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ARTICLE

Bringing *Laboulbeniales* into the 21st century: enhanced techniques for extraction and PCR amplification of DNA from minute ectoparasitic fungi

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Abstract: *Laboulbeniales* is one of the most peculiar orders of *Ascomycota*. These fungi are characterized by an ectoparasitic life-style on arthropods, determinate growth, lack of an asexual stage, high species richness, and intractability to culture. The order *Laboulbeniales*, sister to *Pyxidiophorales*, has only recently been assigned a separate class, the *Laboulbeniomycetes*, based on very few ribosomal DNA sequences. So far, DNA isolations and PCR amplifications have proven difficult. Here, we provide details of isolation techniques and the application of commercially available kits that enable efficient and reliable genetic analyses of these fungi. We provide 43 newly generated *Laboulbeniales* ribosomal DNA sequences, among which are the first published sequences for species in the genera *Gloeandromyces*, *Herpomyces*, *Laboulbenia*, *Monoicomyces*, and *Polyandromyces*. DNA extractions were possible using from 1 to 30 thalli from hosts preserved in ethanol (70–100 %). In two cases, we successfully isolated DNA from thalli on dried insect collections. *Laboulbeniales* molecular systematics could be substantially enhanced through these improved methods by allowing more complete sampling of both taxa and gene regions.

Key words:
Ascomycota
DNA isolation
insect collections
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INTRODUCTION

Laboulbeniales are obligate ectoparasitic *Ascomycota* on arthropods. Over 2100 species in 140 genera are described, but many more species await discovery (Weir & Hammond 1997, Haelewaters & Yaakop 2014). *Laboulbeniales* differ from most other non-yeast *Ascomycota* in that they do not form hyphae but instead form discrete microscopic and multicellular thalli. Their only form of reproduction is sexual, during which they generate sticky ascospores that are usually transmitted directly from infected to uninfected hosts during mating or other contact (De Kesel 1996a). They are moderately to highly host specific; most species are associated with a particular host species (but see, e.g. De Kesel & Haelewaters 2014). It was experimentally shown that this specificity is driven by several factors: the characteristics of the integument and living conditions of the arthropod host, as well as the nature and availability of nutrients in the habitat chosen by the host (De Kesel 1996b). Study of these fungi also needs some expertise in entomology. Correct identification of a host often facilitates identification of its associated fungi, but since fortuitous infections of hosts occur, it is best to identify these fungi based on their morphology or DNA sequence comparisons. Host-parasite lists are available for some countries (Scheloske 1969, Huldén 1983, Majewski 1994, De Kesel 1998, Santamaría 1998, 2003) and regions (Santamaría *et al*. 1991). Useful advice about general methodology and identification of *Laboulbeniales* can be found in Thaxter (1896), Scheloske (1969), Benjamin (1971), Majewski (1994), and Santamaría (1998).

It was only recently that the order *Laboulbeniales* was recognized as a well-supported lineage in *Ascomycota*, as the class *Laboulbeniomycetes* that includes both *Laboulbeniales* and *Pyxidiophorales* (Weir & Blackwell 2001a). This phylogenetic determination was based on four (partial) SSU ribosomal DNA (rDNA) sequences (*Pyxidiophora* sp.1, *Stigmatomyces limnophorae*, *Hesperomyces coccinelloides*, and *Zodiomyces vorticellarius*). Weir & Blackwell's (2001a) phylogeny suggested a close relationship with *Sordariomycetes*. High bootstrap support for this hypothesis

was later achieved by Schoch *et al*. (2009) based on a six-gene phylogeny. The order *Laboulbeniales* was represented in that dataset by only SSU and LSU sequences for two species (*Hesperomyces virescens* and *Stigmatomyces protrudens*).

Molecular studies of *Laboulbeniales* have proven difficult for several reasons. The thalli are microscopic, on average 200-300 µm in length. Among the smallest species known are *Rickia euxesti* (total length 40–68 µm), *R. lenoirii* (45–67 µm), and *Siemaszkoa annae* (47–54 µm) (Thaxter 1896, 1926, Majewski 1994, Santamaría & Espadaler 2015). At the other end of the size spectrum are *Zodiomyces vorticellarius* (to 2.75 mm) and *Laboulbenia kunckelii* (2–4 mm) (Giard 1892, Sugiyama & Phanichapol 1984, Haelewaters unpubl.). For study and extraction of DNA, thalli need to be removed from their host, which requires micro-manipulation techniques and specific tools. Hosts may bear only a few thalli but certain hosts carry multiple, often position-specific species (e.g. *Chitonomyces* spp., De Kesel & Haelewaters 2012, Goldmann & Weir 2012; *Hesperomyces coleomegillae* and *H. palustris*, Goldmann *et al*. 2013). Many species are heavily pigmented with melanin in their cell walls, providing rigidity (Weir & Beakes 1996). This pigment interferes with PCR amplification by binding to the DNA polymerase (Eckhart *et al*. 2000). Thalli are relatively long-lasting and their form is such that they absorb impacts and friction during their entire existence on the hosts' integument. These tough and resilient cells are difficult to break. Because *Laboulbeniales* have not been grown in culture to more than a few cells, obtaining DNA from cultured material has been impossible. Only Whisler (1968) was partly successful in this with *Stigmatomyces ceratophorus*, obtaining 20-celled thalli onto sterile fly wings on brain-heart infusion agar, but perithecia were not produced.

Laboulbeniales are a remarkable clade for their: (1) obligate biotrophy; (2) strictly determinate growth, with development from a two-celled ascospore to a thallus of up to several thousand cells; (3) bilateral symmetry; and (4) loss of germ tubes, hyphae, and conidia. Despite these special features, the order and the class were not included in studies dealing with "major lineages in *Ascomycota*" (Prieto & Wedin 2013) or the subphylum *Pezizomycotina*, to which they belong (Spatafora *et al*. 2006).

Extraction of DNA using a variety of methods and protocols have given poor results or failed. These include prolonged boiling of thalli (Henson 1992), microwave treatment (Goodwin & Lee 1993), immersion in liquid nitrogen (Haugland *et al*. 1999), and direct addition of entire thalli to PCR master mix (Haelewaters 2011). Also, the use of commercial kits (Puregene Kit A, DNeasy Plant Mini Kit, Qiagen; Haelewaters 2011) has so far proven unsuccessful.

The first successful published extraction protocol involved transferring thalli to double distilled (dd) H_20, air drying, and manually crushing thalli between microscope slides (Weir & Blackwell 2001a). The success rate for this protocol was 25 %. Weir & Blackwell (2001b) developed an improved technique in which thalli were manually crushed on a microscope slide and picked up with a micropipette facilitated by the use of a bed of dry ice, a modification from previous endeavors based on Conger & Fairchild (1953) and Lee & Taylor (1990). The technique from Weir & Blackwell (2001b) was successful only when hosts were preserved in 95 % ethanol for not more than six months. Thalli taken from dried insect specimens have not been available for molecular phylogenetic analyses because extractions have been unsuccessful with this type of material (Weir & Blackwell 2001b). This technical difficulty limits both the taxonomical and geographical diversity of species that can be included in phylogenetic studies (e.g. Thaxter 1899, 1900, 1901a, 1901b, 1902, 1905, Weir & Hammond 1997, Haelewaters *et al*. 2014, 2015a, 2015b).

Owing to the difficulties in DNA isolation and amplification of phylogenetically informative genes, the molecular phylogenetic relationships within this group have been understudied. Weir & Hughes (2002) constructed a partial SSU rDNA phylogeny of ten species of *Laboulbeniales*, representing three subfamilies (*Ceratomycetoideae*, *Laboulbenioideae*, *Peyritschielloideae*). A combined dataset of the partial SSU and ITS rDNA regions was used to study the phenomenon of position specificity in 13 species of *Chitonomyces* on *Laccophilus maculosus* (*Coleoptera*: *Dytiscidae*; Goldmann & Weir 2012). Goldmann *et al*. (2013) described two position specific species of *Hesperomyces* on *Coleomegilla maculata* (*Coleoptera*: *Coccinellidae*), again based on partial SSU+ITS rDNA. All these studies used the extraction methodology of Weir & Blackwell (2001b).

We tested more generalized techniques that could be adapted to sample the thalli of *Laboulbeniales*.

MATERIAL AND METHODS

Collection

Insects were collected around the world by ourselves or collaborators using standard entomological methods (sticky traps, light trap, entomological net, and hand collecting) or obtained from the pet store (*Blatta lateralis*). Insects were killed in 70–100 % ethanol, ethyl acetate vapors, or simply by freezing. Screening for *Laboulbeniales* was done using a dissecting microscope at 50x.

Morphological studies

Individual thalli were removed from the host using an entomological pin (self-made, sometimes flattened) or the tip of a scalpel. Slide mounts followed techniques for permanent microscope slides (Benjamin 1971, Haelewaters et al. 2015b). Identification of *Laboulbeniales* followed Thaxter (1908, 1931), Majewski (1994), and De Kesel (2011). Voucher slides are deposited at BP (Botanical Department, Hungarian Natural History Museum), FH (Farlow Herbarium, Harvard University), and WA (Faculty of Biology, University of Warsaw). Herbarium acronyms are according to Thiers 2015.

DNA extraction protocols

Between one and 30 thalli were removed from each host specimen. In this study we wanted to test the efficacy of different commercial and noncommercial DNA extraction protocols. The following were used: (1) QIAamp DNA Micro

Kit (Qiagen, Stanford, CA); (2) Extract-N-Amp Plant PCR Kit (Sigma-Aldrich, St Louis, MO); (3) a heat-extraction protocol; and (4) ISOLATE II Plant DNA Kit (Bioline Reagents, London).

(1) *QIAamp DNA Micro Kit*: DNA was isolated from two to sixteen thalli for each extraction, following the manufacturer's instructions. Some extracts received pre-treatment with liquid nitrogen or two cycles of heating to 95 °C and freezing on liquid nitrogen.

(2) *Modified Extract-N-Amp Plant PCR Kit*: The manufacturer's instructions were followed but with 20 µL of Extraction Solution (EX) and 60 µL of Dilution Solution. One to 20 thalli were removed from the host with the help of a tiny drop of Hoyer's medium (30 g arabic gum, 200 g chloral hydrate, 16 mL glycerol, 50 mL ddH_20) or glycerine at the very end of a micropin and then added to EX-filled 0.5 µL tubes. When hosts were preserved in dried collections, 16–30 thalli were used. Again, the pre-treatment described above was applied for some extracts.

(3) *Heat-extraction protocol*: This method was adapted from a protocol for single-spore extractions and subsequent PCR reactions (Ferreira & Glass 1996, based on Goodwin & Lee 1993). Thalli were removed from the host (3 thalli of *Hesperomyces virescens*, 20–30 thalli of *Rickia wasmannii*) or a ~5 mm portion of a heavily infected *Blatta lateralis* antenna with *Herpomyces stylopygae* thalli was removed, placed in 0.5 mL PCR tubes, and microwave-treated (750 W for 5 min). Then 50 µL ddH_20 was added to the individual tubes, and the thalli (or antennal parts) were manually crushed using a sterile pipette tip under a dissecting microscope. Some loss of material did occur by capillary action, but it was minimal. The PCR tubes were incubated at -20 °C for 10 min. Strong pressure was applied to the ice inside the PCR tubes to further break apart thalli using a sterile pipette tip.

(4) *ISOLATE II Plant DNA Kit*: Up to twenty thalli were removed from the host and transferred to 1.5 mL Eppendorf tubes with 20–50 µL 95 % ethanol. Alternatively, in the case of *Herpomyces ectobiae* on *Blattella germanica*, a piece of an antenna was isolated and transferred altogether. The 1.5 mL tubes were vacuum-dried at room temperature. Thalli were subsequently crushed in liquid nitrogen, using a sterile pipette with melted-closed tip. CTAB-based isolation buffer (PA1, ISOLATE II Plant DNA Kit) was added to the tubes and incubated in liquid nitrogen for 3 min, followed by incubation in a heat block set at 65–90 °C for 3 min. This cycle of freezing/heating was repeated twice. Further steps were performed following the ISOLATE II Plant DNA Kit manufacturer's protocol.

PCR amplification and DNA sequencing

Three gene loci were amplified: partial rDNA SSU (*ca* 1100 bp), rDNA ITS (including ITS1, 5.8S and ITS2; *ca* 500 bp), and partial rDNA LSU (*ca* 1300 bp). PCR amplification was performed using both previously published and newly designed primers (Table 1). *Laboulbeniales*-specific primers were designed for the SSU region based on existing sequences in GenBank (Table 1). PCR reactions were performed according to the protocols listed in the respective reference for mentioned primers, or, in the case of the Extract-N-Amp Plant PCR Kit, according to the suggested protocol in the manufacturer's instructions. When PCR reactions did not produce clear bands during gel electrophoresis, conditions were optimized to include a two-step (60 °C, 55 °C) "touch-down" annealing phase (Sohrabi *et al*. 2010). In some cases, a semi-nested "touch-down" PCR was performed, using the product of the first, unsuccessful PCR reaction (e.g. PCR 1 using primers LR0R and LR5, semi-nested PCR using the product of PCR 1 with primers LR0R and LR3).

Products that showed clear bands on agarose gel were cleaned with Qiaquick PCR Purification Kit (Qiagen, Stanford, CA) or ExtractMe DNA Gel-out kit (Blirt, Gdańsk, Poland) and subsequently sequenced. We prepared 10 µL sequencing reactions containing the same primer pairs and 1 µL of purified PCR product. The sequencing reactions were performed using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA).

Sequences were trimmed, edited and assembled in Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). We performed BLAST searches on all of our sequences at http://ncbi.nlm.nih.gov/blast/Blast.cgi for similar sequences. For genera not yet represented in GenBank we compared sequences with our personal database, which is accessible at the Harvard University Herbaria internal server.

RESULTS

Our study shows that some simple, general DNA extraction protocols work. The commercial kits we tested are widely available.

Table 2 shows the success rates of the individual protocols, per genus extracted. Extractions using the QIAamp DNA Micro Kit yielded the lowest rates of success among the tested protocols, with seemingly no effect of pre-treatment. The overall success rate was 22 % (*n* = 27 extractions total), for *Hesperomyces virescens* extractions the success rate was 35 % (*n* = 17). Overall success of the Extract-N-Amp Plant PCR Kit was 64 % (*n* = 66), with 92 % success for *Herpomyces* spp. (n = 13) and 66 % for *H. virescens* (*n* = 35). For the third, heat-extraction protocol the success rate was 83 % for *Herpomyces ectobiae* (*n* = 6) and 100 % for *H. virescens* (*n* = 3). The ISOLATE II Plant DNA Kit gave an overall success rate of 59 % (*n* = 34), with a 100 % success rate for *H. ectobiae* (*n* = 5) and 86 % for *H. virescens* (*n* = 7). Interestingly, extracting DNA of *Laboulbenia* species was only successful 20 % of the time with the Extract-N-Am Plant PCR Kit and 10 % with the ISOLATE II Plant DNA Kit. Four extraction attempts of *Laboulbenia* species with the QIAamp DNA Micro Kit were unsuccessful.

We generated 43 sequences (SSU, ITS, and/or LSU rDNA) for 18 isolates of the following species: *Gloeandromyces nycteribiidarum*, *Herpomyces chaetophilus*, *H. ectobiae*, *H. periplanetae*, *H. stylopygae*, *Hesperomyces virescens*, *Laboulbenia diopsidis*, *Monoicomyces invisibilis*, *Polyandromyces coptosomalis*, *Rhachomyces philonthinus*, *Rickia wasmannii*, and *Zodiomyces vorticellarius* (Table 3). *Rhachomyces philonthinus* was removed from a specimen

Table 1. List of primers used for PCR amplification of small subunit (SSU), internal transcribed spacer (ITS), and large subunit (LSU) rDNA.

Locus	Primer name		Sequence	Reference
SSU rDNA	NS1	forward	GTAGTCATATGCTTGTCTC	White *et al*. 1990
SSU rDNA	NS2	reverse	GGCTGCTGGCACCAGACTTGC	White *et al*. 1990
SSU rDNA	NS4	reverse	CTTCCGTCAATTCCTTTAAG	White *et al*. 1990
SSU rDNA	SL344	forward	GGTCGCAAGGCTGAAACTTA	Landvik *et al*. 1997
SSU rDNA	NS6	reverse	GCATCACAGACCTGTTATTGCCTC	White *et al*. 1990
SSU rDNA	SL122	forward	AGGCGCGCAAATTACCCAAT	Landvik *et al*. 1997
SSU rDNA	SR4	reverse	AAACCAACAAAATAGAA	R. Vilgalys unpublished
SSU rDNA	NSL1	forward	GTAGTGTCCTCrCATGCTTTTGAC	present study
SSU rDNA	NSL2	reverse	AATCyAAGAATTTCACCTCTGAC	present study
SSU rDNA	L	forward	AACCTGGTTGATCCTGCCAGT	Wrzosek 2000
SSU rDNA	402	forward	GCTACCACATCCAAGGAAGGCA	Wrzosek 2000
SSU rDNA	416	reverse	ATTTGCGCGCCTGCTGCCTTCC	Wrzosek 2000
SSU rDNA	895	forward	GTCAGAGGTGAAATTCTTGGAT	Wrzosek 2000
SSU rDNA	898	reverse	TAAATCCAAGAATTTCACCTCT	Wrzosek 2000
SSU rDNA	1144	forward	GCCTGCGGCTTAATTTGACTCAACA	Wrzosek 2000
SSU rDNA	1308	reverse	CTCGTTCGTTAACGGAATTAACC	Wrzosek 2000
SSU rDNA	R	reverse	TGATCCTTCTGCAGGTTCACCTACG	Wrzosek 2000
ITS rDNA	ITS1f	forward	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns 1993
ITS rDNA	ITS4	reverse	TCCTCCGCTTATTGATATGC	White *et al*. 1990
ITS rDNA	ITS4_kyo1	reverse	TCCTCCGCTTWTTGWTWTGC	Toju *et al*. 2012
ITS rDNA	ITS5	forward	GGAAGTAAAAGTCGTAACAAGG	White *et al*. 1990
ITS rDNA	ITS2	reverse	GCTGCGTTCTTCATCGATGC	White *et al*. 1990
LSU rDNA	LR0R	forward	ACCCGCTGAACTTAAGC	R. Vilgalys unpublished
LSU rDNA	LR1R	forward	AGGAAAAGAAACCAACC	Moncalvo *et al*. 1993
LSU rDNA	LIC24R	forward	GAAACCAACAGGGATTG	Miadlikowska & Lutzoni 2000
LSU rDNA	LR3	reverse	GGTCCGTGTTTCAAGAC	Vilgalys & Hester 1990
LSU rDNA	LR5	reverse	ATCCTGAGGGAAACTTC	Vilgalys & Hester 1990
LSU rDNA	LR7	reverse	TACTACCACCAAGATCT	Vilgalys & Hester 1990
LSU rDNA	NL1	forward	GCATATCAATAAGCGGAGGAAAAG	Kurtzman & Robnett 1997
LSU rDNA	NL4	reverse	GGTCCGTGTTTCAAGACGG	Kurtzman & Robnett 1997

of *Philonthus* that had been collected by Tomasz Majewski in August 2004. The host specimen was preserved for 11 years in 70 % ethanol.

We were able to extract DNA from thalli of *Hesperomyces virescens* from dried insect specimens (with the Extract-N-Amp Plant PCR Kit); on *Cycloneda sanguinea sanguinea* from Guatemala collected in May 2013, and on *Harmonia axyridis* from Massachusetts collected in August 2006 (details in Haelewaters *et al*. 2015b). Extractions were performed from *H. paranensis* on a dried *Archimandrita tessellata* (*Blattodea*: *Blaberidae*) collected in 2001 [deposited at the Harvard Museum of Comparative Zoology] and from *Rodaucea* sp. on a dried *Cholevinae* sp. (*Coleoptera*: *Leiodidae*) collected in 1991 [part of the collection of Invertebrate Zoology at the American Museum of Natural History], but no bands were noted on the agarose gel after PCR.

DISCUSSION

Micromanipulation practices

Laboulbeniales are more problematic to work with than many other groups of fungi. One of the main difficulties is their small size, which requires sterile micromanipulation with precise micropin handling.

It is preferable to separate thalli from the host's body, but minute thalli of *Rickia*, *Herpomyces* or *Siemaszkoa* are hard to detach. Using whole infected body parts in an extraction makes the procedure faster and easier. Most of the primers used in this study do not amplify the host insect's DNA, However, amplification of insect DNA by some primers may happen (as with LR0R/LR7 and the sets of SSU primers used in Wrzosek 2000). Prominent appendages, such as those in many species of *Laboulbenia* or or *Rhachomyces*, pose another difficulty; debris is often observed to stick to the appendages and is very hard to impossible to wash away. In this case contamination with fungal propagules may be inevitable. *Laboulbeniales*-specific primers will serve to reduce the chance of amplifying non-target DNA. Another

Table 2. Success rates per DNA extraction protocol used in this study, for all tested genera. *Laboulbeniales* from dried host insects were only extracted using the Extract-N-Amp Plant PCR Kit.

	QIAamp DNA Micro Kit				Extract-N-Amp Plant PCR Kit			
	# extractions	# success	# failed	% success	# extractions	# success	# failed	% success
Aphanandromyces								
Chitonomyces	4	0	4	0 %				
Gloeandromyces					1	1	0	100 %
Haplomyces	2	0	2	0 %	2	0	2	0 %
Herpomyces					13	12	1	92 %
Hesperomyces	17	6	11	35 %	35	23	12	66 %
Laboulbenia	4	0	4	0 %	5	1	4	20 %
Monoicomyces								
Polyandromyces					2	2	0	100 %
Rhachomyces								
Rickia								
Zodiomyces								
Herpomyces (dried)					2	0	2	0 %
Hesperomyces (dried)					5	3	2	60 %
Rodaucea (dried)					1	0	1	0 %

	Heat-extraction protocol				ISOLATE II Plant DNA Kit			
	# extractions	# success	# failed	% success	# extractions	# success	# failed	% success
Aphanandromyces					1	0	1	0 %
Chitonomyces								
Gloeandromyces								
Haplomyces								
Herpomyces	6	5	1	83 %	5	5	0	100 %
Hesperomyces	3	3	0	100 %	7	6	1	86 %
Laboulbenia					10	1	9	10 %
Monoicomyces					6	4	2	67 %
Polyandromyces								
Rhachomyces					2	1	1	50 %
Rickia	11	5	6	45 %				
Zodiomyces					3	3	0	100 %

option is to simply excise the appendage system prior to extraction.

Preservation techniques

One of the most important concerns regarding successful molecular research is the method employed for preservation of material. The most effective option for extraction of *Laboulbeniales* DNA involves using freshly collected material preferably stored in ≥ 95 % ethanol. These two factors certainly contribute to most of our DNA isolation positive results. Storage in ≥ 95 % ethanol generally provides good DNA preservation for a prolonged period of time. Our DNA extraction protocols enabled us to amplify DNA and generate sequences from *Laboulbeniales* material that was on average 1–2 years old (one specimen was 11 years old), which is a novel development. Conditions that consistently yielded good results included: freshly collected specimens of larger species of *Laboulbeniales*, which provide ample DNA concentration even from a single thallus (e.g. *Zodiomyces vorticellarius*), and mature ascospore-containing thalli, which provide a higher concentration of DNA compared to immature or old thalli (always without ascospores). Many entomological practices involve preservation methods that interfere with successful DNA extraction of either the host or its associated fungi: most insect specimens are pinned in museum collections or preserved on 70 % ethanol.

For morphological study of *Laboulbeniales*, researchers are able to make use of the many excellent systematic insect collections in natural history museums around the world. Such collections of dried pinned insects give relatively easily access to data (e.g. Weir & Hammond 1997, Haelewaters *et al*. 2014). However, to date, extracting DNA from dried specimens has resulted in a 100 % failure rate (Weir & Blackwell 2001b). We present sequences obtained from two

Table 3. Detailed collecting data, method of preservation, number of thalli used in extraction, DNA extraction protocol, and GenBank accession numbers for SSU, ITS, and LSU rDNA sequences of 18 isolates. NB: Table 3 is best viewed in a 2-page view, as it continues on the next page.

Genus	Species	Isolate	Host	COUNTRY: locality	Collector
Gloeandromyces	*nycteribiidarum*	DH619a	Streblidae sp.	TRINIDAD	J.C. Camacho
Herpomyces	*chaetophilus*	DH602b	*Periplaneta americana*	USA: Massachusetts, Cambridge, Kirkland House	T.W. Wang
Herpomyces	*ectobiae*	MG001	*Blattella germanica*	POLAND: Warsaw	M. Gorczak
Herpomyces	*periplanetae*	DH602c	*Periplaneta americana*	USA: Massachusetts, Cambridge, Kirkland House	T.W. Wang
Herpomyces	*stylopygae*	DE_HerpBL1	*Blatta lateralis*	HUNGARY	W.P. Pfliegler
Hesperomyces	*virescens*	DH167e	*Cycloneda sanguinea sanguinea*	GUATEMALA: Huehuetenango Dept., La Laguna	R.S. Zack
Hesperomyces	*virescens*	JP353a	*Olla v-nigrum*	USA: Georgia, Peach County, USDA-ARS	E. Brooks Thompson
Hesperomyces	*virescens*	DH486c	*Harmonia axyridis*	USA: Massachusetts, World's End peninsula	J. Rykken
Hesperomyces	*virescens*	DH646c	*Harmonia axyridis*	GERMANY: Bavaria state, Bereuth	S. Tragust
Hesperomyces	*virescens*	HM497c	*Harmonia axyridis*	USA: Georgia, Peach County, USDA-ARS	E. Brooks Thompson
Hesperomyces	*virescens*	DE_HV01	*Harmonia axyridis*	HUNGARY: Debrecen	W.P. Pfliegler
Hesperomyces	*virescens*	MT001	*Harmonia axyridis*	POLAND: Warsaw	M. Tischer
Laboulbenia	*diopsidis*	DH468a	*Diopsidae* sp.	SIERRA LEONE: Eastern Province, Nemahugoima	W. Rossi
Monoicomyces	*invisibilis*	MT004	*Anotylus sculpturatus*	POLAND: Warsaw	M. Tischer
Polyandromyces	*coptosomalis*	DH313f	*Phoeacia* sp. nov.	ECUADOR: Orellana Province, Quito	D. Forero
Rhachomyces	*philonthinus*	TM10446	*Philonthus* sp.	POLAND: Zachodniopomorskie, Łobżany	T. Majewski
Rickia	*wasmannii*	DE_Rak4	*Myrmica scabrinodis*	HUNGARY: Rakaca	A. Tartally
Zodiomyces	*vorticellarius*	MG003	*Helochares obscurus*	POLAND: Warsaw	M. Gorczak

collections of *H. virescens* from dried ladybirds (DH167e and DH486c) collected in 2013 and 2006, respectively. Often thalli acquired from dried hosts are in poor condition and both identification based on morphological characters and DNA extraction may be a challenge.

Many insects in entomological collections are preserved in 70 % ethanol. This decreases the DNA quality of the insect and its associates – especially after an extended period of storage (e.g. A'Hara *et al*. 1998). Some studies have generated short segments of mitochondrial DNA (< 300 bp) from material in 70 % ethanol (e.g. Colgan *et al*. 2002). For phylogenetic studies, however, longer segments are needed, and these need to be acquired from non-degraded DNA. Non-degraded DNA is also required for PCR amplification of low copy-number nuclear genes commonly used in modern fungal phylogenies (e.g. Hibbett *et al*. 2007, Hansen *et al*. 2013, Wang *et al*. 2014). If 70 % ethanol was used to preserve insect hosts, it comes as no surprise that the DNA of *Laboulbeniales* harvested from them is adversely affected.

When working with *Laboulbeniales* from dried collections, another challenge is that information about the habitat or methods of collection and preservation is typically sparse. The extraction of DNA from insects can be drastically affected by using certain media (such as killing agents in pitfall traps) that degrade DNA. Some commonly used materials such as ethylene glycol or formalin have been linked to considerable DNA degradation (e.g. Dillon *et al*. 1996, Stoeckle *et al*. 2010).

Negative results

Our negative results can be explained based on protocols employed and/or the nature of the fungi that were under investigation. The 100 % failure rate of the QIAamp DNA

Year of collection	Preservation	Number of thalli used	Extraction protocol	SSU	ITS	LSU
2014	95 % EtOH	12 thalli	Extract-N-Amp (with glycerine)			KT800008
2014	95 % EtOH	10 female thalli	Extract-N-Amp (with glycerine)	KT800023	KT800039	KT800009
2014	95 % EtOH	piece of antenna with ± 20 adult thalli	ISOLATE II Plant DNA Kit without freeze/thaw	KT800024	KT800040	
2014	95 % EtOH	11 female thalli	Extract-N-Amp (with glycerine)	KT800025	KT800041	KT800010
2014	80 % EtOH	piece of antenna with ± 30 adult thalli	Heat-extraction	KT800026	KT800042	KT800011
2013	dried	18 adult thalli	Extract-N-Amp	KT800027		KT800012
2014	95 % EtOH	10 adult thalli	QIAamp DNA Micro Kit	KT800028	KT800043	KT800013
2006	dried	16 adult thalli	Extract-N-Amp (with glycerine)	KT800029	KT800044	KT800014
2013	95 % EtOH	2 adult thalli	Extract-N-Amp		KT800045	KT800015
2014	95 % EtOH	15 adult thalli	Extract-N-Amp (with Hoyer's medium)	KT800030	KT800046	KT800016
2014	80 % EtOH	9 adult thalli	Heat-extraction	KT800031	KT800047	KT800017
2015	95 % EtOH	1 adult thallus	ISOLATE II Plant DNA Kit	KT800032	KT800048	KT800018
2013	100 % EtOH	12 adult thalli	Extract-N-Amp	KT800033	KT800049	KT800019
2015	95 % EtOH	1 adult thallus	ISOLATE II Plant DNA Kit	KT800034		
2009	95 % EtOH	7 female and 2 male thalli	Extract-N-Amp	KT800035		KT800020
2004	70 % EtOH	± 15 adult thalli	ISOLATE II Plant DNA Kit	KT800036		
2014	80 % EtOH	30 adult thalli	Heat-extraction	KT800037	KT800050	KT800021
2015	95 % EtOH	1 adult thallus	ISOLATE II Plant DNA Kit	KT800038		KT800022

Micro Kit for *Chitonomyces*, *Haplomyces*, and *Laboulbenia* is largely due to the fact that no pre-treatments were carried out for these extracts. However, for one *Laboulbenia* extraction using this protocol a pre-treatment was done involving two cycles of heating to 95 °C and freezing on liquid nitrogen. Then why was this extraction unsuccessful? *Laboulbenia* species are generally heavily melanized, and the melanin pigment seems to hinder PCR amplification reactions (Eckhart *et al*. 2000). Also in the Extract-N-Amp Plant PCR Kit and the ISOLATE II Plant DNA Kit the success of extracting DNA and subsequent PCR amplification of *Laboulbenia* species is considerably lower compared to other genera. This observation shows that variables other than isolation techniques, such as the presence of pigments, are important to the success of DNA extraction and amplification. The 0 % success rate of *Haplomyces* using both the QIAamp DNA Micro Kit and the Extract-N-Amp Plant PCR Kit probably is due to the combination of two factors: (1) the extract received no pre-treatment; and (2) host insects were collected and preserved (for four to five years) in 70 % ethanol. The relatively low success rate with *Rickia*, with the heat-extraction protocol, may be explained by the fact that these small but very rigid thalli are difficult to break during the treatments that were applied; visual inspection after performing the entire protocol shows many intact thalli. Thus, the amount of DNA available for the *Taq* polymerase during PCR was limited, despite the high number of thalli (20–30) per reaction.

We can only hint at the low success rate of extractions from dried material. The extraction of *Rodaucea* sp. received no pre-treatment and the thalli were removed from a cholevine specimen collected in 1991. It might have been too old for successful DNA extraction. The same may be true

for the unsuccessful attempts to extract DNA of *Herpomyces paranensis* from a pinned specimen of *Archimandrita tessallata* from 2001.

CONCLUSIONS

Even with fresh thalli available, successful extraction of DNA has been one of the greatest obstacles in applying molecular methods to research on *Laboulbeniales*. Their minute size, the difficulty in fracturing thalli to release DNA, and the fact that (to date) they remain resistant to isolation into culture makes molecular protocols applied to *Laboulbeniales* difficult. This is the reason "laboulbeniologists" need: (1) colleagues (entomologists) or museums to provide high-quality, properly prepared samples; and (2) DNA isolation protocols that focus heavily on deep homogenization of the material. Microwave heating, submersion in liquid nitrogen, freeze/thaw cycles, and simple yet effective crushing with pipette tips are all means of destroying the tough cell walls without damaging the DNA.

As stated in previous studies, both the SSU and ITS portions of rDNA are suited for molecular phylogenetics of the *Laboulbeniales* and universal fungal primers for these regions work well for most of the species (Weir & Blackwell 2001b, Goldmann & Weir 2012, Weir & Hughes 2002, Goldmann *et al*. 2013)). We have found that LSU sequences are also easily to obtain. Designing specific primers often facilitates the work. Well-designed primers specific for *Laboulbeniales* may perform better and their specificity helps to avoid contamination. As the number of genes being used in fungal phylogenetic studies increases it will be important that these new genes/regions/markers be explored in the *Laboulbeniales* as well.

We hope that sharing our experience with various techniques for extraction and PCR amplification of *Laboulbeniales* DNA will have a positive effect on present and future molecular biology research of *Laboulbeniomycetes* – the only class among the *Ascomycota* without a reliable multi-gene phylogeny.

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