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BIODIVERSITY OF THE GENUS *LABOULBENIA*

Are there cryptic species?

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Abstract

This contribution is one of the first to focus on molecular aspects of Laboulbeniales, a group of ascomycete fungi obligately associated with living arthropods. The aim of this study is to find whether morphological delimitation of species in the genus *Laboulbenia* is supported using molecular techniques. We chose this genus because 1) it represents about a quarter of all known Laboulbeniales, 2) morphological identification is difficult and 3) only few sequences are available in GenBank. The extraction failure rate is notoriously high in *Laboulbenia*, but we managed to obtain 50 sequences (LSU and ITS) of 19 *Laboulbenia* species, the highest number made available so far. We made a two-locus phylogenetic tree (ITS and LSU). Our preliminary phylogenetic tree shows that the long-standing morphological grouping for *Laboulbenia* of Tavares (1985) is not supported. Species placed together in Tavares' system are phylogenetically separate, indicating that morphological criteria are inadequate for delimiting subgeneric taxa. At species level, we observe that all material identified as *L. flagellata*, a well-known species with wide host range, forms a large clade with separate and highly supported subclades. Each of the subclades corresponds with material obtained from different host taxon (species of genus), indicating that they may represent cryptic and host-specific phylogenetic species. It is suggested that formerly considered useless morphological characters, might in fact be used to formally describe these taxa. Similar observations could be made for *L. vulgaris* and *L. pedicellata* in future research. Species of *Laboulbenia* should no longer be described without molecular support. To better understand the taxonomy, specificity, and diversity of *Laboulbenia*, we need to further elaborate the phylogenetic tree by including more species, sampled worldwide and preferably from as many host groups as possible.

Samenvatting

Deze bijdrage is een van de eerste gericht op de moleculaire aspecten van Laboulbeniales, een groep ascomycete schimmels die geassocieerd worden met levende geleedpotigen. Het doel van deze studie is te achterhalen of morfologische kenmerken van soorten in het genus *Laboulbenia* worden ondersteund met behulp van moleculaire technieken. We kozen het genus *Laboulbenia* omdat het ongeveer een kwart van alle gekende Laboulbeniales vertegenwoordigt, morfologische identificatie moeilijk is en slechts enkele sequenties beschikbaar zijn in GenBank. Het percentage mislukte extracties is notoir hoog in Laboulbenia, we zijn erin geslaagd om 19 sequenties van *Laboulbenia*-soorten te verkrijgen, het hoogste aantal dat tot nu toe beschikbaar is. In combinatie met bestaande sequenties hebben we een fylogenetische boom met twee loci (ITS en LSU) gemaakt. Onze voorlopige fylogenetische boom illustreert dat de morfologische groepering van Tavares (1985) niet wordt ondersteund. Soorten samen geplaatst in het systeem van Tavares zijn fylogenetisch gescheiden, wat aangeeft dat sommige morfologische criteria onvoldoende zijn voor het afbakenen van subgenerische taxa. Op soortniveau zien we dat al het materiaal dat is geïdentificeerd als *L. flagellata*, een bekende soort met een breed gastheerbereik, een grote clade vormt met afzonderlijke en sterk ondersteunde subclades. Elk van de subclades komt overeen met materiaal dat is verkregen van verschillende gastheer-taxons, hetgeen aangeeft dat zij cryptische en gastheer-specifieke fylogenetische soorten vertegenwoordigen. Er wordt gesuggereerd dat voorheen beschouwde nutteloze morfologische karakters, in feite kunnen worden gebruikt om deze taxa te onderscheiden. Soortgelijke waarnemingen kunnen worden gedaan voor *L. vulgaris* en *L. pedicellata* in toekomstig onderzoek. We kunnen in het geslacht *Laboulbenia* geen nieuwe taxa beschrijven zonder moleculaire ondersteuning. Om de taxonomie, maar ook de specificiteit en diversiteit van *Laboulbenia* beter te begrijpen, moeten we de fylogenetische analyse verder uitwerken door meer soorten op te nemen, wereldwijd te verzamelen, bij voorkeur uit zoveel mogelijk gastgroepen

Jargon-free abstract

Living fungi on insects have been found to be complex to distinguish, not only for their microscopic figure but also for their appearance. *Laboulbenia* is an example that is well-known to be very complex because it has many variations in appendage, shape, etc. Species descriptions generally focus on appearance, but this approach may underestimate true diversity. *Laboulbenia* might include species that look the same but do not belong to the same species. We have found material of 19 species of *laboulbenia* to make an analysis of evolutionary relatedness. Tavares (1985) already separated several species in groups on the basis of appearance. However we found that these groups are not molecularly supported. Further we found indeed three species that are lookalikes but do not belong to the same species. These lookalikes all originated from different insect host, it is clear that host relationship plays a role in the relatedness of lookalikes. However, careful analysis of the appearance does show subtle differences in pigmentation or branching of the appendages. Many of such features were previously considered of little value. Thanks to a good match between morphology, host identity and molecular data, we think these subtle morphological features can actually be used to separate and define several of these new taxa.

1 Introduction

This introduction presents general aspects related to the classification, morphology, development, distribution and host specificity of Laboulbeniales, as well as some aspects related to species concept, identification and estimating their diversity. Objectives of the thesis are given at the end of this chapter (1.6).

1.1 Position of Laboulbeniales among the Fungi

Laboulbeniomycetes represent a large class within the phylum Ascomycota. The largest order within this class is Laboulbeniales, counting 142 genera and about 2,200 species (Reboleira *et al.* 2018). Laboulbeniales were discovered by the French entomologist Joseph Alexandre Laboulbène in the 1840s (Thaxter 1896). At first they were considered outgrowths of the insect itself, parasitic worms, or red algae. Eventually they were classified within the fungi (Robin 1853), but with varying affiliations, i.e. in the Ascomycota, Basidiomycota, and even Zygomycota. Thaxter (1896) described their ascogenous spore development, but they were only accepted as Ascomycetes after Blackwell (1994) presented a partial Ascomycota phylogeny based on SSU rDNA including sequences of *Pyxidiophora* Bref. & Tavel (Pyxidiophorales) and *Rickia* Cavara (Laboulbeniales).

The morphology of Laboulbeniales is unique among the ascomycete fungi (see further). The class Laboulbeniomycetes shares some morphological traits with its sister group Sordariomycetes, such as perithecia and unitunicate asci with poricidal dehiscence (Schoch *et al.* 2009). Molecular data support the sister relationship of Sordariomycetes and Laboulbeniomycetes (Goldmann & Weir 2018, Haelewaters *et al.* 2019, Weir & Blackwell 2001a). Three orders can be distinguished within Laboulbeniomycetes: Herpomycetales, Laboulbeniales, and Pyxidiophorales (Haelewaters *et al*. 2019).

1.1.1 Classification within Laboulbeniales

On a finer taxonomic scale, there have been two major classification systems of Laboulbeniales, both of which were based on morphological features: Thaxter (1908) and Tavares (1985). Thaxter (1908) based his classification on antheridial structures and recognized two suborders, the Laboulbeniineae and Ceratomycetineae. This antheridium-based classification was prevailing until Tavares (1985) discovered that the families established based

on antheridial characters were not monophyletic. Her classification includes characters of the perithecium wall and perithecial development. In this classification, she divided Laboulbeniales into the suborders Herpomycetinae (now a separate order; Haelewaters *et al*. 2019) and Laboulbeniinae. The Herpomycetinae suborder includes the single family Herpomycetaceae and the Laboulbeniinae includes three families; Ceratomycetaceae, Euceratomycetaceae, and Laboulbeniaceae.

Recent molecular work of Goldmann & Weir (2018) partly supports the classification proposed by Tavares (1985), but also shows: (1) there is no support for the family Euceratomycetaceae and (2) polyphyly in the tribes Haplomyceteae and Peyritschielleae, and subtribes Amorphomycetinae, Stigmatomycetinae, and Laboulbeniinae. Also Haelewaters *et al.* (2018b) found that the subtribe Stigmatomycetinae is polyphyletic. The phylogeny by Goldmann & Weir (2018) presents five distinct clades based on SSU. As already mentioned above, Haelewaters *et al.* (2019) now accommodate the Herpomycetaceae in a separate order Herpomycetales, based on SSU, ITS, LSU data. The three orders Herpomycetales, Laboulbeniales, and Pyxidiophorales are strongly supported, but several subtribes, tribes and subfamilies are still polyphyletic.

Despite being the most diverse genus within Laboulbeniales, *Laboulbenia* Mont. & C.P. Robin is not well enough sampled in any of these phylogenies to make vast conclusions about taxonomy. *Laboulbenia* counts 650 species worldwide, all based or derived from a single thallus construction pattern with fairly strict receptacular and perithecial features. Subgenera have never been formally described, Tavares (1985) distinguished 20 morph groups in *Laboulbenia*: 1) *L. vulgaris,* 2) *L. flagellata,* 3) *L. rougetii,* 4) *L. luxurians ,* 5) *L. fasciculata ,* 6) *L. proliferans,* 7) *L. variabilis,* 8) *L. orectochili,* 9) *L. quedii,* 10) *L. galeritae,* 11) *L. texana,* 12) *L. longicollis,* 13) *L. rhinoceralis,* 14) *L. diabroticae,* 15) *L. nisotrae,* 16) *L. hermaeophaga,* 17) *L. muscariae,* 18) *L. pectinulifera,* 19) *Ceraiomyces*, and 20) *L. brachyonychi*.

The morphological groups Tavares (1985) recognized in *Laboulbenia* are based on species with similar construction of appendages, receptaculum, and perithecium, or combinations thereof. In some cases, but not all, this purely morphological grouping of species also corresponds with the host range of the species. For example, some morphological groups Tavares (1985) delimited are exclusively found on chrysomelid beetles, others only on ground beetles (e.g., Rossi *et al.* 2016). Recent developments in isolating DNA from representatives of

the genus *Laboulbenia* (Goldmann & Weir 2018, Haelewaters *et al.* 2019, Sundberg *et al.* 2019), make it now possible to test whether this morphology-based classification is supported by molecular data.

Table 1: Classification comparison of Laboulbeniales between Thaxter (1908) and Tavares (1985).

Thaxter's classification (1908)	Tavares' classification
Suborder Laboulbeniineae	Suborder Herpomycetinae
Family Laboulbeniaceae	Family Herpomyceteae
Tribe Herpomyceteae	Tribe Herpomyceteae
Tribe Amorphomyceteae	Suborder Laboulbeniinae
Tribe Stigmatomyceteae	Family Ceratomycetaceae
Tribe Idiomyceteae	Subfamily Tettigomycetoideae
Tribe Coreothromyceteae	Tribe Thaumasiomyceteae
Tribe Laboulbenieae	Tribe Drepanomyceteae
Tribe Rhachomyceteae	Tribe Ceratomyceteae
Tribe Clematomyceteae	Subtribe Helodiomycetinae
Tribe Compsomyceteae	Subtribe Ceratomycetinae
Tribe Chaetomyceteae	Subfamily Ceratomycetoideae
Tribe Ecteinomycetea	Family Euceratomycetaceae
Family Peyritschiellaceae	Family Laboulbeniaceae
Tribe Dimorpomyceteae	Subfamily Zodiomycetoideae
Tribe Rickieae	Subfamily Laboulbenioideae
Tribe Enarthromyceteae	Tribe Compomyceteae
Tribe Haplomyceteae	Subtribe Compsomycetinae
Suborder Ceratomycetineae	Subtribe Kainomycetinae
Tribe Ceratomyceteae	Tribe Hydrophilomyceteae
Tribe Zodiomyceteae	Tribe Coreomyceteae
	Tribe Teratomyceteae
	Subtribe Teratomycetinae
	Subtribe Rhachomycetinae
	Subtribe Chaetomycetinae
	Subtribe Filariomycetinae
	Subtribe Smeringomycetinae
	Subtribe Scelophoromycetinae
	Subtribe Histeridiomycetinae
	Subtribe Rhipidiomycetinae
	Subtribe Amphimycetinae
	SubtribeAsaphomycetinae
	Tribe Laboulbenieae
	Subtribe Laboulbeniinae
	Subtribe Misgomycetinae
	Subtribe Chitonomycetinae
	Subtribe Chaetarhriomycetinae
	Subtribe Stigmatomycetinae
	Subtribe Amorphomycetinae
	Tribe Euphoriomyceteae
	Subtribe Euphoriomycetinae
	Subtribe Aporomycetinae
	Subfamily Peyritschielloideae
	Tribe Peyritschielleae
	Subtribe Peyritschiellinae
	Subtribe Mimeomycetinae
	Subtribe Enarthromycetinae
	Subtribe Diandromycetinae
	Tribe Dimorphomyceteae
	Tribe Haplomyceteae
	Subtribe Haplomycetinae
	Subtribe Kleidiomycetinae
	Subfamily Monoicomycetoideae

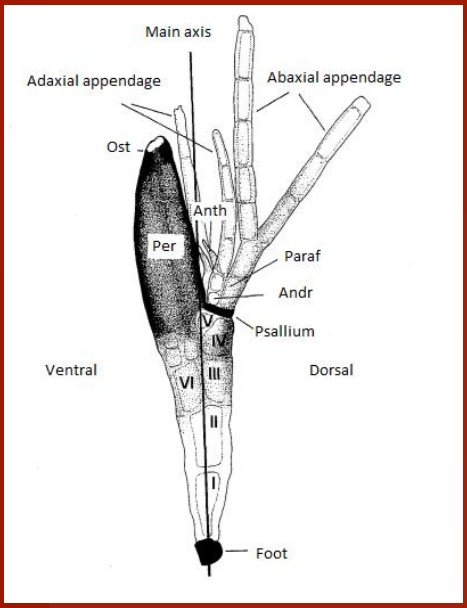

Figure 1: Construction of the *Laboulbenia* thallus. Example: *Laboulbenia Melanaria* Thaxter, abbreviatisions see glossary (De Kesel 1998).

Glossary

For the description of the thalli of *Laboulbenia* and the positions of parts, the terminology is used according to (De Kesel, 1998; Majewski, 1994; Tavares, 1985). The terminology related to the *Laboulbenia* genus is explained below.

Andropodium (Andr): Basic cell from the adaxial appendage.

Androstichum: Thallus above cell II, consisting out of cells III, IV and V.

Antheridia (Anth): Male reproduction structure.

Appendix: Primary appendage.

1.2 Morphology and development of Laboulbenia

The development and morphology of Laboulbeniales is complex and not entirely understood. In this work we will restrict ourselves to *Laboulbenia*. The ontogeny of some *Laboulbenia* representatives has been studied in detail by Tavares (1985) and De Kesel (1998). In the following paragraphs the terminology follows the figure on the left. Different variations in the model lead to different taxonomic groups.

The structure and organisation of *Laboulbenia* thallus is characterised by the suppression of the ascospore germ tube formation and the development of the thallus from a bicellular ascospore by a restricted number of mitotic divisions. The dimensions of *Laboulbenia* can vary approximately between 0.150 and 1 mm (Benjamin 1971, Thaxter 1896). *Laboulbenia kunkelii* (Giard) Thaxt. is the largest Laboulbeniales representative, measuring 4 mm (Giard 1892).

The precise structure, organization, and relative position of the cells of the receptacle provide the main taxonomic characters. The receptacle develops from the foot. The foot is usually pigmented black, except in some species (e.g., *Laboulbenia hyalopoda* De Kesel). Generally, it is accepted that most Laboulbeniales do not penetrate the living tissue of their host. This was shown by electron microscopy for four ant-associated Laboulbeniales species (Tragust *et al.* 2016). A few species, such as *Laboulbenia dahlii* Thaxt. and *Hesperomyces virescens* Thaxt., do penetrate the

Glossary continued

Cell I, II, III, IV, V: Receptacle cells.

Cell VI: Support cell of perithecium.

Cell VII: Secundair support cell of perithecium.

Fialide: Antheridia.

Foot: Basal from cel I, connects with host.

Haustorium: Structure from the foot of the thallus, entering the host.

Hyaline: See through.

Lobs: Vingerlike outgrowths on the ostiolairs lips.

Lower receptaculum: Part of the receptaculum between cell I and the base of the perithecium.

Ostiolum (Ost): Apical inlet of perithecium, surrounding with ostiolair lips with or without lobs.

Parafysopodium (Para): Base cell of abaxiaal appendage.

Perithecium (Per): Female reproduction structure above cell VI and cel VII.

Primair septum : Septum between the two cells of the ascospore.

Psallium: Mostly blacked flattened cell above IV and V.

Spermatia: Reproduction cells from the antheridia.

Suprabasale cel of x: The cell above x.

Trichogyne: Last cel of the young peritchium.

integument of the host by forming a haustorium (Benjamin 1971).

The receptacle consists of three cells that are indicated by Roman numerals: I (basal cell), II (suprabasal cell), and III (referred to as the uppermost cell of the receptacle). Exceptions exist in other genera, including non-divisions or subdivisions. Cell I is the basal cell, forming the only connection with the host's integument. In *Laboulbenia,* cell III divides and forms two additional receptacle cells, i.e. cells IV and V. The complex of cells III-IV-V is called the androstichum. Unlike *Laboulbenia*, some genera develop secondary receptacles that lie outside the primary axis of the original ascospore. Atypical divisions of the primary and secondary receptacles can occur and include development of sterile, antheridial, and perithecial branches from any cell of the receptacle except cell I. The primary appendage is formed by the division of the upper (unattached, shorter) cell of the ascospore and is a direct continuation of the primary receptacle axis. An appendage consists of one or two cells or can be more extensive, like in *Corethromyces* Thaxt. and *Laboulbenia*. Secondary appendages are formed from the lowermost ascospore as sterile or antheridial branches, but these are absent in *Laboulbenia*. In *Laboulbenia* features of appendages are important for identification. Appendages can vary from tiny and small to very elaborate. Based on experimental work it has been suggested that these branches play a role in the water balance of the thallus and the uptake of nutrients from the environment (Cavara 1899, De Kesel 1996).

Antheridial characters are important classification features. In Laboulbeniales antheridia can be present on the appendages, either clustered or regularly distributed along the axis. They can also originate from small corner cells or develop within one or several cells. Three distinct types of spermatial development have been distinguished: exogenous, simple endogenous and compound endogenous. 1) In exogenous development, the spermatia are formed on intercalary cells or terminally from cells of the appendage. Exogenous spermatia production is unusual and is associated with aquatic hosts. 2) Simple endogenous development contains spermatia formation within a flask shaped cell. This simple endogenous type of antheridium is commonly encountered in the Laboulbeniales and typical in *Laboulbenia*. 3) Compound endogenous development is when antheridia cells discharge their spermatia into a common chamber before exiting through a single opening. Compound endogenous antheridia are only known in representatives of the subfamilies *Monoicomycetoideae* and *Peyritschielloideae.*

The perithecium is mostly formed as an outgrowth from the receptacle suprabasal cell (II). In the first type, a single cell arises laterally from the receptacle which divides into a lower and upper cell. 1) The lower cell, by continued divisions, initiates the primary stalk cell (VI), secondary stalk cell (VII) and basal cells m, n and n’ formed from cell VI. The division of cells n and n' results in the formation of three vertical rows of perithecial wall cells. Cell m divides to form the fourth row (Rossi & Weir 1998). The number and arrangement of perithecium wall cells are important taxonomic characters in the classification of Tavares (1985). 2) The upper cell initiates the female sexual organ, which includes three cells: basal carpogenic cell, trichophoric cell and terminal trichogyne. The trichogyne is a thin appendage-like outgrowth that develops into a multicellular simple or branched structure, depending on the species. Its function is to receive spermatia.

Ascospores are formed and organized in the perithecium in an upward direction. The larger cell of the bicellular ascospore is organised upwards and thus the first to be released from the ostiole. The ascospores are hyaline, elongate, spindle-shaped, and surrounded by a thin mucilaginous envelope. The spores are adhesive and immobile, which causes their spread to depend on their host activities. The larger cell of the ascospore initiates contact with a new host by forming the foot.

1.3 Distribution and specificity of Laboulbeniales

General distribution, transmission and specificity are discussed in the following sections. These processes do not only apply to *Laboulbenia*, but are in general for Laboulbeniales.

1.3.1 General distribution on Arthropoda

Laboulbeniales are obligate ectoparasitic on Arthropoda. The host spectrum of Laboulbeniales is broad and includes representatives in Actinotrichida, Anactinotrichida, Diptera, Hymenoptera, Hemiptera, Orthoptera, and Blattodea. The majority of species (80%), however, has been reported on Coleoptera (Rossi & Santamaría 2012, Tavares 1985). From the Coleoptera, representatives of Carabidae, Staphylinidae, Gyrinidae, Chrysomelidae, Elateridae, and Corylophidae are most commonly reported as hosts (De Kesel 1997). The distribution of Laboulbeniales depends from the distribution of the host, although the presence of the host does not guarantee the presence of the parasite (Huldén 1983). Laboulbeniales only occur on adult hosts. Some studies by Benjamin (1971) and Richards & Smith (1955) observed Laboulbeniales in immature stages, but the infection is less abundant and disappears completely with the instars moult.

1.3.2 Transmission

Laboulbeniales spend their entire life cycle on one host. When an ascospore becomes attaches to the outside of the host's integument, the development is fixed on the place of attachment. The immobility and adherence of the ascospores of Laboulbeniales causes them to be exclusively spread by the activities of the host and cannot be spread through the air over long distances (Huldén 1983). Theoretically the transmission of spores is achieved by direct contact (cross- and auto-infection) between hosts or indirectly by the intermediate of spores left on the soil or substrate (Scheloske 1969). Experimental studies from Benjamin & Shanor (1952) De Kesel (1993, 1995, 1996) and Richards & Smith (1955) showed that transmission of Laboulbeniales can take place by contact between at least one infected host.

Experimental data confirmed that direct transmission is the most important way of spore transmission. Transmission was highly promoted by increasing host population density and copulations involving infected hosts (De Kesel 1996). De Kesel (1995) and Scheloske (1976) found that mating was most important for transmission, and that it generates significantly

different infection patterns between male and female hosts. Benjamin (1971), Haelewaters *et al.* (2015), Huldén (1983) and Scheloske (1976) illustrated that auto-infection, caused by grooming, can also be an important way of transmission.

1.3.3 Host and ecological specificity

Most species are associated with a specific host species (or related). Host and parasite phylogenies often align as a result of co-divergence but explaining how morphologically similar Laboulbeniales can occur on multiple phylogenetically unrelated hosts is challenging (De Kesel & Haelewaters 2014). As mentioned before, the transmission of spores is most effective by mating. This leads to isolation of genes when having multiple different unrelated hosts. It was experimentally shown that host specificity is driven by several factors: the characteristics of the integument and living conditions of the host, as well as the nature and availability of nutrients in the habitat chosen by the host (De Kesel 1997). This implies that the influence of the environment on the success of Laboulbeniales is entirely depended on the habitat choices made by the host.

De Kesel (1996) found for *Laboulbenia slackensis* Cépède & F. Picard that population growth was significantly affected by temperature, relative humidity, salinity, soil type, and the mineral richness of the soil moisture. The results indicated that *L. slackensis* depends from the environmental conditions provided by its halobiont carabid host *Pogonus chalceus* Marsham, 1802. It was also shown that under these conditions the parasite is able to develop on other Carabidae, i.e. on hosts from outside its natural host range. Under natural conditions, *Laboulbenia* species adapt to the specific preferences and habitat choices of their host species and incapable of developing successfully on another host species (De Kesel 1996) unless it occupies the same or a suitable environment. In this context, De Kesel & Haelewaters (2014) suggested that the existence of sibling species in *Laboulbenia* (*L. slackensis* and *L. littoralis* De Kesel & Haelew.) on unrelated hosts (Carabidae and Staphylinidae) is the result of a switch between hosts (*Pogonus chalceus* & *Cafius xantholoma* Gravenhorst, 1806) that have the same or similar habitat choice (beaches and saltmarshes). The fungus *Rickia wasmannii* Cavara 1899 is capable of infecting alternative, unrelated host species as they co-occur in the *Myrmica* Latreille, 1804 nest "microhabitat" (Pfliegler *et al*. 2016). Also the fungus *Stichomyces conosomatis* Thaxt. has only been previously found in Staphylinidae beetles of the genus *Sepedophilus* Gistel, 1856. This Laboulbeniales species on a new host, *Speonemadus algarvensis*

of the family Leiodidae is the first case of host shifting following an ecological opportunity in the subterranean environment (Reboleira *et al.* 2017). These observations provide direct evidence for ecological specificity in Laboulbeniales.

Ecological specificity means that a host is essential, but not sufficient for the successful development of Laboulbeniales (De Kesel 1996). This is supported by the fact that controlled changes of environmental conditions, significantly affect the fungal population dynamics on the natural host. Moreover, under suitable environmental conditions, successful development of Laboulbeniales took place on hosts outside the natural host range, indicating that host specificity is fairly independent from host physiology or defence mechanism (De Kesel 1996). Haelewaters *et al.* (2018) found evidence for distinct clades within *Hesperomyces virescens,* each clade corresponding to separate coccinellid genera. These lineages represent separate species, driven by adaptation to different ladybird host genera. Species of the genus *Hesperomyces* Thaxt. develop a haustorium that penetrates the integument of the host, which makes them more prone to likely to be affected by the physiology and immune system of the beetle (Haelewaters & De Kesel 2017).

1.3.4 Island biogeography theory

De Kesel (1996) states that host populations of Laboulbeniales are probably similar to islands in the island biogeography theory of MacArthur & Wilson (1967). The populations on islands/hosts are isolated and therefore also their genes. Isolation/divergence of genes leads to speciation, but this subject is poorly explored worldwide in the *Laboulbenia*. Haelewaters & De Kesel (2017) have discovered worldwide that different species of *Hesperomyces virescens* (Laboulbeniales) have different host. In contrast, Haelewaters *et al*. (2015) found evidence that *Rickia wasmannii* (originated from 4 different countries) is indeed a single phylogenetic species, i.e. Myrmica host populations of different species are co-occuring which causes intermittent gene flow of *R. wasmannii.*

1.4 Species identification and concepts

Species identification in *Laboulbenia* is often problematic. The first reason is the inadequacy of currently available taxonomic keys. Although many of the monographs are monumental works, the taxonomic keys are often not good enough to unambiguously identify specimens to species level. The second reason is the lack of objective criteria for defining an individual specimen as the standard reference holotype for a species. Holotypes have traditionally been designated by taxonomists without an objective argument and sometimes without examination of a sufficient number of specimens to take into account inter-individual variability in different characters. In the absence of other distinguishing characters in the keys, it is essentially impossible to assign such specimens to a given species (Balakrishnan, 2005).

The process of assigning individuals to a given species obviously depends on the criteria by which species are defined and delimited, which are in turn determined by the used concept of what a species is. Different species concepts exist, each with different criteria, resulting in non-universal acceptance (de Queiroz, 1998 & 2007). In addition, not all concepts can be used methodologically. Many species concepts are criticised for several reasons, including its lack of universality, lack of a temporal dimension and the difficulty of applying it to allo/para/sympatric populations. Major problems are the practical impossibility of ascertaining reproductive isolation between large numbers of populations in the wild. Other concepts are criticised due to the high cost and intensive work needed for molecular research. For more information about the variety of concepts see Balakrishnan, 2005 or/and de Queiroz, 2005.

1.5 Estimating diversity

In the following sections we will illustrate the quandaries of estimating real diversity. Further on, the quandaries of different approaches to estimate the diversity through morphology, barcoding or host identification. Finally we will highlight the challenge of DNA based identification of Laboulbeniales.

1.5.1 Underestimating diversity

Traditionally, species descriptions have focused on morphological features, but this approach may underestimate true diversity. Having a broad original description (protologue), i.e. including more morphological variation than the species actually has, can lead to underestimating diversity. Also *Laboulbenia* can infect multiple genera of beetles which increase their distribution and leads to speciation. Laboulbeniales are unlikely to be affected by the phylogeny of their host because most Laboulbeniales do not penetrate the integument except for the genus *Hesperomyces* (Haelewaters & De Kesel 2017). Haelewaters *et al.* (2018) found evidence for distinct clades within *Hesperomyces virescens* using multiple independent methods, each clade corresponding to isolate a diverse *Hesperomyces* from a single host species. These lineages represent separate species, driven by adaptation to different ladybird hosts.

When the species are already variable, inadequate or subjective; acceptance of a new species is hard when it falls within the range of the species description of another species. For example, morphological plasticity of *Laboulbenia flagellata* Peyr. from different carabid hosts (Coleoptera, Carabidae) was studied by De Kesel & Van den Neucker (2005). Their study revealed that environmental factors affect the presence/absence of *L. flagellata* and that the average size of thalli from elytra is significantly affected by the identity of the host. They suggested that populations of *L. flagellata,* found on *Loricera pilicornis* Fabricius, 1775 could be different from those on *Agonum* sp., but refrained from formally separating it from *L. flagellata* in the absence of environmental data and molecular support. Molecular analysis of *Laboulbenia* was not available at that time, but testing the environmental requirements of *L. flagellata* from carabidae, including *L. pilicornis*, was possible. As previously mentioned, ecological specificity is partly explained and determined by the habitat choice of the host. Whether *Laboulbenia flagellata* from *Agonum* sp. differs from the ones associated with *Loricera*, is still undecided because we don't know if and how much host and environmental factors have segregated them.

Formerly, the species *L. coneglianensis* Speg. found on *Harpalus* Latreille, 1802 was not accepted by Balazuc (1974) and Majewski (1994). It was considered a synonym of *L. flagellata.* This implies that *L. flagellata* would have had even a wider host distribution range. Variability in morphology and host range gives indication that there are perhaps cryptic species. Eventually after establishing distinct characters (Terada 1998, Santamaría 1998), Majewski (2003) recognized that *L. coneglianensis* was different from *L. flagellata*

1.5.2 Overestimating diversity

1.5.2.1 Position- and sex related host morphology

Peyritsch (1875) and later Thaxter (1896) were the first to describe that Laboulbeniales can exhibit extreme position specificity. Both suggested that specificity to body parts was associated with mating behaviour of the hosts. Mating explains most gender-related position specificity found in the Laboulbeniales, but not all. Other behaviour patterns resulting in contact transfer must occur. In the past these patterns have been difficult to explain using the combination of position and morphological features of thalli (Goldmann & Weir 2012, Thaxter 1896). Several different Laboulbeniales have the potential to infect the same beetle at the same time. It is not always obvious whether this species is indeed new or is morphologically variable due to the growing place, pairings, history, etc. The theory of 'position specificity' as proposed by Benjamin & Shanor (1952) states that each morphotype displaying position specificity is a distinct species. In contrast, taxa that occupy specific positions on the host are not necessarily individual species; instead, they could represent morphotypes of the same biological species (Scheloske 1976). Scheloske (1969) pointed out that morphological characteristics can be related to the position of growth. Such morphological variations exist in *L. flagellata, L. giardii* Cépède & F. Picard*, L. pedicellata* Thaxt.*, L. polyphaga* Thaxt., and *L. vulgaris* Peyr. (Santamaría *et al.* 1991).

Observing multiple Laboulbeniales in a distinct place on the beetle, may lead to accept the position specificity theory. Gender-related distribution and position-related distribution illustrated by Benjamin & Shanor (1952) may cause to overestimate the diversity. All parts of the insects are potential spots for infections; however certain species exhibit a remarkable preference with regard to their point of attachment. The 'position specificity' is more unlikely and observations are possibly inadequate, yet the importance of molecular research in this

matter has proven to be of great value by Goldmann *et al.* (2013). In their research they showed that *Hesperomyces coleomegillae* W. Rossi & A. Weir and *H. palustris* W. Rossi & A. Weir might have been described as four different species, because the two species have two alternate morphologies (dimorphic). Goldmann *et al.* (2013) used the ITS marker to reveal that these are indeed two species, each with two position-specific morphotypes.

Another example is that 13 species of *Chitonomyces* Peyr. were reported to exhibit position specificity on *Laccophilus maculosus* Say, 1823. Goldmann & Weir (2012) used molecular analysis, ecological data, and video footage of the mating behaviors of *Laccophilus* Leach, 1815 and confirmed that sexual transmission is the mechanism to speciation. They placed the 13 species into pairs of morphotypes, resulting in synonymies and recognition of six phylogenetic species (one species is a triplet). Each phylogenetic species was located at corresponding positions on male and female beetles that make contact during mating.

As shown before position dependent morphology can occur and is easily explained with gender related behaviour (mating and male on male mounting). But position dependent morphology easily leads to assume speciation instead of intraspecific variation. The importance of molecular and ecological research is therefore still necessary to clarify these issues.

1.5.3 Morphological identification versus barcoding

DNA barcoding is highly accurate for species identification if used correctly (Schoch *et al.* 2012). Yet it may be too expensive to be used in biodiversity studies, where the number of specimens to be identified can be very large. Further, field identification and non-invasive sampling, two increasingly important requirements in biodiversity studies, are not always compatible with molecular methods. Identification keys using non-molecular data will therefore remain crucial to the process of species identification, particularly in the tropics. However, further detailed studies with barcoding will be required to clarify some diversity issues regarding species concepts and degree of specificity as mentioned above.

1.5.4 Host identity based identifications

Hosts give an easy prediction of which *Laboulbenia* could be infecting them, but this is not always reliable. For example: *Laboulbenia calathi* Majewski and *L. polyphaga* are both associated with *Calathus melanocephalus* Linnaeus, 1758. These are quite similar and might

represent the same species with interspecific variability (Majewski 1994, Weir & Beakes 1993). Two different species on the same host, each consisting of two quite different morphotypes can co-exist (Goldmann *et al.* 2013). On species in the genus *Philonthus* Sharp, 1874, the most common species is *L. philonthi* Thaxt. but other species have also been described on *Philonthus*: *L. dubia* Thaxt. and *L. cafii* Thaxt. (Majewski 1994, Santamaría 1998). Within these species their morphology is noticeably different.

For hosts with multiple infections a host-based identification key would be useless and leads to confusion, for example *L. cafii* and *L. littoralis* are both occurring on *Cafius xantholoma* (De Kesel & Haelewaters 2014). Spontaneous new infections or unknown parasite-host combinations can occur, which would not be recognised when using a purely host-based identification key.

Further, a host-based identification key would require intensive knowledge about the class Insecta. Over the years, the host species are better phylogenetically organised, which causes changes in names. For example: *Agonum assimile* Motschulsky, 1865 was recombined in *Limodromus assimilis* Paykull, 1790. Majewski (1994) used *Bembidion ustulatum* Duftschmid, whereas the recent name for this host is now *B. tetracolum* Say, 1823. Yet, host identification can be useful when it is used in combination with other significant features. But identification of the host is still important to make speciation assumptions and to determine the distribution of the *Laboulbenia*.

1.5.5 The challenge of DNA based identification

The first successful extraction protocols were published by Weir & Blackwell (2001b). This protocol was time-consuming, showed a low success rate (25%) and required substantial amounts of thalli. Their minute size, the difficulty in fractioning thalli to release DNA, and the impossibility to obtain cultures, makes it difficult to develop molecular extraction protocols for *Laboulbenia* (Haelewaters & Yaakop 2015). Research by (Haelewaters *et al.* 2018) used the REPLI-g Single Cell Kit, including a whole-genomic amplification step as part of the DNA extraction, prior to targeted PCR. (Sundberg *et al.* 2018) also suggested a whole-genome amplification to increase the success rate. So far it is the most efficient protocol when only few thalli are available. Yet this protocol is extremely sensitive to contamination when low quantities of target DNA is available. Low amplification success for genes of *Laboulbenia* is

possible because of using general fungal-specific primers (e.g., Walker *et al.* 2018). To construct primers specific to *Laboulbenia* for the different marker is a difficult task (Haelewaters *et al.* (2015) because of the substantial genetic variation in the group, especially in the ITS region. Molecular studies of *Laboulbenia* are still challenging and successful extractions of DNA have been one of the greatest obstacles.

1.6 Objectives

We have reviewed in the previous paragraphs that specificity and diversity of Laboulbeniales are poorly studied and related to a complex of factors. Variability in morphology, wide distributional ranges, and a diversity of hosts indicate that there are perhaps cryptic species. Can molecular techniques help us to detect cryptic or near-cryptic taxa in *Laboulbenia* species infesting Carabidae from different genera? This may be the case in *Laboulbenia pedicellata*, which infects carabid beetles in the genera *Bembidion, Pogonus*, *Patrobus, Pterostichus* and *Dyschirius*. Also for *L. vulgaris*, which infects the genus *Bembidion* Latreille, 1802*; Trechus* Clairville, 1806 and *Ocys* Stephens, 1828; as well for *L. pseudomasei* Thaxt., which infects the genus *Loricera*, *Patrobus* and *Pterostichus* and finally *L. flagellata*, which infects the genera *Agonum; Limodromus* Motschulsky 1850; *Oxypselaphus Chaudoir, 1843; Pterostichus* Stephens, 1827 and *Loricera* Latreille, 1802 (De Kesel 1997; De Kesel & De Kesel 2006; De Kesel & Rammeloo 1991; Majewski 1994, Santamaría 1998).

Detailed studies with barcoding will be required to clarify some diversity issues regarding species concepts and degree of specificity. Though the solution is simple, molecular studies of Laboulbeniales are still challenging. We aim to make a phylogenetic tree on the basis of the internal transcribed spacer (ITS) and large subunit (LSU) regions of the ribosomal DNA. Our goal is to sequence as many different species of *Laboulbenia*, from multiple hosts and localities, to support our phylogenetic tree. It would be interesting to discover if the phylogeny of *Laboulbenia* follows the classical subgeneric grouping proposed by Tavares (1985). In this case the morphological features pointed out by Tavares give support to a morphological/phenetic species concept.

Our hypothesis is that there are indeed hidden cryptic species present and that those cryptic species correlate with the phylogeny of the beetle due to sympatric speciation. We expect this for large morphological plasticity species like: *Laboulbenia flagellata, L. vulgaris, L. pedicellata* and *L. pseudomasei*. Precisely, we expect that *Laboulbenia flagellata* on *Agonum micans* Nicolai, 1822 and *Limodromus assimilis* (syn. *Agonum assimilis*) shall differ on species level from *Laboulbenia flagellata* on the other beetle species, as already suggested by De Kesel & Van den Neucker (2005). Further, we expect that *L. vulgaris* on *Trechus* shall be different from *Bembidion* and *Ocys*. Both *Bembidion* and *Ocys* are members of the subtribe Bembidiina (tribe

Bembidiini) while Trechus is a member of the tribe Trechiini. Based on the assumption of existing cryptic species, we expect that the classification of Tavares is not supported.

2 Material & Methods

2.1 Collecting samples and photography

Insects were collected in the Scheldeschorren (Bornem, Hingene) and at the Meise Botanic Garden. Besides fresh material, preserved material from other localities in Belgium, France, The Netherlands, Latvia, DR Congo and Benin collected by Dr. A. De Kesel (stored at Meise Botanic Garden, Belgium) were used. At the Bornem site, the insects were mostly found under bark of rotten logs. While removing the loose bark, the insects were collected in a wide container placed under the logs. At Meise Botanic Garden insects were collected with pitfall traps placed in an Alder forest and in a rivulet associated meadow.

Insects were transferred into a container with >98% ethanol. Potential carabid hosts were sorted and identified using Muilwijk *et al.* (2015). Screening for infection and removal of thalli was done at 50x magnification with an Olympus SZ61 stereo microscope. Infected insects were given a unique herbarium number. The non-infected insects were stored at Meise Botanic Garden, Belgium.

All insect were cleaned by sonicating (https://www.sigmaaldrich.com/labware/labware-products) the insects in a 2ml plastic tube filled with distilled water. This step was done to give minimal debris that is possible attached to the appendage of the thalli. Removal of thalli was done by placing the infected insect in a petri dish filled with distilled water. The insects was stabilised by a fine tweezers and a small needle (minuten 0) was used to dislodge the basal cell of the thallus from the integument. Free thalli were picked up with the same needle and transferred to a microscope slide with a small droplet of water. The transferred thalli were covered with a cover slip and photographed using an Olympus BX51 light microscope with digital camera and AnalySIS 5 imaging software (Soft Imaging System GmbH). Measurements of *Laboulbenia* are performed by FIJI ImageJ program (Schindelin *et al.,* 2012). Identifications of thalli were done using the keys in De Kesel 1998, Majewski 1994, Santamaría 1998 and Tavares 1985.

Quickly after photography, coverslips were gently removed from the slides. Thalli were then cut in half using a sterile scalpel. Since the *Laboulbenia* cell walls do not disintegrate in chemical lysis, we used this step to ensure cell walls are physically broken. The cut pieces of thalli were then placed in a PCR tube with 2 µl phosphate buffered saline (PBS) from the REPLI-g

Single Cell Kit (QIAGEN, St. Louis, MO). To facilitate manipulation and reduce the influence of electrostatic forces on the tiny pieces of thallus, the latter were transferred with a dissection needle previously dipped in a sterile 1:1 glycerine:water solution.

An alternative way to avoid extra steps was to photograph the *Laboulbenia* of a permanent slide and use another *Laboulbenia* from the same beetle for the extraction. In this way laboulbenia(s) are less prone to contamination because directly isolated for extraction.

2.2 Extraction and whole genome amplification

Extraction and whole genome amplification from single cells was performed with REPLI-g Single Cell Kit (QIAGEN, St. Louis, MO), following Haelewaters *et al.* (2019). The REPLI-g Single Cell Kit provides highly uniform amplification across the entire genome, with negligible sequence bias (Hosono *et al.*, 2003). The method is based on Multiple Displacement Amplification (MDA) technology, which carries out isothermal genome amplification utilizing a uniquely processive DNA polymerase capable of replicating up to 100 kb without dissociating from the genomic DNA template (Dean *et al.*, 2002).

In the first step of the procedure, the cell sample was lysed by adding 1,5 lysis buffer (D2). D2 consist out of 3µl of 1M DTT (Dithiothreitol) and 33 µl reconstituted DLB (direct lysis buffer, contains potassium hydroxide), this provides 24 reactions. The DNA was denatured by incubating at 65° for approximately 30 minutes in the thermocycler. For all amplifications and incubation a T100™ Thermal Cycler (Bio-Rad, Temse and California) was used. After denaturation has been stopped by the addition of neutralization buffer, a master mix containing buffer and DNA polymerase is added. The master mix consisted of 4,5 µl water, 14,5 µl REPLI-g Reaction Buffer and 1 µl REPLI-g DNA polymerase for each sample. The isothermal amplification reaction ran for 8 hours at 30°C and 3 min for 65°C to inactivate the DNA polymerase.

2.3 PCR amplification

We used ribosomal markers due to higher PCR amplification and sequencing success compared to protein-coding gene regions (Conrad *et al.*, 2012). The internal transcribed spacer (ITS) region has the highest probability of successful identification with clearly defined barcode gap between inter- and intraspecific variation. The nuclear ribosomal large subunit (LSU) is a marker with resolution on species-level in some taxonomic groups. We used these two markers

because they have been found to be useful as a barcode in previous publications (Goldmann *et al.* 2013, Haelewaters *et al.* 2018a, 2019; Sundberg 2018; Walker *et al.* 2018).

Goldmann & Weir 2012; etc.). In some of these publications, also the nuclear ribosomal small subunit (SSU) was used, but in comparison to the other markers it has poor species-level resolution in fungi (Conrad *et al.* 2012, Goldmann *et al.* 2013, Goldmann & Weir 2018). We choose not to work with SSU due inferior resolution and limited time.

2.3.1 LSU amplification

Amplification was performed of the ribosomal large subunit (LSU) according to Fraiture *et al.* (2019). Primers used for the large subunit were LIC15R (20 µM forward primer) and LR6 (20 µM reversed primer). In each PCR tube 20 µl was added of the Master Mix. The mix for 24 reactions contained 297 µl water, 60 µl buffer, 48 µl BSA, 48 µl dNTPs, 12 µl forward primer, 12 µl reverse primer and 3 µl TAQ. Then, 5 µl of diluted sample (1 on 20) was added in each tube. The PCR program ran a pre-denaturation at 95 °C for 3:00 min; followed by 26 cycles with denaturation at 95°C for 45 sec, followed by 40 sec annealing at 52°C and elongation at 72°C for 2:30 min; then 15 cycles of identical condition, except for the elongation +5 sec/cycle; and a final extension at 72°C for 10 min.

2.3.2 ITS Amplification

Gene amplification was performed of the internal transcribed spacer (ITS) according to Fraiture *et al.* (2019). Primers used for the internal transcribed spacer were ITS1F (20 µM forward primer) and ITS4 (20 µM reversed primer). In each PCR tube, 20 µl was added of the Master Mix. The mix for 24 reactions contain 297 µl water, 60 µl buffer, 48 µl BSA, 48 µl dNTPs, 12 µl forward primer, 12 µl reverse primer and 3 µl TAQ. Then, 5 µl of diluted sample (1:20) was added in each tube. The PCR program contained an initial denaturation step at 94 °C for 3:00 min; followed by 30 cycles of denaturation at 94 °C for 90 sec, annealing at 55 °C for 90 sec, and elongation at 72 °C for 2:00 min; and a final extension at 72 °C for 7:00 min.

2.4 Quality check, purification and sequencing

The *Fragment Analyzer* by Agilent formerly Advanced Analytical (AATI) quantified and qualified our results. Successful results were purified; unsuccessful results were redone if needed, by changing the concentration or using new uncontaminated water. PCR products were purified by adding 0.5 U of Exonuclease I and 0,5 µl shrimp Alkaline Phosphatase (SAP) -buffer and 1U SAP (Thermo Scientific, St. Leon-Rot, Germany) and incubating at 37°C for 1 h, followed by inactivation at 80°C for 15 min. Sequencing was performed by Macrogen Inc., Korea, with the primers LIC15R, LR3R, LR3, LR6, NS7, ITS1f, and ITS4. Newly produced sequences were checked and modified using Geneious Pro v. 6.0.6 (Biomatters, https://www.geneious.com).

2.5 Phylogenetic analyses

Fifty sequences of *Laboulbenia* species were used for this study (Table 1A). Twenty-eight (including the outgroup) sequences were newly generated. Other sequences are originated from NCBI GenBank or sent by Dr. Haelewaters. Initial BLAST searches (http://blast.ncbi.nlm.nih.gov) of both LSU and ITS sequences were performed to estimate similarity with *Laboulbenia* sequences already in Genbank database. A combined dataset of LSU and ITS sequences was constructed with PhyDE2 0.2.0-90 alpha (http://bioinfweb.info/PhyDE2) to use in the phylogenetic analyses.

We were not able to generate ITS sequences for all species (Table 1A). Sequences were automatically aligned using the MUSCLE algorithm (Edgar, 2004) with default settings. The alignment was further optimized and manually adjusted as necessary by direct examination with the software Se-Al v. 2.0a11 (University of Oxford). Ambiguously aligned segments were detected by Gblocks v0.91b (Castresana, 2000; Talavera & Castresana, 2007), with the following parameter settings: minimum number of sequences for a conserved position = 24 (minimum possible); minimum number of sequences for a flank position = 24 (minimum possible); maximum number of contiguous non-conserved positions = 4 bp, minimum block size = 4 bp, and gaps allowed within selected blocks in half of the sequences.

Models of evolution for Bayesian Inference (BI) were estimated using the Akaike information criterion (AIC) as implemented in Modeltest 3.7 (Posada and Crandall, 1998). Phylogenetic analyses were performed separately for each individual and loci using (BI) as implemented in MrBayes v3. 2 (Ronquist *et al.,* 2012) and Maximum likelihood (ML) as

implemented in RAxML 7.2.7 (Stamatakis *et al.,* 2008). The best-fit models for each partition were implemented as partition specific models within partitioned mixed-model analyses of the combined dataset.

Bayesian analysis was performed with two independent runs, each with four independently chains for 10,000,000 generations, starting from random trees, and sampling trees every 1000 generations. All constructed trees were converted into a 50% majority-rule consensus tree (BC) and used to calculate Bayesian posterior probabilities (BPP). BPP of each node was estimated based on the frequency at which the node was resolved among the sampled trees with the consensus option of 50% majority-rule (Simmons *et al.,* 2004). A probability of 0,90 was considered significant.

Maximum likelihood (ML) searches conducted with RAxML involved 1000 replicates under the GTR+I+G model. In addition, 1000 bootstrap (ML BS) replicates were run with the same model. Clades with Maximum likelihood bootstrap values of 75% or greater were considered supported. Sequence data and statistical analysis are illustrated in Table 2A.

3 Results

Thirty-nine species of Laboulbeniales were identified: *Chitonomyces aethiopicus, Haplomyces teanus, Hesperomyces coccinelloides* Thaxt., *H. virescens, Laboulbenia argutoris* Cépède & F. Picard, *L. benjaminii* Balazuc, *L. bertiae* Balazuc, *L. blanchardii* Cépède, *L. bruchii* (Speg.) Thaxt., *L. calathi, L. clivinalis* Thaxt., *L. collae* Majewski, *L. coneglianensis, L. cristata* Thaxt., *L. dactyliophorus* Thaxt., *L. elaphricola* Siemaszko, *L. elaphri* Speg., *L. fasciculata* Peyr., *L. flagellata, L. hyalopoda, L. littoralis, L. longicollis* Thaxt., *L. luxurians* Peyr., *L. notiophili* Cépède & F. Picard, *L. ophoni* Thaxt., *L. palmella* Thaxt., *L. partita* Thaxt., *L. pasqueti* F. Picard, *L. pedicellata,, L. philonthi, L. proliferans* Thaxt., *L. pseudomasei, L. slackensis, L. thaxteri* Cépède & F. Picard, *L. vulgaris, Rickia wasmanii* and *R. laboulbenioides* De Kesel. We managed to obtain sequences of 11 species: *H. virescens, L. bruchii, L. collae, L. coneglianensis, L. clivinalis, L. fasciculata, L. flagellata, L. littoralis, L. pedicellata, L. proliferans, L. pseudomasei, L. notiophili, L. vulgaris, Rickia laboulbenioides* and *R. wasmannii*. Beside our own data, 9 different species sequences on GenBank and personal send sequences from Haelewaters were used: *L. bruchii, L. collae, L. coneglianensis, L. diopsidis* Thaxt., *L. flagellata, L. oioveliicola* nom. prov., *L. pedicellata, L. stilicicola* Speg. and *L. systenae* Speg.

Our phylogenetic inferences (fig 2) recovered at least three phylogenetic species (PS), previously identified as *L. flagellata* by the morphological examination. The first PS has *Limodromus assimilis* as host. The second PS has *Agonum nigrum* Dejean, 1828 as host. The last PS has *Agonum emarginatum* Gyllenhal, 1827; *A. micans, Loricera pilicornis*, and *Oxypselaphus obscurus* Herbst, 1784 as host. These three phylogenetic species are placed in a sister position to *L. pseudomasei* (grey dot on fig 2). *Laboulbenia littoralis* clusters in a sister position to *L. clivinalis* in a well-supported clade. So is *L. stilicicola* in a sister position to *L. notiophili*.

In our phylogenetic tree (fig 2) all morphological defined species of *L. vulgaris* cluster together as one OTU. However, our *L.* sp. nov. was originally identified as *L. vulgaris*, but after contemplation molecular and morphological, it was considered to be an undescribed species. *Laboulbenia* on Chrysomelidae do cluster together with the exception of *L. systenae*. However the position of *L. systenae* is not supported in our tree. Other not well supported species are *L. diopsidis* and *L. fasciculata*. All other species identified have strong molecular support, i.e. a total of 16 OTUs. However the lineages between them are poorly supported. The genus *Laboulbenia* as currently circumscribed is well supported by our analyses

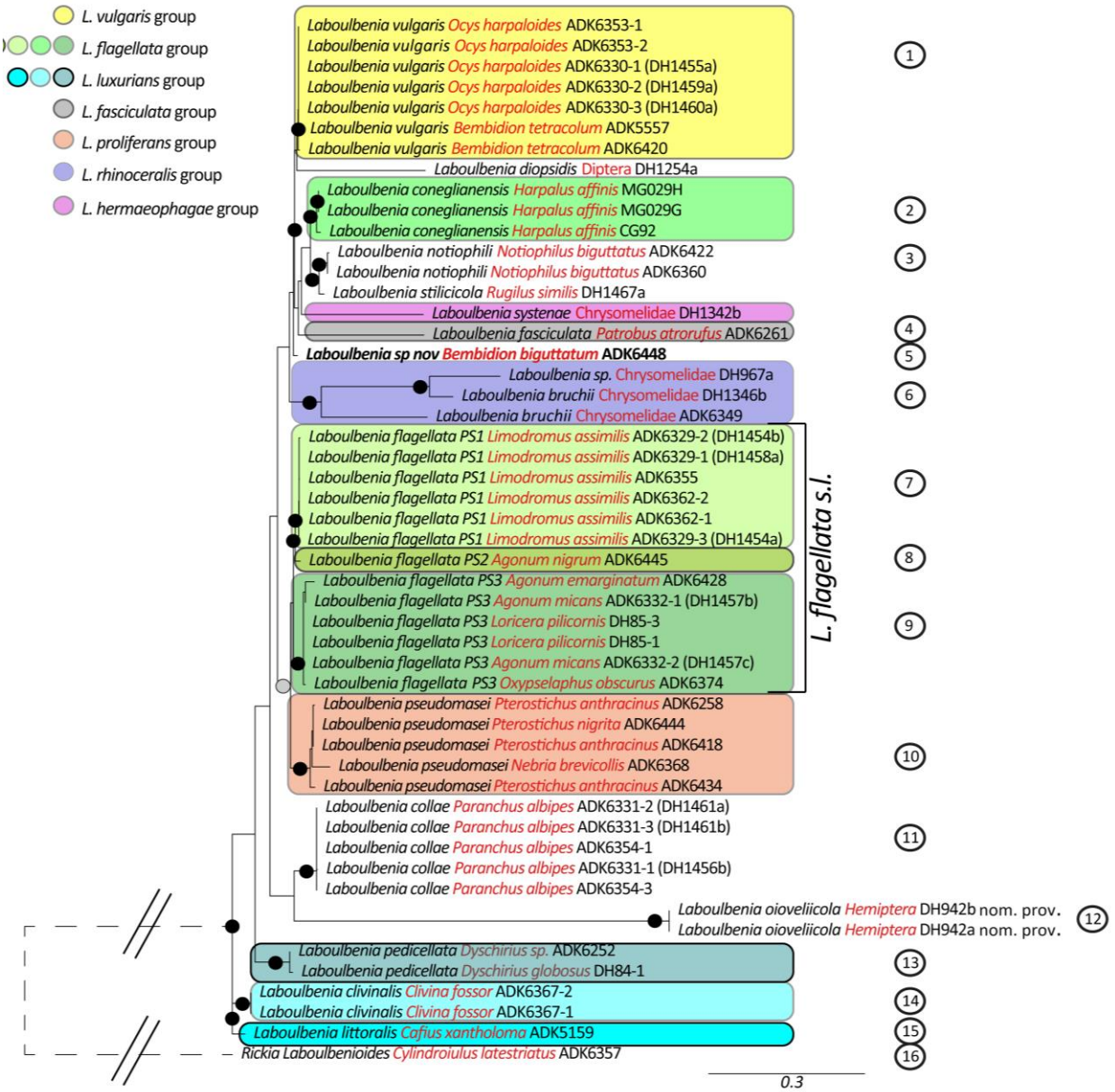

Figure 2: Maximum likelihood tree from combined ITS-LSU datasets. Species name is represented in black followed by the host in red and the collector number in black. Black dots on branches represent BPP and ML BS of 99% or higher; grey dots on branches denote BPP greater than 85% and ML BS greater than 75%. Numbers on the right refer to illustrated species, used in our experiment, on plate I.

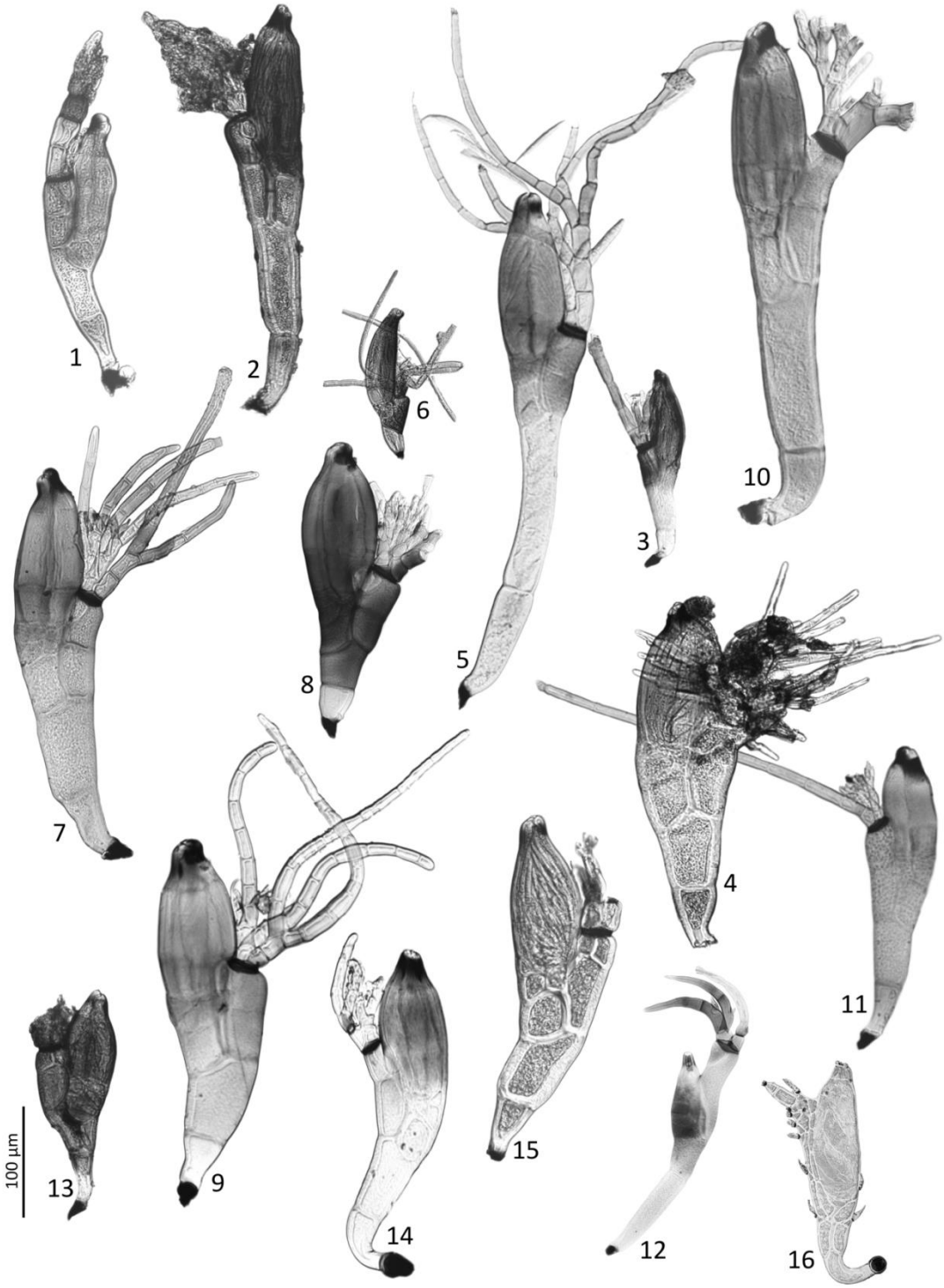

Plate I: Thalli of the genus *Laboulbenia* representing the several species of our study. 1) *L. vulgaris* ADK5557, 2) *L. coneglianensis* CG92, 3) *L. notiophili* ADK6360, 4) *L.* fasciculata ADK6261, 5) *L.* sp. nov. ADK6448, 6) *L. bruchii*, ADK6349 7) *L. flagellata* PS1 ADK6352, 8) *L. flagellata* PS2 ADK6445, 9*) L. flagellata* PS3 ADK 6428, 10) *L. pseudomasei* ADK6418, 11) *L. collae* ADK6354, 12) *L. oioveliicola* nom. prov. DH942a, 13) *L. pedicellata* ADK6252, 14) *L. clivinalis* ADK6367, 15) *L. littoralis* ADK5159, and the outgroup 16) *Rickia laboulbenioides* ADK5533 (not the same as the one used in this study).

4 Discussion

Our phylogenetic work suggests that *L. flagellata* is not a single phylogenetic species. Isolates removed from different host species form three separate clades. To be able to find the true *L. flagellata, L. flagellata* sensu stricto, thalli should be collected of *Paranchus albipes* Fabricius, 1796 (formely named *Anchomenus albipes*), *Bembidion lunatum* Geoffroy, 1785, or *Agonum emarginatum*, based on which the species was described (Peyritsch 1873). However on *Bembidion lunatum* he already found extremities and the species described on *Agonum marginatum* was young (Peyritsch 1873). Therefore to distinguish *L. flagellata* from the other phylogenetic species, sequences of *L. flagellata* preferably on *Paranchus albipes* are required.

Limodromus assimilis used to be named *Agonum assimilis*, the placement of the beetle into another genus reflects that *L. flagellata* is indeed further related than thought due host specificity on genus level. Moreover, our *L. flagellata* isolates were taken from *Agonum micans* and *Limodromus assimilis*, both collected from under bark of the same log. This result is still unexpected, because under controlled conditions and in confined areas, carabidicolous *Laboulbenia* are known to easily transmit and successfully develop on several host species (De Kesel 1996). Since both host species share the same micro-habitat, our preliminary molecular results point towards sympatry. This suggests that there is little to no spore transmission between the *Laboulbenia* populations from *L. assimilis* and *A. micans.*

A fourth, morphologically unrelated, clade is placed between these phylogenetic species of the *L. flagellata* species complex in the preliminary analysis. Given that *L. flagellata* on *Limodromus assimilis* is phylogenetically more related to *L. pseudomasei* than to the *L. flagellata* on *Agonum, Loricera*, and *Oxypselaphus*. In our final results, this fourth clade that represents the species *L. pseudomasei* is located outside the clade but this support is only supported for >85% BPP and >75% ML BS. More species of *Laboulbenia* should be included to confirm the position of *L. pseudomasei*

In addition to molecular phylogenetic work, more research needs to be done under controlled conditions, i.e. with both these hosts kept in containers (with stacks of bark), it has been observed that they stay separate (De Kesel, unpubl. data). Separation under natural conditions is harder to demonstrate, but *L. assimilis* is significantly larger than *A. micans* and occupies areas with more space between bark and wood. In the field it also seems to select the

bigger and drier parts of the log. In contrast, *Agonum micans* is smaller and able to use much narrower spaces from the same logs. In the field it seems to prefer the damper zones of the logs, often closer to the ground. Considering both these carabids are predators, they probably also avoid contact. As stated earlier, more work is needed, although both hosts occupy the same habitat they have different micro-habitat preferences, which ultimately leads to reduced interspecific parasite transmission and isolation (*sensu* De Kesel 1996).

As suspected, sequences of *L. vulgaris* removed from *Bembidion* and *Ocys* do not differ. Both *Bembidion* and *Ocys* are members of the subtribe Bembidiina (tribe Bembidiini). However, we could not find any cryptic species of *L. vulgaris* due the inability to obtain a sequence from *L. vulgaris* from more divers hosts like *Trechus*, a member of the tribe Trechiini. *Laboulbenia vulgaris* is also common on *Bembidion*. The thalli from *Bembidion biguttatum* (ADK6448) are exceptional in the sense that they combine features from *L. vulgaris* (first identification) and *L. flagellata*. In the phylogenetic tree this material takes a separate position which is a strong argument to treat this material as potentially new. *Laboulbenia* sp. nov. (Plate I, thalli 5) is characterized by branched outer appendage, these outer appendage have pigmentation apically. The first and only septum of this species is also rather low in comparison with *L. flagellata* (Plate I, thalli 7, 8, and 9) and *L. vulgaris* (Plate I, thalli 1). We did not worked on a species description due the time availability. Further, *Laboulbenia pseudomasei* which infects Carabidae are all considered as the same species in our phylogeny. For *Laboulbenia pedicellata*, there were no cryptic species found as we suspected due to insufficient success in sequencing material.

Based on thallus morphology from *Laboulbenia* obtained from all over the world, Tavares (1985) distinguished about twenty morphological groups within *Laboulbenia*. Our analysis has not as many representatives, but several of Tavares' (1985) subgeneric groups also appear in our phylogeny. The first group, "*Laboulbenia vulgaris* group", includes species with a basal cell of the inner appendage that is much smaller than the outer appendage basal cell. The outer appendage is usually simple and unbranched. The inner appendage is usually small, i.e. not extending beyond the tip of the perithecium. Cell V is short and wedge-shaped while the thallus is often enlarged above the lower receptacle (Tavares 1985). Two representative species are found in this study: the *Laboulbenia vulgaris* and *Laboulbenia stilicicola.* Yet these species are not clustered together therefore do not support the "*Laboulbenia vulgaris* group" classification of Tavares.

The group "*Laboulbenia flagellata* group" includes all the *L. flagellata* on different beetles and *L. coneglianensis* on *Harpalus*. In this group the specimen have equal-sized basal cells of the inner and outer appendage. The appendages are highly branched and cell V is short and wedge-shaped. Although *L. coneglianensis* corresponds with this description, our material clusters in a different clade. Formerly, the species *L. coneglianensis* was considered it a synonym of *L. flagellata* (Balazuc 1974, Majewski 1994). Santamaría (1998) and Terada (1998) distinguishes *L. coneglianensis* from *L. flagellata* based on the former's dark perithecium with nearly straight anterior margin and almost free posterior margin, as well as a dark receptaculum with pale contrasting cells II, III, V and VI.. Also, *L. coneglianensis* grows exclusively on hosts from the subfamily Harpalinae (Terada 1998), which suggest specialization and speciation based on host differences. *Harpalus tardus* prefers sandy soils and shady, sometimes moist, habitats (Turin, 2000). There is little doubt that the definition of the "*Laboulbenia flagellata* group" is purely morphological, i.e. not reflecting parasite-host relationships.

The "*Laboulbenia hermaeophagae* group" has *Laboulbenia systenae* as representative species. This group is characterised by a short cell V that reaches cel III. The cell IV extends upwards and outwards. Also this group species have a small basal cell of the inner appendage. The separate position of this material supports Tavares (1985) classification however this clade is not enough supported and needs more diverse material from this morph group.

The next group presented in the tree, is the "*Laboulbenia fasciculata* group" from which the most typical representative, *Laboulbenia fasciculata,* is sequenced. The separate position of this material in the tree was expected. This small group of species is recognised by a linear series of cells replacing the solitary cell V seen in most of the other *Laboulbenia*. Moreover the appendages are typically made of a series of cells each with constricted dark septa in the lower part.

The "*Laboulbenia rhinoceralis* group" that grows on Chrysomelidae (Coleoptera) and from which we only have *L. bruchii* and *Laboulbenia sp*. in our phylogeny. This group is characterised by a short cell V that reaches cell III. Cell IV typically extends upwards and outwards. The basal cell of the inner appendage is small. Tavares (1985) stated that *L. bruchii* lacks the terminal perithecial spines in reference with the representative *L. rhinoceralis*. In spite of being obtained in Africa and Panama, the two representatives from Chrysomelidae form a separate clade. However, L. *sp.* is placed between these two species which may due the wide geographical

distribution and insufficient resolution. It may be early to conclude, but in contrast with the "*Laboulbenia flagellata* group", the "*Laboulbenia rhinoceralis* group" does reflect both morphology and host relationship.

The following group is the "*Laboulbenia proliferans* group", recognised by a secondary appendage arising above a series of cells formed by cell V, possibly with some black septa on the appendage. In this group only *L. pseudomasei* complies with Tavares' (1985) concept. However, in our phylogenetic analysis *L. pseudomasei* falls within the "*Laboulbenia flagellata* group". More material with proliferating cell V should be sequenced, but for now it looks like this feature is not important. In fact, both *L. pseudomasei* and *L. flagellata* have a very similarly constructed inner and outer appendage.

The last group is the "*Laboulbenia luxurians* group", which have a tall V cell that is equal in height to cell IV and short abundantly branched appendages. *Laboulbenia pedicellata*, *L. clivinalis* and *L. littoralis* belong to this group and their sequences also clustered paraphyletic together.

It is not clear in which morphological group(s) the following taxa belong: *L. diopsidis, L. notiophili, L.* sp. nov*., L. collae* and *L. oioveliicola* nom. prov. Thaxter (1896) pointed out that the structure of *L. diopsis* perithecium and receptacle resembles that of *L. subterranea* Thaxt., a species Tavares (1985) placed in the "*Laboulbenia vulgaris* group". By this the *Laboulbenia vulgaris* group (*L. vulgaris*, *L. cristata, L. subterranea L. diopsis*) seems populated with species that infect hosts from quite different orders (Coleoptera, Diptera). However the description for *L. diopsidis* matches the Tavares classification, except cell IV is not short and has the same size as cell V.

Goldmann & Weir (2018) made a phylogenetic analysis with only SSU rDNA sequences for Laboulbeniomycetes focusing on higher-level taxonomy. In their tree the genus Botryandromyces was placed within the clade *Laboulbenia*. This makes *Laboulbenia* in their analysis paraphyletic. However the locus that they used is not conserved enough to distinguish the different genera, besides that, they only used one marker. Haelewaters *et al.* (2019) used 3 different makers (SSU, ITS and LSU) and found maximum support in a monophyletic *Laboulbenia* clade in their phylogeny of Laboulbeniomycetes focusing on higher-level taxonomy. Despite being the most diverse genus within the order Laboulbeniales, Goldmann & Weir (2018) and

Haelewaters *et al.* (2019) used limited species to support the clade *Laboulbenia*. This genus has been known to be difficult to sequence and has never been elaborated until now.

To increase the robustness of our phylogenetic analysis more samples and species of *Laboulbenia* should be included. This requires locating, sampling, identifying, and screening a full range of specific host populations in the hope they are infected. Part of this work was done during this masters project, but further extending the number of species was unfortunately impossible given the time available. During this research we also needed to test and improve a number of protocols that never were tested on a larger number of *Laboulbenia* representatives. In fact, by the time we started this research, GenBank contained only few sequences of *Laboulbenia*.

Most effective DNA extraction of Laboulbeniales involves using freshly collected material preferably stored in ≥ 95 % ethanol. Collecting fresh material is intensive, time consuming and not always successful. It is possible to sequence old herbarium material that is preserved correctly and if conditions during the conservation are also favourable. Still the success rate is low and contamination is still a problem. Prominent appendages, such as those in many species of *Laboulbenia*, catch debris and are very hard to impossible to wash away. The REPLI-g Single Cell Kit is a very powerful way to obtain a lot of DNA from such small amounts. But in disadvantage, other unwanted material will also be sequenced in the whole genome amplification. This can be resolved with specific primers for a given taxonomic group in the gene/loci amplification step. However if the extraction kit was cheaper, more valuable samples could be sequenced at low costs. In spite of this, we managed to obtain ITS and LSU sequences of *Hesperomyces virescens*, *Rickia laboulbenioides* and 11 *Laboulbenia* species: *L. flagellata, L. vulgaris, L. collae, L. notiophilli, L. pseudomasei, L. clivinalis, L. littoralis, L. pedicellata, L. coneglianensis, L. fasciculata,* as well as several ones that are potentially new to science.

Some of these new taxa are morphologically difficult to separate from existing species. However, they are not truly cryptic taxa since careful analysis of their morphology does show subtle differences in pigmentation or branching of the appendages. Many such features were previously considered of little taxonomic value. Thanks to a good match between morphology, host identity and molecular data, we think these subtle morphological features can actually be used to separate and define several of these new taxa (Haelewaters *et al.*, 2018a).

5 Conclusions

Based on the studied material we found that *L. flagellata* is not a single phylogenetic species but represents at least three phylogenetic species. Each of these seems host-specific, i.e. associated to a host genus (*Agonum*) or to a single species. Formal description of these new taxa can only be done after we determine the sequence of *L. flagellata* sensu stricto. This species was described from thalli taken from *Paranchus albipes*, which we unfortunately failed to sequence. Thalli removed from *Bembidion biguttatum* (ADK6448) combine morphological features from *L. vulgaris* and *L. flagellata*, which may indicate a new species. The phylogenetic species and the thalli from *Bembidion biguttatum* are not truly cryptic taxa since careful analysis of their morphology does show subtle differences in pigmentation and branching of the appendages. Many features were previously considered of little taxonomic value. New morphological/morphometric features, correct host identity, and molecular phylogenetic data are necessary to separate and define several of these new taxa. Further we found that both host species of *L. flagellata* share the same micro-habitat, which leads towards sympatry.

Tavares (1985) distinguished about twenty morphological groups within *Laboulbenia*. Our analysis includes material from several of Tavares' (1985) subgeneric groups but did not entirely support her classification. *Laboulbenia* is known to be difficult to sequence and has never been elaborated until now. Still more samples and species of *Laboulbenia* should be included because this is the most diverse genus of the order Laboulbeniales, with over 650 accepted species.

6 References

Balakrishnan, R. (2005). Species Concepts, Species Boundaries and Species Identification: A View from the Tropics. *Systematic Biology,* **54**: 689-693.

Balazuc, J. (1974). Laboulbeniales de France (suite). *Publications de la Société Linnéenne de Lyon,* **43**: 295-315.

Benjamin, R.K. (1971). Introduction and Supplement to Roland Thaxter's Contribution Towards A Monograph of the Laboulbeniaceae. *Bibliotheca Mycologica,* **30**: 1-155.

Benjamin, R.K. and Shanor, L. (1952). Sex of host specificity and position specificity of certain species of Laboulbenia on Bembidion picipes. *American Journal of Botany,* **39**: 125-131.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution,* **17**: 540-552.

De Kesel, A. (1993). Relations between host population density and spore transmission of Laboulbenia slackensis (Ascomycetes, Laboulbeniales) from Pogonus chalceus (Coleoptera, Carabidae). *Belgian Journal of Botany,* **126**: 155-163.

De Kesel, A. (1995). Relative importance of direct and indirect infection in the transmission of Laboulbenia slackensis (Ascomycetes, Laboulbeniales). *Belgian Journal of Botany,* **128**: 124-130.

De Kesel, A. (1996). Host specificity and habitat preference of Laboulbenia slackensis. *Mycologia,* **88**: 565-573.

De Kesel, A. (1997). Contribution towards the study of the specificity of Laboulbeniales (Fungi, Ascomycetes), with particular reference to the transmission, habitat preference and host-range of Laboulbenia slackensis. (Universitaire Instelling Antwerpen).

De Kesel, A. (1998). Identificatie en gastheerspectrum van het genus Laboulbenia in België (Ascomycetes, Laboulbeniales). *Sterbeeckia,* **18**: 13-31.

De Kesel, A. and De Kesel, I.K. (2006). Laboulbeniales (Ascomycetes) from Latvia. Acta *Mycologica,* **41**: 55-64.

De Kesel, A. and Haelewaters, D. (2014). Laboulbenia slackensis and L. littoralis sp. nov. (Ascomycota, Laboulbeniales), two sibling species as a result of ecological speciation. *Mycologia,* **106**: 407-414.

De Kesel, A. and Rammeloo, J. (1991). Checklist of the Laboulbeniales (Ascomycetes) of Belgium. *Belgian journal of botany,* **124**: 204-214.

De Kesel, A. and Van den neucker, T. (2005). Morphological variation in Laboulbenia flagellata (Ascomycetes, Laboulbeniales). *Belgian Journal of Botany,* **138**: 165-172.

de Queiroz, K. (2005). A unified concept of species and its consequences for the future of taxonomy. *Proceedings of the California Academy of Sciences,* **56**: 196–215.

de Queiroz, K. (1998). The general lineage concept of species, species criteria, and the process of speciation: A conceptual unification and terminological recommendations in Endless forms. *Species and speciation* (Oxford University Press), 57-75.

de Queiroz, K. (2007). Species concepts and species delimitation. *Systematic Biolog,* **56**: 879–886.

Dean, F.B., Hosono, S., Fang, L., Wu, X., Faruqi, A.F., Bray-Ward, P., Sun, Z., Zong, Q., Du, Y. and Du, J. (2002). Comprehensive human genome amplification using multiple displacement amplification. *Proceedings of the National Academy of Sciences of the United States of America,* **99**: 5261-5266.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research, **32***: 1792-1797.

Fraiture, A., Amalfi, M., Raspé, O., Kaya, E., Akata, I. and Degreef, J. (2019). Two new species of Amanita sect. Phalloideae from Africa, one of which is devoid of amatoxins and phallotoxins. *MycoKeys*, **53**: 93–125.

Giard, A. (1892). Sur une Laboulbéniacée (Thaxteria küncheli nov. gen. et sp.), parasite de Mormolyce phyllodes Hagenbach. *Comptes Rendus Hebdomadaires des Séances et Mémoires de la Société de Biologie,* **4**: 156–158.

Goldmann, L. and Weir, A. (2012). Position specificity in Chitonomyces (Ascomycota, Laboulbeniomycetes) on Laccophilus (Coleoptera, Dytiscidae): a molecular approach resolves a century-old debate. *Mycologia,* **104**: 1143-1158.

Goldmann, L., and Weir, A. (2018). Molecular phylogeny of the Laboulbeniomycetes (Ascomycota). *Fungal Biology,* **122**: 87-100.

Goldmann, L., Weir, A. and Rossi, W. (2013). Molecular analysis reveals two new dimorphic species of Hesperomyces (Ascomycota, Laboulbeniomycetes) parasitic on the ladybird Coleomegilla maculata (Coleoptera, Coccinellidae). *Fungal Biology,* **117**: 807 - 813.

Haelewaters, D., Boer, P., Gort, G. and Noordijk, J. (2015). Studies of Laboulbeniales (Fungi, Ascomycota) on Myrmica ants (II): variation of infection by Rickia wasmannii over habitats and time. *Animal Biology,* **65**: 219-231.

Haelewaters, D., De Kesel, A. (2017). De schimmel Hesperomyces virescens, een natuurlijke vijand van lieveheersbeestjes. *Entomologische Berichten,* **77**: 106-118

Haelewaters, D., De Kesel, A. and Pfister, D.H. (2018a). Integrative taxonomy reveals hidden species within a common fungal parasite of ladybirds. *Scientific reports,* **8**: 1-16.

Haelewaters D., Page R.A. and Pfister D.H. (2018b). Laboulbeniales hyperparasites (Fungi, Ascomycota) of bat flies: Independent origins and host associations. *Ecology and Evolution,* **8**: 8396-8418.

Haelewaters, D., Pfliegler, W.P., Gorczak, M. and Pfister, D.H. (2019). Birth of an order: Comprehensive molecular phylogenetic study excludes Herpomyces (Fungi, Laboulbeniomycetes) from Laboulbeniales. *Molecular phylogenetics and evolution,* **133**: 286-301.

Haelewaters, D. and Yaakop, S. (2015). New and interesting Laboulbeniales from southern and southeastern Asia. *Mycotaxon,* **129**: 439-454.

Hosono, S., Faruqi, A.F., Dean, F.B., Du, Y., Sun, Z., Wu, X., Du, J., Kingsmore, S.F., Egholm, M. and Lasken, R.S. (2003). Unbiased whole-genome amplification directly from clinical samples. Genome research, **13**: 954-964.

Huldén, L. (1983). Laboulbeniales (Ascomycetes) of Finland and adjacent parts of the USSR. *Karstenia,* **23**: 31–136.

Majewski, T. (1994). The Laboulbeniales of Poland. *Polish Botanical Studies,* **7**: 1–466.

Majewski T. (2003). Distribution and ecology of Laboulbeniales (Fungi, Ascomycetes) in the Bialowieza Forest and its western foreland. *Phytocoenosis* (S.N.), **16**: 1-144.

Muilwijk, J., Felix, R.F., Dekoninck, W. and Bleich, O. (2015). De loopkevers van Nederland en België (Carabidae). *Nederlandse Entomologische Vereniging,* **9**: 1-125.

Peyritsch, J. (1873). Beitrage zur Kenntnis der laboulbenien.Sitzungerber. Akademie der Wissenschaften. *Mathematische-Naturwissenschaftliche Classe*, **68**:247.

Peyritsch, J. (1875). Sitzungsberichte der Kaiserlichen Akademie der Wissenschaften. *Mathematische-Naturwissenschaftliche Classe*, **72**: 247-385.

Pfliegler, W.P., Báthori, F., Haelewaters, D. and Tartally, A. (2016). Studies of Laboulbeniales on Myrmica ants (III): myrmecophilous arthropods as alternative hosts of Rickia wasmannii. *Parasite,* **23**: 1-7.

Posada D. and Crandall K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics,* **14**: 817–818

Reboleira, A.S., Enghoff, H., and Santamaría, S. (2018). Novelty upon novelty visualized by rotational scanning electron micrographs (rSEM): Laboulbeniales on the millipede order Chordeumatida. *PloS one*, **13**: e0206900.

Reboleira, A.S., Fresneda, J. and Salgado, J. M. (2017). A new species of Speonemadus from Portugal with the revision of the escalerai-group (Coleoptera: Leiodidae*). European Journal of Taxonomy,* **261**: 1–23.

Richards, A.G., and Smith, M.N. (1955). Infection of cockroaches with Herpomyces (Laboulbeniales). I. Life history studies. *The Biological Bulletin,* **108**: 206-218.

Robin, C. P. (1853). Histoire Naturelle des Végétaux Parasites qui Croissent sur l'Homme et sur les Animaux Vivants. *J.-B. Baillière* (Paris), **1**.

Ronquist F., Teslenko M., Van Der Mark P., Ayres D.L., Darling A., Höhna S., Larget B., Liu L., Suchard M.A. and Huelsenbeck J.P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic biology,* **61**: 539-542.

Rossi, W., and Santamaría, S. (2012). Rodaucea, a new genus of the Laboulbeniales. *Mycologia,* **104**: 785-788.

Rossi, W., and Weir, A. (1998). Triainomyces, a new genus of Laboulbeniales on the pill-millipede Procyliosoma tuberculatum from New Zealand. *Mycologia,* **90**: 282-289.

Rossi, W., Bernardi M., and Torres J.A. (2016). New species of Laboulbenia parasitic on leaf beetles. *Mycological Progress,* **15**: 4.

Santamaría, S. (1998). Laboulbeniales, 1. Laboulbenia. *Flora Mycologica Iberica,* **4**: 1-187.

Santamaría, S., Balazuc, J., and Tavares, I.I. (1991). Distribution of the European Laboulbeniales (Fungi, Ascomycotina), an annotated list of species. *Treballs de l'Institut Botànic de Barcelona,* **14**: 5-233.

Scheloske, H.-W. (1969). Beitrage zur Biologie, Okologie und Systematik der Laboulbeniales (Ascomycetes) unter besonderer Berucksichtigung des Parasit-Wirt-Verhaltnisses. *Parasitol. Schriftenreihe,* **19**: 1-176.

Scheloske, H. (1976). Vergleichend-morphologische und funktionelle Untersuchungen am Magen von Asellus aquaticus (L.)(Asellidae, Isopoda). *Zool Jahrb Abt Anat Ontog Tiere,* **95**: 519-573.

Schindelin, J., Arganda-Carreras, I. and Frise, E. (2012), "Fiji: an open-source platform for biological-image analysis", *Nature methods,* **9**: 676-682.

Schoch C.L., Seifert K.A., Huhndorf S., Robert V., Spouge J.L., Levesque C.A. and Chen W. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences,* **109**: 6241-6246.

Schoch, C.L., Sung, G.-H., López-Giráldez, F., Townsend, J.P., Miadlikowska, J., Hofstetter, V., Robbertse, B., Matheny, P.B., Kauff, F. and Wang, Z. (2009). The Ascomycota tree of life: a

phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. *Systematic biology,* **58**: 224-239.

Simmons MP, Pickett KM, Miya M. (2004). How meaningful are Bayesian support values? *Molecular Biology and Evolution*, **21**:188–199.

Stamatakis A., Hoover P. and Rougemont J. (2008). A Rapid Bootstrap Algorithm for the RAxML Web Servers. *Systematic Biology,* **57**:758–771

Sundberg, H., Ekman, S., and Kruys, Å. (2018). A crush on small fungi: An efficient and quick method for obtaining DNA from minute ascomycetes. *Methods in Ecology and Evolution,* **9**: 148-158.

Sundberg, H. 2018. Contributions to the understanding of diversity and evolution in the genus Coreomyces. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology,* **1703**: 1-47.

Talavera, G., and Castresana, J. (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology,* **56:** 564-577.

Tavares, I.I. (1985). Laboulbeniales (Fungi, Ascomycetes). Mycol. Mem. **9:** 431-434.

Terada, K. (1998). Notes on Laboulbenia coneglanensis (Ascomycetes, Laboulbeniales) in Japan. *Mycoscience,* **39**: 425-431.

Thaxter, R. (1896). Contribution Toward A Monograph Of The Laboulbeniaceae Part I. *In Memoirs of The American Academy of Arts and Sciences* (Cambridge: Wheeler E.W.), 241-250.

Thaxter, R. (1908). Contribution Toward A Monograph Of The Laboulbeniaceae Part II. *In Memoirs of The American Academy of Arts and Sciences* (Cambridge: Wheeler E.W.), 230-235.

Tragust, S., Tartally, A., Espadaler, X. and Billen, J. (2016). Histopathology of Laboulbeniales (Ascomycota: Laboulbeniales): ectoparasitic fungi on ants (Hymenoptera: Formicidae). *Myrmecological News,* **23**: 81-89.

Turin, H. (2000). De Nederlandse loopkevers: verspreiding en oecologie (Coleoptera: Carabidae). *Nationaal Natuurhistorisch Museum* (Leiden: KNNV-Uitgeverij & EIS-Nederland), **3**: 1-666.

Walker, M. J., Dorrestein, A., Camacho, J. J., Meckler, L. A., Silas, K. A., Hiller, T., and Haelewaters, D. (2018). A tripartite survey of hyperparasitic fungi associated with ectoparasitic flies on bats (Mammalia: Chiroptera) in a neotropical cloud forest in Panama. *Parasite,* **25**: 19.

Weir, A., and Beakes, G. (1993). New British Laboulbeniales*. Mycological Research,* **97**: 1045-1055.

Weir, A., and Blackwell, M. (2001a). Molecular data support the Laboulbeniales as a separate class of Ascomycota, Laboulbeniomycetes. *Mycological Research,* **105**: 1182-1190.

Weir, A., and Blackwell, M. (2001b). Extraction and PCR amplification of DNA from minute ectoparasitic fungi. *Mycologia,* **93**: 802-806.

Weir, A., and Blackwell, M. (2005). Fungal biotrophic parasites of insects and other arthropods. *Insect-Fungal Associations: ecology and evolution* (Oxford University Press), 119-145.

7 Appendix

Table 1A. Summary of data sets used for phylogenetic inferences using Rickia laboulbenioides ADK 6357 as outgroup.

Datasets		
Properties	LSU	ITS
Model selected	GTR+I+G	GTR+I+G
-Likelihood score	5055,9004	3691,7512
Base frequencies		
Freq. A =	0,2326	0,2049
Freq. C =	0,2545	0,301
Freq. G =	0,3204	0,3068
Freq. T =	0,1925	0,1872
Proportion of invariable sites	0,3179	0,3191
Gamma shape	0,2986	0,3782

Table 2A. Summary of species used for phylogenetic tree using Rickia laboulbenioides ADK 6357 as outgroup.

Species			References	
specimen name	Locality	Host	LSU	ITS
Genus *Laboulbenia*				
Laboulbenia bruchii				
DH1346b	South America, Panama	Chrysomelidae	Danny Haelewaters	-
ADK6349	Africa, Benin, Niaouli	Chrysomelidae	This study	-
Laboulbenia clivinalis				
DH6367-1	-	*Clivina fossor*	Danny Haelewaters	-
DH6367-2	-	*Clivina fossor*	Danny Haelewaters	-
Laboulbenia collae				
ADK6331-1 (DH1461a)	Belgium, Hingene	*Paranchus albipes*	Danny Haelewaters	Danny Haelewaters

ADK6331-2 (DH1461b	Belgium, Hingene	*Paranchus albipes*	Danny Haelewaters	Danny Haelewaters
ADK6331-3 (DH1461c)	Belgium, Meise	*Paranchus albipes*	Danny Haelewaters	Danny Haelewaters
ADK6354-1	Belgium, Hingene	*Paranchus albipes*	This study	This study
ADK6354-3	Belgium, Hingene	*Paranchus albipes*	This study	This study
Laboulbenia coneglianensis				
MG029H	-	*Harpalus affinis*	MG029H	-
MG029G	-	*Harpalus affinis*	MG029G	-
CG92	Belgium, Anhée	*Harpalus affinis*	This study	-
Laboulbenia diopsis				
DH1254a	Africa, Sierra Leone	Diptera	MG68738	MG68738
Laboulbenia fasciculata				
ADK6261	Latvia, Valmiera	*Patrobus atrorufus*	This study	-
Laboulbenia flagellata PS1				
ADK6329-2 (DH1454b)	Belgium, Hingene	*Limodromus assimilis*	Danny Haelewaters	Danny Haelewaters
ADK6329-1 (DH1454a)	Belgium, Hingene	*Limodromus assimilis*	Danny Haelewaters	Danny Haelewaters
ADK6355	Belgium, Bornem	*Limodromus assimilis*	This study	This study
ADK6362-1	Belgium, Meise	*Limodromus assimilis*	This study	This study
ADK6362-2	Belgium, Meise	*Limodromus assimilis*	This study	This study
ADK6329-3 (DH1454a)	Belgium, Bornem	*Limodromus assimilis*	Danny Haelewaters	Danny Haelewaters
Laboulbenia flagellata PS2				
Danny Haelewaters	Belgium, Meise	*Agonum nigrum*	This study	-
Laboulbenia flagellata PS3				
ADK6428	Belgium, Meise	*Agonum*	This study	-

		emarginatum		
ADK6332-1 (DH1457b)	Belgium, Bornem	*Agonum micans*	Danny Haelewaters	-
DH85-1	Belgium, Meise	*Loricera pilicornis*	KY350538.1	-
DH85-3	Belgium, Meise	*Loricera pilicornis*	KY350539.1	-
ADK6332-2 (DH1457c)	Belgium, Bornem	*Loricera pilicornis*	Danny Haelewaters	-
ADK6374	Latvia, Valmiera	*Loricera pilicornis*	This study	-
Laboulbenia littoralis				
ADK5159	Belgium, Knokke-Heist	*Cafius xantholoma*	This study	This study
Laboulbenia notiophili				
ADK6422	Belgium, Meise	*Notiophilus biguttatus*	This study	-
ADK6360	Belgium, Meise	*Notiophilus biguttatus*	This study	-
Laboulbenia oioveliicola nom. prov.				
DH942a	Brazil, Sao Paulo State	Hemiptera	Danny Haelewaters	-
DH942b	Brazil, Sao Paulo State	Hemiptera	MF314142.1	-
Laboulbenia pedicellata				
DH84-1	Sweden, Hjaelstaviken	*Dyschirius globosus*	KY350537.1	-
ADK6252	Latvia	*Dyschirius sp.*	This study	-
Laboulbenia pseudomasei				
ADK6258	Latvia, Valmiera	*Pterostichus anthracinus*	This study	-
ADK6444	Belgium, Meise	*Pterostichus nigrita*	This study	-
ADK6418	Belgium, Meise	*Pterostichus anthracinus*	This study	-
ADK6368	Belgium, Meise	*Nebria brevicollis*	This study	This study

ADK6434	Belgium, Meise	*Pterostichus anthracinus*	This study	-
Laboulbenia sp.				
DH967a	-	Chrysomelidae	Danny Haelewaters	-
Laboulbenia sp. nov				
ADK6448	Belgium, Meise	*Bembidion biguttatum*	This study	This study
Laboulbenia stilicicola				
DH1467	-	*Rugilus similis*	Danny Haelewaters	-
Laboulbenia systenae				
DH1342b	-	*Chrysomelidae*	Danny Haelewaters	-
Laboulbenia vulgaris				
ADK6353-1	Belgium, Bornem	*Ocys harpaloides*	This study	This study
ADK6353-2	Belgium, Bornem	*Ocys harpaloides*	This study	This study
ADK6330-1 (DH1455a)	Belgium, Bornem	*Ocys harpaloides*	Danny Haelewaters	Danny Haelewaters
ADK6330-2 (DH1459a)	Belgium, Bornem	*Ocys harpaloides*	Danny Haelewaters	Danny Haelewaters
ADK6330-3 (DH1460a)	Belgium, Bornem	*Ocys harpaloides*	Danny Haelewaters	Danny Haelewaters
ADK5557	Latvia, Rauna's staburags	*Bembidion tetracolum*	This study	-
ADK6420	Belgium, Meise	*Bembidion tetracolum*	This study	-
Genus *Rickia*				
Rickia Laboulbenioides				
ADK6357	Belgium, Meise	*Cylindroiulus latestriatus*	This study	-