Org Divers Evol (2015) 15:447–458
DOI 10.1007/s13127-015-0219-5

ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

Hidden diversity in the morphologically variable script lichen (*Graphis scripta*) complex (Ascomycota, Ostropales, Graphidaceae)

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Received: 20 August 2014 / Accepted: 11 May 2015 / Published online: 17 May 2015

Abstract *Graphis scripta*, or script lichen, is a well-known species of crustose lichenized fungi, widely distributed in the temperate region of the Northern Hemisphere. It is now considered to be a species complex, but because of the lack of secondary chemistry and paucity of measurable morphological characters, species delimitation within the complex has been challenging and is thus far based on apothecium and ascospore morphology. In this study, we employed molecular as well as morphological data to assess phylogenetic structure and delimitation of lineages within the *G. scripta* complex. We generated sequences for four genetic markers (mtSSU, nuLSU, *RPB2*, and *EF-1*) and performed phylogenetic analyses. The resulting trees were used to determine the number of distinct lineages by applying a general mixed Yule-coalescent (GMYC) model and species tree estimation through maximum likelihood (STEM). Our analyses suggest between six and seven putative species within the *G. scripta* complex. However, these did not correspond to the taxa that were recently distinguished based on apothecium morphology and could not be circumscribed with the morphological characters that were traditionally used in the classification of the complex. Any formal taxonomic treatment will require additional sampling and evaluation of additional traits that potentially can characterize these clades.

Keywords Crustose lichens · General mixed Yule-coalescent method · Species delimitation · Species trees · Taxonomy

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Introduction

Since the time of Darwin, the concept of species remains central to our understanding of evolutionary processes (Cracraft 1983; Mayr 1963; Wiens and Penkrot 2002). For centuries, biologists relied on morphological characters as a principal tool for delimiting species boundaries. While this method has led to a tremendous growth in biodiversity knowledge and conservation, it is particularly challenging for organisms with very few morphological characters available for delimitation, such as lichenized fungi. Morphological characters have been shown to also vary with environmental factors or converge under the same set of conditions (Blanco et al. 2004; Högnabba and Wedin 2003; Parnmen et al. 2010; Stenroos and DePriest 1998; Tehler and Irestedt 2007; Wedin and Döring 1999), making it difficult to use them for rigorous

species delimitation. It also presents problems for many so-called cryptic species which are reproductively isolated from one another but do not exhibit any obvious phenotypic differences (Bickford et al. 2007). With these species, morphological-based delimitation methods often fail to identify relevant units for study of evolution and conservation.

Recent advances in molecular biology have allowed systematists to use DNA sequence data as an additional tool to understand phylogenetic relationships and use them to inform classifications (Divakar and Crespo 2015; Goldstein et al. 2000; Leavitt et al. 2015; Vogler and Monaghan 2007; Wiens and Penkrot 2002). In organisms with a relatively simple morphology, molecular data have revealed a tremendous amount of genetic diversity within many formerly known taxonomic units. When combined with other types of data (morphological, ecological, anatomical), molecular evidence has also led to dramatic changes in classification. Such integration also allows for discoveries of numerous previously unrecognized lineages, many of which can be distinguished by previously overlooked or undervalued characters (Crespo and Lumbsch 2010; Fontaneto et al. 2011; Lendemer and Hodkinson 2013; Lumbsch and Leavitt 2011; Lücking et al. 2013; Moncada et al. 2013; Parnmen et al. 2010). However, not all newly recognized lineages characterized by molecular data have been shown to be correlated with phenotypic characters or ecology (Bickford et al. 2007; Leavitt et al. 2011).

Lichenized fungi in the family Graphidaceae provide an excellent opportunity for an investigation surrounding the issue of species delimitation, as these are crustose lichens with a limited set of morphological characters. Traditional taxonomic treatments within the family largely rely on chemistry, apothecium shape, and ascospore morphology (Staiger 2002). Molecular data on other lichenized fungal lineages have shown that the classification based on these characters usually underestimates the number of distinct lineages under a single taxonomic name (Crespo and Lumbsch 2010; Crespo and Ortega 2009; Hodkinson and Lendemer 2011; McCune and Schoch 2009; Ruprecht et al. 2010; Spribille et al. 2011; Vondrák 2012; Wedin et al. 2009). Within Graphidaceae, traits traditionally used for classification were shown to have evolved independently several times (Lumbsch et al. 2014; Rivas Plata and Lumbsch 2011). Consequently, a number of phylogenetic analyses have led to substantial changes in classification at the family and generic levels (Parnmen et al. 2013; Rivas Plata et al. 2012, 2013) and to the identification of previously unrecognized species complexes (Kraichak et al. 2014; Lumbsch et al. 2008). Genetic variation within a single species complex, however, has not yet been studied in any detail.

In this study, we focus on a widespread complex representing one of the best-known crustose lichens—*Graphis scripta*, commonly known as the "script lichen." This complex represents one of a few extratropical members of the predominantly tropical family Graphidaceae (Lücking et al. 2014). Tropical specimens had sometimes been identified as *G. scripta*, but Staiger (2002) recognized these tropical specimens as *G. furcata* Fée. Following this treatment, we considered only the specimens from temperate areas for the *G. scripta* complex. The species complex is characterized by a suite of phenotypic characters: simple to sparsely branched, elongated black apothecia with black labia and a laterally carbonized excipulum, clear hymenium, transversely septate, small ascospores, and the lack of secondary substances (Lücking et al. 2009). While all the members within the complex share these features, many additional characters—such as branching of the lirellae, visibility of the disc, absence or presence of pruina, and ascospore size and morphology—vary tremendously within the complex. This large variation has led to proliferation, as well as confusion, of numerous names, particular at the infraspecific level, since the time that Linnaeus first introduced the name *Lichen scriptus* (Linnaeus 1753). Acharius transferred the epithet to the genus *Graphis* and synonymized it with 30 other available names (Acharius 1809). In 1923, Zahlbruckner adopted an even broader circumscription and synonymized over 100 names with *G. scripta* (Zahlbruckner 1923). Since then, the broad concept of *G. scripta* proposed by Zahlbruckner has been widely accepted and maintained for almost a century. Only recently, a study of European materials recognized four species within the *G. scripta* complex (Fig. 1). These taxa were distinguished based on the co-occurrence of multiple morphologically distinct individuals on the same trunk, particularly in their apothecium morphology (Neuwirth and Aptroot 2011). However, the authors did not provide molecular evidence to support their classification.

Adopting the general lineage concept (de Queiroz 2007), we used DNA sequences and morphological data to evaluate the diversity within the *G. scripta* complex. More specifically, we employed phylogenetic and coalescent-based methods, including the general mixed Yule-coalescent (GMYC) model (Zhang et al. 2013) and species tree estimation with maximum likelihood (STEM) (Kubatko et al. 2009), to identify distinct lineages within the group. We also measured five morphological characters from apothecia and ascospores to determine the differences among identified clades, using phylogenetic analysis of variance to account for the effects of non-independence among data points. More specifically, we addressed the following questions: (1) How many genetically distinct clades exist within the *G. scripta* complex? (2) Do these clades correspond to previously described species? (3) Can the distinct lineages be characterized by morphological characters?

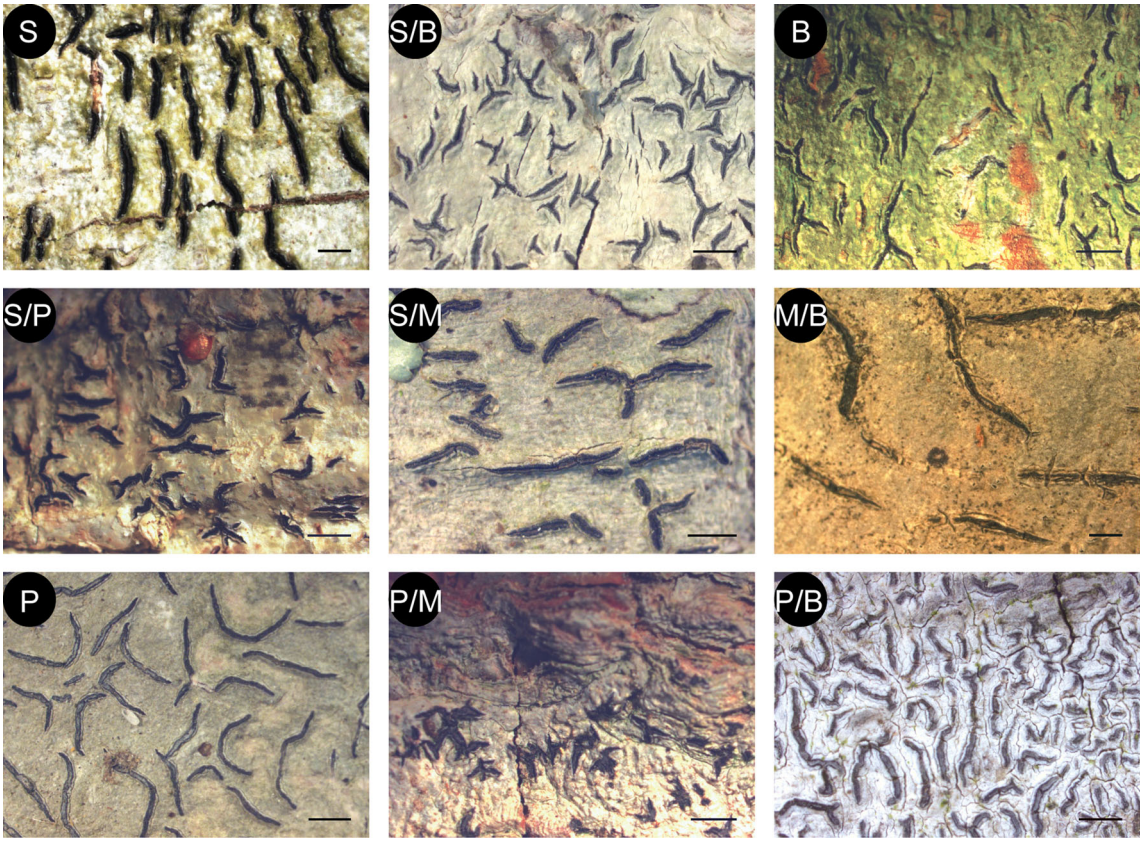


Fig. 1 Morphotypes within *Graphis scripta* complex. The identification of morphotypes followed Neuwirth and Aptroot (2011). *S Graphis scripta* morphotype (Bachmann 8.210), *S/B G. scripta-betulina* intermediate morphotype (Dornes K_OA 4411), *B G. betulina* morphotype (John 8.144), *S/P G. scripta-pulverulenta* morphotype (John 8.150), *S/M G. scripta-macrocarpa* morphotype (Lendemer 32854), *M/B G. macrocarpa-betulina* morphotype (Sohrabi 16438), *P G. pulverulenta* morphotype (Lendemer 33481), *P/M G. pulverulenta-macrocarpa* morphotype (Nelsen 498), *P/B G. pulverulenta-betulina* morphotype (Dornes 21304.006). A *black bar* at the bottom right of each picture indicates 1 mm

Materials and methods

Molecular methods

A total of 56 recently collected samples of the *Graphis scripta* complex (*G. scripta*, *G. betulina*, *G. macrocarpa*, and *G. pulverulenta*) were obtained through various herbarium loans and personal collections from North America, Europe, and China (Table 1). The identification of samples in this study followed a recently published description of morphological species (Table 1) (Neuwirth and Aptroot 2011). Total genomic DNA was extracted from the specimens using the REDExtract-N-Amp PCR Plant Kit (Sigma-Aldrich) following the instructions of the manufacturer, except that we used 1:10 dilution of the total DNA extract and obtained the genomic DNA with the final concentration between 50 to 200 ng/μL. Data matrices of 203 sequences from these samples were generated using sequences of mitochondrial small subunit rRNA (mtSSU), nuclear large subunit rRNA (nuLSU), the RNA polymerase II second largest subunit (*RPB2*), and translation elongation factor (*EF-1*) sequences (Table 1). We also included sequences of *Graphis implicata* and *Graphis librata* as outgroups based on previous studies (Rivas Plata et al. 2013). Nuclear intergenic spacer (nuITS)—the conventional barcoding locus for fungi—was not included in the study, because of the low amplification rate in this group of samples. All DNA extraction vouchers are housed at the Pritzker Laboratory of Molecular Systematics in the Field Museum.

Genomic DNA extracts were used for PCR amplifications. Primers for amplification included (a) for mtSSU: mrSSU1 and mrSSU3R (Zoller et al. 1999), (b) for nuLSU: AL2R (Mangold et al. 2008) and LR3 (Vilgalys and Hester 1990), and (c) for EF-1: EFdF and 1567R (Rehner 2001). For *RPB2*, we designed a set of primers to target samples of Graphidaceae: GD1-RPB2-7cF (forward: GAGCGAATGG ATACCATGGCGA) and GD-RPB2-11aR (reverse: GCTT ACGCCCGGTGTGACCATTGT). The 10-μL PCR reactions contained 2.5 μL of REDExtract-N-Amp PCR Ready Mix (Sigma-Aldrich), 0.5 μL of each primer (10 μM), 2 μL genomic DNA extract, and 4.5 μL nuclease-free water. The PCR conditions were as follows: (1) for mtSSU, initial denaturation for 5 min at 95 °C, followed by 35 cycles of 45 s at 94 °C,

Table 1 List of taxa with their collection data and GenBank accession numbers for the sequences used in this study

Voucher information	DNA voucher	mtSSU	nuLSU	*RPB2*	*EF-1*	Morphotype
Costa Rica, *Lücking 16103* (F)	3194	DQ431978	DQ431939	JF828947	**KJ441071**	*Graphis imbricata*
El Salvador, *Lücking 28001* (F)	3188	HQ639621	HQ639636	JF828945	**KJ441070**	*Graphis librata*
Austria, *Neuwirth 11808* (F)	6812	KF875564	KF875543	KF875526	**KJ441073**	P
Austria, *Neuwirth 11834* (F)	6814	KF875565	KF875544	KF875527	**KJ441077**	S
Canada, British Columbia, *Tønsberg 42518* (BG)	8611	**KJ440969**	**KJ440922**	**KJ441017**	**KJ441061**	S
Canada, British Columbia, *Tønsberg 42519* (BG)	8612	**KJ440970**	**KJ440923**	–	**KJ441062**	S
Canada, British Columbia, *Tønsberg 42520* (BG)	8613	**KJ440971**	**KJ440924**	**KJ441018**	**KJ441063**	S/B
Canada, British Columbia, *Tønsberg 42522* (BG)	8615	**KJ440972**	**KJ440925**	**KJ441019**	**KJ441064**	S/B
China, *Sohrabi 16429* (F)	6464	KF875562	KF875541	KF875524	–	P
China, *Sohrabi 16438* (F)	6450	KF875563	KF875542	KF875525	**KJ441072**	M/B
China, *Sohrabi 16579* (F)	6454	KF875561	KF875540	KF875523	**KJ441030**	P/B
Germany, *Bachmann 8.210* (POLL)	7507	**KJ440955**	**KJ440894**	**KJ440994**	**KJ441028**	S
Germany, *Bachmann 8.208* (POLL)	7505	**KJ440954**	**KJ440893**	**KJ440993**	**KJ441039**	S
Germany, Baden-Württemberg, *Dornes 21212.136* (M)	8274	**KJ440967**	**KJ440911**	**KJ441006**	**KJ441053**	S/B
Germany, Baden-Württemberg, *Dornes 21212.151* (M)	8275	**KJ440968**	**KJ440912**	**KJ441007**	–	S/B
Germany, Baden-Württemberg, *Dornes 21304.006* (M)	8286	–	**KJ440920**	**KJ441015**	**KJ441059**	P/B
Germany, Baden-Württemberg, *Dornes 21304.007* (M)	8287	–	**KJ440921**	**KJ441016**	**KJ441060**	P/B
Germany, Baden-Württemberg, *Dornes 21304.008* (M)	8276	–	**KJ440913**	**KJ441008**	**KJ441054**	P/B
Germany, Baden-Württemberg, *Dornes 21304.012 (*M)	8277	–	**KJ440914**	**KJ441009**	**KJ441055**	S/B
Germany, Baden-Württemberg, *Dornes 21304.015* (M)	8278	–	**KJ440915**	**KJ441010**	**KJ441056**	P/B
Germany, Bayern, *Dornes K_OA 4410* (M)	8271	–	**KJ440909**	**KJ441004**	**KJ441051**	S
Germany, Bayern, *Dornes K_OA 4411* (M)	8272	–	**KJ440910**	**KJ441005**	**KJ441052**	S/B
Germany, Bayern, *Dornes K_OA 4413* (M)	8280	–	**KJ440916**	**KJ441011**	–	P
Germany, Bayern, *Dornes K_OA 4433* (M)	8281	–	**KJ440917**	**KJ441012**	**KJ441057**	S/B
Germany, Bayern, *Dornes K_OA 4442* (M)	8282	–	**KJ440918**	**KJ441013**	**KJ441074**	P
Germany, Bayern, *John 8.051* (POLL)	6999	**KJ440950**	**KJ440889**	–	**KJ441035**	S
Germany, Rheinland-Pfalz, *John 8.140* (POLL)	6991	**KJ440942**	**KJ440881**	**KJ440984**	**KJ441026**	B
Germany, Rheinland-Pfalz, *John 8.144* (POLL)	6992	**KJ440943**	**KJ440882**	–	**KJ441031**	B
Germany, Rheinland-Pfalz, *John 8.149* (POLL)	6993	**KJ440944**	**KJ440883**	**KJ440985**	**KJ441027**	P
Germany, Rheinland-Pfalz, *John 8.150* (POLL)	6994	**KJ440945**	**KJ440884**	**KJ440986**	**KJ441032**	S/P
Germany, Rheinland-Pfalz, *Werth K_OA 9237* (M)	8284	–	**KJ440919**	**KJ441014**	**KJ441058**	P/B
The Netherlands, *Aptroot 11808* (ABL)	8018	**KJ440956**	**KJ440895**	–	**KJ441040**	P
Switzerland, Ticino, *John 8.172* (POLL)	7000	**KJ440951**	**KJ440890**	**KJ440990**	**KJ441036**	P
Switzerland, Ticino, *John 8.173* (POLL)	7001	**KJ440952**	**KJ440891**	**KJ440991**	**KJ441037**	P
Switzerland, Ticino, *John 8.174* (POLL)	7002	**KJ440953**	**KJ440892**	**KJ440992**	**KJ441038**	S/P
Switzerland, Ticino, *John 8.175* (POLL)	6995	**KJ440946**	**KJ440885**	**KJ440987**	**KJ441033**	S/P
Switzerland, Ticino, *John 8.176* (POLL)	6996	**KJ440947**	**KJ440886**	**KJ440988**	–	S/P
Switzerland, Ticino, *John 8.177* (POLL)	6997	**KJ440948**	**KJ440887**	–	**KJ441029**	S/P
Switzerland, *John 8.178* (POLL)	6998	**KJ440949**	**KJ440888**	**KJ440989**	**KJ441034**	S/P
USA, Maryland, *Harris 57934* (NY)	8270	**KJ440966**	**KJ440908**	**KJ441003**	**KJ441050**	S
USA, Delaware, *Harris 57956* (NY)	8236	–	**KJ440900**	**KJ440999**	**KJ441044**	S
USA, Delaware, *Harris 57982* (NY)	8237	–	**KJ440901**	**KJ441000**	**KJ441045**	S
USA, North Carolina, *Lendemer 31033* (NY)	8264	**KJ440964**	**KJ440906**	–	**KJ441048**	S
USA, Delaware, *Lendemer 32055* (NY)	8239	**KJ440960**	**KJ440902**	–	**KJ441046**	S/P
USA, Delaware, *Lendemer 32104* (NY)	8240	**KJ440961**	**KJ440903**	–	**KJ441075**	S
USA, Delaware, *Lendemer 32154* (NY)	8242	**KJ440962**	**KJ440904**	**KJ441001**	**KJ441047**	S/P
USA, Maine, *Lendemer 32300* (NY)	8226	–	**KJ440896**	**KJ440995**	**KJ441041**	P
USA, North Carolina, *Lendemer 32854* (NY)	8255	**KJ440963**	**KJ440905**	–	–	S/M
USA, Maryland, *Lendemer 33481* (NY)	8268	**KJ440965**	**KJ440907**	**KJ441002**	**KJ441049**	P
USA, Delaware, *Lendemer 35766* (NY)	8233	**KJ440958**	**KJ440898**	**KJ440997**	**KJ441042**	S/P
USA, Delaware, *Lendemer 35829* (NY)	8235	**KJ440959**	**KJ440899**	**KJ440998**	**KJ441043**	S/P

Table 1 (continued)

Voucher information	DNA voucher	mtSSU	nuLSU	*RPB2*	*EF-1*	Morphotype
USA, Pennsylvania, *Lendemer 37782* (NY)	8232	**KJ440957**	**KJ440897**	**KJ440996**	–	P
USA, Michigan, *Nelsen MN184* (F)	MN184	**KJ461719**	**KJ440933**	**KJ441020**	**KJ441065**	P
USA, Wisconsin, *Nelsen MN498* (F)	MN498	**KJ440978**	**KJ440934**	**KJ441021**	**KJ441066**	P/M
USA, Wisconsin, *Nelsen MN499* (F)	MN499	**KJ461720**	**KJ440935**	**KJ441022**	**KJ441076**	S
USA, Illinois, *Nelsen MN503* (F)	MN503	**KJ461721**	**KJ440936**	**KJ441023**	**KJ441067**	S/P
USA, Illinois, *Nelsen MN507* (F)	MN507	**KJ461722**	**KJ440937**	**KJ441024**	**KJ441068**	S
USA, Illinois, *Nelsen MN559* (F)	MN559	**KJ461723**	**KJ440938**	**KJ441025**	**KJ441069**	P
	Number of new sequences	37	51	42	52	
	Total number of sequences	44	58	49	52	
	Original alignment length (bp)	831	935	966	400	
	Adjusted alignment length (bp)	759	483	879	357	
	Number of identical sites	608	176	603	169	

Bold accession numbers indicate sequences generated for this study. Morphotype column refers to the identification of the specimen based on the most recent classification. A morphotype with two letters indicates an intermediate form between two morphotypes

B Graphis betulina, *M G. macrocarpa*, *P G. pulverulenta*, *S G. scripta*

– missing data

1 min at 50 °C, 1 min 30 s at 72 °C, and a final elongation for 10 min at 72 °C; (2) for nuLSU, initial denaturation for 1 min 15 s at 94.5 °C, followed by 35 cycles of 35 s at 95 °C, 55 s at 55 °C, 42 s at 72 °C, and a final elongation for 10 min at 72 °C; (3) for *RPB2*, initial denaturation for 3 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 1 min at 52 °C, 1 min 30 s at 72 °C, and a final elongation for 10 min at 72 °C; and (4) for *EF-1*, initial denaturation for 5 min at 95 °C, followed by a cycle of 45 s at 95 °C, 45 s at 60 °C, 30 min at 72 °C. For the following cycles, the annealing temperature was reduced at 1 °C per cycle until it reached 50 °C and increased it back up to 55 °C for 30 additional cycles with a final elongation for 10 min at 72 °C.

Amplification products were visualized on 1 % agarose gels stained with ethidium bromide and subsequently isolated using GELase™ Agarose Gel-Digesting Preparation (Epicentre). Fragments were sequenced using the Big Dye Terminator Reaction Kit (ABI PRISM, Applied Biosystems) and the same sets of primers from the amplification. Cycle sequencing was performed with the following program: 25 cycles of 95 °C for 30 s, 48 °C for 15 s, 60 °C for 4 min. Sequenced products were precipitated with sterile dH_2O, EDTA, and 70 % EtOH before they were loaded on an ABI 3100 (Applied Biosystems) automatic sequencer. Sequence fragments obtained were assembled with Geneious 5.5.8 (Drummond et al. 2014) and manually adjusted.

Sequence alignments and phylogenetic analysis

Alignments were initially performed using Geneious (Drummond et al. 2014). Individual single-gene alignments were then optimized by removing regions lacking sequence conservation using Gblocks (Castresana 2000; Talavera and Castresana 2007), using options for a "less stringent" selection on the Gblocks web server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). The alignments were then analyzed using maximum likelihood (ML) and Bayesian approaches (B/MCMC). Individual gene trees were reconstructed under the ML approach and visually inspected for incongruences before combining the data. Namely, we looked for any conflict in well-supported clades (posterior probability≥0.95 in B/MCMC analysis an MP bootstrap ≥70 %) and did not perform any formal statistical test.

The ML analysis was performed on a partitioned alignment with the program RAxML-HPC2 (version 7.3.1) on XSEDE (Stamatakis 2006), using the default rapid hill-climbing algorithm and the GTRGAMMA model of nucleotide substitution. The partitions were set for each of the loci. Rapid bootstrap estimates were carried out for 1000 pseudoreplicates (Stamatakis et al. 2008).

For the B/MCMC analysis, the data set was also partitioned into four parts (one for each locus) and then analyzed using MrBAYES 3.1.2 (Huelsenbeck and Ronquist 2001). The model testing was performed for each locus, using the program jmodeltest2 (Darriba et al. 2012; Guindon and Gascuel 2003). For all loci, the GTR+I+G was chosen as the appropriate substitution model both using likelihood ratio tests and the AIC. Two parallel runs with 20,000,000 generations, starting with a random tree and employing four simultaneous chains, were executed. Heating of chains was set to 0.2. Posterior probabilities were estimated by sampling trees using a variant of Markov chain Monte Carlo (MCMC) method. Every 1000th tree was sampled to avoid sample autocorrelation. Based on the likelihood profile, the first 4000 trees were

discarded as burn in. A 50 % majority-rule consensus tree with average branch lengths was computed from the remaining trees, using the sumt option of MrBAYES. Posterior probabilities were obtained for each clade. Only clades with bootstrap support equal or above 70 % under ML and posterior probabilities equal or above 0.95 in a Bayesian framework were considered as supported. Both analyses were performed using the online supercomputer facility CIPRES (www.phylo.org) (Miller et al. 2010). Phylogenetic trees were visualized using the program FigTree 1.4.0 (Rambaut 2012). The alignment and tree were submitted to the public database, TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S15336). The nodes with strong support values from both the ML and Bayesian analyses (i.e., PP≥0.95 in B/MCMC analysis and MP bootstrap ≥70 %) are indicated with thickened branches in Fig. 2.

Generalized mixed Yule-coalescent method for species delimitation

In order to identify the number of genetically distinct clusters within the *G. scripta* s. lat. group, we employed the GMYC (Fujisawa and Barraclough 2013; Pons et al. 2006). This method uses a chronogram to define genetic clusters by detecting nodes where the shifts between intraspecific (neutral coalescence, Hudson 1990) and interspecific (Yule model, Nee et al. 1994) diversification occur and calculated likelihood of the model. This likelihood is then compared with the likelihood from a null model, which assumes that all samples come from the same species and only follow within-species processes. A likelihood ratio test (LRT) is used to evaluate whether the null model can be significantly rejected. If the null model is rejected, the threshold allows the estimation of the number of genetic clusters and thus putative species present in the data set.

The ML tree obtained from a RAxML search using the combined data set was used for the analysis. The two outgroup samples (*G. librata* and *G. implicata*) were excluded from the data set using the drop.tip command in ape (Paradis et al. 2004). A chronogram was calculated from the ML tree using a variant of the penalized likelihood method to calculate relative time to the root with the chronos command (Paradis 2013; Sanderson 2002) in ape. This chronogram was then analyzed using the gmyc command in SPLITS package (Ezard et al. 2009) in R (version 2.10, www.cran.r-project.org), using the single and multiple threshold methods. Finally, we used the summary command to summarize the output statistics, including the results of the LRT and the indication of the numbers of clusters and entities. We also used LRT to determine which method (single vs. multiple threshold) was more likely for the data set.

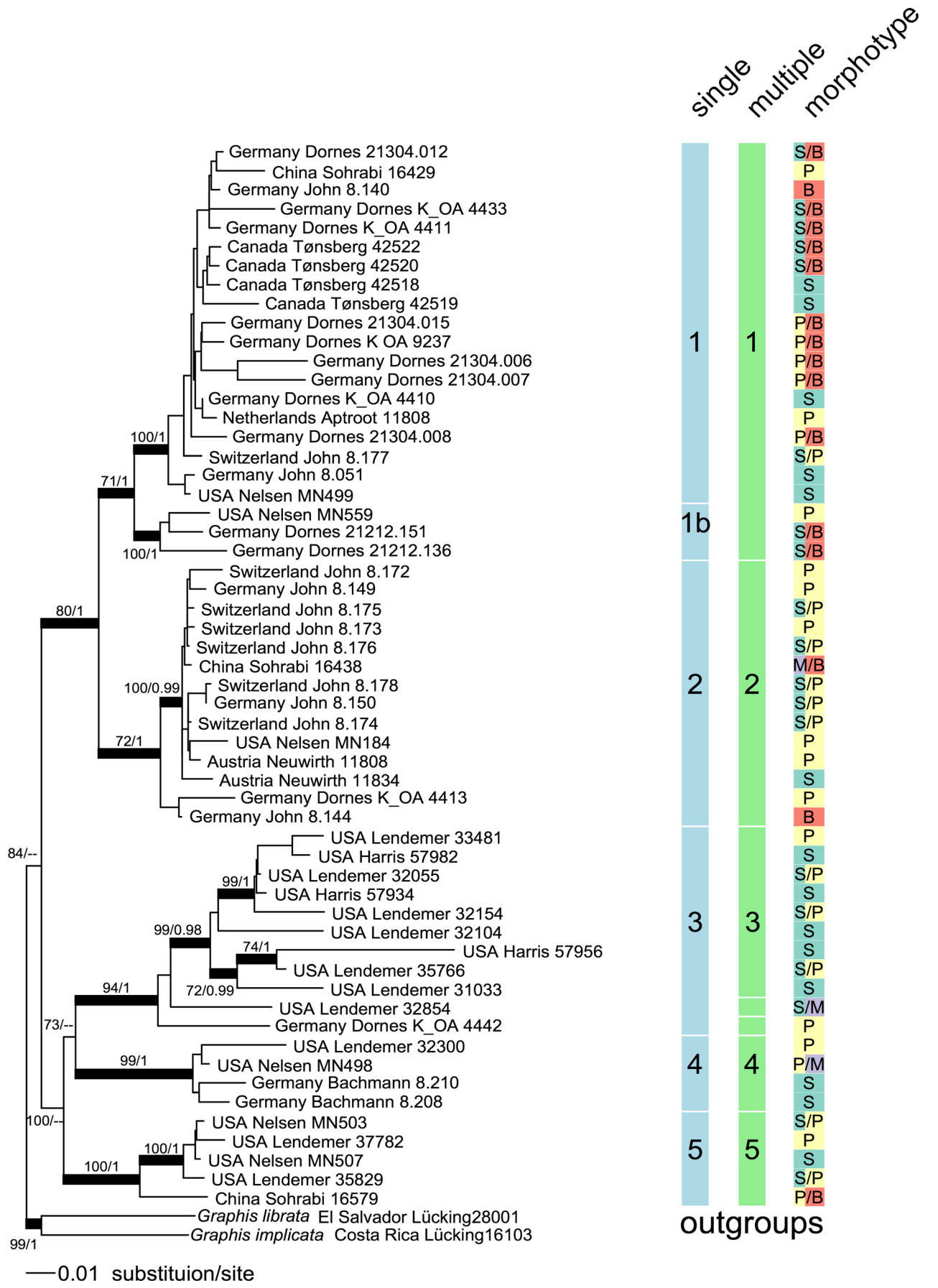
Fig. 2 Maximum likelihood (ML) tree of the *Graphis scripta* complex ▶ from a concatenated data set (mtSSU, nuLSU, *RPB2*, and *EF-1*) under a partitioned ML analysis and resulting delimitation by general mixed Yule-coalescent (GMYC) method. The *numbers at the nodes* indicate the bootstrap support from ML analysis and posterior probability from Bayesian analysis, respectively. The names of specimens are color-coded based on their identification following Neuwirth and Aptroot (Neuwirth and Aptroot 2011) (see text). The *light blue bars* represent genetic clusters recognized by the single threshold method and the *light green bars* by the multiple threshold method

Species tree estimation

We also estimated species trees to compare alternative species delimitation scenarios, using species tree estimation using maximum likelihood program (Kubatko et al. 2009). The program uses multiple gene trees of individual samples to estimate a species tree under a coalescent model using user-defined groupings of species and then to compute the likelihood of the resulting tree. We performed two analyses for each of the two scenarios suggested by the single and multiple methods of GMYC. As the analysis can be sensitive to the input theta value (Carstens and Dewey 2010), we calculated the theta value from our data set, using the R-package "pegas" (Paradis 2010). Then, we used the LRT to compare the likelihood of resulting species trees of these two scenarios.

Morphological characteristics of clades

All specimens were photographed under a dissecting microscope at 10× magnification. For each image, five apothecia were randomly chosen and measured for their width, length, and thalline margin width. When available, images of ascospores from each specimen were also obtained under a compound microscope at 400× magnification and measured for their width and length. All measurements were performed using ImageJ software (Schneider et al. 2012). These characters were some of the few measurable characters in this group and was used in previous taxonomic treatments (Lücking et al. 2009; Neuwirth and Aptroot 2011). The five morphological measurements were averaged for each specimen. For each character, we then performed a phylogenetic analysis of variance (ANOVA) (Garland et al. 1993), using the phylANOVA command in the phytools package in R (Revell 2011), in order to determine the differences among genetic clusters suggested by the best performing GMYC analysis. When a data point was missing, the specimen with no data was removed from the tree prior to the analysis with the drop.tip command in ape. For all *P* values, we set the alpha=0.05 for significant differences from a null model or hypothesis.


single
multiple
morphotype
Germany Dornes 21304.012
China Sohrabi 16429
Germany John 8.140
Germany Dornes K_OA 4433
Germany Dornes K_OA 4411
Canada Tønsberg 42522
Canada Tønsberg 42520
Canada Tønsberg 42518
Canada Tønsberg 42519
Germany Dornes 21304.015
Germany Dornes K OA 9237
Germany Dornes 21304.006
Germany Dornes 21304.007
Germany Dornes K_OA 4410
Netherlands Aptroot 11808
Germany Dornes 21304.008
Switzerland John 8.177
Germany John 8.051
USA Nelsen MN499
USA Nelsen MN559
Germany Dornes 21212.151
Germany Dornes 21212.136
Switzerland John 8.172
Germany John 8.149
Switzerland John 8.175
Switzerland John 8.173
Switzerland John 8.176
China Sohrabi 16438
Switzerland John 8.178
Germany John 8.150
Switzerland John 8.174
USA Nelsen MN184
Austria Neuwirth 11808
Austria Neuwirth 11834
Germany Dornes K_OA 4413
Germany John 8.144
USA Lendemer 33481
USA Harris 57982
USA Lendemer 32055
USA Harris 57934
USA Lendemer 32154
USA Lendemer 32104
USA Harris 57956
USA Lendemer 35766
USA Lendemer 31033
USA Lendemer 32854
Germany Dornes K_OA 4442
USA Lendemer 32300
USA Nelsen MN498
Germany Bachmann 8.210
Germany Bachmann 8.208
USA Nelsen MN503
USA Lendemer 37782
USA Nelsen MN507
USA Lendemer 35829
China Sohrabi 16579
Graphis librata El Salvador Lücking28001
Graphis implicata Costa Rica Lücking16103
100/1
71/1
100/1
80/1
100/0.99
72/1
84/--
99/1
99/0.98
74/1
72/0.99
94/1
73/--
99/1
100/--
100/1
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1
1b
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S/B
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P/M
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S/P
P
S
S/P
P/B
outgroups
0.01 substituion/site

Results

We generated 182 new sequences and aligned them with sequences obtained from GenBank (Table 1). A matrix of 2478 unambiguously aligned nucleotide position characters was produced, of which 1556 were constant (Table 1). In the B/MCMC analysis, the mean log-likelihood of sampled trees was −12,587.60, while the best ML tree had a log-likelihood of −13,957.77. Since the topologies of the ML and B/MCMC analyses did not show any conflicts, only the topology of ML tree from the concatenated data set is shown here with bootstrap support and PP values. The inspection of individual gene trees did not reveal any discordance among the gene trees.

The resulting phylogenetic tree revealed several distinct clades within what has been traditionally classified as *G. scripta* (Fig. 2). The clade numbers follow the results of the GMYC analysis with multiple thresholds (described below). Clade 1 and clade 2 consisted mostly of the specimens from Europe with seven samples from North America (USA and Canada) and two samples from China. Clades 3, 4, and 5, on the other hand, included the majority of specimens from the USA with three samples from Germany nested within. Many specimens had intermediate morphology between two described morphospecies (Table 1). Although *G. betulina* and its intermediate form appeared to cluster within clade 1, none of the four species proposed by Neuwirth and Aptroot (2011) formed a monophyletic group.

The GMYC analyses with single and multiple thresholds yielded different results in the number of detected genetic clusters. In the single threshold analysis, the GMYC model performed significantly better than the null model (L_{GMYC}= −177.92, L_{null}=−173.037, P_{LRT}=0.007). This model resulted in six genetic clusters with no singletons. In the multiple threshold analysis, the GMYC model also performed significantly better than the null model (L_{GMYC}=−177.73, L_{null}= −173.037, P_{LRT}=0.009). Unlike the single threshold method, this model yielded five genetic clusters with two singletons at the base of cluster 3 (Fig. 2). Under the LRT, the likelihood of the multiple threshold method was not significantly better than that of the single threshold (P_{LRT}=0.94). In STEM analysis, the seven-species scenario (from the multiple threshold method) produced a ML tree with a slightly better likelihood (LnL=−2850.93) than the six-species scenario, suggested by the single threshold method (LnL=−2850.90, P_{LRT}=0.19).

Using the five major clades recognized in the multiple threshold method, we found no significant differences among clusters in their morphological measurements ($P \geq 0.36$, Fig. 3), except for spore length (phylogenetic ANOVA, P= 0.04; Fig. 3). Post hoc tests showed that only spores in clade 1 were significantly longer than the spores in clade 5 (P=0.01; Fig. 3). Other pairwise comparisons did not exhibit a significant difference in spore length ($P \geq 0.09$; Fig. 3).

Discussion

The current study provides another example of cryptic diversity under a single name of a widespread lichenized fungal species complex (Lumbsch and Leavitt 2011). Phylogenetic and species delimitation analyses revealed multiple distinct lineages. These lineages, however, do not correspond to the four species recently recognized in the *G. scripta* complex (Neuwirth and Aptroot 2011) or exhibit differences in the majority of the studied morphological characters. While spore length appears to differ significantly between clade 1 and the other clades, it does not provide enough resolution to circumscribe all of these clades with morphological characters alone.

Both ML and B/MCMC analyses provide strong support for monophyly of the *G. scripta* complex, as well as the five major clades that were identified by GMYC models. With the exception of clade 3, the branch lengths within each clade are relatively short, suggesting close relationship among the samples. However, the backbone relationships among these clades are still not as clear. While clade 1 and clade 2 are well-supported sister clades, their relationships with other clades are not strongly supported. The circumscription of the *G. scripta* complex also requires further studies. A previous phylogenetic study of the family reveals that several tropical species *Graphis*, such as *Graphis japonica* (Müll. Arg.) A.W. Archer and Lücking and *Graphis pseudoserpens* Chaves and Lücking, are closely related to *G. scripta* and may potentially have complicated relationships with this complex (Rivas Plata et al. 2013).

Two methods of the GMYC analysis returned slightly different results. Overall, the single threshold supported six-species delimitation, whereas the multiple threshold yielded seven species. Clades 2, 4, and 5 are recovered with both of the methods. However, the single threshold method detected two distinct groups (1a and 1b) within clade 1 but included the two singletons from the multiple threshold method as part of clade 3. The LRT did not show significant differences in the performance of these two models in either GMYC or STEM analyses. Two singletons at the base of clade 3 may actually represent their own independent lineages, but the current sampling did not include more than one of their members. Because these clades are not characterized by any known morphological characters (see below), it is challenging to have an even sampling for all the clades in this study, since we had no a priori knowledge of genetic clusters of the complex.

It has been long suspected that the *G. scripta* complex contains more than just a single-named species (Lücking et al. 2009). Even without thallus and chemical characters, lichenologists observed a tremendous amount of variation in apothecia and ascospores within this complex and attempted to use these characters for species (or subspecies) delimitation (Erichsen 1957). The latest attempt at classification within this complex used aspects of apothecia (length, width, thallus

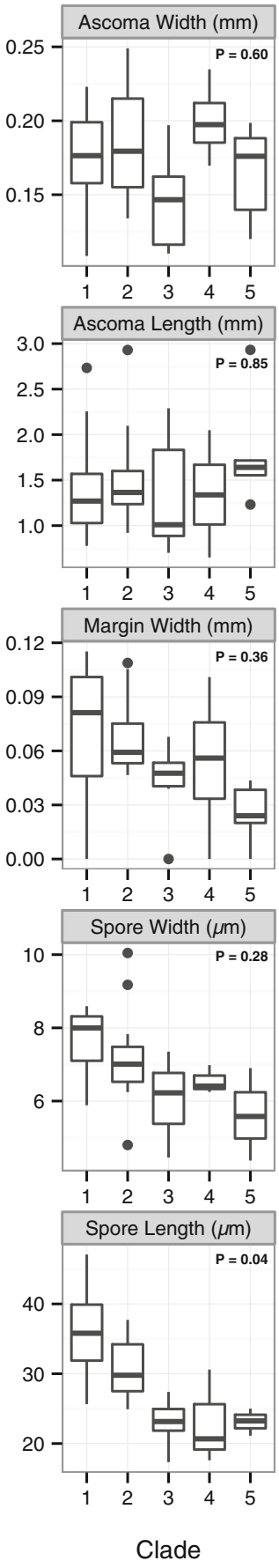

Fig. 3 Differences in morphological characters among genetic clusters of the *Graphis scripta* complex as recognized by the general mixed Yule-coalescent (GMYC) method with multiple thresholds. *P* values from phylogenetic ANOVA are indicated at the upper right corner of the panels. Only spore length exhibits a significant difference among cluster (phylogenetic ANOVA, $P=0.04$)

margin, disc exposure, pruinosity), as well as shapes of ascospore, and resurrected three additional species besides *G. scripta* s.str.: *G. betulina*, *G. macrocarpa*, and *G. pulverulenta* (Neuwirth and Aptroot, 2011). In our phylogenetic analyses, these four morphospecies do not correlate with the phylogenetic structure. While our data support that even more than four species in the complex, the current set of morphological characters is not effective in delimiting species within this complex.

While four out of five measured morphological characters do not correlate with the clades identified by GMYC, spore length exhibits significant differences among clades. Specimens in clade 1 and clade 2 have a longer spore than in other clades. The other studied characters are highly variable within each clade. This may be because these characters are highly plastic, changing in responses to different ecological conditions and especially substrate texture. We currently know relatively little about variation of apothecium size and spore characters, compared to other characters (Lumbsch et al. 2010; Tehler and Irestedt 2007). In the current study, we could not gather sufficient ecological data from the voucher specimens to allow inferences about potential correlations of morphology and ecological conditions.

Without any obvious morphological differences, these distinct lineages may be referred to as cryptic species (Bickford et al. 2007). However, a speciation event can happen with a change of any phenotypic character that leads to differences in reproductive success and subsequently increases reproductive isolation (Coyne and Orr 2004). Therefore, these cryptic lineages may have differentiated in ways that did not involve changes in morphology of apothecia and thallus. In many cases, the discovery of cryptic lineages leads to discovery of other subtle, previously unknown characters that characterize those lineages. Such characters may include differences in ecology, chemistry, physiology, microstructures, biogeography, or even a combination of these traits (Arup and Åkelius 2009; Czarnota and Guzow-Krzemińska 2010; Lendemer 2011; Parnmen et al. 2012; Pillon et al. 2014; Vondrák et al. 2009; Yost et al. 2012).

The clades in the *G. scripta* complex exhibit very little biogeographical structure. In clades of mostly European specimens (clades 1 and 2), several North American samples are nested within, and vice versa for clades 3, 4, and 5. While biogeographical structure within a large continent, such as North America, may exist, the current set of samples only

comes from a relatively small number of localities, mostly from Eastern and Midwestern North America (Table 1), making it difficult to discern the pattern at this point. In general, the clades in the *G. scripta* complex have a wide distribution, which is the case with many other lichenized fungi (Printzen and Ekman 2002; Printzen et al. 2003; Wirtz et al. 2008). At least one intercontinental dispersal event exists in all of the clades. At the same time, samples with similar morphology from the same locality, such as Dornes K_OA 4413 and Dornes K_OA 4442, are placed in different clades.

Our phylogenetic analysis reveals at least five putative species in the *G. scripta* complex under the general lineage concept of species (de Queiroz 2007). However, we currently do not have sufficient morphological evidence to formally recognize these lineages at the species level. Further detailed investigations on additional morphological, anatomical, and ecological characters may find a set of phenotypes that correlate with genetic diversity we observe in this famous group of crustose lichens. In the meanwhile, the results provide another example of high genetic diversity within a species complex and how we can discover this hidden diversity with helps from molecular and computational tools.

Acknowledgments We would like to thank Kyle Huff (Roosevelt University) for helping with morphological measurements. The molecular work was performed at the Pritzker Laboratory for Molecular Systematics and Evolution at The Field Museum (Chicago).

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