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Molecular analyses of first collections of *Elaphomyces* Nees (Elaphomycetaceae, Eurotiales, Ascomycota) from Africa and Madagascar indicate that the current concept of *Elaphomyces* is polyphyletic

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Abstract – First collections are reported for *Elaphomyces* species from Africa and Madagascar. On the basis of an ITS phylogeny, the authors question the monophyletic nature of family Elaphomycetaceae and of the genus *Elaphomyces*. The objective of this preliminary paper was not to propose a new phylogeny for *Elaphomyces*, but rather to draw attention to the very high dissimilarity among ITS sequences for *Elaphomyces* and to the unfortunate choice of species to represent the genus in most previous phylogenetic publications on Elaphomycetaceae and other cleistothecial ascomycetes. Our study highlights the need for examining the monophyly of this family and to verify the systematic status of *Pseudotulostoma* as a separate genus for stipitate species. Furthermore, there is an urgent need for an in-depth morphological study, combined with molecular sequencing of the studied taxa, to point out the phylogenetically informative characters of the discussed taxa.

Elaphocordyceps* / Elaphomycetaceae / Eurotiales / ITS / *Pseudotulostoma* / phylogeny / *Tolypocladium

INTRODUCTION

The ca. 60-70 accepted species of the cosmopolitan genus *Elaphomyces* Nees (type species: *E. granulatus* Fr. = *E. officinalis* Nees) produce 0.5-4 cm wide,

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hypogeous fruiting bodies which produce small, scattered, globose and evanescent asci containing thick-walled, dark-coloured and strongly ornamented spores (Castellano *et al*. 2012b). The genus is ecologically important as its species are obligatory root symbionts forming ectomycorrhizal associations with trees. It shares several parallel evolutions with the independent lineage of the gastronomically reputed and equally ectomycorrhizal, truffle forming ascomycetes of *Tuber*, Pezizomycetidae (Quandt *et al*. 2015).

The resemblance of *Elaphomyces* fruiting bodies to those of *Tuber* was responsible for referring family Elaphomycetaceae initially to the Tuberales (Saccardo, 1889; Ainsworth, 1961), a placement that was still adopted for practical reasons by Korf (1973), although Korf stated that "their true relationship may possibly be with the Eurotiales". Dodge (1929) had already transferred the Elaphomycetaceae to the Plectomycetes (as "Plectascales") due to similar internal morphological characters of the ascomata. He accepted two genera in the family: *Elaphomyces* with a cottony central core and *Mesophellia* Berkeley with a corky or woody core. The genus *Mesophellia*, however, was rapidly moved to the Basidiomycota, first to Lycoperdales (Cunningham 1932, 1944), although intact basidia have never been observed as they disintegrate very quickly (Trappe *et al*. 1996). More careful microscopic examination then demonstrated that the genus grouped probably unrelated species because of the very heterogeneous spore characters and was later indeed proven to be polyphyletic using molecular approaches. The species of *Mesophellia* sensu stricto are now placed in Hysterangiales (Phallomycetidae) as the sister family to the Hysterangiaceae (Hosaka *et al*. 2006).

With the exclusion of *Mesophellia*, family Elaphomycetaceae has remained for a long time a monotypic family, and was at some point even considered a separate order, Elaphomycetales (Trappe 1979). The first phylogenetic analyses comprising species of *Elaphomyces* were based on Small Subunit ribosomal DNA sequences (SSU rDNA). These confirmed (1) that Elaphomycetales were indeed closely related to Eurotiales and Onygenales (Landvik *et al*. 1996), and (2) that the genus was unrelated to the asexual mycorrhizal ascomycete *Cenococcum* (Lobuglio *et al*. 1996), previously assumed to represent the anamorph of *Elaphomyces* based on co-occurrence and some shared hyphal morphologies (Trappe 1971). The intimate co-occurrence of both genera was confirmed recently in a study interested in the identification of fungal sequences recovered from *Cenococcum* sclerotia (Obase *et al*. 2014), several sequences of which seem to represent *Elaphomyces* species. As a result, Elaphomycetales has been abandoned as an independent order to be maintained at family level, although of uncertain position, within Eurotiales, an order mainly composed of saprotrophs and some mammalian pathogens (Geiser *et al*. 2006, 2015).

With the discovery of the epigeous, stipitate and volvate *Pseudotulostoma* O.K. Miller & T. Henkel in the lowland Caesalpiniaceae forest patches of tropical Guyana (Miller *et al*. 2001), the morphological variation in Elaphomycetaceae was considerably widened, although the phylogenetic analyses, still only based on SSU rDNA sequences, lacked significant support for the monophyletic grouping of this new genus with *Elaphomyces*. A few years later, the much larger, Japanese *Battarrea japonica* (Kawam.) Otani was recombined into *Pseudotulostoma*, again on the basis of SSU sequence data (Asai *et al*. 2004; Masuya & Asai, 2004). Within Eurotiales, species of *Pseudotulostoma* and *Elaphomyces* share the evanescent nature of their asci, the similarity of their unopened fruiting bodies, as well as their unique ectomycorrhizal habit (Henkel *et al*. 2006), although the latter remains to be verified for *P. japonicum* (Masua & Asai, 2004). Henkel *et al*. (2006) maintained *Pseudotulostoma* as sister to *Elaphomyces* based on their SSU sequence data,

although this position was later challenged by Reynolds (2011) who suggested that *Pseudotulostoma* might be nested within *Elaphomyces*. If true, this hypothesis implies that the wind-mediated spore dispersal for the stipitate *Pseudotulostoma*, as suggested by Reynolds, would have evolved from animal-mediated spore dispersal in *Elaphomyces*, which – to the best of our knowledge – is exactly the opposite of what is observed in all other groups of ectomycorrhizal fungi, whether ascomycetes or basidiomycetes, especially since animal mediated dispersal is believed to be much more efficient than wind dispersal (Johnson 1996). Field observations during the sample collection in Central African Republic showed that these African *Elaphomyces* were indeed consumed and dispersed by several animals including different species of duikers, primates and wild pigs.

Elaphomyces has longtime been considered an ectomycorrhizal genus of predominantly northern hemisphere distribution in association with Pinaceae, Betulaceae and Fagaceae (Castellano *et al*. 1989). Recent inventories in New Zealand (Castellano *et al*. 2012a) and Australia (Castellano *et al*. 2011) have demonstrated the importance of the genus in the southern hemisphere forests of Myrtaceae, Casuarinaceae and, more occasionally, also with *Nothofagus*. The first record of the genus from lowland tropical forest dates back to Corner & Hawker (1953), who described *E. singaporensis* and *E. carbonaceus* from near Singapore, possibly associated with Dipterocarpaceae. Much more recently, *Elaphomyces* has also been shown to grow in association with Caesalpiniaceae in the neotropical lowlands of Guyana (Castellano *et al*. 2012b). So far, no species have been described from the African continent, although the */elaphomyces* lineage was recently reported (Tedersoo *et al*. 2011) as a variable but relatively important component retrieved from molecular soil samples in several African and Malagasy woodlands and rain forests or erroneously relate the genus to introduced eucalypts in Madagascar (e.g. GenBank FR731296 which is not an ECM fungus in Eurotiales but *Penicillium*). When checking on GenBank (not shown), it appears in fact that very few of these sequences correspond to *Elaphomyces* (see also discussion below) and most concern either other Eurotiales or refer even to various ectomycorrhizal basidiomycetes on GenBank.

The here newly reported ITS sequences in the */elaphomyces* lineage have been obtained from the first African collections of fruiting bodies for this lineage: one species has been collected in a monospecific *Uapaca* (Phyllantaceae) pocket of the mountain forest crest in Madagascar, while the other species was found in the *Gilbertiodendron dewevrei* rain forest in the Central African Republic. Although both specimens correspond to the morphological concept of *Elaphomyces*, their ITS sequences were genetically so different that they seemed to challenge our identification. Apart from ITS sequences, hardly any other sequence data are publicly available for *Elaphomyces* at the moment. This paper discusses the results of our phylogenetic analyses which place the sequences of our African specimens in two very different clades of a representative set of publicly available ITS sequence data for *Elaphomyces*. Detailed descriptions of these collections will be published elsewhere.

MATERIAL AND METHODS

Sampling. – The Malagasy *Elaphomyces* was collected by the first author in a monospecific *Uapaca ferruginea* vegetation on the crest of mountain rain forest near

Ranomafana on the Eastern escarpment in February 1999 (Buyck 99.404, 99.406). These are the only collections we ever made of this genus in Madagascar during the past twenty years. The specimens from the Central African Republic were collected in 2009 by S. Masi in the *Gilbertiodendron dewevrei* forest in the Dzanga-Ndoki National Park where they appear to be very common and abundant. For the *Elaphomyces* and *Pseudotulostoma japonicum*, the fungal material was conserved in CTAB and genomic DNA isolation and amplification of the internal transcribed spacers region (ITS1-5,8S-ITS2) were performed as described in Hofstetter *et al.* (2014) using primers ITS1F-ITS4. In addition, for the specimen from Central African Republic, some twenty direct amplifications from spore material were performed following Hofstetter *et al.* (2012). Apart from the newly produced sequences, we sampled representative ITS sequences available in GenBank for *Elaphomyces* and *Pseudotulostoma*, as well as selected members of Eurotiales (*Trichocoma, Talaromyces, Rasamsonia, Thermoascus, Geosmithia*) and Onygenales (*Parananizziopsis, Gymnoascus*)

Capronia pilosella (P. Karst.) E. Müll., Petrini, P.J. Fisher, Samuels & Rossman (Eurotiomycetes, Chaetothyriales) was selected as outgroup.

Phylogenetic analyses – Sequence alignment was done by eye using MacClade v.4.06 (Maddison and Maddison 2002) and is available from the authors (VH). Analyses were performed using the software PhyML 2.4.4 (Guindon and Guascuel, 2003). Search for the best tree used 10 independent runs, a GTR model of substitution with a number of substitution rate categories = 4. Other parameters were estimated during search. Branch bootstrap support (MLBS) was assessed based on 500 bootstrap replicates using the same substitution model and settings as for the best tree search. The threshold considered for MLBS significant support was ≥ 70% (Hillis and Bull, 1993).

RESULTS

Eight ITS sequences were newly produced for this study: for *Elaphomyces* sp. (one sequence; GenBank accession number [GenBank] KU934219), for *Elaphomyces* aff. (four identical sequences from the same collection; GenBank KU934220), and for *Pseudotulostoma japonicum* (three sequences; GenBank KU934216, KU934217, KU934218; see Fig. 1).

The alignment used for phylogenetic analyses included 66 sequences and 311 characters after exclusion of 479 characters that could not be unambiguously aligned in ITS1 & 2. The most likely tree obtained by maximum likelihood analyses (-ln = 1994.99706) is depicted in Fig. 1. This ITS-based phylogeny suggests that *Elaphomyces* is polyphyletic, with species clustering into four distinct clades: (1) a 'core' clade (MLBS = 84%) including North-American, European and Australasian species and our Malagasy specimen. Two 'uncultured fungus' sequences cluster sister to this 'core clade' (MLBS = 100%) on a very long branch; (2) an 'Afro-Neotropical' clade (MLBS = 74%) including the only South-American *Elaphomyces* and the newly sequenced Central African species together with another Central African sequence. This clade is suggested to be monophyletic and sister to the *Pseudotulostoma* clade (3), however without significant support; and (4), a 'Guangdongensis clade'(MLBS = 99%) including three European species of *Elaphomyces* together with the Asian *E. guangdongensis*.

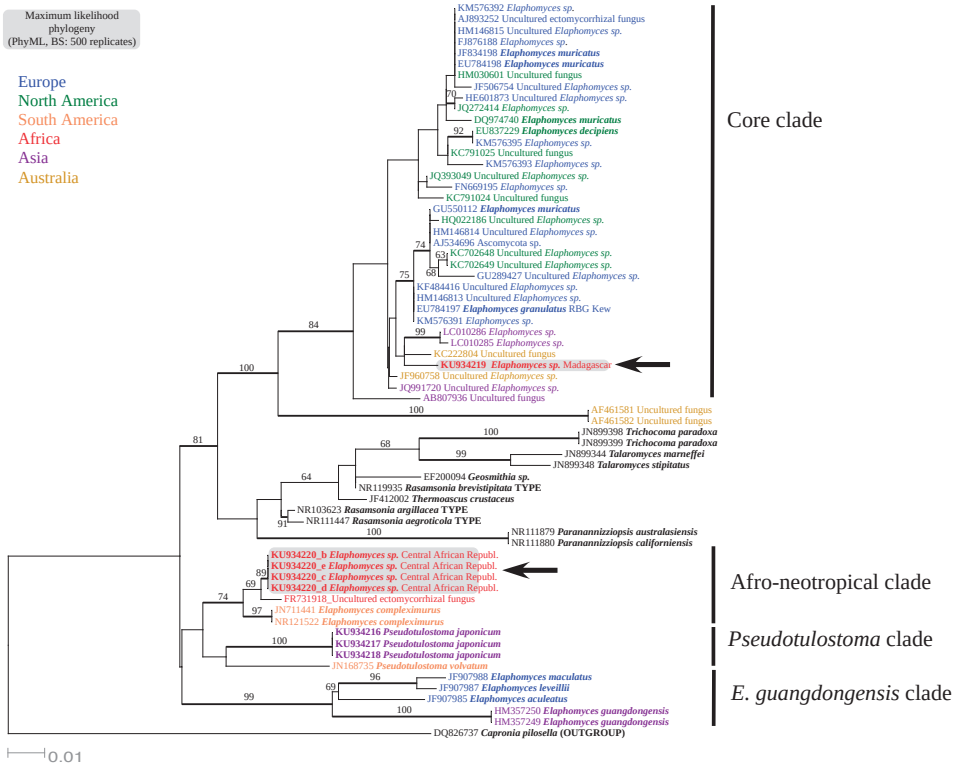


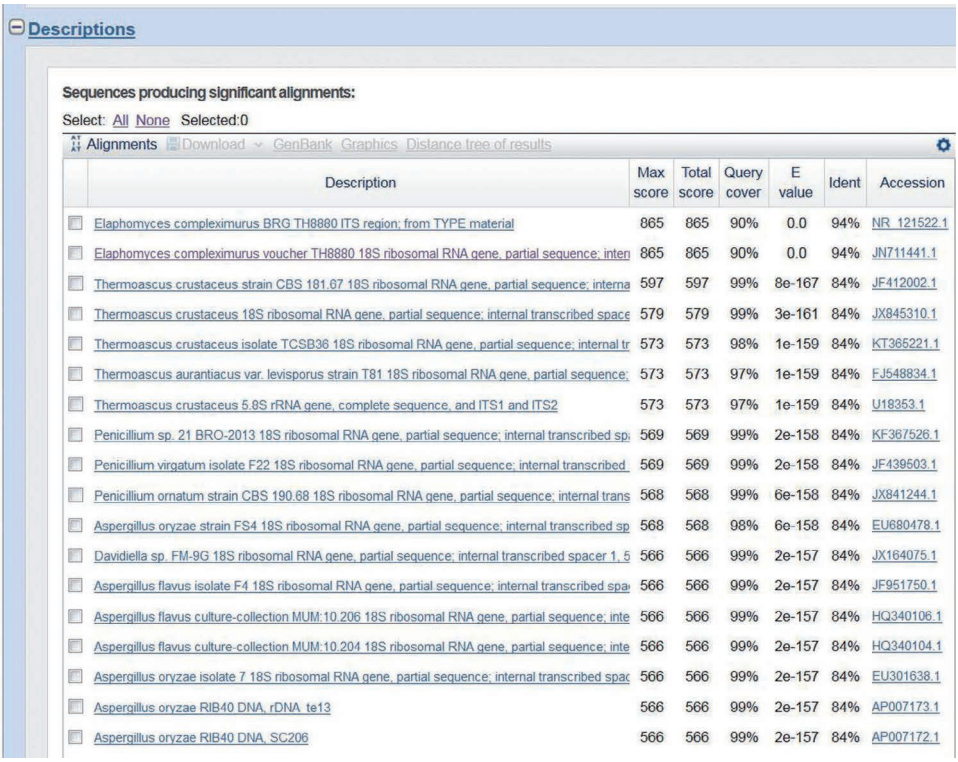
Fig. 1. ML phylogeny based on ITS sequences showing the position for species in Elaphomycetaceae among selected genera of Eurotiales and Onygenales. Colors correspond to continents (see insert). Genera from outside the Elaphomycetaceae, including the outgroup (*Capronia pilosella*), are left in black. Arrows indicate the newly sequenced *Elaphomyces* from Africa and Madagascar. Numbers above branches give bootstrap support.

DISCUSSION

When checking for the presence of ITS sequences for *Elaphomyces* on GenBank and other public sequence databases, it becomes immediately clear that the bulk of the sequences relate to environmental samples generated by fungal community studies based on soil or root samples. Only part of these sequences indicates *Elaphomyces* as corresponding identity and far less relate a sequence to a specific species. The 28 proposed *Elaphomyces* species hypotheses (SH, when applying a 1.5% divergence threshold on Unite [https://unite.ut.ee]) reflect the unreliability and lack of identifications for the few named sequences (4 SH for *E. muricatus*, 2 SH for E. *decipiens*, 2 SH for *E. leveillii*, most other SH remaining unnamed). Exception made for the recently described *E. complexiumurus* and for *E. digitatus* Castellano, T.W. Henkel & S.L. Mill., no other sequence on public depositories is based on type material. Moreover, except for both latter species and for *E. guangdongensis*, no other sequences have been produced as the result of a

taxonomic study that focuses on the genus. Nevertheless, taxonomic expertise on *Elaphomyces* exists on both sides of the Atlantic, in Europe almost exclusively among amateur scientists as opposed to the few North American professional mycologists working on these fungi.

Another unexpected finding when starting this study was that BLAST searches on ITS sequences in GenBank systematically excluded part of the deposited sequences. Indeed, when describing the new *Pseudotulostoma volvatum* (as *P. volvata*) from Guyana, the produced ITS sequences for this new genus were at first seriously questioned by the authors (Miller *et al*. 2001) and were initially presumed to have resulted from mold contamination (Redhead 2001). When performing BLAST searches in GenBank for our newly produced ITS sequences from Africa, one sequence came up as being closely related to *E. compleximurus*, a newly described *Elaphomyces* from Guyana (Castellano *et al*. 2012b), but not one single other *Elaphomyces* was even close (Fig. 2). These neotropical Elaphomycetaceae sequences are so much more similar to ITS sequences of *Thermoascus, Penicillium*, *Aspergillus, Rasamsonia* and other Eurotiales, that those (<80% similarity, Fig. 3) from species that constitute the core clade of *Elaphomyces* in our phylogeny (Fig. 1) never showed up in any BLAST search. Castellano *et al*. (2012b) provided the partial LSU and ITS sequences for both new *Elaphomyces* species in their paper, but do not produce a phylogeny, nor do they mention the high dissimilarity of the



Descriptions

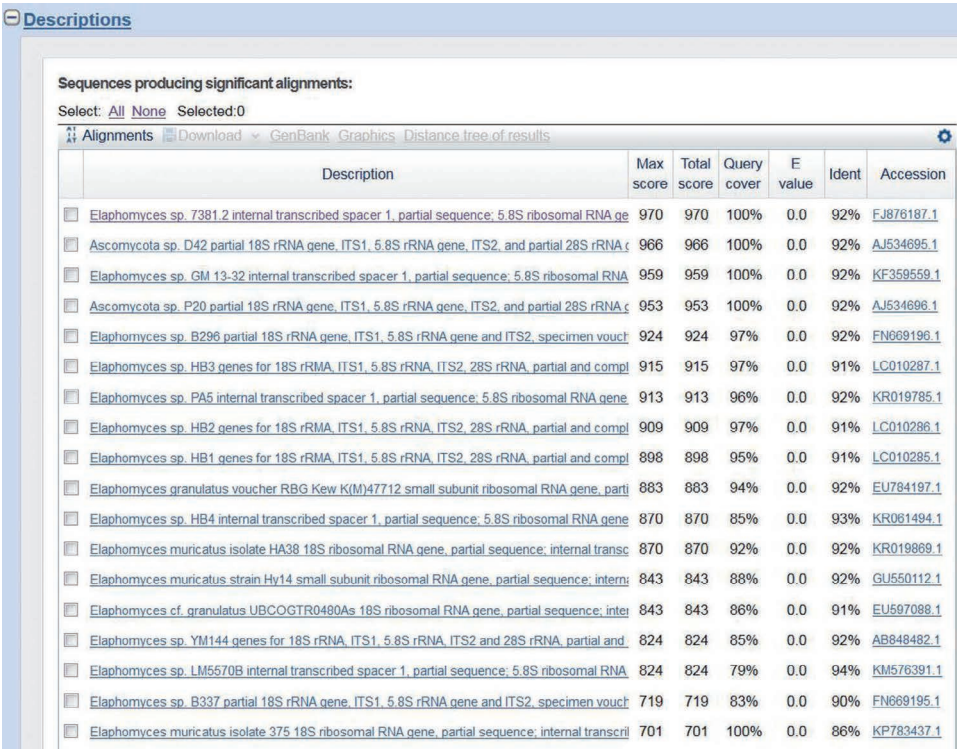
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Elaphomyces compleximurus BRG TH8880 ITS region; from TYPE material	865	865	90%	0.0	94%	NR_121522.1
Elaphomyces compleximurus voucher TH8880 18S ribosomal RNA gene, partial sequence; inter	865	865	90%	0.0	94%	JN711441.1
Thermoascus crustaceus strain CBS 181.67 18S ribosomal RNA gene, partial sequence; interna	597	597	99%	8e-167	84%	JF412002.1
Thermoascus crustaceus 18S ribosomal RNA gene, partial sequence; internal transcribed space	579	579	99%	3e-161	84%	JX845310.1
Thermoascus crustaceus isolate TCSB36 18S ribosomal RNA gene, partial sequence; internal tr	573	573	98%	1e-159	84%	KT365221.1
Thermoascus aurantiacus var. levisporus strain T81 18S ribosomal RNA gene, partial sequence;	573	573	97%	1e-159	84%	FJ548834.1
Thermoascus crustaceus 5.8S rRNA gene, complete sequence, and ITS1 and ITS2	573	573	97%	1e-159	84%	U18353.1
Penicillium sp. 21 BRO-2013 18S ribosomal RNA gene, partial sequence; internal transcribed sp	569	569	99%	2e-158	84%	KF367526.1
Penicillium virgatum isolate F22 18S ribosomal RNA gene, partial sequence; internal transcribed	569	569	99%	2e-158	84%	JF439503.1
Penicillium ornatum strain CBS 190.68 18S ribosomal RNA gene, partial sequence; internal trans	568	568	99%	6e-158	84%	JX841244.1
Aspergillus oryzae strain FS4 18S ribosomal RNA gene, partial sequence; internal transcribed sp	568	568	98%	6e-158	84%	EU680478.1
Davidiella sp. FM-9G 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5	566	566	99%	2e-157	84%	JX164075.1
Aspergillus flavus isolate F4 18S ribosomal RNA gene, partial sequence; internal transcribed spa	566	566	99%	2e-157	84%	JF951750.1
Aspergillus flavus culture-collection MUM:10.206 18S ribosomal RNA gene, partial sequence; inte	566	566	99%	2e-157	84%	HQ340106.1
Aspergillus flavus culture-collection MUM:10.204 18S ribosomal RNA gene, partial sequence; inte	566	566	99%	2e-157	84%	HQ340104.1
Aspergillus oryzae isolate 7 18S ribosomal RNA gene, partial sequence; internal transcribed spac	566	566	99%	2e-157	84%	EU301638.1
Aspergillus oryzae RIB40 DNA, rDNA_te13	566	566	99%	2e-157	84%	AP007173.1
Aspergillus oryzae RIB40 DNA, SC206	566	566	99%	2e-157	84%	AP007172.1

Fig. 2. Typical BLAST result for species in the Afro-neotropical clade, in this case for our specimen from Central African Republic.



Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

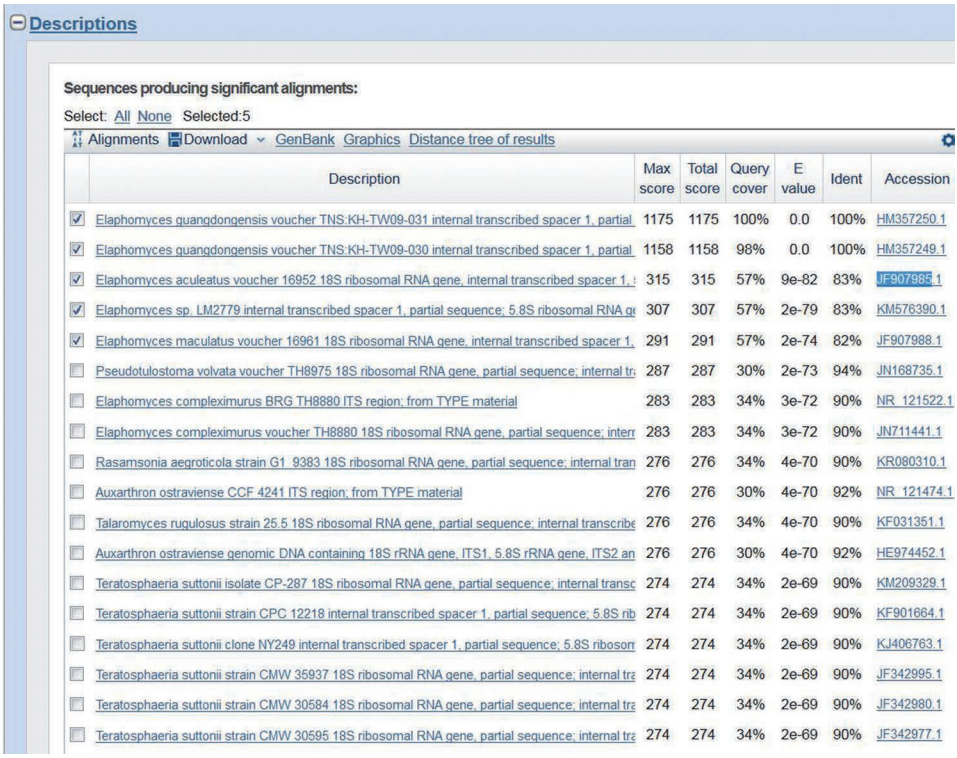
Description	Max score	Total score	Query cover	E value	Ident	Accession
Elaphomyces sp. 7381.2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge	970	970	100%	0.0	92%	FJ876187.1
Ascomycota sp. D42 partial 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, and partial 28S rRNA (	966	966	100%	0.0	92%	AJ534695.1
Elaphomyces sp. GM 13-32 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA	959	959	100%	0.0	92%	KF359559.1
Ascomycota sp. P20 partial 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, and partial 28S rRNA (	953	953	100%	0.0	92%	AJ534696.1
Elaphomyces sp. B296 partial 18S rRNA gene, ITS1, 5.8S rRNA gene and ITS2, specimen vouch	924	924	97%	0.0	92%	FN669196.1
Elaphomyces sp. HB3 genes for 18S rRMA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and compl	915	915	97%	0.0	91%	LC010287.1
Elaphomyces sp. PA5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene	913	913	96%	0.0	92%	KR019785.1
Elaphomyces sp. HB2 genes for 18S rRMA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and compl	909	909	97%	0.0	91%	LC010286.1
Elaphomyces sp. HB1 genes for 18S rRMA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and compl	898	898	95%	0.0	91%	LC010285.1
Elaphomyces granulatus voucher RBG Kew K(M)47712 small subunit ribosomal RNA gene, parti	883	883	94%	0.0	92%	EU784197.1
Elaphomyces sp. HB4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene	870	870	85%	0.0	93%	KR061494.1
Elaphomyces muricatus isolate HA38 18S ribosomal RNA gene, partial sequence; internal transc	870	870	92%	0.0	92%	KR019869.1
Elaphomyces muricatus strain Hy14 small subunit ribosomal RNA gene, partial sequence; intern	843	843	88%	0.0	92%	GU550112.1
Elaphomyces cf. granulatus UBCOGTR0480As 18S ribosomal RNA gene, partial sequence; inter	843	843	86%	0.0	91%	EU597088.1
Elaphomyces sp. YM144 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA, partial and	824	824	85%	0.0	92%	AB848482.1
Elaphomyces sp. LM5570B internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA	824	824	79%	0.0	94%	KM576391.1
Elaphomyces sp. B337 partial 18S rRNA gene, ITS1, 5.8S rRNA gene and ITS2, specimen vouch	719	719	83%	0.0	90%	FN669195.1
Elaphomyces muricatus isolate 375 18S ribosomal RNA gene, partial sequence; internal transcril	701	701	100%	0.0	86%	KP783437.1

Fig. 3. Typical BLAST result for species in the core clade, in this case the BLAST of our Malagasy specimen.

obtained ITS sequences to the rest of the genus and even between both new species (in the case of *E. digitatus*, the deposited holotype sequence (JQ657705) was unalignable, being either of very bad quality or corresponding to a contamination). In the absence of other *Elaphomyces* LSU sequences deposited on GenBank, this gene gives no relevant BLAST results).

The hypothesis of fungal contamination to explain the unexpected PCR results therefore popped up again. Did we sequence perhaps specific endophytic or parasitic microfungi that inhabit the tissues of tropical *Elaphomycetaceae?* Multiple ITS (ca 20) were therefore produced by direct PCR of spore tissue of our African *Elaphomyces*. We retrieved the same ITS in all cases, except for one ITS with 95% similarity to *Tolypocladium japonicum* (previously *Elaphocordyceps japonica*, see Quandt *et al*. 2014) which is another first African record for a genus that is reputedly parasitic on *Elaphomyces* species. We therefore assumed that the obtained ITS was correct, which brought us to the second hypothesis: the genus *Elaphomyces*, as currently circumscribed, is polyphyletic and corresponds to a convergent evolution that characterizes several independent lineages in Eurotiales.

As mentioned before, the systematic position of Elaphomycetaceae is nearly entirely based on SSU sequence data and SSU might not be the best gene for resolving relationships within a fungal family or even beyond (see Bruns & Taylor 2016). Reynolds (2011) did sequence quite some species for part of the LSU and mitSSU loci (sequences not publicly available), but neither ML/MP bootstrap nor



Descriptions

Sequences producing significant alignments:

Select: All None Selected:5

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Elaphomyces guangdongensis voucher TNS:KH-TW09-031 internal transcribed spacer 1, partial	1175	1175	100%	0.0	100%	HM357250.1
Elaphomyces guangdongensis voucher TNS:KH-TW09-030 internal transcribed spacer 1, partial	1158	1158	98%	0.0	100%	HM357249.1
Elaphomyces aculeatus voucher 16952 18S ribosomal RNA gene, internal transcribed spacer 1,	315	315	57%	9e-82	83%	JF907985.1
Elaphomyces sp. LM2779 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g	307	307	57%	2e-79	83%	KM576390.1
Elaphomyces maculatus voucher 16961 18S ribosomal RNA gene, internal transcribed spacer 1,	291	291	57%	2e-74	82%	JF907988.1
Pseudotulostoma volvata voucher TH8975 18S ribosomal RNA gene, partial sequence; internal tr	287	287	30%	2e-73	94%	JN168735.1
Elaphomyces compleximurus BRG TH8880 ITS region; from TYPE material	283	283	34%	3e-72	90%	NR_121522.1
Elaphomyces compleximurus voucher TH8880 18S ribosomal RNA gene, partial sequence; interr	283	283	34%	3e-72	90%	JN711441.1
Rasamsonia aegroticola strain G1_9383 18S ribosomal RNA gene, partial sequence; internal tran	276	276	34%	4e-70	90%	KR080310.1
Auxarthron ostraviense CCF 4241 ITS region; from TYPE material	276	276	30%	4e-70	92%	NR_121474.1
Talaromyces rugulosus strain 25.5 18S ribosomal RNA gene, partial sequence; internal transcribe	276	276	34%	4e-70	90%	KF031351.1
Auxarthron ostraviense genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 an	276	276	30%	4e-70	92%	HE974452.1
Teratosphaeria suttonii isolate CP-287 18S ribosomal RNA gene, partial sequence; internal transc	274	274	34%	2e-69	90%	KM209329.1
Teratosphaeria suttonii strain CPC 12218 internal transcribed spacer 1, partial sequence; 5.8S rib	274	274	34%	2e-69	90%	KF901664.1
Teratosphaeria suttonii clone NY249 internal transcribed spacer 1, partial sequence; 5.8S riboson	274	274	34%	2e-69	90%	KJ406763.1
Teratosphaeria suttonii strain CMW 35937 18S ribosomal RNA gene, partial sequence; internal tra	274	274	34%	2e-69	90%	JF342995.1
Teratosphaeria suttonii strain CMW 30584 18S ribosomal RNA gene, partial sequence; internal tra	274	274	34%	2e-69	90%	JF342980.1
Teratosphaeria suttonii strain CMW 30595 18S ribosomal RNA gene, partial sequence; internal tra	274	274	34%	2e-69	90%	JF342977.1

Fig. 4. Typical BLAST result for one of the two public *E. guangdongensis* sequences. Note that the coverage of the BLAST drops abruptly for all other species, while from the sixth result downwards, the query coverage (ca 30%) corresponds only to the 5.8S.

Bayesian PP allowed to retrieve significant support for the monophyly of *Elaphomyces* in any of Reynold's single gene phylogenetic analyses. Using a combined analysis of a subset of only 11 taxa, she recovered *P. volvatum* with strong BPP and moderate ML support as derived from within one clade of *Elaphomyces* (in particular related to *E. fallax* which has a sterile base, and *E. queenslandicus* which has not).

When Miller *et al*. (2006) argued that *Pseudotulostoma volvatum* was sister to *Elaphomyces* and that both genera constituted family Elaphomycetaceae in Eurotiales (although with an insufficient 56% ML bootstrap support), the former species was on a long branch in their phylogeny and the only three European *Elaphomyces* in their SSU phylogeny are genetically very different from the core *Elaphomyces* and are here (Fig. 1) grouped with very strong support with the Asian *E. guangdongensis* (for a description of the latter species, see Hosaka *et al*. 2010). Also the SSU phylogeny by Masuya & Asai (2004) obtained a largely insufficient 51% MP bootstrap support for the grouping of *Pseudotulostoma* with the same three European *Elaphomyces* sequences, but groups both *Pseudotulostoma* as monophyletic with very high support (96%). One remark needs to be made about these three European specimens (two from collections made in Italy and one from Switzerland) in the *E.guangdongensis* clade: all three sequences were produced by the same sequencing project of the Venice Herbarium (Osmundson *et al*., 2013), and there is no morphological trait that seems to set them apart from the core-clade *Elaphomyces*

(Paz *et al*. 2012) except that they all form black or very dark, verrucose fruiting bodies, just like *E. guangdongensis* (Hosaka *et al*. 2010) and most species of the Afro-neotropical clade. We suggest that future phylogenetic analyses of the Elaphomycetaceae should include *Elaphomyces* species representative of the core clade.

Our ITS phylogeny (Fig. 1) does not provide significant support for the monophyly of the two *Pseudotulostoma* species, but it does present high support for the monophyly of the core *Elaphomyces* clade, the *E. guangdongensis* clade and the Afro-neotropical clade. It seems, therefore, that both the systematic position and monophyly of Elaphomycetaceae, *Elaphomyces* and *Pseudotulostoma* all remain unresolved at the moment. We performed several analyses with different samplings (not shown), for example by taking out the long branches in our phylogeny (Fig. 1) or by modifying outgroup choices. This added support to the core clade, but did not substantially modify the arrangement among the various supported clades in our phylogeny.

The strong geographical signal in our phylogeny not only adds support to the fact that the obtained ITS sequences are indeed correct, but also suggests that our Afro-neotropical *Elaphomyces* clade is clearly a candidate for an old Gondwanan vicariance between African and neotropical lowland ectomycorrhizal species as suggested repeatedly for other ectomycorrhizal fungi (e.g. Moyersoen 2012). The *Elaphomyces* core clade appears here to represent a clade of Australasian origin that migrated into the northern hemisphere. The presence of *Eucalyptus* at our Malagasy collecting site is highly unlikely (a very dense, monospecific *Uapaca* vegetation at a mountain crest in the middle of virgin forest) and we therefore assume that the Malagasy *Elaphomyces* is a native species or perhaps a species of African origin. The closest sequences in our phylogeny (Fig. 1) were associated with eucalypt stands in Australia (GenBank KC222804, Bastias *et al*. 2006) and Tasmania (GenBank JF960758, Horton 2011) and with dipterocarp rain forest in Indonesia (GenBank LC010285, LC010286, Sukarno *et al*. 2015). Our sequence demonstrates at least that the *Elaphomyces* core clade is represented in Madagascar by species occupying rather basal positions. The only other mentions of the /*elaphomyces* lineage in Madagascar (Tedersoo *et al*. 2011, supplementary material S2) correspond in reality to *Cortinarius* on GenBank (UDB007644=FR731466) and to *Penicillium* (GenBank FR731296).

Basal to our core clade, but on a very long branch, are two environmental sequences from Australia (Chen & Cairney 2002). Eliminating these sequences from the analysis results in 100% bootstrap for the core clade, but hardly influences our analysis in other ways. We have no idea what species these sequences correspond to, but BLAST results (not shown) list the entire core clade, although only for the 5.8S at ca 80% similarity.

The only other African sequence in our alignment corresponds to UDB004498|FR731918|L6519a_EurM_Gab02 from Gabon (Tedersoo *et al*. 2011) and is closely related to our sequence from the Central African Republic (UDB004497|FR731919|L6539a_EurM_Gab01 is probably the same but BLAST lists only 5.8S similarities). As for the other mainland African sequences related to the /*elaphomyces* lineage (Tedersoo *et al*. 2011), several sequences were either too short, chimeric or of too low quality to include them in our analysis, e.g. UDB004315|FR731781|L5402_Elaph_Cam02 (chimeric, blasts on *Saccharomyces*), UDB004314|FR731782|L5339_Elaph_Cam01 (chimeric, blasts on *Russula*),UDB004316|FR731779|L5282_Elaph_Cam03, UDB004496|FR731921|L6488_Elaph_Gab01 (too short, no results or 40bp BLAST results), UDB004317|FR731780|L5356_Elaph_

Cam04 (chimeric, BLAST on *Tomentella*). Whether or not the /*elaphomyces* lineage is common or not in Africa remains therefore an open question. Yet, our field observations demonstrate at least that the genus can be a locally abundant ectomycorrhizal partner for tropical rain forest trees in Central Africa. This Afro-neotropical clade is suggested as being sister to the *Pseudotulostoma* clade but lacks bootstrap support in all of our analyses. Whether or not they represent separate genera remains to be established, but morphological features might support such a viewpoint. In any case, both latter clades appear to be genetically too distant from the core clade to represent *Elaphomyces* sensu stricto.

Finally, the *E. guangdongensis* clade corresponds to species that have been used to represent *Elaphomyces* in all previous analyses on cleistothecial ascomycetes, but clearly cannot represent *Elaphomyces* sensu stricto. As mentioned above, it is difficult to understand why these species are genetically so different from the core clade. BLAST results (Fig. 4) list the Afro-neotropical clade and *Pseudotulostoma* clade, but then leave only the 5.8S for BLAST results on various ascomycetes such as *Auxarthron* (Onygenales), *Teratosphaeria* (Pleosporales), *Mycosphaerella* (Capnodiales)… but no *Elaphomyces* sensu stricto. When introducing *Gymnoascus* sequences in our alignment (not shown), we received 84% BS support for the grouping of the Afro-neotropical, *Pseudotulostoma* and *E. guangdongensis* clades, but lost most of BS support on other branches.

CONCLUSION

We are fully aware that ITS sequence data cannot resolve the systematics and monophyly of *Pseudotulostoma* and *Elaphomyces* (*sensu lato*) and that multigene approaches using non-ribosomal loci are required to answer these questions. Instead, the objective of this preliminary paper was not to propose a phylogeny for *Elaphomyces*, but rather to draw attention to the high dissimilarity among ITS sequences for *Elaphomyces* and to the likely unfortunate choice of species from outside the core clade to represent the genus in all of the previous phylogenetic publications on Elaphomycetaceae and cleistothecial ascomycetes. Our study highlights the need for examining the monophyly of the family Elaphomycetaceae and to verify the systematic status of *Pseudotulostoma* as a separate genus for stipitate species. Furthermore, there is an urgent need for an in-depth morphological study, combined with molecular sequencing of the studied taxa, to point out the phylogenetically informative characters of *Elaphomyces*.

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