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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, *Laboulbeniales*-specific primers, ribosomal DNA, unculturable fungi

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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 readily available at 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 Siamaskoza annae (47–54 μm) (Thaxter 1896, 1926, Majewski 1994, Santamaria & 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 & Phanichapal 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 subphyllum 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) H₂O, 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 (Ceratomyctoideae, Laboulbenioideae, Peyritschioideae). 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 ddH₂O) or glycerine at the very end of a micropip 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 ddH₂O 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, Gdańska, 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 Sequencer 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-Amp 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 nycteribiardum, 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
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 viridescens from dried insect specimens (with the Extract-N-PS3ODQW3&5.LW... from Cycloneda sanguinea sanguinea and Harmonia axyridis from Massachusetts collected in August 2006 (details in Haelewaters et al.)).

**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, and prominent appendages, such as those in many species of Laboulbenia or Rhachomyces, are very hard to wash away. In this case contamination with fungal propagules may be inevitable.

| Locus | Primer name | Sequence | Reference |
|-------|-------------|----------|-----------|
| SSU rDNA | NS1 forward | GTAGTCATATGCTTGTCTC | White et al. 1990 |
| SSU rDNA | NS2 reverse | GGCCTGCTGGCACCAGACTTGC | White et al. 1990 |
| SSU rDNA | NS4 reverse | CTTCCGTCATTCTTTAAG | White et al. 1990 |
| SSU rDNA | SL344 forward | GTTCGCAAGGCCTGAACTTA | Landvik et al. 1997 |
| SSU rDNA | NS6 reverse | GCATCACAGACCTTTATTGCTC | White et al. 1990 |
| SSU rDNA | SL122 forward | AGGGCGCGCAAATACCCAAT | Landvik et al. 1997 |
| SSU rDNA | SR4 reverse | AAACCAAACAAATAGAA | R. Vilgalys unpublished |
| SSU rDNA | NSL1 forward | GTAGTGTCCTCCTAGCTTTTGAC | present study |
| SSU rDNA | NSL2 reverse | AATCyAAGAATTTCACTCTGAC | present study |
| SSU rDNA | L forward | AACCGGTGTAGCTGCGGCAG | Wrzosek 2000 |
| SSU rDNA | 402 forward | GCTACCCATCCAAGGACGA | Wrzosek 2000 |
| SSU rDNA | 416 reverse | ATTTGCCGCGCAGCTGCTTCC | Wrzosek 2000 |
| SSU rDNA | 895 forward | GTACAGAGTGAAATTTCTTGAT | Wrzosek 2000 |
| SSU rDNA | 898 reverse | TAAATCCGAATTTACCTC | Wrzosek 2000 |
| SSU rDNA | 1144 forward | GCTTCGGCTTAATTGACTCAACA | Wrzosek 2000 |
| SSU rDNA | 1308 reverse | CTCTGCCTGTAACGAATACC | Wrzosek 2000 |
| SSU rDNA | R reverse | TGATCTCTCTGCAGGTTCACCTACG | Wrzosek 2000 |
| ITS rDNA | ITS1f forward | CTGGTGCAATTATAGAGAGAT | Gardes & Brun 1993 |
| ITS rDNA | ITS4 reverse | TCCCTCCGTTATGTAT | White et al. 1990 |
| ITS rDNA | ITS4_kyo1 reverse | TCCCTCGCTTTTGGTWTG | Toju et al. 2012 |
| ITS rDNA | ITS5 forward | GGAGTAAAGATCGTAACAGG | White et al. 1990 |
| ITS rDNA | ITS2 reverse | GTCGCGTCTCTGACAGCT | White et al. 1990 |
| LSU rDNA | LR0R forward | ACCCGCTGAACCTAAG | R. Vilgalys unpublished |
| LSU rDNA | LR1R forward | AGGAAAAGAACAACC | Moncalvo et al. 1993 |
| LSU rDNA | LIC24R forward | GAAAACACAGGATTT | Miadlikowska & Lutzoni 2000 |
| LSU rDNA | LR3 reverse | GTCCCTGTTTCAAGAC | Vilgalys & Hester 1990 |
| LSU rDNA | LR5 reverse | ATCTCGAGGAAACCTT | Vilgalys & Hester 1990 |
| LSU rDNA | LR7 reverse | TACTACCCACGAGCT | Vilgalys & Hester 1990 |
| LSU rDNA | NL1 forward | GCATATCAATAAGCCGGGAAAAG | Kurtzman & Robnett 1997 |
| LSU rDNA | NL4 reverse | GGTCGCGTGGCTTCAAGG | Kurtzman & Robnett 1997 |
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
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. 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

### 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. Pfiegler      |
| *Hesperomyces* | *virescens*                      | DH167e  | Cycloneda sanguinea sanguinea | GUATEMALA: Huehuetenango Dept., La Laguna | R.S. Zack          |
|                 |                                   | JP353a  | Olla v-nigrum         | USA: Georgia, Peach County, USDA-ARS | E. Brooks Thompson |
| *Hesperomyces* | *virescens*                      | DH486c  | Harmonia axyridis     | GERMANY: Bavaria state, Bereuth | S. Tragust        |
| *Hesperomyces* | *virescens*                      | DH646c  | Harmonia axyridis     | USA: Georgia, Peach County, USDA-ARS | E. Brooks Thompson |
| *Hesperomyces* | *virescens*                      | HM497c  | Harmonia axyridis     | HUNGARY: Debrecent | W.P. Pfiegler      |
| *Hesperomyces* | *virescens*                      | DE_HV01 | Harmonia axyridis     | POLAND: Warsaw    | M. Tischer        |
| *Laboulbenia*  | *diipsidis*                      | DH468a  | Diopsidae sp.         | SIERRA LEONE: Eastern Province, Nemahugoma | 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, Lobžany | T. Majewski       |
| *Rickia*       | *wasmannii*                      | DE_Rak4 | Myrmica scabrinodis   | HUNGARY: Rakaca   | A. Tartally       |
| *Zodiomyces*   | *vorticellarius*                 | MG003   | Helocharas obscurus   | POLAND: Warsaw    | M. Gorczak        |
| 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       | QI Amp 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*, *Haploomyces*, 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 *Haploomyces* 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 *Rodauceae* 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 from 2001.

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 suitable 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 design. 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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