbsch, 2011; Parmen et al., 2012a, 2013; Sipman et al., 2012;). For chemistry, instead of coding each substance separately, we coded depsidones according to the groups at positions 1 and 6, which might be either carboxyl groups (COOH), hydroxyl group (OH) or methyl group (ME). This was done to emphasize chemical relationships between substances. The three types of depsidones distinguished here include stictic acid for (1’-6’-OH) depsidones, protocetraric acid for (1’-COOH-6-ME) depsidones, and psoromic acid for (6’-COOH) depsidones.

Maximum likelihood (ML) reconstructions were carried out on each individual tree using an unrestricted
Table 1. List of taxa with their collection data and GenBank accession numbers for the sequences used in this study. Bold accession numbers indicate sequences generated for this study. Dashes (–) indicate missing data.

| Species                      | Subfamily   | Tribe                        | Collection data                  | mtSSU       | nuLSU      | RPB1      | RPB2      |
|------------------------------|-------------|------------------------------|----------------------------------|-------------|------------|-----------|-----------|
| *Acanthothecis hololeucoides* | Graphidoideae| Thelotremateae               | Brazil, Lücking 31303 (F)        | JX420952    | JX421423   | –         | JX420938  |
| *Acanthothecis perplorhola*  | Graphidoideae| Thelotremateae               | USA, Common 9126A                | JX420954    | JX421424   | KF688524  | –         |
| *Acanthothecis sarcographoides* | Graphidoideae| Thelotremateae               | Brazil, Cáceres 6785 (F)         | JX420957    | –          | KF875493  | –         |
| *Acanthotrema alboisidiatum*  | Graphidoideae| Thelotremateae               | USA, Rivas Plata 0808Da (F)      | KF688506    | KF688492   | KF688526  | KF688542  |
| *Acanthotrema frischii*       | Graphidoideae| Thelotremateae               | Cameroon, Frisch 99kA40          | DQ384916    | DQ343192   | –         | –         |
| *Ampliotrema oculatum*        | Graphidoideae| Thelotremateae               | Australia, Lumbsch 19160Wa (F)   | EU075565    | EU075612   | –         | –         |
| *Astrostigma leucophthalum*   | Graphidoideae| Thelotremateae               | USA, Lücking 26573 (F)          | JX421007    | JX421460   | –         | JX420830  |
| *Astrostigma platycarpum*     | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Asteristion alboisidiatum*   | Graphidoideae| Thelotremateae               | Peru, Rivas Plata 0808Da (F)     | JX420958    | JX421429   | –         | JX420876  |
| *Asteristion mastersonii*     | Graphidoideae| Thelotremateae               | Australia, Lumbsch 19160Wa (F)   | EU075565    | EU075612   | –         | –         |
| *Astrostigma pseudophlyctis*  | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma thyttocarpum*    | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma zahlbruckneri*   | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma plactocarpum*    | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma mastersonii*     | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma pseudophlyctis*  | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma thyttocarpum*    | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma zahlbruckneri*   | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma plactocarpum*    | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma mastersonii*     | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma pseudophlyctis*  | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma thyttocarpum*    | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma zahlbruckneri*   | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma plactocarpum*    | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma mastersonii*     | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma pseudophlyctis*  | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma thyttocarpum*    | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma zahlbruckneri*   | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma plactocarpum*    | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma mastersonii*     | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma pseudophlyctis*  | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma thyttocarpum*    | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma zahlbruckneri*   | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma plactocarpum*    | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma mastersonii*     | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma pseudophlyctis*  | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma thyttocarpum*    | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma zahlbruckneri*   | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| *Astrostigma plactocarpum*    | Graphidoideae| Thelotremateae               | USA, Mercado 290 (F)            | JX420954    | JX421429   | –         | JX420876  |
| Species              | Subfamily       | Tribe             | Collection data                     | mtSSU       | nLSU         | RPB1         | RPB2         |
|----------------------|-----------------|-------------------|-------------------------------------|-------------|--------------|--------------|--------------|
| ** species **        |                 |                   |                                     |             |              |              |              |
| Diorygma antillarum  | Graphidoideae   | Graphideae        | USA, Nelsen 322 (F)                 | JX046452    | JX046465     | –            | –            |
| Diorygma junguhnnii  | Graphidoideae   | Graphideae        | FIJI, Lumbsch 205391 (F)            | JX421023    | JX421474     | –            | –            |
| Diorygma miniisporum | Graphidoideae   | Graphideae        | Kenya, Lumbsch 19543v (F)           | HQ639598    | HQ639626     | –            | –            |
| Diorygma poitaei     | Graphidoideae   | Graphideae        | Nicaragua, Lücking 28538 (F)       | HQ639596    | HQ639627     | **KF875497** | **JF828942** |
| Diploschistes cinereocaesius | Graphidoideae       | Thelotremateae    | Costa Rica, unknown 24 (F)         | DQ912306    | DQ883799     | –            | –            |
| Diploschistes diploschistoides | Graphidoideae       | Thelotremateae    | Australia, Lumbsch 19073b (F)      | KF688500    | KF688513     | KF688533     | KF688544     |
| Diploschistes euganeus | Graphidoideae   | Thelotremateae    | Swizerland, Lumbsch 20605g (F)     | KF688508    | KF688494     | KF688528     | KF688544     |
| Diploschistes muscorum | Graphidoideae   | Thelotremateae    | Ecuador, Paloe 2805 (F)            | AY300886    | AY300836     | –            | –            |
| Diploschistes rampodensis | Graphidoideae   | Thelotremateae    | Papua New Guinea, Aptroot 39679 (F) | AF431954    | AF274094     | –            | –            |
| Diploschistes thunbergianus | Graphidoideae   | Thelotremateae    | Australia, Lumbsch 19109b (F)      | KF688499    | JX421482     | KF688512     | KF688533     |
| Dyplolabia azellii   | Fissurinoideae   |                   | Australia, Eldridge 3800 (F)       | AY779296    | KF874095     | –            | –            |
| Fibrillithecis gibbosa | Fissurinoideae  | Ocellularieae     | Australia, Mangold 31g 36v (F)     | EU075573    | JX421485     | **KF875498** | –            |
| Fissurina aff. dumastii | Fissurinoideae  |                   | Thailanad, Kahl 38899 (F)          | JX421034    | JX421487     | –            | –            |
| Fissurina aff. humilis | Fissurinoideae  |                   | Perun, Rivas Plata 107C (F)        | JX421036    | JX421490     | –            | –            |
| Fissurina astroisidiata | Fissurinoideae  |                   | Mexico, Lücking RLD057a (F)        | JX421040    | JX421491     | –            | –            |
| Fissurina bullata    | Fissurinoideae   |                   | Australia, Mangold 6f (F)          | JX421041    | KF875537     | –            | –            |
| Fissurina comparimuralis | Fissurinoideae  |                   | El Salvador, Lücking 28103 (F)     | JX421042    | KF875538     | –            | –            |
| Fissurina crassiflora | Fissurinoideae   |                   | USA, Mercado 4462 (F)              | JX421034    | KF875521     | –            | –            |
| Fissurina insidiosa  | Fissurinoideae   |                   | USA, Lendener 4793 (F)             | DQ972958    | DQ973045     | –            | –            |
| Fissurina margiatica | Fissurinoideae   |                   | Thailand, Lücking 24122 (F)        | HQ639613    | JX421493     | –            | –            |
| Fissurina monticarpa  | Fissurinoideae   |                   | USA, Mercado 156 (F)               | **KF875506** | **KF875539** | –            | –            |
| Fissurina nigroalbata | Fissurinoideae   |                   | Philippines, Rivas Plata 1198B (F) | JF828961    | JF828976     | –            | –            |
| Fissurina pseudostromatica | Fissurinoideae  |                   | Thailand, Kahl 3827 (F)            | –           | JX421495     | –            | JX420929     |
| Fissurina rufula     | Fissurinoideae   |                   | Fiji, Lumbsch 20522 (F)            | JX421053    | JX421497     | –            | –            |
| Gintarasia lamellifera | Graphidoideae   | Thelotremateae    | Australia, Lumbsch 20009b (F)      | JX420990    | JX421494     | –            | –            |
| Gintarasia megalophthalma | Graphidoideae   | Thelotremateae    | Australia, Mangold 4989 (F)        | –           | JX421456     | **KF875519** | –            |
| Glacotorema glaucophaeum | Graphidoideae   |                   | Philippines, Rivas Plata 1099 (F)  | JX421061    | JX421501     | KF875499     | JX420862     |
| Glyphis cicatricosa  | Graphidoideae   | Graphideae        | El Salvador, Lücking 28047 (F)     | HQ639610    | JX421505     | KF875500     | –            |
| Glyphis substratula  | Graphidoideae   | Graphideae        | El Salvador, Lücking 28014 (F)     | –           | JX421506     | **KF875501** | **JF828944** |
| Graphis aff. caesicola | Graphidoideae   | Graphideae        | Australia, Kahl 33919 (F)          | DQ431977    | DQ431938     | –            | –            |
| Graphis aff. epimelaena | Graphidoideae   | Graphideae        | China, Sohrabi, 16579 (F)          | **KF875561** | **KF875540** | **KF875502** | **KF875523** |
| Graphis angustata    | Graphidoideae   | Graphideae        | El Salvador, Lücking 28102 (F)     | HQ639612    | HQ639632     | –            | –            |
| Graphis betulina     | Graphidoideae   | Graphideae        | China, Sohrabi, 16429 (F)          | **KF875562** | **KF875541** | **KF875503** | **KF875524** |
| Graphis chlorocarpa  | Graphidoideae   | Graphideae        | Guatemala, Lücking 25522 (F)       | HQ639595    | –            | –            | JF828946     |
| Graphis gracicansens | Graphidoideae   | Graphideae        | Australia, Kahl 33942B (F)         | DQ431976    | DQ431936     | –            | –            |
| Graphis illinata     | Graphidoideae   | Graphideae        | Mexico, Lumbsch 19109b (F)         | HQ639614    | JX421508     | –            | –            |
| Graphis implicata    | Graphidoideae   | Graphideae        | Costa Rica, Lücking 16103 (F); El Salvador, Lücking 28039 (F) | DQ431978    | DQ431939     | –            | –            |
| Graphis leptocladus  | Graphidoideae   | Graphideae        | Fiji, Lumbsch 20532b (F)           | JX421068    | JX421509     | –            | –            |
| Graphis librata      | Graphidoideae   | Graphideae        | El Salvador, Lücking 28001 (F)     | HQ639621    | HQ639636     | –            | JF828945     |
| Graphis macrocarpa   | Graphidoideae   | Graphideae        | China, Sohrabi, 16438 (F)          | **KF875563** | **KF875542** | **KF875504** | **KF875525** |

(continued)
| Species                  | Subfamily                  | Tribe       | Collection data               | mtSSU      | nuLSU      | RPB1      | RPB2      |
|-------------------------|----------------------------|-------------|-------------------------------|------------|------------|-----------|-----------|
| Graphis oshioi          | Graphidoideae              | Graphideae  | Costa Rica, Lücking 16100c (F) | DQ431986  | DQ431946  |           |           |
| Graphis pseudocinerea   | Graphidoideae              | Graphideae  | USA, Lücking 26537 (F)        | HQ639620   | HQ639639   |           |           |
| Graphis pulverulenta    | Graphidoideae              | Graphideae  | Austria, Neilworth 11808 (UPS) | **KF875564** | **KF875543** | –         | –         |
| Graphis raiziana        | Graphidoideae              | Graphideae  | Costa Rica, Lücking 14009 (F) | DQ431985  | DQ431945  |           |           |
| Graphis scripta         | Graphidoideae              | Graphideae  | Austria, Neilworth 11834 (UPS) | **KF875565** | **KF875544** |           | –         |
| Gyalecta flooti       | Outgroup                  |             |                               |            |            |           |           |
| Gyalecta hypoleuca     | Outgroup                  |             |                               |            |            |           |           |
| Gyalecta jenensis      | Outgroup                  |             |                               |            |            |           |           |
| Gyalecta ulmi          | Outgroup                  |             |                               |            |            |           |           |
| Kalbographa lückingii  | Graphidoideae              | Graphideae  | Dominican Republic, Kalb 33152 | DQ431959   | DQ431926   |           |           |
| Leiorreuma hypomelaenum| Graphidoideae              | Graphideae  | Australia, Kalb 33916 (Herb. Kalb) | DQ431971  | DQ431933  |           |           |
| Leucoedecton anamaliense| Graphidoideae             | Thelotrematae | El Salvador, Lücking 28095 (F) | –          | EU075622   | –         | –         |
| Leucoedecton compunctellum| Graphidoideae             | Thelotrematae | Venezuela, Lücking 32120 (F) | JX421077   | JX421512   |           |           |
| Leucoedecton occulturn  | Graphidoideae              | Thelotrematae | Australia, Lumbsch 19161vA (F) | FJ708501   | FJ708491   |           |           |
| Leucoedecton sordidenes| Graphidoideae              | Thelotrematae | Venezuela, Lücking 28098 (F)  | HQ639611   | HQ639657   |           | –         |
| Melanotrema lynceodes  | Graphidoideae              | Ocellulariae | Australia, Lumbsch 191160vA (F) | EU075575   | EU075623   |           |           |
| Myriochapsa psoromica  | Graphidoideae              | Thelotrematae | Brazil, Cáceres s.n. (F)      | JX421009   | JX421461   | **KF875451** | JX420848 |
| Myriotrema eorotellum  | Graphidoideae              | Ocellulariae | Venezuela, Lücking 32113 (F)  | JX421091   | JX421522   | –         | –         |
| Myriotrema laeviusculum| Graphidoideae              | Ocellulariae | Venezuela, Lücking 32039 (F)  | JX421092   | JX421526   | –         | –         |
| Myriotrema microporum  | Graphidoideae              | Ocellulariae | Australia, Lumbsch 19092vA (F) | EU075578   | EU075626   |           |           |
| Myriotrema myrioporoides| Graphidoideae              | Ocellulariae | Venezuela, Lücking 32025 (F)  | –          | JX421529   | –         | –         |
| Myriotrema subcompactum| Graphidoideae              | Ocellulariae | Australia, Lumbsch 19113vA (F) | EU075579   | EU075627   | –         | –         |
| Myriotrema filicola    | Graphidoideae              | Ocellulariae | USA, Mangold 36vA (F)         | HQ639616   | –         |           | –         |
| Myriotrema peninsulare | Graphidoideae              | Ocellulariae | USA, Lücking 26542 (F)        | HQ639616   | –         |           | –         |
| Nadvornikia hawaiiensis| Graphidoideae              | Thelotrematae | Australia, Mangold 36vA (F)   | EU075581   | AY605080   | –         | –         |
| Nitidochapsa leprieuri | Graphidoideae              | Ocellulariae | Thailand, Kalb 38882 (F)      | JX420991   | JX421451   | –         | –         |
| Ocellularia aff. laevisculai | Graphidoideae          | Ocellulariae | Thailand, Lumbsch 19756vA (F) | JX421010   | JX421534   | –         | –         |
| Ocellularia aff. dolichotata | Graphidoideae          | Ocellulariae | India, Lumbsch 19730vA (F)    | JX421092   | JX421537   | –         | –         |
| Ocellularia aff. fumosa | Graphidoideae              | Ocellulariae | Perú, Rivas Plata 809 (F)    | JX421105   | –         |           | –         |
| Ocellularia aff. laevisculae | Graphidoideae          | Ocellulariae | Thailand, Lumbsch 20200vA (F) | JX421106   | JX421540   |           |           |
| Ocellularia aff. obtusascens | Graphidoideae           | Ocellulariae | USA, Lücking 26553vA (F)      | JF828967   | JF828979   | –         | –         |
| Ocellularia aff. rhabdospora | Graphidoideae        | Ocellulariae | USA, Mercado F76 (F)          | **KF875546** | –         | **KF875453** | **KF875505** |
| Ocellularia albocincta | Graphidoideae              | Ocellulariae | Perú, Rivas Plata 801vA (F)   | JX421113   | JX421543   | –         | –         |
| Ocellularia austratiana | Graphidoideae              | Ocellulariae | Australia, Lumbsch 19151vA (F) | EU075595   | EU075641   |           |           |
| Ocellularia australiana | Graphidoideae              | Ocellulariae | Australia, Lumbsch 19151vA (F) | EU075582   | EU075629   | –         | –         |
| Ocellularia cima       | Graphidoideae              | Ocellulariae | Perú, Rivas Plata 108vA (F)   | JX421138   | JX421552   | JX420869  |           |

(continued)
### Table 1. (Continued)

| Species               | Subfamily       | Tribe           | Collection data | mtSSU   | nuLSU   | RPB1   | RPB2   |
|-----------------------|-----------------|-----------------|-----------------|---------|---------|--------|--------|
| **Ocellularia**       |                 |                 |                 |         |         |        |        |
| crocea                | Graphidoideae   | Ocellulariae    | USA, Mercado F16 (F) | KF875548 | KF875529 | –      | KF875507 |
| diacida               | Graphidoideae   | Ocellulariae    | Australia, Lumbsch 19120jb (F) | EU075583 | EU075630 | –      | –      |
| dolichotata           | Graphidoideae   | Ocellulariae    | Thailand, Kalb 38892 (F) | JX421146 | JX421554 | –      | –      |
| domingensis           | Graphidoideae   | Ocellulariae    | Venezuela, Lücking 32233 (F) | JX421151 | JX421560 | –      | JX420918 |
| ficunda               | Graphidoideae   | Ocellulariae    | Venezuela, Lücking 32162 (F) | JX421155 | JX421562 | –      | –      |
| garoana               | Graphidoideae   | Ocellulariae    | Peru, Rivas Plata 801A (F) | JX421157 | JX421563 | KF875457 | JX420872 |
| gerardii              | Graphidoideae   | Ocellulariae    | Peru, Rivas Plata 1canopy (F) | JX421159 | JX421564 | –      | –      |
| henatomma             | Graphidoideae   | Ocellulariae    | Peru, Rivas Plata 809canopy (F) | JX421162 | –      | –      | JX420881 |
| sorediate             |                 |                 |                 |         |         |        |        |
| interposita           | Graphidoideae   | Ocellulariae    | USA, Mercado F16 (F) | KF875548 | KF875529 | –      | KF875507 |
| laeviusculoides       | Graphidoideae   | Ocellulariae    | Australia, Lumbsch 19120jb (F) | EU075577 | EU075625 | –      | –      |
| massalongoi           | Graphidoideae   | Ocellulariae    | Australia, Mangold 36n (F) | EU075584 | EU075631 | –      | –      |
| microacidum           | Graphidoideae   | Ocellulariae    | Peru, Rivas Plata 809canopy (F) | JX421171 | –      | –      | JX420878 |
| microsorediata        | Graphidoideae   | Ocellulariae    | Peru, Rivas Plata 107C (F) | JX421172 | JX421572 | –      | –      |
| microstoma            | Graphidoideae   | Ocellulariae    | Japan, Lumbsch 19056h2 (F) | JX421173 | JX421573 | –      | –      |
| minutula              | Graphidoideae   | Ocellulariae    | USA, Lumbsch 19167 (F) | JX421175 | –      | –      | JX420877 |
| natashae              | Graphidoideae   | Ocellulariae    | Peru, Rivas Plata 1canopy (F) | JX421177 | –      | –      | JX420888 |
| percolumellata        | Graphidoideae   | Ocellulariae    | Brazil, Cáceres 6002a (F) | JX421180 | –      | –      | –      |
| perforata             | Graphidoideae   | Ocellulariae    | Australia, Lumbsch 19120ja (F) | EU075587 | EU075634 | –      | –      |
| petrinensis           | Graphidoideae   | Ocellulariae    | Venezuela, Lücking 32024 (F) | JX421158 | JX421532 | –      | JX420910 |
| pluriporoides         | Graphidoideae   | Ocellulariae    | Brazil, Cáceres sn (F) | JX421190 | JX421580 | –      | –      |
| portoricensis         | Graphidoideae   | Ocellulariae    | USA, Mercado F64 (F) | KF875547 | –      | KF875455 | KF875506 |
| praestans             | Graphidoideae   | Ocellulariae    | Venezuela, Lücking 32239 (F) | JX421195 | JX421583 | –      | JX420911 |
| profunda              | Graphidoideae   | Ocellulariae    | Australia, Lumbsch 19100p (F) | JX421198 | JX421585 | KF875459 | KF875508 |
| psorbarroensis        | Graphidoideae   | Ocellulariae    | Peru, Rivas Plata 801D (F) | JX421202 | JX421588 | –      | JX420874 |
| rhodospora            | Graphidoideae   | Ocellulariae    | USA, Mercado F75 (F) | KF875459 | KF875530 | KF875460 | KF875508 |
| rhiocoporoides        | Graphidoideae   | Ocellulariae    | Thailand, Lumbsch 19750e (F) | JX421208 | JX421592 | –      | –      |
| rimosa                | Graphidoideae   | Ocellulariae    | Thailand, Kalb 38767 (Herb. Kalb) | JX421211 | –      | –      | JX420927 |
| spec.                 | Graphidoideae   | Ocellulariae    | USA, Mercado 4451 (F) | KF875550 | KF875531 | –      | –      |
| thelotremoides        | Graphidoideae   | Ocellulariae    | Australia, Lumbsch 191081 (F) | EU075592 | EU075638 | –      | –      |
| Palidogramme          | Graphidoideae   | Graphideae      | Australia, Kalb 33926 (Herb. Kalb); Phillipines, Rivas Plata 2008a,2005a (F) | JX421232 | DQ431932 | KF875461 | JF828951 |
| chlorocarposoides     |                 |                 |                 |         |         |        |        |
| Phaeographis aff. hypoglaucoides | Graphidoideae | Graphideae    | Thailand, Kalb 39068 (Herb. Kalb) | KF875552 | KF875532 | KF875462 | KF875509 |
| Phaeographis intricans | Graphidoideae   | Graphideae      | Thailand, Kalb 38646 (Herb. Kalb) | JX421254 | JX421602 | KF875463 | JX420924 |
| Phaeographis lecanophora | Graphidoideae   | Graphideae      | Mexico, Lücking RLD071 (F) | DQ431983 | DQ431943 | –      | –      |
| Phaeographis lobata   | Graphidoideae   | Graphideae      | Bermuda, Berger 19598 (F) | DQ431984 | DQ431944 | –      | –      |
| Phaeographis platycarpa | Graphidoideae   | Graphideae      | Australia, Mangold 30a (F) | JX421263 | –      | –      | KF875510 |
| Phaeographis spec.    | Graphidoideae   | Graphideae      | Brazil, Cáceres sn (F) | JN127363 | JN127365 | –      | –      |
| Phlegographa leprieurii | Graphidoideae   | Graphideae      | Kenya, Lumbsch F2388 (F) | JN127362 | JN127364 | –      | –      |
| Platygramme caesiopruinosa | Graphidoideae | Graphideae    | Mexico, Lumbsch 19636b (F); USA, Common 9084F (F) | JX421286 | JX421611 | KF875465 | –      |

(character transformations in Graphidaceae 277)
| Species                        | Subfamily         | Tribe           | Collection data                     | mtSSU   | nuLSU   | RPB1   | RPB2   |
|-------------------------------|-------------------|-----------------|-------------------------------------|---------|---------|--------|--------|
| *Platygramme impudica*        | Graphidoideae     | Graphideae      | Phillipines, Rivas Plata 1021C (F); Thailand, Kalb 38818 (Herb. Kalb) | JX421288 | JX421612 | –      | –      |
| *Platygramme praestans*       | Graphidoideae     | Graphideae      | USA, Lücking 9129 (F)               | JX421291 | –       | KF875466 | –      |
| *Platythycteum dimorphodes*   | Graphidoideae     | Graphideae      | USA, Mercado F31 (F)                | KF875554 | –       | KF875467 | KF875512|
| *Pseudochapsa dilatata*       | Graphidoideae     | Thelotremateae  | Venezuela, Lücking 26143 (F)       | JX420982 | JX421447 | –      | –      |
| *Pseudochapsa esslingeri*     | Graphidoideae     | Thelotremateae  | Peru, Rivas Plata 809A (F)         | JX420986 | JX465294 | KF875468 | JX465321|
| *Pseudochapsa phlyctidioides* | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 1900f (F)       | EU075569 | –       | KC020291 | JX465325|
| *Pseudoramonia richiae*      | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 19977a (F)      | KF875555 | KF875534 | –      | –      |
| *Pseudotepiopsis laceratula*  | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 19139s (F)      | JX420988 | JX421448 | –      | –      |
| *Reinimella glaucoglyphica*   | Graphidoideae     | Ocellularieae   | Peru, Rivas Plata 0810extra (F)     | JX421296 | JX421618 | –      | –      |
| *Reinimella heterochroma*     | Redonographoideae | Chile, Mehler 3870 (F) |                         | JX890304 | JX890301 | JX989305 | –      |
| *Reinimella saxonensis*       | Redonographoideae | Chile, Mehler 3870 (F) |                         | JX890304 | JX890301 | JX989305 | –      |
| *Rhabdodiscus crassus*        | Graphidoideae     | Ocellularieae   | Fiji, Lumbsch 20501p (F)           | JX420987 | JF828952 | –      | –      |
| *Rhabdodiscus granulosus*     | Graphidoideae     | Ocellularieae   | Peru, Rivas Plata 1054A (F)        | JX421298 | –       | KF875471 | –      |
| *Sarcographa aff. phlyctidioides* | Graphidoideae     | Graphideae      | Venezuela, Lücking 32212 (F)       | JX421300 | JX421621 | –      | –      |
| *Schistophoron tenue*         | Graphidoideae     | Graphideae      | Australia, Kalb 33920 (Herb. Kalb) | DQ431967 | DQ431931 | –      | –      |
| *Sarcographa aff. schizoloma* | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 20501p (F)      | JX421323 | KF875473 | –      | –      |
| *Sarcographa aff. zebrinum*   | Graphidoideae     | Thelotremateae  | Ecuador, Lühler 8796 (F)           | EU544933 | EU544932 | –      | –      |
| *Schizotrema anthracosporinaceum* | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 19983f (F)      | JX421326 | JX421631 | KF875474 | –      |
| *Schizotrema adjectum*        | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 1908f (F)       | EU075594 | EU075640 | –      | –      |
| *Schizotrema crespoae*        | Graphidoideae     | Thelotremateae  | USA, Lumbsch 20501p (F)            | JX421323 | JX421625 | –      | –      |
| *Schizotrema diplotrema*      | Graphidoideae     | Thelotremateae  | Australia, Kalb 33913 (Herb. Kalb) | DQ431972 | DQ431934 | –      | –      |
| *Schizotrema gallowayanum*    | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 1908f (F)       | EU075594 | EU075640 | –      | –      |
| *Schizotrema inspersoporinaceum* | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 1908f (F)       | EU075594 | EU075640 | –      | –      |
| *Stegobolus anamorphus*       | Graphidoideae     | Ocellularieae   | Peru, Rivas Plata 0810extra (F)     | JX421331 | JX421634 | KF875475 | –      |
| *Stegobolus radians*          | Graphidoideae     | Ocellularieae   | Peru, Rivas Plata 0810extra (F)     | JX421331 | JX421634 | KF875475 | –      |
| *Stegobolus subwrightii*      | Graphidoideae     | Ocellularieae   | Peru, Rivas Plata 0810extra (F)     | JX421331 | JX421634 | KF875475 | –      |
| *Thecaria montagnei*          | Graphidoideae     | Graphideae      | Phillipines, Rivas Plata 2083 (F)  | JX644422 | HQ639666 | KF875476 | –      |
| *Thecaria quassiicola*        | Graphidoideae     | Graphideae      | Puerto, Rivas Plata 0810extra (F)   | JX421320 | JX421634 | –      | –      |
| *Thecaria subquassiicola*     | Graphidoideae     | Graphideae      | Puerto, Rivas Plata 0810extra (F)   | JX421320 | JX421634 | –      | –      |
| *Thelotrema adjectum*         | Graphidoideae     | Thelotremateae  | India, Lumbsch 1973o (F)           | JX421344 | JX421642 | KF875478 | JX420851|
| *Thelotrema crepuscule*       | Graphidoideae     | Thelotremateae  | Australia, Mangold 27v (F)         | EU075606 | FJ708493 | –      | –      |
| *Thelotrema fuscospinata*     | Graphidoideae     | Thelotremateae  | Australia, Mangold 3j, 1d (F)      | JX421357 | JX421650 | KF875479 | JX420827|
| *Thelotrema gallowayanum*     | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 1915k (F)       | EU075600 | EU075653 | –      | –      |
| *Thelotrema inspersoporinaceum* | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 1908f (F)       | EU075594 | EU075640 | –      | –      |
| *Thelotrema jugale*           | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 19100yB (F)     | JX421360 | –       | –      | –      |
| *Thelotrema lepadinum*        | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 19977a (F)      | JX421360 | –       | –      | –      |
| *Thelotrema macrosporum*      | Graphidoideae     | Thelotremateae  | Scotland, Lumbsch 20100f (F)       | JX465291 | JX465313 | KF875483 | JX420890|
| *Thelotrema monosporoides*    | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 1915k (F)       | EU075601 | EU075646 | –      | –      |
| *Thelotrema monosporum*       | Graphidoideae     | Thelotremateae  | Australia, Lumbsch 1915k (F)       | EU075601 | EU075646 | –      | –      |

(continued)
(2-parameter) model of character evolution. In order to include topological uncertainty into the ancestral state reconstruction, we used the ‘Trace character over trees’ method of Mesquite (Maddison & Maddison, 2011). One thousand trees were randomly sampled from the post-burning of the Bayesian sampling (described above) of the concatenated dataset using the program RT (http://www.lutzonilab.net/downloads/), and Mesquite displayed a summary for the probability for each node and each character, indicating the probability for the different states, and also taking into account ambiguous reconstructions and the percentage of Bayesian trees in which the given node was present (Table S2, see supplemental material online).

In contrast to ML, maximum parsimony (MP) does not take into account branch lengths when reconstructing ancestral states. The reconstructions were performed over the same 1000 randomly sampled trees as in the ML analysis. We used Mesquite 2.75 (Maddison & Maddison, 2011) to carry out both ML and MP reconstructions for the character datasets.
Character state transformations

We used MP to estimate the number of character state transformations over the tree using Mesquite (Maddison & Maddison, 2011). Parsimony potentially underestimates the true amount of changes in a character over a tree (Huelsenbeck & Lander, 2003) but it gives the minimum amount of changes. To test for phylogenetic signals of the characters studied, we used two different approaches. First, we employed a randomization test (Archie, 1989) in Mesquite to calculate the number of parsimony steps from 999 randomizations of character states on the optimal ML tree. The mean number of parsimony steps and standard deviation were calculated. Then the numbers from the randomization and the observed number of parsimony steps were used to calculate the probability that the observed number of parsimony steps is higher or equal than expected from the randomization. Second, we used the fitDiscrete program in the R package ‘geiger’ (Yang, 2006) to calculate Pagel’s $\lambda$ value (Pagel, 1999). For this, the likelihood values of the tree with the actual data were calculated and a tree transformed to have no phylogenetic signal ($\lambda = 0$). High phylogenetic signal of a character is indicated by $\lambda = 1$. Significant departure from lack of phylogenetic signal was calculated using a likelihood ratio test.

Results

Phylogenetic analysis

The aligned 4-gene matrix contained 3274 unambiguously aligned nucleotide positions (945 in nuLSU, 836 in mtSSU, 607 in RPB1 and 886 in RPB2), with a total of 1009 constant characters. Topologies of single-locus analyses did not show conflict and hence combined analyses were performed. Since the topologies of the ML and Bayesian analyses did not show any supported conflict, only the phylogram obtained from the ML analysis is shown with branches in bold that received strong support in Bayesian analyses (i.e. PP above 0.95 and ML bootstrap values equal or above 70% indicated at branches) (Figs S1–S3, see supplemental material online). The topology obtained from the 4-gene dataset is similar to previously published phylogenies (Lucking et al., 2013; Rivas Plata et al., 2013). All subfamilies, tribes and genera as previously delimited were recovered (Fig. 1).

Ancestral character state reconstructions

The characters studied and their states are listed in Table 2. Results of the ancestral character state reconstructions are listed in Tables S2–S4 (see supplemental material online), and summarized in Figs 2–7 and Figs S4–S7 (see supplemental material online). The node numbers in the supplementary tables correspond to the clade numbers given in Fig. 1 Tables S3 and S4 (see supplemental material online), Figs 2–7 and Figs S4–S7 (see supplemental material online) only specify characters for which both MP and ML reconstructions gave strong support for the same character state.

In the ancestral character reconstruction for the character ‘habitat’ (Fig. 2), most ancestral nodes were reconstructed as being tropical, with the exception of Redonographa, which was reconstructed as subtropical, and the ancestral nodes of the genera Diploschistes, Schizotrema and Topeliopsis, which were reconstructed as being non-tropical. The reconstructions estimated a tropical habitat as ancestral for Graphidiaceae and subfamily Graphidoideae. All reconstruction methods estimated a trentepohlioid photobiont as ancestral state for all nodes, with the exception of the base of the genus Diploschistes (Fig. S4, see supplemental material online), for which a chlorococcoid photobiont was reconstructed.

Regarding the presence or absence of cortex, the ancestral character state reconstruction for the basal nodes in Graphidiaceae, with the exception of node 1 (corticated) did not yield conclusive results (Fig. 3). Within tribes Graphiheae and Ocellulariaeae, most nodes were reconstructed as being corticate. In contrast, in tribe Thelotremaeae, most nodes were reconstructed as being eocorticate, including the basal nodes of the genera Chapsa, Diploschistes, Leucodecton, Pseodochapsa and Thelotrema. The ancestral state for the genera Acanthotrema, Chroodiscus and the clade including Gintarasia and Pseudoramonia were reconstructed as corticate.

Absence of secondary metabolites was reconstructed for the basal nodes in subfamily Fissurinoideae and tribe Graphiheae and the base of Acanthotrema and Topeliopsis (Fig. 4). Due to the variability of presence of secondary metabolites among species, there was a higher incidence of inconclusive results compared with other characters. However, presence of (1′-6′-OH) depsidones, e.g. stictic acid and related substances, was reconstructed for the base of Chroodiscus, Leucodecton, Phaeographis, Pseodochapsa, Redonographa, Wirthiotrema and the clades including Asteristion, Austotrema, Nadvornikia and Myriotrema peninsulae, as well as this group plus Wirthiotrema, corresponding to tribe Thelotremaeae. The presence of (6′-COOH) depsidones, mainly psoromic acid, was reconstructed as ancestral trait for the base of the genera Compositrema, Myriotrema, Stigobolus and the genus Rhagodiscus. For all examined nodes in Ocellularia s. lat. and s. str., presence of (1′-COOH-6′-ME) depsidones, such as protocetraric acid, was reconstructed as the ancestral state.

The ancestral character state for ascoma persistence for all nodes studied was reconstructed as non-mazaediate with persistent hymenium (Fig. S5, see supplemental material online), strongly suggesting that the mazaediate
ascomata found in the genera *Nadvornikia* and *Schistophoron* originated independently from ascomata with persistent hymenium. Ascoma shape for the basal node of the family and the clade including the two subfamilies Graphidoideae and Redonographidoideae was not reconstructed with certainty, whereas the base of Graphidoideae was reconstructed as apothecioid (Fig. 5). Within Graphidoideae, most nodes in tribes Ocellulariae and Thelotremateae were reconstructed as apothecioid, except for *Acanthothecis*. In contrast, most nodes within tribe Graphideae were reconstructed as lirellate, except for the *Phaeographis lobata* + *P. spondaica* clade.
Figs. 2–4. Ancestral state reconstruction at the major nodes for (2) habitat, (3) cortex and (4) secondary chemistry of lichenized fungi family Graphidaceae. Node colours represent the reconstructed states (see legend). Inconclusive reconstructions are indicated in grey circles.
Figs. 5–7. Ancestral state reconstruction at the major nodes for (5) shape of ascoma, (6) the presence of lateral paraphyses and (7) ascospore amyloidity of lichenized fungi family Graphidaceae. Node colours represent the reconstructed states (see legend). Inconclusive reconstructions are indicated in grey circles.

Character transformations in Graphidaceae
 Almost all reconstructions suggested solitary ascomata as ancestral state (Suppl. Fig. S6). Only for the base of Compositrena (tribe Ocellulariae) the analysis reconstructed pseudostromatic ascomata as the ancestral character state. For all nodes outside tribe Ocellulariae, the analyses reconstructed absence of a columella as ancestral states (Fig. S7, see supplemental material online). Within Ocellulariae, the bases of the Macropyrumen and Stigmagora groups, Ocellularia s. str. and related taxa, and Stegalobolus were reconstructed as having a columella.

The ancestral character state reconstructions for lateral paraphyses showed these to be absent at numerous basal nodes, as well as at all nodes in Fissuroinoideae, Ocellulariae and Graphideae, except for Schizotrema and Topeliopsis (Fig. 6). In addition, the analysis suggests absence of lateral paraphyses at certain nodes within tribe Thelotrematae, including the clades containing the genera Chroodiscus, Gintarasia, Leucodencton and Pseudaramonia. Presence of lateral paraphyses was reconstructed for the bases of the genera Acanthotrema, Acanthothecis, Astrochapsa, Chapsa, Diplochistes, Pseudochapsa, Schizotrema, Thelotrema, Topeliopsis and the clade including the genera Astrochapsa, Pseudochapsa and Pseudotopeliopsis. In general, species with a columella lack lateral paraphyses. The ancestral state of ascospore amyloidity was reconstructed as non-amyloid for the base of Graphidaceae (Fig. 7) and several basal nodes in the family, e.g. subfam. Graphioideae and Redonografoideae. Non-amyloidity was also reconstructed as ancestral state for the base of Thelotrematae, the genera Acanthothecis, Acanthotrema, Astrochapsa, Chroodiscus, Diplochistes, Gintarasia and Pseudaramonia. Ascospores were reconstructed as being amyloid for the base of the genera Diorygma, Graphis, the Graphis scripta group in Graphideae, all nodes in Ocellulariae and two nodes in Thelotrematae (Leucodencton and Thelotrema). Within Phaeographis s. lat., all nodes were reconstructed as hemi-amyloid.

We also tested whether the observed number of transformations was significantly different from expectation based on randomized data. Table 4 summarizes the results and show that for all 10 characters, both the randomization test of parsimony steps and Pagel’s $\lambda$ suggest high phylogenetic signal of the characters, except for ascomata persistence.

In addition, the nature of transformations was found to be highly phylogenetically structured even for the fastest evolving character, secondary chemistry. Transitions between no substances and (1’ 6’ OH) depsidones and vice versa were almost entirely restricted to tribes Graphideae and Thelotrematae, and the subfamily Redonografoideae, whereas transitions between no substances and (1’ COOH 6’ ME) and (6’ COOH) and vice versa were restricted to tribe Ocellulariae in this dataset.

**Discussion**

Our phylogenetic study is based on a broad taxon sampling of Graphidaceae including the entire range of morphological and chemical diversity in three of the four subfamilies currently accepted, even if the species studied here represent only slightly more than 10% of the total species diversity in this lineage. The overall phylogeny is congruent with previously published analyses which are discussed elsewhere in detail (Rivas Plata et al., 2013). Hence, we anticipate that including a larger number of species will not change the overall structure of the results, with the exception of possible new lineages to be discovered. Rather, we project that with increased taxon and gene sampling, higher resolution and backbone support will resolve ancestral character reconstruction for most of the presently unresolved nodes. In the current study the subfamily Gomphilloideae was not included and this might potentially influence the results on character evolution, especially the basal nodes of Graphidaceae and hence we have refrained from discussing the character states obtained for node 1 at the base of the family in detail.

We found support for the ancestor of subfamily Graphioideae to be a tropical species with a trentepohlioid photobiont, apothecoid, solitary ascomata with persistent hymenium lacking a columella and lateral paraphyses, and having non-amyloid ascospores. Among extant lineages, the taxon that comes closest to this circumscription is Acanthotrema. This genus indeed appears to be the relict of an ancient lineage going back almost 100 million years and usually is positioned close to the base of the subfamily (Lücking et al. 2013).

Reconstructions of the ancestral character state of the cortex and chemistry did not yield conclusive results for the ancestor of the subfamily. Regarding ecology, our results suggest that subtropical and non-tropical

**Character state transformations**

The minimal numbers of transformations under MP are shown in Table 2. The most conserved characters were photobiont, lateral paraphyses, ascoma aggregation and persistence, and habitat, whereas by far the fastest evolving was chemistry.

Direction of these changes varies among the characters (Table 3). A few characters were reconstructed to change unidirectionally, such as the switch from trentepohlioid to chlorococcoid photobiont, from persistent to mazaediate ascomata, and solitary to pseudostromatic ascomata. In contrast, transformations from apothecoid to lirellate and vice versa were reconstructed as being almost equally common (53% vs. 46%).

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Table 3. Proportion of changes between character states under maximum parsimony summing to 1 for ten studied characters in lichenized fungi family Graphidaceae. The second line contains maximum a posteriori value of number of transitions and 95% equal-tail credible interval in the parenthesis.

| Character | Description |
|-----------|-------------|
| Character 1 | Habitat; 0 = tropical, 1 = subtropical, 2 = non-tropical |
| 0 | 1 | 2 |
| 0 | – | 0.09 | 0.73 |
| 1 | 0.00 | – | 0.00 |
| 2 | 0.18 | 0.00 | – |
| Character 2 | Photobiont; 0 = trentepohlioid, 1 = chlorococcoid |
| 0 | 1 |
| 0 | – | 1.00 |
| 1 | 0.00 | – |
| Character 3 | Cortex; 0 = corticate, 1 = ecorticate |
| 0 | 1 |
| 0 | – | 0.68 |
| 1 | 0.32 | – |
| Character 4 | Chemistry; 0 = no substances, 1 = depsidones (1'-6'-OH), 2 = depsidones (1'-COOH-6'-ME), 3 = depsidones (6'-COOH), 4 = depsides |
| 0 | 1 | 2 | 3 | 4 |
| 0 | – | 0.36 | 0.09 | 0.03 | 0.04 |
| 1 | 0.13 | 20(14-27) | 5(3-8) | 1.8(1-3) | 2.3(1-3) |
| 3 | 0.07 | 0.01 | 0.05 | – | 0.00 |
| 4 | 0.01 | 0.00 | 0.00 | 0.00 | – |
| Character 5 | Ascoma persistence; 0 = persistent, 1 = mazaediate |
| 0 | 1 |
| 0 | – | 1.00 |
| 1 | 0.00 | – |
| Character 6 | Ascoma shape; 0 = apothecioid, 1 = lirellate |
| 0 | 1 |
| 0 | – | 0.53 |
| 1 | 0.46 | – |
| Character 7 | Ascoma aggregation; 0 = solitary, 1 = pseudostromatic |
| 0 | 1 |
| 0 | – | 1.00 |
| 1 | 0.00 | – |
| Character 8 | Columella; 0 = absent, 1 = present |
| 0 | 1 |
| 0 | – | 0.63 |
| 1 | 0.36 | – |

(continued)
species in this lineage are derived from tropical taxa. However, none of these lineages re-adapted to strictly tropical rain forest vegetation, where the majority of Graphidaceae occurs. This is consistent with a previous study with a smaller taxon sampling (Lücking et al., 2013).

A chlorococcoid photobiont is restricted to the non-tropical genera Diploschistes and Xalocoa (formerly D. ocellatus; Kraichak et al., 2014). Within those clades, there was no transformation back to trentepohlioid photobionts, which are the dominant type of photosynthetic partners in this family, including other extratropical groups within the family such as Schizotrema and Topeliopsis (Nakano, 1988; Nelsen et al., 2011). Species of Diploschistes differ from other Graphidaceae in their peculiar ecology, being most common and diverse in subtropical, semi-arid regions in both hemispheres and growing mostly on soil and rock substrata. Other, unrelated lichenized fungi sharing these habitats almost exclusively have chlorococcoid photobions. Since Diploschistes species do not produce vegetative propagules but exclusively propagate by means of ascospores, new thalli can only be formed through resymbiosis with the appropriate photobiont, and it is highly unlikely in these habitats to encounter Trentepohlia algae. This is recognized by some species of Diploschistes being juvenile parasites on other lichen species (Hawksworth, 1982; Friedl, 1987; Friedl & Gärtnert, 1988). We therefore conclude that the switch to a chlorococcoid photobiont enabled Diploschistes to diversify in these unusual habitats for Graphidaceae and with no reversal to a trentepohlioid photobiont in this lineage. Notably, subfamily Gomphilloideae, which was not included in this study, also is characterized by a chlorococcoid photobiont. However, this subfamily is predominantly tropical and mostly foliicolous, sharing niches with other lichens that do have trentepohlioid photobionts, so the advantage of the photobiont in this lineage is unclear.

Table 4: Observed numbers of parsimony steps of transitions and phylogenetic signals of ten studied characters in the lichenized fungal family Graphidaceae from randomization tests (Archie 1989) and Pagel’s Lambda (1999). Mean_{random} and SD_{random} refer to the mean and standard deviation of the number of parsimony steps from 999 randomizations of character states. P_{random} is the probability that the observed number is higher than expected from the randomization. Thus, a low P_{random} suggests high phylogenetic signal. Lambda = 1 indicates high phylogenetic signal, and P_{LTR} is the result from the Likelihood Ration Test when compared the likelihoods of data against the tree transformed to lambda = 0 (no signal). Therefore, a low P_{LTR} indicates a significant deviation from the model with no signal.
Our study suggests that a cortex was gained twice as often as lost, and that this character has switched repeatedly over the evolution of the family (15–29 times). As discussed elsewhere (Lakatos et al., 2006; Rivas Plata & Lumbsch, 2011), a dense cortex could be an adaptation to avoid oversaturation with water in understory crusts (the cortex is water-repellent) or to provide protection against high light levels and damaging UV radiation, as well as against herbivores and fungivores. However, eocarticulate thalli might also be water-repellent, but respond to hydration differently (Lakatos et al. 2006). Given these multiple functions of the cortex and the fact that closely related lineages may show different habitat preferences, it is not surprising that a cortex has been gained or lost several times independently. Despite the fact that we coded secondary metabolites as substance classes (Huneck & Yoshimura, 1996), rather than individual substances, we reconstructed a high number of transformations. Secondary metabolites are well-known in lichenized fungi, with over 1000 substances recorded (Huneck & Yoshimura, 1996; Lumbsch, 1998; Nash, 2008) and their potential ecological importance, such as sunscreens, protection against herbivores and control of carbon diffusion, has been discussed in a number of studies (Lawrey, 1983; Goloujch & Lawrey, 1988; Emmerich et al., 1993; Giez et al., 1994; Rikkinen, 1995; Lange et al., 1997; Nybakken et al., 2004, 2010; Gauslaa, 2005; McEvoy et al., 2007; Solhaug et al., 2009). Our study showed that the majority of the transformations (86%) are between having no substance and producing a given substance class, while transformations between different substance classes are much less common. In addition, substance classes appear to be largely conserved within major clades. Thus, basal nodes of tribe Ocellulariaeae (e.g. Rhabdodiscus, Myriotrema, Stegobolus) are reconstructed as having (6′–COOH) depsidones, i.e. psoromic acid and relatives, while the majority of clades within Ocellularia are reconstructed as having (1′–COOH–6′–ME) depsidones, i.e. protocetraric acid and relatives. The high level of conservation among major clades suggests that transformations between substance classes are the result of constrained evolution. The pattern of occurrence and absence of secondary metabolites within each clade suggests that there is no strong selective advantage in having medullary secondary substances, since species with and without substances often grow side by side in the same conditions, e.g. in the genera Graphis and Ocellularia.

Our analyses suggest that the transformation of ascomata with persistent hymenium to mazaediate ascomata happened at least twice in Graphidaceae and that there was no reversal from this trait back to persistent ascomata. Mazaediate ascomata are characteristic for the genera Nadvornikia and Schistophoron (Lumbsch et al., 2004; Tehler et al., 2009). Both genera have few species, with rather deep phylogenetic relationships, with lack of evidence for radiation after the transformation, contrary to other mazaediate groups such as calicioid species in Physciaceae, which are very species-rich. Mazaedia are considered an adaptation to wind dispersal of ascospores, and species with such ascomata often grow in open microhabitats and particularly well on old trees with weathered bark or on wood. This is also the case with species of Nadvornikia and Schistophoron, hence representing a case of convergent evolution with unrelated lineages also producing mazaedia, such as Heterocyphellum and Tylophoron (Arthoniales), Mazaediothecium and Pyrgillus (Pyrenulales), and many of the species in Caliciales.

Ascoma shape was traditionally used to distinguish the families Graphidaceae and Thelotremataceae, now included in a single family, as well as groups of genera within Graphidaceae. Our study confirms previous results demonstrating that ascoma shape does not constitute a synapomorphic trait characterizing monophyletic groups within Graphidaceae (Staiger et al., 2006; Mangold et al., 2008b; Rivas Plata et al., 2013). Transformations from apotheciate to lirellate ascomata and vice versa are almost equally probable in the family. Despite the number of transformations in this character, most nodes – except the majority of basal nodes – were reconstructed with strong support as being either apothecoid or lirellate. While the character is variable at a deeper phylogenetic level, it is mostly invariant among genera and tribes (Rivas Plata et al., 2012a). This suggests a high level of evolutionary plasticity at the more basal nodes but subsequent stabilization at nodes leading to major lineages. This is consistent with the observation that this character is more variable in smaller clades, such as Acanthothecis, which includes species with both types of ascomata (perfectly round and strongly lirellate ascomata). The reason for the observed variation and its relatively high level of conservation is unknown, but likely this character is somehow involved in protecting the hymenium and ascospores from fungivores or exposure to UV radiation. Since most species reproduce through ascospores, reproductive success depends in part on the number of ascospores produced, which is in turn a function of the hymenium surface. In lineages with rounded ascomata, the hymenium has to expand radially in order to produce a large number of ascospores, exposing a large surface area to the environment and thus making it more vulnerable. To still protect the hymenium, one solution is to maintain the ascomata relatively closed, which is observed in many lineages in tribe Ocellulariaeae and in the genera Thelotrema and Leucodecton (Thelotremataeae). Another solution is to cover the hymenium with dead hyphal material and crystals for protection, as found in species with chroodiscoid ascomata in tribe Thelotremataeae. Another alternative is to maintain the ascomata completely closed, in which case a lirellate shape is of advantage since the ascoma can grow without the necessity to expose the hymenium.
Although we found evidence for at least 10 transformations in ascoma aggregation, we found no evidence for transformations from pseudostromatic to solitary ascomata. This is consistent with results in other groups that in part produce pseudostromatic ascomata, such as Arthoniales (Ertz et al., 2009).

The higher number of transformations in gaining a columnella and lateral paraphyses in comparison to losing them can be interpreted as evidence for an adaptive value of these structures. A columnella is a sterile tissue in ascomata that can sometimes cover large parts of the ascomata, potentially preventing fungivores from feeding on hymenial structures (Lücking & Bernecker-Lücking, 2000; Rivas Plata & Lumbsch, 2011). The adaptive value of lateral paraphyses, however, is currently not understood. The character might represent a conserved relict of the ascoma ontogeny, rather than an ecological adaptation, since the lateral paraphyses are ontogenetically a part of the generative hymenium (Henssen, 1976, 1995). It appears that in lineages forming lateral paraphyses, the hymenium originally develops throughout most of the cavity, and upon maturity and opening of the ascoma, the lateral portions of the hymenium that originally developed in the upper parts of the cavity remain vertical and sterile.

Ascospore amyloidity showed a high number of transformations (16–28), especially gains and losses of amyloidity. Similar to what we observed for secondary chemistry, in spite of the high number of transformations, this character is usually conserved at the generic and often also the tribe level, with the notable exception of tribe Thelotrematae (and subfamily Fissurinoideae). Amyloid ascospores are also found in other groups of lichen-forming and non-lichenized ascomycetes, but the ecological importance of this character is not known.

In summary, it appears that while there are a large number of transitions between phenotype character states in Graphidaceae, more than any other crustose lichen group with the exception of Arthoniales, these transitions are highly structured phylogenetically and also provide evidence to formulate hypotheses on ecological functions versus evolutionary constraints. Our results are consistent with previous studies on character evolution in the family focusing on striking cases of parallel evolution and evolutionary plasticity in closely related lineages (Rivas Plata & Lumbsch, 2011). However, our extended sampling of loci and taxa improved the confidence in the phylogenetic estimate (Rivas Plata et al., 2013) and allowed for a more solid statistical approach, also demonstrating an even higher number of character transformations than previously assumed. This study hence provides an ideal base for addressing the question whether and how specific traits are correlated with ecological conditions and how these traits go along with diversification, in order to test hypotheses of adaptive radiations.

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