Lactarius megalopterus, a new angiocarpous species from a tropical rainforest in Central Africa, shows adaptations to endozoochorous spore dispersal

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Abstract A new sequestrate *Lactarius* species was found in a humid evergreen tropical rainforest dominated by Fabaceae of the subfamily Caesalpinoideae in Cameroon, Central Africa. It is described here as new to science and is named *Lactarius megalopterus*, referring to its spore ornamentation of extraordinarily high wings. Anatomical characters and molecular systematic analyses confirm its relationship to *Lactarius* subgenus *Plinthogali*. Phylogenetic analyses based on two nuclear DNA regions revealed its close relationship to *Lactarius angiocarpus*, which is also an angiocarpous species from Zambia in Africa. Molecular studies have shown that tuber-like, sequestrate sporocarps evolved independently in several lineages of Basidiomycota. The findings of sequestrate fungi in tropical rainforests raise questions regarding the evolutionary benefit of enclosing the spore-producing hymenium. The enclosure of spore-producing tissue has often been associated

with the protection of the delicate hymenium against desiccation in arid habitats or against frost in cold habitats. However, these cannot be the selective factors in warm and humid areas like the tropics. This controversy is exemplarily studied and discussed in the family of Russulaceae, especially in the genus *Lactarius*. Characters shown by the angiocarpous sporocarp of the new *Lactarius*, such as thick-walled statismospores, an aromatic smell and mild taste, can be interpreted as adaptations to endozoochorous spore dispersal by mammals. Therefore, here we prefer the alternative hypothesis that sequestrate sporocarps are the result of adaptation to endozoochorous spore dispersal.

Keywords Russulaceae · *Lactarius* subgenus *Plinthogali* · Mycophagy · Endozoochory syndrome · Cameroon

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Introduction

The milk-cap genera *Lactarius* Pers. and *Lactifluus* (Pers.) Roussel show high diversity in tropical regions, especially in Africa, where about 100 species are identified up to now (Van Rooij et al. 2003; Douanla-Meli and Langer 2009; Van de Putte et al. 2009; Verbeken and Walley 2010; Maba et al. 2014) and even more species are to be expected (Verbeken and Buyck 2002). They are among the most important and diverse genera of ectomycorrhizal fungi in this region (Verbeken and Buyck 2002; Verbeken and Walley 2010). Thirteen *Lactarius* and *Lactifluus* species have been reported from Cameroon, and four of these (*Lactarius desideratus* Verbeken & Stubbe, *L. dewevrei* Douanla-Meli, *L. uapacae* Verbeken & Stubbe, *L. undulatus* Verbeken) have recently been described as new to science (Verbeken et al. 2008; Douanla-Meli and Langer 2009). Until now, only two sequestrate *Lactarius* species were known from sub-Saharan tropical Africa: the secotioid *Lactarius dolichocaulis* (Pegler)

Verbeken & Eberhardt and the angiocarpous *L. angiocarpus* Verbeken & Eberhardt (Verbeken and Walley 2010). Both were collected in the relatively dry Zambezian miombo woodlands (Eberhardt and Verbeken 2004; Pegler 1982). In the present study, a third, new sequestrate *Lactarius* species is described from a very humid tropical rainforest in Southwest Cameroon.

Sequestrate sporocarps are well known from truffles and other Ascomycota, but have evolved fairly often in several clades within the Basidiomycota as well (e.g. Bruns et al. 1989; Læssøe and Hansen 2007; Wilson et al. 2011; Smith et al. 2015). Among Basidiomycota, sequestrate sporocarps have been detected in the family Russulaceae (e.g. Miller et al. 2001; Lebel and Tonkin 2007), especially in the genus *Lactarius* (e.g. Verbeken et al. 2014). Such an evolutionary transition to sequestrate sporocarps enclosing the delicate hymenium can be interpreted as a mechanism of protection against desiccation in arid habitats (e.g. Thiers 1984; Bruns et al. 1989) or against frost in cold areas (Trappe 1988; Maser et al. 2008). However, recent records of angiocarpous fungi from humid tropical rainforests contradict these climate-based explanations (e.g. Verbeken et al. 2014; Smith et al. 2015). Hence, in tropical regions and in humid temperate climates, other factors may trigger the evolution to angiocarpous sporocarps (e.g. Bougher and Lebel 2001; Trappe and Claridge 2005).

A special kind of sequestrate fungi are those that are adapted to mycophagous animals that feed from the sporocarps and transport the spores within their intestines. This endozoochorous spore dispersal (endozoochory) requires the evolution of several characters that attract feeding animals, and at the same time requires that fungi develop protective features against destructive digestion of the spores (Fogel and Trappe 1978; Johnson 1996; Maser et al. 2008). The angiocarpous sporocarp of the newly described *Lactarius* has such features and may be suited to endozoochorous spore dispersal.

To elucidate diagnostic characters of this new species, we conducted a detailed descriptive anatomical study along with a molecular systematic study of two nuclear DNA sequence regions to obtain phylogenetic information in a wider *Lactarius* context. We also reviewed the available literature on endozoochorous spore dispersal and examined established views on the evolution of this feature.

Materials and methods

Morphological analysis

The description of the macroscopic features is based on field observations of a fresh fruit body discovered during an expedition to Cameroon in 2002. The new species was collected only once at the type locality, and thus all data presented here are from the type specimen. Microscopic characters are based

on dried material. Spores were described and drawn after treatment with Melzer's reagent. The measurements (without ornament) are based on 25 observed spores. Length, width, length/width ratio (Q) and volume (V) are given as minimum–mean–maximum values. The volumes are calculated using the formula of the rotational ellipsoid.

Basidia were stained with ammoniacal Congo red following brief aqueous potassium hydroxide (KOH) preparation. Basidia lengths exclude sterigmata. Peridiovellis and subperidial and hymenophoral tramas were studied from radial hand sections in 2 % KOH. In addition, cryosections were obtained from the dried basidiocarp and the adherent rhizomorph, which had been previously macerated in aqueous KOH and incubated in glycerol water. The sections, 30–60 µm thick, were stained with cotton blue/lactic acid. The terminology for peridiovellis (as pileipellis) structures and hymenial elements is according to Heilmann-Clausen et al. (1998) and Verbeken and Walley (2010). Scanning electron microscopy (SEM) images were obtained for air-dried material after sputtering with gold.

Molecular analysis

DNA was isolated from a small piece of gleba from the dried fruit body using the Dynabeads DNA DIRECT Universal kit (Dynal Biotech), according to the instructions for fungal tissue. The ITS1, 5.8S and ITS2 regions of the nuclear rDNA were amplified by polymerase chain reaction (PCR) using the primers ITS1F and ITS4B (Gardes and Bruns 1993). Cycle sequencing reactions were carried out with primers ITS1 and ITS4 (White et al. 1990) using the BigDye Terminator kit v3.1 (Applied Biosystems, Foster City, CA, USA). The LSU was amplified and sequenced using the primers LR0R and LR3 (Vilgalys and Hester 1990). Processed cycle sequencing products were run on an ABI PRISM 3100-Avant Genetic Analyzer capillary sequencer (Applied Biosystems).

Sequences were edited with Sequencher 4.10 software (Gene Codes, Ann Arbor, MI, USA) and deposited at GenBank. The ITS and LSU sequences were compared with accessions deposited at GenBank by applying the Basic Local Alignment Search Tool (BLAST) with the nucleotide search option (blastn) to classify the *Lactarius* species. Subsequently, ITS and LSU *Lactarius* sequences were obtained from GenBank in order to compile independent alignments to test for the phylogenetic position of the new *Lactarius* species (for GenBank accession numbers see Fig. 1). Alignments were performed using MUSCLE 3.8.31 (Edgar 2004). All of the ingroup taxa belong to *Lactarius* subg. *Plinthogali*. The non-*Plinthogali* *L. hispidulus* was used as outgroup (derived from Verbeken et al. 2014 and Stubbe and Verbeken 2012). Ambiguously aligned regions were delimited and excluded from phylogenetic analyses with Gblocks version 0.91b (Castresana 2000).

Fig. 1 Maximum-likelihood tree of *Lactarius* subg. *Plinthogali* obtained from the combined datasets of ITS + LSU. The phylogenetic position of the newly described *Lactarius megalopterus* is indicated in bold letters. Angiocarpous species are identified by black dots. Bars denote geographic origin of samples: black, Africa (Z = Zambia, C = Cameroon, G = Guiana, M = Malawi); white, Asia (S = Sri Lanka,

M = Malaysia, T = Thailand, I = India); light grey, Europe (N = North Europe, E = Central Europe); dark grey, North America (U = USA). Numbers above branches indicate RAxML bootstrap support > 50 %/ Bayesian posterior probabilities > 0.90. With the exception of *L. megalopterus*, all sequences were obtained from GenBank. GenBank accession numbers of ITS + LSU are given behind the species names

These two datasets resulted in the same tree topology; hence, they were combined and analyzed with maximum likelihood methods implemented in RAxML version 7.2.8 (Stamatakis 2006). Analysis was performed assuming a general time-reversible (GTR) model of nucleotide substitution, estimating a discrete gamma distribution (GTRGAMMA option in RAxML) with partitions according to the sub-matrices, allowing for multiple models of substitution. One thousand runs with distinct starting trees were completed for each dataset using the rapid bootstrap (BS) algorithm of RAxML (Stamatakis et al. 2008). Bayesian analysis was

performed with MrBayes 3.2.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2005) on the same dataset as the maximum likelihood analysis. Independent GTR models with gamma distribution approximated by four categories were implemented for all data partitions, with four chains and ten million generations, sampling every 100th tree. Post-burn-in trees were collected and the summarizations calculated only when the standard deviation of split frequencies reached levels below 0.01. Posterior probability (PP) values equal to or greater than 0.95 were considered significant. The phylogenetic ML tree was visualized

and rooted using the Dendroscope program (Huson et al. 2007).

Results

Molecular phylogeny

ITS1-5.8S-ITS2 and LSU of *Lactarius megalopterus* had lengths of 661 bp and 581 bp, respectively. When compared with sequences deposited at GenBank, both sequences showed the highest similarity to the sequences of *L. angiocarpus* of *L.* subgenus *Plinthogali* (blastn: ITS [AY606942] 97 % identity, LSU [AY606970] 99 % identity). The computed phylogeny was therefore restricted to species of *L.* subg. *Plinthogali*. Both types of analyses (RaxML, MrBayes) resulted in the same tree topology (Fig. 1). The two sequestrate species from Africa, *L. megalopterus* and *L. angiocarpus*, paired in a maximally supported clade (100 % BS, 1.00 PP). The four African species and the seven European, one North American and eight Asian species, respectively, appeared as polyphyletic geographic groups. The six angiocarpous *Lactarius* species appeared as polyphyletic.

Taxonomy

Lactarius megalopterus Beenken & Sainge, sp. nov.
Mycobank MB 803213; Figs. 2, 3, 4 and 5

Etymology: The name *megalopterus* refers to the highly winged spores (from Greek: μέγας πτερόν = large wing).

Macroscopic features – Basidiocarp (Fig. 2) 55 × 45 × 40 mm, tuberous, consisting of subglobose to irregular parts, epigeous. Peridial surface smooth, dry, buff-yellow to orange with pinkish tinge after bruising, paler at base. Gleba pale pinkish-brown, lacunose, with 0.5–1(2) mm wide labyrinthine locules. Stipe and columella absent. Rhizomorphs at base of basidiocarp, up to 0.5 mm thick. Smell aromatic, rubber-like, with fruity components. Taste mild. Latex abundant, close to the peridia, sparse or absent in the gleba, white, pale pink when dried, mild.

Microscopic features – Hymenium: Basidiospores (Figs. 3c and 4) globose to subglobose ($Q = 1\text{--}1.07\text{--}1.18$), $8.5\text{--}9.6\text{--}11 \times 8\text{--}9.0\text{--}10.5 \mu\text{m}$, $V = 285\text{--}411.2\text{--}632 \mu\text{m}^3$, symmetric and orthotropic; spore wall 0.75–1 μm thick; ornamentation amyloid, highly winged, ridges up to 3.5(4) μm high, often encompassing half or more of the spore circumference, rarely branched but never reticulate, isolated shorter ridges, spines and warts between the main ridges; hilar appendix 2–2.5 × 1.5–2 μm ; suprahilar plage absent. Basidia (Fig. 3b) 45–60 × 12–16 μm , occasionally up to 80 × 25 μm , 4-spored, clavate, thin-walled; sterigmata 5–6(8) μm long. True cystidia and pseudocystidia (gloeocystidia) absent. Sterile elements not found. Hymenophoral trama 30–100 μm wide, composed of net-like, loosely arranged hyphae, 3–6 μm in diam., hyphal walls 0.3 μm thick, close to the hymenium (subhymenium), with spherocytes arranged candelabra-like (Fig. 5b), not gelatinized; lactiferous hyphae absent or very rare. Peridiopellis (Figs. 3a and 5a) palisade-like, 30–70 μm thick, two-layered; (i) upper layer pseudoparenchymatous, cells ± isodiametric, (5) 10–30(40) in diam., walls gelatinized, 0.5–2 μm thick; bearing short, septate ascending hyphae (15–50 × 4–7 μm) sparsely scattered over the surface, walls 0.3–0.5 μm thick; (ii) underlying hyphae interwoven, distinctly gelatinized, 3–6(7) μm in diam. Subperidial trama 200–300 μm thick, composed of subparallel, colorless, hyaline hyphae 3–6(7) μm in diam., hyphal walls 0.3–0.5 μm thick; lactiferous hyphae abundant, 5–20 μm in diam., walls 0.5–1(1.5) μm thick, gelatinization weak to absent.

Rhizomorphs (Fig. 3d) two-layered; centrally composed of four distinct types of hyphae: (i) parallel colorless, hyaline hyphae 3–6 μm in diam., hyphal walls 0.3–0.5 μm thick; (ii) lactiferous hyphae with granulose content, 5–10 μm in diam., walls 0.5–1(1.5) μm thick, septa in long distances; (iii) vessel-like hyphae similar to lacitfers but without content, 5–20 μm in diam; (iv) hyphae with numerous arched septa, distance between septa 10–20 μm , 10–15 μm in diam., walls 1–1.5 μm thick; outer layer similar to the peridiopellis, 30–40 μm thick, cells up to 20 × 30 μm , walls gelatinized, 0.5–2 μm thick, short ascending hyphae not observed. Clamp connections in all hyphae absent.

Type: CAMEROON, Southwest Region, Ndian Division, Diongo Community Forest, peripheral zone of the Onge Forest Reserve, SE of Bamusso, close to Diongo village,

Fig. 2 *Lactarius megalopterus* (holotype). Basidiocarp ca. 55 × 45 × 40 mm (**b** in section), with feeding mark on the bottom right

Fig. 3 *Lactarius megalopterus* (holotype). **a** Section through the outer peridiopellis with septate ascending hyphae. **b** Basidia, on the left an extremely large one. **c** Basidiospores (on the bottom right, optical section showing the thick spore wall). **d** Longitudinal section through a rhizomorph with cellular outer surface and with vessel-like hypha, ladder-like hypha and lactifer in the inner part (top to bottom). Scale bars = 10 µm

4°25'N, 8°57'E, alt c. 200 m, 17 Oct. 2002, leg. M. N. Sainge and L. Beenken, no. K02/79 (holotype in YA, isotype in M). GenBank accession numbers: ITS1-5.8S-ITS2 sequence KC492994; LSU partial sequence KC492995.

Habitat – *Lactarius megalopterus* was fruiting above ground in the litter of a dense rainforest. The Onge area at the western side of Mount Cameroon is one of the most humid regions in Africa (6–7 m of rain per year) with major rainfall from June to October and a drier period from November to May (Cable and Cheek 1998). *Lactarius megalopterus* was collected in October at the end of the rainy season. The area is part of the Lower Guinean phytochorion and one of the plant species-richest areas in western Africa, with a high rate of endemism (Beentje et al. 1994; Cable and Cheek 1998; Mutke et al. 2001). The type locality of *L. megalopterus* is a humid evergreen rainforest dominated mainly by ectomycorrhiza-forming trees such as the genera *Anthonotha*, *Gilbertiodendron*, *Microberlinia*, *Tetraberlinia* (Fabaceae of subfamily Caesalpinioideae) and *Uapaca* (Phyllanthaceae) (Smith and Read 2008). Thus, species of these genera may be ectomycorrhizal partners of *L. megalopterus*, although it was not possible to assign the

Fig. 4 *Lactarius megalopterus* (holotype). Basidiospores (SEM). Scale bars = 1 µm

Fig. 5 *Lactarius megalopterus* (holotype). **a** Section through the peridiopellis. **b** Section through gleba. Scale bars = 20 μ m

fungus to a specific tree. Located close to the village of Diongo, the forest suffers from human impact of logging and hunting.

Discussion

Taxonomy and morphology

Molecular phylogenetic studies have shown that *Arcangeliella*, *Gastrolactarius* and *Zelleromyces* represent artificial genera for sequestrate *Lactarius* species, and may be better subsumed in the amplified genus *Lactarius* (e.g. Nuytinck et al. 2003; Verbeken et al. 2014; Kirk 2015; Pierotti 2015). To date, sequestrate forms are not reported from the genus *Lactifluus* (Verbeken et al. 2014). As the sequestrate life habit evolved several times independently within various subgenera of *Lactarius*, this character is the result of convergent evolution (Miller et al. 2001; Peter et al. 2001; Desjardin 2003; Nuytinck et al. 2003; Eberhardt and Verbeken 2004; Verbeken et al. 2014). The newly described *Lactarius megalopterus* supports this presumption. According to our molecular data, *L. megalopterus* is closely related to *L. angiocarpus* from Zambia, an also angiocarpous species belonging to *Lactarius* subg. *Plinthogali* (Fig. 1; Eberhardt and Verbeken 2004; Verbeken et al. 2014). Our tree

topology matches well with the corresponding subclades in the more extensive analyses of Verbeken et al. (2014) and with the analysis of Stubbe and Verbeken (2012), respectively.

The placement of *L. megalopterus* within *L.* subg. *Plinthogali* is also well supported by morphological characters. A spore ornamentation composed of highly winged ridges is characteristic of this subgenus. Also, the pileipellis is often palisade-shaped (Heilmann-Clausen et al. 1998; Verbeken 2000; Verbeken and Walley 2010; Stubbe and Verbeken 2012). White latex, turning reddish when exposed to air, is characteristic of many species of *L.* subg. *Plinthogali* as well (De Bernadi et al. 1992; Sterner 1995; Verbeken 2000).

The sister species of *L. megalopterus* is *L. angiocarpus*. The new species is well differentiated from *L. angiocarpus* in various morphological and anatomical characters. Although both species have subglobose to irregularly shaped fruit bodies, without stipe and columella, the sporocarp of *L. angiocarpus* is smaller, and its peridia and gleba are both light-cream, without yellow, pinkish or brown colors. Laticifers and pseudocystidia occur in the hymenophoral trama of *L. angiocarpus*, and thus the latex is abundant in the whole gleba, not only near the peridia, and remains white. The spores of *L. angiocarpus* bear shorter wings, forming an incomplete net. Its peridiopellis is a trichoderm and not palisade-like. These pronounced morphological differences split *L. megalopterus* well from *L. angiocarpus*.

The secotioid *L. dolichocaulis* from Zambia, thus far not sequenced, differs from *L. megalopterus* in its rather agaricoid habit, with cap and stipe and reticulate spore ornamentation (Pegler 1982; Verbeken 2000; Verbeken and Walley 2010). *L. dolichocaulis* is only similar to *L. megalopterus* in the gleba-like hymenium without true cystidia and pseudocystidia (Verbeken 2000; Verbeken and Walley 2010). It is worth mentioning that all known sequestrate *Lactarius* species from tropical Africa belong to *L.* subg. *Plinthogali* (Eberhardt and Verbeken 2004; Verbeken and Walley 2010).

Additional angiocarpous representatives of *L.* subg. *Plinthogali* from tropical Sri Lanka are reported to be associated with Dipterocarpaceae (Verbeken et al. 2014). Of these, *L. saturnisporus* Verbeken & Stubbe and *L. shoreae* Stubbe & Verbeken have like *L. megalopterus* highly winged spores, whereas *L. echinellus* Verbeken & Stubbe and *L. echinus* Stubbe & Verbeken differ from *L. megalopterus* by echinate spore ornamentation. *Arcangeliella beccarii* (Petri) Zeller & C.W. Dodge and *A. malaiensis* Corner & Hawker from Malaysia (Corner and Hawker 1953) and Australia (Beaton et al. 1984, as *Zelleromyces malaiensis* [Corner & Hawker] A.H. Sm.), respectively, are also angiocarpous lactarioid species that have globose spores ornamented with very high ridges forming incomplete nets. They may also belong to *L.* subg. *Plinthogali*, but a recombination to *Lactarius* should not be done without having studied their type specimens.

The rhizomorph of *L. megalopterus* has a typical anatomy, which is otherwise known only from the rhizomorphs of *Lactarius*, *Lactifluus* and *Russula* (Russulaceae) (Beenken 2001a, b; 2004; russuloid rhizomorphs *sensu* Agerer 1999): it is divided into an outer layer and an inner part containing lactifers, vessel-like hyphae and densely septate hyphae with arched septa (ladder-like hyphae *sensu* Agerer 1999). Lactiferous hyphae are common in the rhizomorphs of *Lactarius* but are absent in *Russula* species and *Lactifluus piperatus* (Beenken 2001a, b, 2004).

The endozoochory syndrome

Lactarius megalopterus shows all morphological features characteristic of truffle-like fungi with spore dispersal by mycophagous mammals via endozoochory. The characteristics of this endozoochorous spore dispersal syndrome include sporocarps with a peridium enclosing a fleshy, chambered gleba bearing the hymenium, thick-walled statismospores, and a smell attracting animals (Fogel and Trappe 1978; Trappe and Cázares 1990; Johnson 1996; Claridge 2002; Trappe and Claridge 2005). Therefore, we hypothesize that *L. megalopterus* has an endozoochorous dispersal mode, and we justify this assumption in detail below.

The first hint was that the field-collected sporocarp showed parallel tooth-marks, which are typical indications of the feeding habits of small rodents (Fig. 2). Hastings and Mottram (1916) described and illustrated similar tooth-marks of rodents on European mushrooms. Furthermore, an aromatic smell of *L. megalopterus* was detected in the field that did not resemble any smell we know from lamellate *Lactarius* species in Africa or Europa. This special smell may attract mammals. The spore morphology of *L. megalopterus* is the main indication of dispersal by means other than air. In contrast to air-dispersed, thin-walled and asymmetric ballistospores, which are known from lamellate Russulaceae (e.g. Nilsson 1983, Heilmann-Clausen et al. 1998), the basidiospores of *L. megalopterus* are radially symmetrical, orthotropic and without suprahilar plage. Thus, they show the characters of so-called statismospores, having lost their features of ballistic spore dispersion (Pegler and Young 1979; Miller 1988; Miller and Miller 1988). In addition, the walls of these spores are much thicker (0.75–1 µm) than those of airborne ballistospores of *Lactarius* spp. (<0.5 µm, authors' own unpublished observations, microphotographs in Nilsson 1983) reported for many sequestrate genera or species of several other fungal families with references to animal mycophagy (e.g. Castellano et al. 1989). These thick spore walls, and likely the unusual high ornaments, may protect the spores against digestion during their intestinal passage (Trappe and Claridge 2005). Lescourret and Génard (1986) showed that the digestive enzymes of small mammals affect the spore surfaces. Thus, germination of spores adapted to endozoochory may be promoted

even after passing through a mammal's gut (Kotter and Farentinos 1984; Cork and Kenagy 1989; Claridge et al. 1992; Johnson 1996; Reddell et al. 1997; Colgan and Claridge 2002; Trappe and Claridge 2005). In many agaricoid *Lactarius* species, the sporocarp exudes an acrid-tasting latex as a mechanism for fending off several predators, especially from the lamellae with the hymenium (Camazine and Lupo 1984; Sterner 1995; Stadler and Sterner 1998). However, the pungent latex ingredients can be an antifeedant against mammals (Camazine et al. 1983). In the case of endozoochorously dispersed *Lactarius* species, such an antifeedant would be counterproductive. Hence, this could explain the milder taste of the latex of *L. megalopterus* and *L. angiocarpus*, and the reduction of lactiferous hyphae as well as the lack of cystidia in the gleba of *L. megalopterus*. Various species of the *L.* subg. *Plinthogali* are acrid and toxic, but there are also many species with a sweet taste. Some of these are edible for humans (Heilmann-Clausen et al. 1998, Karhula et al. 1998; Verbeken 2000). However, the edibility of *L. megalopterus* for humans is unknown, as the natives of Diongo village and its environs do not consume this species.

In conclusion, we think that there is a strong indication of endozoochoric spore dispersal of *L. megalopterus* provided by the indirect morphological evidence explained above, even though we were not able to prove our hypothesis through direct observation of animals feeding on *L. megalopterus* or the spores passing through the digestive tract of an animal in the field.

The habitat of *L. megalopterus* supports the hypothesis that its angiocarpous sporocarp is not an adaptation to extreme climate conditions, as the specimen was found in one of the wettest regions of tropical Africa at the end of the rainy season. The type locality lies in the western foothills of Mount Cameroon, a hilly area belonging to a glacial refuge that was continuously covered by rainforest in the past, even during the dry periods of the Quaternary (Maley 1996; Sosef 1996; Maley and Brenac 1998). Therefore, the sequestrate habit of *L. megalopterus* has obviously evolved under wet conditions and not in a dry environment. However, *L. megalopterus* is not an isolated case of a sequestrate fungus from humid tropical Africa (Dring and Pegler 1978; Verbeken and Walley 2003). The two first authors (L.B., M.N.S.) have found two other angiocarpous fungi in the Cameroon rainforests showing similar adaptations to endozoochorous spore dispersal (*Gelopellis rufus* Dring [Phallaceae], *Octaviania* sp. [Boletaceae]; unpublished data). In addition, the sporocarps of the closely related *L. angiocarpus* and of the secotioid *L. doliocaulis* were collected from the miombo woodlands in Zambia in December at the peak of the rainy season (Eberhardt and Verbeken 2004; Pegler 1982; <http://en.climate-data.org>). Outside tropical Africa, angiocarpous *Lactarius* species occur in the tropical rainforests of Southeast Asia (Verbeken et al. 2014). Smith et al. (2015)

described three angiocarpous taxa of Boletaceae (Boletales) from tropical South America, which were collected during rainy seasons in a primary rainforest dominated by ectomycorrhizal tree species in Guyana. Sequestrate habits have been found to occur even in moderate, humid to wet climates of Europe, North America, Australia and New Zealand (e.g. Jülich 1984; Bougher and Lebel 2001; Trappe and Claridge 2005). Lastly, Reddell et al. (1997) detected spores of several ectomycorrhizal fungi in scats of 17 mammal species in tropical forests of northeastern Australia, including basidiospores of sequestrate Russulaceae.

These observations of sequestrate fungi from wet tropical and temperate areas contradict the general assumption that the sequestrate habit is an adaptation of fungal sporocarps to dry conditions as protection of the hymenium against desiccation or against other climatic stress such as heat or cold (e.g. Thiers 1984; Trappe 1988; Bruns et al. 1989; Maser et al. 2008). Hence, the adaption to endozoochorous spore dissemination is a more plausible explanation for the evolution of sequestrate fungi, particularly in humid forest habitats.

Regardless of temperature and humidity, endozoochory may be a more effective strategy than aerial transport for spore dissemination in closed-canopy forests with no winds close to the ground (Trappe and Claridge 2005). This type of dispersal is particularly important for ectomycorrhizal fungi (Johnson 1996; Frank et al. 2006, 2009; Maser et al. 2008; Schickmann et al. 2012).

Our hypothesis that endozoochorous spore dissemination plays a major role in the evolution of sequestrate fungi is supported by the fact that even some agaricoid species show an affinity for endozoochorous spore dispersal (Castillo-Guevara et al. 2011). Such adaptation is a necessary precondition for successful spore dissemination after an enclosed hymenium has evolved.

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