# Species Diversity and Production of Antimicrobial Compounds by Pacific Northwestern Clavicipitalean Entomogenous Fungi (Cordyceps spp.) 

by

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#### Abstract

Members of the fungal genus Cordyceps and its anamorphic genera are mostly pathogens of arthropods (insects, spiders, mites) with a few species parasitizing hypogeous fungi (Elaphomyces spp.). They produce numerous secondary metabolites with a wide range of biological activities which facilitate their life cycle and ecological roles. Examples include immunosuppressant, antibacterial, antifungal, antiviral, cytotoxic, and insecticidal compounds. This research focused on entomogenous fungi collected from British Columbia, Oregon, and Washington.

Twenty-eight isolates were selected for DNA sequencing and phylogenetic analysis. Genomic DNA was extracted from mycelium grown on agar media. PCR was used to amplify the ribosomal ITS and LSU regions which were then sequenced. Examination of morphology and DNA sequences grouped the isolates into twelve distinct taxa within the family Clavicipitaceae (Ascomycota). Most were from the teleomorphic genus Cordyceps and its anamorphic genera Beauveria, Isaria, Tolypocladium, and Metarhizium. One isolate was in Lecanicillium, an anamorph of the spider and scale pathogen Torrubiella.

To screen for antibacterial secondary metabolites, seven isolates from four species of entomogenous fungi were grown on different liquid media. Cultures were filtered through a fine mesh screen to separate the mycelium from the liquid broth. They were then frozen, freeze dried, and extracted in 100\% methanol. Antibacterial activity was tested using the disc-diffusion bioassay. The crude extracts from five of six fungi showed significant inhibition of the gram-positive bacteria Staphylococcus aureus, $S$. aureus methicillin resistant, Bacillus subtilis, and Enterococcus faecalis. None showed


activity against the gram-negative Escherichia coli, and Salmonella typhimurium. The minimum inhibition concentration for extracts showing bioactivity was determined using serial dilutions in a ninety-six well plate. The phenol-red overlay method allowed for the integration of thin layer chromatography and bioactivity. One fungus was selected for further chemical investigation. Using a combination of liquid-liquid partitioning, preparative column chromatography, and preparative thin layer chromatography, three compounds showing antimicrobial activity were isolated. Structural elucidation of these compounds using MS and NMR is currently underway.

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## Chapter 1 INTRODUCTION

### 1.1 History and current taxonomy of Cordyceps and related genera

Insect pathogens are found in all four divisions of the true fungi
(Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota) (Goettel, Inglis \& Wraight 2000). The genus Cordyceps (Ascomycota: Clavicipitaceae) is of particular interest, and is the major focus of this study. Cordyceps species are cosmopolitan in distribution, and are mostly pathogens of arthropods (insects, spiders, and mites) with a few species occurring on hypogeous fungi (Elaphomyces spp.) (Kobayasi 1941; Mains 1954, 1957; Kobayasi \& Shimizu 1977; Kobayasi 1982; Hawksworth et al. 1995). These fungi have attracted much attention from mycologists, entomologists, chemists, and ecologists. They are most curious fungi and have highly unique life cycles involving complex chemical and biological interactions. Studies by several researchers in the early twentieth century laid the foundation for the current understanding of species diversity within Cordyceps (Berkeley 1843; Atkinson 1894; Massee 1895; Lloyd 1915b, 1915a, 1916d, 1916c, 1916b, 1916a, 1916e, 1917, 1918, 1920; Speare 1920). By 1915, around 160 species were named, although many were considered dubious (Lloyd 1915b). Subsequent work by Petch further described species of Cordyceps and related genera (such as the teleomorphic genus Torrubiella and various asexual, anamorphic genera) (Petch 1921, 1923, 1931, 1934, 1937, 1939, 1942, 1944). At the time of Kobayasi's 1941 monograph of the genus Cordyceps and its allies, over 200 species of Cordyceps had been proposed. He transferred 20 species to new genera, excluded 16 due to lack of information, synonymized 60 names, and added 13 new species. Ultimately he
recognized 124 species (Kobayasi 1941). Work by Mains in the 1930s, 40 s , and 50 s addressed many of the species occurring in North America (Mains 1939b, 1939a, 1940, 1947, 1949, 1950, 1951, 1954, 1957, 1958). In his 1982 key to the genera Cordyceps and Torrubiella, Kobayasi recognized 282 species of Cordyceps, 59 species of Torrubiella, and 75 species of other genera, and this scheme is generally accepted today (Kobayasi 1982).

Taxonomy of and relationships among Cordyceps species are complicated by the fact that these fungi exist in both teleomorphic (sexual) and anamorphic (asexual) states. A single teleomorph genus (Cordyceps spp.) has anamorph species in different genera (such as Beauveria bassiana, Isaria japonica, Lecanicillium militaris, Metarhizium anisopliae, Tolypocladium niveum, etc.), while several teleomorph species in different genera (Cordyceps militaris, Torrubiella confragosa) have anamorphs in the same genus (Lecanicillium militaris, Lecanicillium lecanii) (Zare \& Gams 2001a; Stensrud, HywelJones \& Schumacher 2005). Before molecular techniques became widely available, to sufficiently prove teleomorph-anamorph connections it was necessary to either produce mature stromata from an anamorphic isolate or produce conidia from cultures established from teleomorphic spores or tissue. Currently, DNA evidence is aiding the understanding of teleomorph-anamorph connections, and has supported the placement of many hyphomyceteous entomogenous fungi within the family Clavicipitaceae (Liu et al. 2002; Yokoyama, Yamagishi \& Hara 2004). A number of species occur in nature almost exclusively in either teleomorphic (C. militaris) or anamorphic (B. bassiana, M. anisopliae) forms. There is also evidence supporting heteroxenous life cycles, where a single species has two or more synanamorphs. An example is Harposporum and its

Hirsutella-like synanamorph which infect nematodes and insects respectively (Hodge 2003).

Substantial effort has been placed on developing a phylogenetic description of Cordyceps and related genera based on DNA sequence analysis (Gams et al. 1998; Nikoh \& Fukatsu 2000; Artjarlyasripong et al. 2001; Stensrud, Hywel-Jones \& Schumacher 2005). Recent molecular-based revisions of the genera Verticillium and Metarhizium and current studies on Beauveria and Isaria have attempted to provide nomenclature that reflects monophyletic groupings (Curran et al. 1994; Driver, Milner \& Trueman 2000; Zare, Gams \& Culham 2000; Gams \& Zare 2001; Obornik, Jirku \& Dolezel 2001; Sung et al. 2001; Zare \& Gams 2001a, 2001b; Zare, Gams \& Evans 2001; Gams \& Zare 2002; Luangsa-Ard, Hywel-Jones \& Samson 2004; Gams et al. 2005; Hodge et al. 2005; Luangsa-Ard et al. 2005; Rehner \& Buckley 2005).

Although the genus Cordyceps and allied genera are cosmopolitan in distribution (Hawksworth et al. 1995) and a number of studies have looked at diversity in North America (Mains 1939b, 1939a, 1951; Ginns 1988; Guzman, Moron \& Ramirez-Guillen 2001; Sung \& Spatafora 2004), only a small number of studies have examined specimens from northwestern North America (Mains 1940, 1947, 1950, 1957, 1958; Jovel 2002).

To date, at least 23 species of clavicipitalean entomogenous fungi are known from the Pacific Northwest. Twelve species were collected in this study, and eleven additional species are known from previous work in our lab, the literature, culture collections, and herbarium specimens. Previous studies grouped the twelve species from this study into three clades (Nikoh \& Fukatsu 2000; Stensrud, Hywel-Jones \& Schumacher 2005).

Clade I contains B. bassiana, B. brongniartii, C. militaris, I. cicadae, I. farinosa, Isaria
sp., and L. muscarium; clade II is represented in this study by only M. anisopliae; clade III contains C. capitata, C. heteropoda, C. ophioglossoides, and T. cylindrosporum. In addition to taxa included in this study, the Cordyceps species C. myrmecophila (Mains 1940, 1947, 1958), C. ravenelii (Massee 1895; Mains 1940; Jovel 2002), C. subsessilis (Mains 1958), and C. washingtonensis (Mains 1947, 1958) are known from the Pacific Northwest. The anamorphic species Akanthomyces aculeata (Mains 1950), Gibellula pulchra, Gibellula sp. (Jovel 2002), L. lecanii, M. album (Humber \& Hansen 2005), and Paecilomyces marquandii (Jovel 2002) have also been reported. Additionally, a Torrubiella sp . has been reported (Jovel 2002) This region is still relatively unexplored, and continued investigation will surely describe further diversity.

### 1.2 Infection mechanisms

Entomopathogenic have been successfully employed as biological control agents, and elaborate relationships between the fungus and insect have been described (Goettel \& Inglis 1997; Goettel, Inglis \& Wraight 2000; Inglis et al. 2001). A majority of hypomycetous entomopathogenic fungi infect their hosts by direct penetration of the external cuticle. This is in contrast to most bacterial and viral insect pathogens, which invade via the alimentary canal. After spore attachment to the cuticle, the fungus penetrates and proliferates within the host insect using a variety of enzymatic, mechanical, and chemical means. Appressoria are sometimes formed to facilitate penetration, and thigmotropic signals are often required for induction of appressoria. In order to successfully establish within the insect, the fungus must proliferate within the haemocoel. The insect typically responds to infection through humoral and cellular
mechanisms (Gillespie et al. 2000). To evade detection, the fungus grows as hyphal bodies, which often lack cell walls and/or specific surface residues and are not recognized by the insect. They may also mimic surface epitopes on insect haemocytes (Inglis et al. 2001). The recent discovery of a group of yeast-like endosymbionts derived from a Cordyceps lineage adds a new dimension of complexity to the interplay of fungi and insects (Suh, Noda \& Blackwell 2001).

Environmental conditions are very important for successful infection. Even under appropriate conditions, the fungus may be present in sub-lethal quantities or may not infect at all. After the fungus has become established in the insect, death may be rapid or can take much longer. Death is facilitated by depletion of nutrients, physical obstruction or invasion of vital organs, and toxicosis. Following insect death, the fungus switches to mycelial growth and proliferates saprotrophically, subsisting on the remaining nutrients from the insect cadaver (Inglis et al. 2001). The fungus may then reproduce asexually, producing conidia arising directly from the mycosed cadaver or on synnemata. Under appropriate environmental and host conditions, ascomyceteous entomogenous fungi may produce a telomorphic form and reproduce via ascospores produced within perithecia, usually formed on an aboveground stroma. The infectious spores or spore fragments are then dispersed to other insects and the cycle repeats itself. Some members of the Clavicipitaceae are plant endophytes, and a few of the entomopathogenic species also show endophytic characteristics. B. bassiana has been documented to persist as an endophyte in maize and infect insects infesting the plant (Wagner \& Lewis 2000). Furthermore, some species of entomopathogenic anamorphs may use scale insets as a
gateway to endophytic colonization of plants (like Hyperdermium) or access to plant phloem via the dead insects' mouthparts (such as Hypocrella) (Hodge 2003).

### 1.3 Secondary metabolism of Cordyceps and related anamorphs

Insect pathogenic fungi, particularly those in the family Clavicipitaceae, are a rich source of biologically active natural products (Isaka et al. 2005). These compounds have diverse structures and biological activities, and arise from five major sources: 1) amino acids, 2) the shikimic acid pathway, 3) the polyketide pathway, 4) the mevalonic acid pathway, and 5) polysaccharides and peptidopolysaccharides (Vey, Hoagland \& Butt 2001). Examples include immunosuppressant, cytotoxic, antimicrobial, antiviral, and neuritogenic compounds (Table 1.1). Generally, these compounds may facilitate fungal penetration and proliferation, disrupt host defense responses, protect the fungus from host defenses, or play roles in signal transduction pathways (Yoder \& Turgeon 2001). They are also likely involved in defense against, and competition with, other microorganisms. The cyclic nonribosomal peptide cyclosporine was first isolated by researchers at Sandoz Ltd of Basel, Switzerland from T. niveum (syn. T. inflatum, B. niveum), isolated from a Norwegian soil sample. Cyclosporine showed limited antifungal activity, but strong immunosuppressive activity and low animal toxicity. It later became the first metabolite from a microorganism to be clinically used to regulate the growth and function of a normal mammalian cell and revolutionized the field of organ transplantation (Hassan \& Al-Yahya 1987; Tribe 1998). A quarter of a century later, it was determined that $T$. niveum is the anamorph of the insect pathogen C. subsessilis (Hodge, Krasnoff \& Humber 1996), and the chemical ecological role of the immunosuppressive compound

Table 1.1: Some previously reported metabolites and their biological activities from clavicipitalean entomogenous fungi and other organisms.

| fungus | chemical | type | activity | references | date |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Tolypocladium inflatum Verticillium lecanii Beauveria spp. | cyclosporine | cyclic peptide | immunosuppressant, insecticidal (mosquito, but not Galleria mellonella) | (Hassan \& Al-Yahya 1987; Tribe 1998; Vey, Hoagland \& Butt 2001) | 1973 |
| Metarhizium anisopliae <br> Trichotecium roseum <br> Alternaria brassicae <br> Opheosphaerella herpotricha Aschersonia sp. | destruxins | cyclic peptide ( $\alpha$ hydroxy acid + 5 AA $=$ hexadepsipeptide) | insecticidal, phytotoxic, cytotoxic (L1210 leukemia cells), antiviral (hepB), immunodepressant, antifeedant, antifungal, prevent metamorphosis, in hibit vacuolar type $\mathrm{H}+$-translocating ATPase - acidify phagocytic vaccuoles) | (Vey, Hoagland \& Butt 2001; Pedras, Zaharia \& Ward 2002) | 1961 |
| Metarhizium anisopliae | swainsinone | indolizidine alkaloid | anti-viral, anti-tumour | (Tamerler et al. 1998; Vey, Hoagland \& Butt 2001) |  |
| Metarhizium anisopliae | cytochalasin C |  |  | (Vey, Hoagland \& Butt 2001) |  |
| Beauveria bassiana | bassianin |  | inhibit ATPase, disrupt erythrocyte membranes | (Jeffs \& Khachatourians 1997; Vey, Hoagland \& Butt 2001) | 1968 |
| Beauveria bassiana <br> Paecilomyces fumosoroseus <br> Fusarium spp. <br> Polyporus sulphureus | beauvericin | cyclic peptide | insecticidal, antiplasmodial | (Gupta et al. 1991; Vey, Hoagland \& Butt 2001) |  |
| Beauveria bassiana | bassianolide |  | insecticidal | (Vey, Hoagland \& Butt 2001) |  |
| Beauveria bassiana <br> Beauveria brongniartii <br> Paecilomyces fumosoroseus | beauverolide | cyclic tetradepsipeptide $(\mathrm{MW}=516)$ | insecticidal?, immunosuppressant? | (Vey, Hoagland \& Butt 2001) |  |
| Beauveria bassiana | tenellin |  | inhibit ATPase, disrupt erythrocyte membranes | (Vey, Hoagland \& Butt 2001) | 1968 |
| Beauveria brongniartii Beauveria spp. <br> Chaetomium trilaterale | oosporein | dibenzoquinone | antiviral, antibacterial, plant growth inhibitor, phytotoxic, animal toxin | (Jeffs \& Khachatourians 1997; Vey, Hoagland \& Butt 2001) | 1955 |
| Paecilomyces fumosoroseus | pyridine-2,6-dicarboxylic acid |  |  | (Vey, Hoagland \& Butt 2001) |  |
| Verticillium lecanii | dipcolonic acid |  |  | (Vey, Hoagland \& Butt 2001) |  |
| Verticillium lecanii | hydroxycarboxilic acid |  |  | (Vey, Hoagland \& Butt 2001) |  |
| Hirsutella thompsonii | hirsutellin A, B | $\begin{aligned} & 34 \mathrm{AA} \text { peptide (HA) } \\ & (\mathrm{MW}=12 \mathrm{kDa}) \end{aligned}$ | miticidal, insecticidal | (Mazet \& Vey 1995; Vey, Hoagland \& Butt 2001) | 1995 |
| Hirsutella thompsonii | phomalactone |  |  | (Vey, Hoagland \& Butt 2001) |  |
| Cordyceps militaris | cordycepin | 3'-deoxyadenosine | insecticidal, antiviral, anti-tumour, cytotoxic, inhibits RNA transcription, antibacterial (Clostridium perfringens, Bacillus subtilis) | (Kim et al. 2002) |  |
| Isaria japonica | AETD | trichthecene | apoptosis inducer | (Pae et al. 2003) | 2003 |
| Isaria japonica | (3R,6R)-4-Methyl-... |  | apoptosis inducer | (Oh et al. 2002) | 2002 |
| Isaria sinclairiii | Myriocin |  | immunosuppressant ( $10-100 \mathrm{x} \mathrm{CsA}$ ), antifungal | (Fujita et al. 1996) | 1972 |
| Paecilomyces tenuipes Fusarium spp. | acetoxyscirpenediol | trichothecenes | cytotoxic, antitumour | (Nam et al. 2001) | 1978 |


| Paeciomyces sp. | Saintopin, UCE 1022 |  | antitumor, antibacterial (gram-positive) | (Yamashita et al. 1990) | $\begin{aligned} & \hline 1990, \\ & 1994 \\ & \hline \end{aligned}$ |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Paecilomyces farinosus | paecilosetin | tetramic acid derivative | antibacterial, anti-leukemia (P388 cell line) | (Lang et al. 2005) | 2005 |
| Tolypocladium niveum T. cylindrosporum | efrapeptins | 16 AA linear peptides | antibacterial, antifungal, insect toxin, antimalarial (anti Plasmodium falciparum), anti-Trypanosoma cruzi | (de Flonbaum \& Stoppani 1981; Krasnoff et al. 1991; Krasnoff \& Gupta 1992; Bandani et al. 2000; Nagaraj et al. 2001) | 1991 |
| Tolypocladium extinguens | 6-methoxymethyleugenin (1) 2-hydroxymethyl-6methoxymethyleugenin (2) | chromone derivatives | cytotoxic (leukemia P388 cells) | (Feng et al. 2002) | 2002 |
| Akanthomyces gracilis | akanthomycin |  | antibacterial | (Wagenaar, Gibson \& Clardy 2002) | 2002 |
| Paecilomyces tenuipes | spirotenuipesine A and B | trichothecanes | neurotrophic factor biosynthesis | (Kikuchi et al. 2004) | 2004 |
| Paecilomyces farinosus | farinosone A-C | pyridone alkaloid |  | (Cheng et al. 2004) | 2004 |
| Paecilomyces militaris | militarinones A-D | pyridone alkaloid | neuritogenic | (Schmidt et al. 2002; Schmidt et al. 2003) | 2002 |
| Cordyceps sinensis |  | sterols | anticancer | (Bok et al. 1999) | 1999 |

became more clear. In the early 1970s, the alkylaminopropanediol compound myriocin (identical with thermozymocidin and ISP-I) was isolated from several fungi, and has recently been isolated from I. sinclairii, an entomopathogen. Although its structure and mechanism of action is different from the cyclosporines, this compound also shows antifungal and immunosuppressive activity (Fujita et al. 1996). Another well characterized family of cyclic peptides are the destruxins, produced by several species of plant and insect pathogenic fungi including the entomopathogenic M. anisopliae. In addition to suppressing immune response in insect cells, they are also insecticidal, phytotoxic, cytotoxic, antiviral, and act as virulence factors (Pedras, Zaharia \& Ward 2002). They may be useful as targets for selection or engineering on hyper-virulent strains of Metarhizium. Efrapeptins are linear peptides produced by Tolypocladium spp., and show insecticidal, antifungal, antibacterial, and antimalarial activity (Krasnoff et al. 1991; Bandani et al. 2000; Nagaraj et al. 2001). In terms of chemical ecology, the antimalarial activity is particularly interesting, since T. cylindrosporum is a common pathogen of Aedes mosquitoes, the vectors of Plasmodium spp. causing malaria. Furthermore, efrapeptins are inhibitory to mitochondrial ATPase from Trypanosoma cruzi, the protozoan responsible for Chagas' disease, also vectored by insects (de Flonbaum \& Stoppani 1981). I. japonica produces the cytotoxic compound 4-acetyl-12,13-epoxyl-9-trichothecene-3,15-diol which induces apoptosis in human leukemia HL60 cells (Pae et al. 2003). I. japonica also produces the structurally related trichothecanes spirotenuipesine A and B. These compounds show potent activity in neurotrophic factor biosynthesis in glial cells, causing them to branch and divide in cell cultures (Kikuchi et al. 2004). Several pyridone alkaloids have been isolated from

Beauveria and Isaria spp. which differ in the length of their acyl side chains and functional groups. These include bassianin, tenellin, pyridovericin, pyridomacrolidin, militarinones A-D, and farinosones A-C (Wat et al. 1977; Takahashi et al. 1998; Schmidt et al. 2002; Schmidt et al. 2003; Cheng et al. 2004). Although structurally unrelated to spirotenuipesine A and B , militarinone A and farinosone A and C also showed neuritogenic activity (Schmidt et al. 2002; Cheng et al. 2004). These compounds may be partly responsible for altered behavior such as the "summit syndrome," where infected insects climb to the top of surrounding vegetation (Evans 1982; Inglis et al. 2001). They may also prove useful in the treatment of neurodegerative disorders such as Alzheimer's disease (Cheng et al. 2004). Militarinone D is cytotoxic (Schmidt et al. 2003), while the biological functions of the other pyridone alkaloids remain undetermined. Clearly, clavicipitalean entomogenous fungi produce many secondary metabolites with diverse structures and biological functions.

### 1.4 Screening for natural products

Successful natural product screening programs are dependant on a combination of extract library generation, rapid bioassay methods, and chemical characterization. In order to maximize metabolic diversity from existing genetic diversity, it is important to systematically culture each isolate under varied conditions. Important variables include:

- multiple isolates from the same species
- shaken liquid vessels, solid media, or in vivo inoculation of insects
- media of different composition
- media with at least two pH levels
- incubation at multiple temperatures
- incubation at multiple shaker speeds
- incubation for different lengths of time (Knight et al. 2003)

In this thesis, I explored strain-to-strain variation within a species, the effect of solid versus liquid media, and the effect of media composition on metabolite production.

Once a diverse extract library has been generated, it is necessary to have a fast, efficient, and biologically significant assay technique. Many medically relevant screens employ high-throughput automated strategies. Such assays are conducted in cell-free or whole-cell conditions, and monitor receptor-ligand interactions, enzymatic activities, or cellular integrity (Ireland et al. 2003). Antibacterial susceptibility tests provide a fast, clear, effective and well established technique for screening of chemical or extract libraries (Lennette 1985; Omar et al. 2000). Many of these methods involve the diffusion of active compounds through agar. Extracts and compounds are introduced to an inoculated bacterial lawn via a small paper disk (Bauer et al. 1966; Hamburger \& Cordell 1987). Phenol-red combined with an MTT spray or other indicators are sometimes employed to aid visualization (Hamburger \& Cordell 1987; Rios, Recio \& Villar 1988). To more carefully quantify the minimum inhibitory concentration, serial dilution of extracts in liquid media and 96-well plates is an effective method. Using a combination
of chromatographic techniques, and bioassay-guided fractionation, pure compounds are isolated. Their structures can then be elucidated using nuclear magnetic resonance (NMR), mass spectrometry (MS), and other physico-chemical techniques.

### 1.5 Thesis objectives

The major objectives of this thesis were to:

- further knowledge of clavicipitalean entomogenous fungal species diversity in the Pacific Spirit Regional Park (Vancouver, BC), with additional collections from other sites in British Columbia, Washington, and Oregon,
- evaluate phylogenetic relationships among collected fungi based on nrDNA sequence analysis,
- screen selected northwestern isolates for production of antimicrobial compounds, and - use bioassay-guided fractionation to separate novel antimicrobial compounds from crude fungal extracts.

Previous work in our laboratory has explored diversity of these fungi in the Pacific Northwest and documented biological activity in extracts of these fungi through antiviral, antifungal, antibacterial, antioxidant, and fatty acid assays (Jovel 2002). The current work described in this thesis builds on this work and addresses species diversity, phylogenetic relationships, and production of antimicrobial compounds from additional collections of Pacific Northwestern entomogenous fungi.

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# Chapter 2 DIVERSITY, PHYLOGENETIC RELATIONSHIPS, AND GEOGRAPHICAL DISTRIBUTION OF THE GENUS CORDYCEPS AND RELTATED GENERA FROM PACIFIC NORTWESTERN NORTH AMERICA ${ }^{1}$ 

### 2.1 Introduction

The fungal genus Cordyceps (Ascomycota; Clavicipitaceae) is cosmopolitan in distribution, and consists mostly of pathogens of arthropods (insects, spiders, and mites) with a few species occurring on hypogeous fungi (Elaphomyces spp.) (Kobayasi 1941; Mains 1954, 1957; Kobayasi \& Shimizu 1977; Kobayasi 1982; Hawksworth et al. 1995). Early research on Cordyceps recognized that many of the species occur in both an ascigerous (teleomorph) and a conidial (anamorph) state (Massee 1895). This has caused a substantial amount of confusion in the taxonomy of these organisms. A single teleomorph genus (Cordyceps) has multiple anamorph genera (Beauveria, Isaria, Lecanicillium, Metarhizium, Tolypocladium, etc.), while several teleomorph genera (Cordyceps, Torrubiella) share a common anamorph genus (Lecanicillium) (Zare \& Gams 2001a; Stensrud, Hywel-Jones \& Schumacher 2005). DNA sequence analysis has helped clarify teleomorph-anamorph connections for a growing number of taxa (Liu et al. 2002; Yokoyama, Yamagishi \& Hara 2004). Another major issue regarding the taxonomy of Cordyceps and related genera is that many anamorphs were originally classified as Fungi Imperfecti and their taxonomic positions did not necessarily reflect common ancestry. Several DNA sequence-based studies have addressed the phylogenetic relationships among Cordyceps and related genera (Gams et al. 1998;

[^0]Nikoh \& Fukatsu 2000; Artjarlyasripong et al. 2001; Stensrud, Hywel-Jones \& Schumacher 2005). Additionally, the relationships of the anamorphs formerly in Verticillium sect. Prostrata (Zare, Gams \& Culham 2000; Gams \& Zare 2001; Sung et al. 2001; Zare \& Gams 2001a, 2001b; Zare, Gams \& Evans 2001; Gams \& Zare 2002), Paecilomyces sect. Isarioidea (Obornik, Jirku \& Dolezel 2001; Luangsa-Ard, HywelJones \& Samson 2004; Gams et al. 2005; Hodge et al. 2005; Luangsa-Ard et al. 2005), Metarhizium (Curran et al. 1994; Driver, Milner \& Trueman 2000), and Beauveria (Rehner \& Buckley 2005) have been or are currently being re-evaluated.

There have been numerous publications on the occurrence and diversity of Cordyceps and related genera in North America (Mains 1939b, 1939a, 1951; Ginns 1988; Guzman, Moron \& Ramirez-Guillen 2001; Sung \& Spatafora 2004), however most focus on Eastern collections and only included a limited number of specimens from the Pacific Northwest (Mains 1940, 1947, 1950, 1957, 1958; Jovel 2002). The major objective of this study was to continue a survey of the diversity of clavicipitalean entomopathogenic fungi in northwestern North America (Jovel 2002). Fungi were collected throughout the region, and species were identified using macro- and micro-morphologies and ribosomal internal transcribed spacer (ITS) and large subunit (LSU) DNA sequences. Inclusion of GenBank sequences helped place the collections from this study in the context of previously mentioned phylogenetic studies.

### 2.2 Materials and methods

### 2.2.1 Site descriptions and collection of specimens

Samples were collected at eight locations throughout the Pacific Northwest (Fig.
2.1). Specimens were collected by visual encounter. The major collection site was the Pacific Spirit Regional Park (PSRP), which surrounds the main campus of the University of British Columbia in Vancouver, BC, Canada. Four additional sites in BC were sampled in addition to the PSRP: Campbell River, Roberts Creek, Squamish, and the Queen Charlotte Islands. Three sites in the United States were sampled: Detroit, OR, Trout Lake, WA (near Gifford Pinchot National Forest and Mt. Adams), and Renton, WA


Figure 2.1: Locations of collection sites in the Pacific Northwest
(near Seattle). A review of the literature, local herbaria (University of British Columbia, University of Washington, Oregon State University), the ARSEF culture collection at Cornell University, and several personal communications helped to identify further taxa known from the Pacific Northwest, although no specimens or sequences from these taxa were examined.

### 2.2.2 Isolation of fungi

Mycosed insect cadavers and parasitized Elaphomyces sp. were carefully collected in the field and placed in individual, sterile containers. Most specimens were anamorphic, and were producing conidia either directly from the insect host or on synnemata. Conidia were transferred under sterile conditions to nutrient agar, either malt-yeast-peptone (MYP) [half-strength Bandoni formulation; 7 g malt extract (Becton Dickenson), 1 g peptone (Difco), 0.5 g yeast extract (Becton Dickenson), 17 g agar (Sigma), 11 distilled $\mathrm{H}_{2} \mathrm{O}$ ], Sabaraud dextrose (SD) (Becton Dickenson), or potato dextrose (PD) (Becton Dickenson) and incubated at $25^{\circ} \mathrm{C}$. In most cases, this resulted in a single, axenic culture. In some cases, subculturing was necessary to isolate a pure culture. For Cordyceps where fresh material was available, the stroma was carefully torn lengthwise, and a small piece of mycelium was transferred to nutrient agar from the freshly exposed tissue. For Cordyceps specimens where only dried material was available, ascospores were transferred to nutrient agar using a sterile inoculation needle. Fungi were identified via morphological characters and taxonomic keys (Kobayasi 1941; Mains 1957, 1958; Kobayasi 1982; Bissett 1983; Ginns 1988; Humber 1998). These
identifications were complemented by a GenBank BLAST search using ITS and LSU nrDNA sequences.

After isolation of pure cultures, working cultures were established on MYP, SD, and PD agar, incubated at $25^{\circ} \mathrm{C}$ until colonies reached a diameter of 2 cm , and then kept at $10-15^{\circ} \mathrm{C}$. Stock cultures were established on quarter-strength MYP agar slants in 20 ml vials at $25^{\circ} \mathrm{C}$, then kept at $4^{\circ} \mathrm{C}$. For those fungi producing conidia in culture, conidial suspensions were prepared using a cryopreservation medium [15 g trypticase soy broth, 75 ml glycerol ( $15 \%$ ), $100 \mu \mathrm{l}$ TWEEN-80 ( $0.02 \%$ ), 500 ml distilled $\mathrm{H}_{2} \mathrm{O}$ ]. Cultures were mostly grown on PD agar. After a few weeks, cultures were washed with 3-5 ml of cryopreservation medium and transferred to 1.5 ml cyropreservation vials, which were then kept at $-80^{\circ} \mathrm{C}$.

### 2.2.3 DNA extraction, PCR, and sequencing

DNA extraction and PCR amplification was done in collaboration with the Breuil lab (Faculty of Forestry, University of British Columbia) using a method adapted from previous studies (Lee \& Taylor 1990). DNA was extracted from mycelium cut from the surface of agar cultures and placed into micro-centrifuge tubes. To each tube was added $600 \mu \mathrm{l}$ of cell lysis buffer [0.5 M Tris- $\mathrm{HCl}(\mathrm{pH} 7.6)$ ]. The mixture was gently vortexed for 10 s and then incubated in a water bath at $75^{\circ} \mathrm{C}$ for 1 h . Tubes were then vortexed at $12,000 \mathrm{rpm}$ at room temperature for an additional 5 minutes. DNA was purified via addition of $500 \mu$ of phenol-chloroform-isoamylalcohol (25:24:1) to each tube and weakly vortexed until the layers mixed. Tubes were then centrifuged at $12,000 \mathrm{rpm}$ for 10 minutes at $4{ }^{\circ} \mathrm{C}$. The upper $500 \mu$ of the supernatant containing DNA was transferred
to a new tube, and the purification was repeated a second time. Then $300 \mu 1$ of the final supernatant was transferred to a new tube and DNA was precipitated with $200 \mu 1$ isopropanol. Tubes were then centrifuged at $12,000 \mathrm{rpm}$ for 10 minutes. After removing the supernatant, the pellet was washed with $70 \%$ ethanol, allowed to air dry, and resuspended in $50 \mu$ l of distilled $\mathrm{H}_{2} \mathrm{O}$. DNA was stored at $-20^{\circ} \mathrm{C}$ until further use.

To achieve PCR amplification of the ITS and LSU regions, fungal universal primers ITS5/ITS4 and LROR/LR3were used respectively. Reactions were carried out in a volume of $25 \mu \mathrm{l}$ and consisted of $5 \mu \mathrm{l}$ of 5 x thermal buffer containing 5 mM dNTPs, 0.3 $\mu 1$ of each primer ( 20 pmol ), 1 U Taq polymerase, $1 \mu \mathrm{l}$ of $1 / 10$ dilution DNA, and $18.3 \mu \mathrm{l}$ $\mathrm{ddH}_{2} \mathrm{O}$. Thermocycler conditions consisted of a 4:00 min denaturation step at $95^{\circ} \mathrm{C}$, followed by 29 cycles of 40 sec at $95^{\circ} \mathrm{C}, 50 \mathrm{sec}$ at $52^{\circ} \mathrm{C}$, and $1: 00 \mathrm{~min}$ at $72^{\circ} \mathrm{C}$, with a final extension time of $10: 00 \mathrm{~min}$ at $72^{\circ} \mathrm{C}$. To confirm amplification, $3 \mu 1$ of each PCR product was used for electrophoresis on agarose gel containing EtBr in Tris-acetate EDTA (TAE) buffer. The PCR product sizes were determined by comparison to a 1 kb DNA marker (Gibco BRL, USA). The PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen Inc.). Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer Inc. USA) at the DNA Sequence and Sequencing Facility, MACROGEN (Seoul, Korea).

### 2.2.4 Sequence analysis

Sequences were aligned using ClustalX. The quality of sequence data was determined by a visual comparison of each sequence to the original chromatogram.

Duplicate sequences were eliminated from the alignment. Additional sequences were
included from GenBank by perfoming BLAST and Entrez Nucleotide searches for each sequence. Most GenBank sequences had previously been published in journals, and•an effort was made to select the most representative sequences. In a few cases, there was either no close match or only unpublished sequences were available. Trees were generated using PAUP.

### 2.3 Results

### 2.3.1 Fungal diversity

Morphological and molecular characters grouped 37 fungal isolates into 12 distinct taxa (Table 2.1, Appendix I). ITS and LSU sequences were invariable within taxa and divergent among taxa. Four taxa were in the teleomorphic genus Cordyceps (the mycopathogenic C. capitata and C. ophioglossiodes and the entomopathogenic $C$.
heteropoda and C. militaris). The other 8 taxa were entomopathogenic anamorphs of either the genus Cordyceps (Beauveria bassiana, B. brongniartii, Metarhizium anisopliae, Isaria cicadae, I. farinosa, Paecilomyces sp., and Tolypocladium cylindrosporum) or the closely related genus Torrubiella (Lecanicillium muscarium).

Within the PSRP, 8 taxa were collected. An unidentified Paecilomyces sp. was the most common species. I. farinosa was the next most common species. Both species were often found in groups, but were occasionally found singly. When producing synnmata from buried host insects, the two species were very similar in macromorphology. However, when Paecilomyces sp. was found on insects not buried, wrapped in leaf material, or inside small ( 1 cm diameter) branches, they produced conidia either directly on the surface of the host or from much reduced synnmata. Micro-morphologies

Table 2.1: List of fungi isolated in this study, culture numbers, hosts, geographic location, and results from BLAST search.

| Fungi | Culture No. | Host ${ }^{\text { }}$ | Site ${ }^{2}$ | Closest match in BLAST search | NT differences [\% homology] $^{3}$ |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Beauveria bassiana | 52 | CA | A | Beauveria bassiana AY532023 (ITS) | 0/532 (100.00\%) |
| Beauveria brongniartii | $\begin{aligned} & 42 \\ & 74 \\ & 77 \end{aligned}$ | $\begin{aligned} & \mathrm{CA} \\ & \mathrm{U} \\ & \mathrm{U} \end{aligned}$ | $\begin{aligned} & \text { A } \\ & \text { B } \\ & \text { A } \end{aligned}$ | Beauveria brongniartii AB027381 (LSU) | 0/555 (100.00\%) |
| Cordyceps capitata | Cc1p | E | C | Cordyceps capitata AY489721 (LSU) | 4/554 (99.28\%) |
| Cordyceps heteropoda | Cg | 0 | D | Cordyceps heteropoda AB027373 (LSU) | 15/578 (97.40\%) |
| Cordyceps militaris | CmE | LP | A | Cordyceps militaris AB027379 (LSU) | 0/553 (100.00\%) |
| Cordyceps ophioglossoides | Co | E | E | Cordyceps ophioglossoides AJ309360 (ITS) | $0 / 538(100.00 \%)$ |
| Isaria cicadae | 53 | U | F | Isaria cicadae AB086631 (ITS) | 1/553 (99.82\%). |
| Isaria farinosa | $\begin{aligned} & 7 \\ & 10 \\ & 11 \\ & 12 \\ & 22 \\ & 43 \\ & 47 \\ & 75 \end{aligned}$ | $\begin{aligned} & \mathrm{LP} \\ & \mathrm{U} \\ & \mathrm{U} \\ & \mathrm{U} \\ & \mathrm{U} \\ & \mathrm{U} \\ & \mathrm{U} \\ & \mathrm{U} \end{aligned}$ | $\begin{aligned} & \mathrm{A} \\ & \mathrm{~A} \\ & \mathrm{~A} \\ & \mathrm{~A} \\ & \mathrm{~A} \\ & \mathrm{~A} \\ & \mathrm{~A} \\ & \mathrm{~B} \end{aligned}$ | Isaria farinosa AY624181 (ITS) | 2/529 (99.62\%) |
| Lecanicillium muscarium | 20 | U | A | Torrubiella confragosa AJ292388 (ITS) | 0/561 (100.00\%) |
| Metarhizium anisopliae | 41 | O | A | Metarhizium anisopliae AB027383 (LSU) | 0/558 (100.00\%) |
| Paecilomyces sp. | $\begin{aligned} & 2 \\ & 6 \\ & 15 \end{aligned}$ | LP LP | $\begin{aligned} & \mathrm{A} \\ & \mathrm{~A} \\ & \mathrm{~A} \end{aligned}$ | Paecilomyces farinosus AY624179 (ITS) | 0/562 (100.00\%) |


|  | 16 | U | A |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: |
|  | 18 | U | A |  |  |
|  | 23 | U | A |  |  |
|  | 27 | LL | A |  |  |
|  | 46 | U | G |  |  |
|  | 48 | U | H |  |  |
|  | 51 | U | A |  |  |
|  | 55 | U | C |  |  |
|  | 63 | U | A |  |  |
|  | 68 | U | A |  |  |
|  | 69 | W | A |  |  |
|  | 70 | W | A |  |  |
|  | 71 | CA | A |  |  |
|  | 73 | H | A |  |  |
| Tolypocladium cylindrosporum | 4 | B | A | Tolypocladium cylindrosporum AB208110 (LSU) | 0/558 (100.00\%) |

${ }^{1}$ CA, coleoptera adult; E, Elaphomyces sp.; LP, lepidoptera pupa; LL, lepidopteran larva; O, orthoptera; W, wasp; H, homoptera; B, bee; U, unknown. ${ }^{2}$ Collection Site: A, Pacific Spirit Regional Park, Vancouver, BC, Canada; B, Renton, WA, USA; C, Breitenbush, OR, USA; D, Trout Lake, WA, USA; E, Campbell River, BC, Canada; F, Queen Charlotte Islands, BC, Canada; G; Robert's Creek, BC, Canada; H, Squamish, BC, Canada.
${ }^{3}$ Nucleotide differences [\% homology] was derived from matched nucleotide/compared nucleotide in GenBank
and DNA sequence analysis clearly separate the two taxa. The ITS sequence of $T$. cylindrosporum matched GenBank sequences of C. subsessilis/T. niveum as well as $T$. cylindrosporum, but morphology and the LSU sequence identified the collection as $T$. cylindrosporum. Two collections of $B$. brongniartii were made at separate locations within the park, and only single collections of B. bassiana, C. militaris, L. muscarium, M. anisopliae, and T. cylindrosporum were made, although numerous specimens of wireworms (Agriotes lineatus and $A$. obscurus) infected with $M$. anisopliae have been collected at the UBC farm, adjacent to the park. It was not uncommon to find multiple species occurring very near to each other. At one location, I. farinosa, Paecilomyces sp., M. anisopliae, and T. cylindrosporum were found within several meters of each other.

Outside of the PSRP eight species were collected. Four species (C. capitata, C. heteropoda, C. ophioglossoides, and I. cicadae) were not found within the PSRP, while four other species (B. brongniartii, I. farinosa, M. anisopliae, and Paecilomyces sp.) were found within the PSRP. The most abundant species were the two truffle (Elaphomyces spp.) pathogens C. capitata and C. ophioglossoides, which were each collected at two separate sites and often found in large groupings. C. heteropoda was found growing on buried orthopteran insects, in small groupings, dry, volcanic soil and sunny, open areas. This is in contrast to virtually every other collection described in this work, which were typically found in damp, shaded, forest soils. Three collections of the unidentified Paecilomyces sp. were made from three different locations outside of the PSRP. Only single collections of B. brongniartii, I. cicadae, and I. farinosa were made outside the park.

### 2.3.2 Phylogenetic analysis

ITS and LSU nrDNA sequences were obtained from each of the 12 taxa collected (Table 2.1). ITS sequences from 25 isolates and LSU sequences from 27 isolates were analyzed. Sequences were found to be invariable within taxa and divergent among taxa. Hypocrea rufa and H. lutea were selected as non-clavicipitalean outgroups, as in previous studies (Nikoh \& Fukatsu 2000; Artjarlyasripong et al. 2001; Stensrud, Hywel-Jones \& Schumacher 2005). Including sequences from GenBank, the ITS data matrix contained 37 taxa, 590 characters, 329 constant characters, 49 parsimony-uninformative variable characters, and 212 parsimony-informative characters. The LSU data matrix contained 30 taxa, 523 characters, 410 constant characters, 23 parsimony-uninformative variable characters, and 90 parsimony-informative characters (Appendix III). Trees were generated using parsimony analysis. ITS and LSU data sets were analyzed individually. Heuristic searches were performed using random stepwise addition of taxa. The best tree score for the ITS matrix was 558 , shared by 192 trees. The LSU search found a single tree with a score of 201. Branch support was generated using 100 bootstrap replicates. Among taxa collected in this study, three distinct clades were formed: clade I contains Beauveria bassiana, B. brongniartii, Cordyceps militaris, Isaria cicadae, I. farinosa, Isaria sp., and Lecanicillium muscarium; clade II consists of only Metarhizium anisopliae; clade III consists of C. capitata, C. heteropoda, C. ophioglossoides, and Tolypocladium cylindrosporum (Figs. 2.2, 2.3). Bootstrap supports below 60 are not presented.


Figure 2.2: Strict consensus most parsimonious tree of ITS sequences from the Pacific Northwest and GenBank; taxa from this study are indicated by "PNW" and representative strain number; taxa from GenBank are designated by species name and strain number (where available).


5 changes

Figure 2.3: Strict consensus most parsimonious tree of LSU sequences from the Pacific Northwest and GenBank; taxa from this study are indicated by "PNW" and representative strain number; taxa from GenBank are designated by species name and strain number (where available).

### 2.4 Discussion

Although the genus Cordyceps has been extensively studied worldwide, this study and previous work in our laboratory (Jovel 2002) are the first to explicitly examine the occurrence, diversity, and DNA sequences of the genus and its related anamorphic genera in northwestern North America. Four species of Cordyceps and 8 species of anamorphic genera were collected in this study. DNA sequence analysis largely agrees with previous studies on the phylogenetic relationships of clavicipitalean entomogenous fungi. ITS sequences were more variable than LSU sequences, and provided greater resolution of relationships among taxa, particularly in clade I.

Seven taxa from clade I were collected, representing $58 \%$ of the taxa included in this study. Teleomorphic species from this clade all have perithecia born in brightly colored (yellow to orange) acicular to clavate stromata (Stensrud, Hywel-Jones \& Schumacher 2005). C. militaris was collected in the Pacific Spirit Regional Park and kindly donated by Eduardo Jovel. This species is common and distributed worldwide (Mains 1958). The anamorph belongs to Lecanicillium (Zare, Gams \& Culham 2000; Sung et al. 2001; Zare \& Gams 2001a).
L. muscarium was also collected in the PSRP. A GenBank BLAST search found a $100 \%$ match to an ITS sequence published in the Verticillium revision (Zare, Gams \& Culham 2000). Additionally, searches using both ITS and LSU sequences found a $100 \%$ match to sequences labeled Torrubiella confragosa. Torrubiella is a clavicipitalean teleomorph genus, which is closely related to Cordyceps and is mostly pathogenic on spiders and scale insects (Mains 1954; Kobayasi \& Shimizu 1977; Kobayasi 1982). However, these sequences were likely from a related Torrubiella sp., since the anamorph
of $T$. confragosa is $L$. lecanii. Although ITS and LSU sequences from this study were clearly distinct from L. lecanii (Zare, Gams \& Culham 2000; Sung et al. 2001), it is reasonable that $L$. muscarium is a Torrubiella anamorph. $L$. muscarium has been collected on a variety of insects throughout the northern hemisphere (Humber \& Hansen 2005).

The two species of Isaria collected in this study are restricted to a well supported Isaria clade, despite a tenuous link to C. militaris and its Lecanicillium anamorph (Obornik, Jirku \& Dolezel 2001; Luangsa-Ard et al. 2005). Several collections of I. farinosa were made, and ITS sequences matched closely with the ex-epitype culture. This sequence also matches a sequence labeled Paecilomyces sp., but do not match closely with any other ITS or LSU sequences in GenBank. It would be useful to compare further sequences from the ex-epitype and related cultures. I. farinosa was collected at two locations, inside the PSRP and near Seattle (specimen donated by Alissa Allen). A specimen of I. cicadae (or a closely related species) grouped closely with I. farinosa as previously described (Luangsa-Ard et al. 2005). I. cicadae was only collected a single time from a northern location on the Queen Charlotte Islands, BC and was donated by Bryce Kendrick.

An unidentified species of Paecilomyces was the most common entomopathogen collected within the PSRP, and the most widely distributed outside the park. Both ITS and LSU sequences matched a number of sequences in GenBank labeled as $P$. farinosus, but not the sequence from the ex-epitype culture of I. farinosa (formerly P. farinosus). Morphologically, it is slightly different from I. farinosa. This sequence appears to be more closely related to C. militaris and Lecanicillium than to Isaria, suggesting that it
may be a Lecanicillium sp., and not an Isaria sp. Because there are numerous sequences in GenBank labeled $P$. farinosus that do not match the recently typified I. farinosa, this raises questions about the identification and classification of many isolates in culture collections and studies identified as P. farinosus (Obornik, Jirku \& Dolezel 2001; Luangsa-Ard et al. 2005). The teleomorph of $P$. farinosus was believed to be $C$. memorabilis (Pacioni \& Frizzi 1978), but this needs re-evaluation due to the questions concerning the typification of I. farinosa and identification of many P. farinosus isolates. It may be that this Paecilomyces sp., and not I. farinosa, is the anamorph of $C$. memorabilis.

Beauveria is allied to Lecanicillium and Isaria and may be monophyletic, however species within the genus are non-monophyletic and may include cryptic species (Rehner \& Buckley 2005). In this study, two species of Beauveria were collected and formed a well-supported group. B. bassiana is common worldwide, and has a wide host range. $B$. brongniartii on the other hand appears to be more restricted in known geographical distribution, occurring primarily in Europe and Asia (Rehner \& Buckley 2005). This may be the first report of B. brongniartii in North America, and was collected in the PSRP at two locations and was also collected near Seattle, WA (specimen donated by Alissa Allen). Although this isolate closely matched several B. brongniartii sequences in GenBank and falls within the $B$. brongniartii clade, it is slightly different than several of the isolates in that clade described by Rehner \& Buckley (2005), represented by relatively low bootstrap support.

Metarhizium anisopliae forms a separate clade, and is the only species from this clade collected in this study. M. anisopliae is a common entomopathogen widely used as
a biocontrol agent and model organism. It has significant genetic diversity, and $M$. anisopliae. var. anisopliae has a wide host range and is distributed worldwide (Driver, Milner \& Trueman 2000; Humber \& Hansen 2005).

The third clade in this study is formed by 4 species, C. capitata, C. heteropoda, C. ophioglossoides, and T. cylindrosporum. This is the only clade to contain species of Cordyceps pathogenic on Elaphomyces sp. (false truffles) (Mains 1957; Kobayasi 1982; Ginns 1988). C. capitata and C. ophioglossoides are both common in the northwest. C. ophioglossoides is widely distributed across North America, Europe, and Asia, and is the most common species occuring on Elaphomyces. C. capitata has a similar distribution (Kobayasi 1941; Mains 1957). Both species were collected near Campbell River, BC by Paul Kroeger. C. capitata was also collected near Detroit, OR. Although cultures and sequences were obtained from the dried C. ophioglossoides specimens, cultures and sequences from C. capitata were only obtained from the Oregon collection.
C. heteropoda strongly resembles C. capitata (yellow stroma with a red-ochre capitate head bearing perithecia), however it is smaller than C. capitata and infects insects, not Elaphomyces. C. heteropoda is often confused with C. gracilis and C. entomorrhiza, and is relatively rare. It is rarely reported from North America and these may be the first collections in the northwest. Several collections were made at different sites near the Gifford Pinchot National Forest, the active volcano Mt. Adams, and Trout Lake, WA.

A single collection of T. cylindrosporum was made in the PSRP. This species is believed to be a Cordyceps anamorph, and is often reported as a soil fungus as well as a pathogen of insects (Bissett 1983). The ITS sequence matched sequences from T. niveum
and its teleomorph C. subsessilis, however morphology and LSU sequences distinguished it from T. niveum. It may be that the ITS sequences in GenBank are mislabeled, or that ITS variation is insufficient to discriminate the taxa at a species level. It is interesting that the insect pathogenic C. heterpoda and T. cylindrosporum ally with the Elaphomyces (false truffle) pathogens C. ophioglossoides and C. capitata respectively. It is not clear to what degree species in this clade survive saprophytically in the soil, but the number of Tolypocladium isolates from soil would suggest some degree of persistence. If members of this clade can survive as non-entomogenous soil saprophytes, it may enhance infection of hypogeous fungi and buried insects. Phylogenetic relationships strongly suggest the ancestral host of this clade was an insect, and that similar host ecological niches (root associates) facilitated interkingdom host jumps once or multiple times within the clade (Nikoh \& Fukatsu 2000).

Numerous other species of entomogenous fungi have been reported in North America, and several are known or suspected to occur in the Pacific Northwest. Species of Cordyceps reported from the northwest but not collected in this study include, $C$. myrmecophila (Mains 1940, 1947, 1958), C. ravenelii (Massee 1895; Mains 1940; Jovel 2002), C. subsessilis (Mains 1958), and C. washingtonensis (Mains 1947, 1958). The anamorphic species Akanthomyces aculeata (Mains 1950), Gibellula pulchra, Gibellula sp. (Jovel 2002), L. lecanii, and M. album (Humber \& Hansen 2005), and P. marquandii have also been reported from the northwest. A Torrubiella sp. was also collected in the northwest (Jovel 2002)

Many researchers have studied the genus Cordyceps and its related anamorphic genera worldwide. The southern Appalachian Mountains of North America are generally
considered to be the richest region of the United States for Cordyceps diversity (Mains 1958). While this region is doubtless a Cordyceps hotspot, part of the diversity can be attributed to the large number of studies, both formal and informal, conducted in that area. Conversely, information on the occurrence of Cordyceps in Pacific Northwestern North America is relatively sparse. There are many regions in Oregon, Washington, British Columbia, and Alaska that are largely unexplored in terms of Cordyceps diversity. Further investigation in this area is warranted, and would significantly increase the knowledge of northwestern North American species diversity.

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# Chapter 3 EFFECT OF MEDIA ON PRODUCTION OF ANTIBACTERIAL COMPOUNDS BY NORTHWESTERN NORTH AMERICAN ENTOMOGENOUS FUNGI 

### 3.1 Introduction

Many pathogenic fungi utilize secondary metabolites to facilitate infection of their hosts. These compounds may remain within or attached to fungal cells, or they may be secreted to enhance direct interaction with the host. Although most of these compounds are not essential for growth in vitro (hence the term "secondary" metabolites), some have been proven to be necessary for successful infection of their hosts. Generally, these compounds may facilitate fungal penetration and proliferation, disrupt host defense responses, protect the fungus from host defenses, or play roles in signal transduction pathways (Yoder \& Turgeon 2001).

Insect pathogenic fungi, particularly those in the family Clavicipitaceae, are a rich source of biologically active natural products (Isaka et al. 2005). These compounds are diverse in structures and biological activities. Examples include the immunosuppressant cyclopeptide cyclosporine (Tribe 1998), the cytotoxic tetramic acid derivative paecilosetin (Lang et al. 2005), the antimicrobial napthacene-dione saintopin (Yamashita et al. 1990), the antiviral dibenzoquinone oosporein (Terry et al. 1992), and the neuritogenic pyridone alkaloid militarinone A (Schmidt et al. 2002).

Although many bioactive compounds have already been isolated from clavivipitalean entomogenous fungi, new compounds are discovered on a regular basis, even from well-studied organisms. A recent study of Japanese isolates showed that of 47 strains tested, $38(81 \%)$ produced anti-Bacillus compounds, and $30(64 \%)$ produced anti-

Staphylococcus compounds, and that media composition influenced production of antibacterial compounds in some isolates (Lee et al. 2005). Previous studies in our lab showed extracts from cultures of entomopathogenic fungi collected in the Pacific Northwest exhibit a range of biological activities, including antibacterial and ultraviolet light photoactivation (Jovel 2002). In this study, we screened five species of fungi isolated from insect cadavers for production of antibacterial metabolites, and note strain-to-strain variation and effects of growth medium on antibiotic production. Using bioassay-guided fractionation, two novel antibacterial peptides were isolated.

### 3.2 Materials and methods

### 3.2.1 Strain selection

Over 37 isolates from 12 species of clavicipitalean entomogenous fungi were isolated mostly from southern coastal British Columbia, with other sites in Pacific Northwestern North America (Chapter 2). Eight isolates from five of these species were screened for the production of antibacterial compounds. Strains were selected partly on their ability to produce abundant conidium on potato dextrose agar to serve as inoculum of liquid cultures. The species Beauveria bassiana, Lecanicillium muscarium, Metarhizium anisopliae, Paecilomyces sp., and Tolypocladium cylindrosporum were selected. One isolate from each species was collected in the Pacific Spirit Regional Park in Vancouver, BC. Three additional isolates of Paecilomyces sp. were included from Roberts Creek, BC (\#46), Squamish, BC (\#48), and Detroit, OR (\#55) Phylogenetic relationships among the taxa were inferred from analysis of ribosomal ITS and LSU DNA sequences as described in a previous study (Chapter 2, Fig. 3.1).

### 3.2.2 Strain isolation and maintenance

Mycosed insect cadavers and parasitized Elaphomyces sp. were carefully collected in the field and placed in individual, sterile containers. Most specimens were anamorphic, and were producing conidia either directly from the insect host or on synnemata. Conidia were transferred under sterile conditions to nutrient agar, either malt-yeast-peptone (MYP) [half-strength Bandoni formulation; 7 g malt extract (Becton Dickenson), 1 g peptone (Difco), 0.5 g yeast extract (Becton Dickenson), 17 g agar (Sigma), 11 distilled $\mathrm{H}_{2} \mathrm{O}$ ], Sabaraud dextrose (SD) (Becton Dickenson), or potato dextrose (PD) (Becton Dickenson) and incubated at $25^{\circ} \mathrm{C}$. In most cases, this resulted in a single, axenic culture. In some cases, subculturing was necessary to isolate a pure culture. For Cordyceps where fresh material was available, the stroma was carefully torn lengthwise, and a small piece of mycelium was transferred to nutrient agar from the freshly exposed tissue. For Cordyceps specimens where only dried material was available, ascospores were transferred to nutrient agar using a sterile inoculation needle. Fungi were identified via morphological characters and taxonomic keys (Kobayasi 1941; Mains 1957, 1958; Kobayasi 1982; Bissett 1983; Ginns 1988; Humber 1998). These identifications were complemented by a GenBank BLAST search using ITS and LSU nrDNA sequences.

After isolation of pure cultures, working cultures were established on MYP, SD, and PD agar, incubated at $25^{\circ} \mathrm{C}$ until colonies reached a diameter of 2 cm , and then kept at $10-15^{\circ} \mathrm{C}$. Stock cultures were established on quarter-strength MYP agar slants in 20 ml vials at $25^{\circ} \mathrm{C}$, then kept at $4^{\circ} \mathrm{C}$. For those fungi producing conidia in culture,


Figure 3.1: Phylogenetic relationships of collected taxa inferred from parsimony analysis of ITS nrDNA sequences; taxa selected for antimicrobial screening are indicated with arrows.
conidial suspensions were prepared using a cryopreservation medium [15 g trypticase soy broth, 75 ml glycerol ( $15 \%$ ), $100 \mu \mathrm{l}$ TWEEN-80 ( $0.02 \%$ ), 500 ml distilled $\mathrm{H}_{2} \mathrm{O}$ ].

Cultures were mostly grown on PD agar. After a few weeks, cultures were washed with $3-5 \mathrm{ml}$ of cryopreservation medium and transferred to 1.5 ml cyropreservation vials, which were then kept at $-80^{\circ} \mathrm{C}$.

### 3.2.3 Liquid cultures

Liquid MYP and SD media were prepared $(\mathrm{pH}=6.8)$ and 75 ml was added to individual Erlenmeyer flasks ( 250 mL ) and autoclaved at 15 PSI for 20 minutes. Each flask containing media was inoculated with $5 \times 10^{7}$ conidia, taken from cryopreserved conidial suspensions. Cultures were incubated at room temperature (around $20^{\circ} \mathrm{C}$ ), in the dark, on a rotary shaker ( 40 rpm ). After 15 days, cultures were filtered through fine mesh to separate mycelium from culture broth, and then freeze dried.

### 3.2.4 Extraction and bioassay

Freeze dried cultures were extracted using $100 \%$ methanol (MeOH) ( 5 ml MeOH : 1 g dried culture). Fungal material was extracted for 24 hours. To remove coarse material, soaked fungal material was loaded into a syringe, and extract was collected in microfuge tubes by compressing the material with the plunger. Extracts were centrifuged at $12,000 \mathrm{rpm}$ for 5 minutes to precipitate any remaining fine solids.

Gentamicin and MeOH were used as controls in all antibacterial bioassays. Both disc-diffusion (Fig. 3.2) and 96 well minimum inhibitory concentration (MIC) assays (Fig 3.3) were performed in duplicate, unless noted otherwise. Six strains of bacteria were used as indicators to identify extracts producing antimicrobial compounds. Four strains were gram-positive (Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus, and S. aureus Methicillin resistant) and two were gram-negative (Escherichia coli and Salmonella typhimurium).

The disc-diffusion method was used for initial screening of extracts (Bauer et al. 1966; Lennette 1985). Bacterial inoculum was incubated in 3 ml of Mueller-Hinton


Figure 3.2: Example of disc-diffusion antibacterial bioassay; here B. subtilis is inhibited by extracts 8, 104,105 and 106, but not 3 or the MeOH control.


Figure 3.3: Example of MIC assay. Numbers down the left hand side represent different extracts, MeOH , and gentamicin controls. Numbers across the top indicate final test concentrations (in $\mu \mathrm{l}$ extract $/ \mathrm{ml}$ media), with the first column containing only extract and media, and the final two columns containing only bacteria (no extract) and only media (no bacteria).
(MH) broth overnight at $37^{\circ} \mathrm{C}$ on a shaker. Overnight cultures were diluted with MH broth (1:9). A sterile cotton swab was used to streak inoculum on MH agar plates. Sterile paper discs ( 6 mm ) were impregnated with $20 \mu \mathrm{l}$ of extract, allowed to dry, and placed on the inoculated plate. Plates were incubated at $37^{\circ} \mathrm{C}$. Zones of inhibition were measured after 18 h .

For extracts showing activity in disc-diffusion assays, the MIC was determined by serial dilution of extracts in 96 well plates. In column one, $230 \mu \mathrm{l} \mathrm{MH}$ broth was added. In columns $2-10,100 \mu \mathrm{l}$ broth was added. Each row in column one had $20 \mu \mathrm{l}$ of a different extract or control (Gentamicin, MeOH ) added, for a final concentration of $80 \mu \mathrm{l}$ extract $/ \mathrm{ml}$ media. Serial dilutions were made by transferring $100 \mu \mathrm{l}$ from column one to column $2,100 \mu \mathrm{l}$ from column 2 to column 3, etc. for final concentrations of 40, 20, 10, $5,2.5,1.25,0.625,0.313$, and $0.156 \mu \mathrm{l} / \mathrm{ml}$ in columns $2-10$ respectively. Bacterial inoculum was prepared as in the disc bioassay. Overnight cultures were diluted (1:999) to approximately $10^{5} \mathrm{cfu} / \mathrm{ml}$. From this, $100 \mu \mathrm{l}$ was added to all rows, columns $2-10$ and column 12. Column 11 contained only broth. Plates were incubated at $37^{\circ} \mathrm{C}$ for 18 hours. MIC was determined to be the lowest concentration with a clear well.

### 3.2.5 Chromatography

Several solvent systems were tried, and $\mathrm{CH}_{2} \mathrm{Cl}_{2}$ : acetone: $\mathrm{MeOH}(3: 2: 0.5)+3$ drops of $90 \%$ formic acid gave the best resolution for most extracts. Plates were visualized under 254 nm ultraviolet light, and then developed by dipping in a solution of phosphomolybdic acid hydrate and heating in an oven at $100^{\circ} \mathrm{C}$ for 5 minutes. To test for the presence of peptides, 0.35 g ninhydrin was dissolved in 100 ml EtOH and used as
a dip. Plates were heated at $100^{\circ} \mathrm{C}$ for 10 minutes. The phenol-red overlay bioassay was used to attribute antibacterial activity to a spot on a TLC plate (Hamburger \& Cordell 1987). Media was prepared by autoclaving MH agar containing 0.02 g phenol red/L in 20 ml aliquots in large test tubes and kept in the refrigerator until needed. TLC plates were prepared in duplicate; one was developed in phosphomolybdic acid hydrate, and the other was used for the overlay bioassay. Phenol-red agar was warmed in a microwave oven until liquified, and then cooled until nearly solidifying. A sterile cotton swab was used to inoculate agar from liquid cultures of $S$. aureus. Inoculated medium was poured over one TLC plate, and incubated at $37^{\circ} \mathrm{C}$ for 18 hours. Plates were then removed and sprayed with the tetrazolium salt MTT to enhance color change. Yellowish areas indicated areas where bacterial growth was inhibited by compounds on the TLC plate, while bacterial colonies stained purple.

### 3.2.6 Fractionation of crude extract

T. cylindrosporum was cultured by inoculating 125 ml flasks containing 50 ml MYP broth with $3.3 \times 10^{7}$ conidia. Seed cultures were incubated on a rotary shaker at 40 rpm for three days at room temperature. Large flasks containing 1800 ml MYP broth were inoculated with 200 ml seed culture, and placed on a rotary shaker at 30 rpm . Cultures were grown for 14 days to allow sufficient biomass production, and then freeze dried. Dried cultures were extracted three times with $100 \% \mathrm{MeOH}$. Solvent was removed using a rotary evaporator. Dried extract was resuspended in water to remove sugars, and liquid-liquid partitions were made by sequential extractions with hexanes, ethyl acetate (EtOAc), and butanol (BuOH). The EtOAc and BuOH fractions contained
the most activity. The EtOAc fraction was dissolved in a small amount of MeOH , and mixed with silica. After the MeOH had evaporated, silica containing extract was loaded into a normal phase open column and the column was developed with $\mathrm{CH}_{2} \mathrm{Cl}_{2}$ : acetone: MeOH (160: 80: 20) +10 drops per 260 ml of formic acid from a glass pipet. When the lead band reached the end of the column it was stopped, and distinct bands were marked and allowed to dry overnight. Ten fractions were collected, extracted with MeOH , and dried in a rotary evaporator. Fractions 1 and 2 from the top of the column contained most of the activity. These were loaded onto preparatory TLC plates, which were developed two times with the same solvent system as the open column, with 25 drops formic $\mathrm{acid} / 100 \mathrm{ml}$. Three major bands were visualized under 254 nm ultraviolet light and were scraped off the glass plate with a razor blade. Compounds were extracted from silica with MeOH , which was removed in a rotary evaporator. These samples were used for MS and NMR analysis. MS-MS was performed at the Core Proteomics Facility at the Michael Smith Laboratories, University of British Columbia. NMR was conducted in the University of British Columbia Faculty of Chemistry's Laboratory of Molecular Biophysics NMR Hub.

### 3.3 Results

### 3.3.1 Antibacterial screening

About half of all extracts screened in this study showed some degree of antibacterial activity (Tables 3.1,3.2). Of the five species of clavicipitalean entomogenous fungi screened in this study, four produced antibacterial compounds. Due to the relative insensitivity of the disc-diffusion assay, some extracts showed weak

Table 3.1: Fungal isolates, culture fraction, zones of inhibition, and MIC when grown on liquid MYP medium; first number indicates diameter of zone of inhibition ( mm ) and the second number (in parentheses) indicates MIC ( $\mu \mathrm{l}$ extract $/ \mathrm{ml}$ media $/ 10^{5} \mathrm{CFU}$ ).

| Fungal species (isolate \#) | Fraction | Diameter of zone of inhibition (mm) (MIC ( $\mu \mathrm{l}$ extract/ml media/ $10^{5} \mathrm{CFU}$ ) ) |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | S. aureus | S. aureus MR | B. subtilis | E. faecalis | E. coli | S. typhimurium |
| Clade I |  |  |  |  |  |  |  |
| Beauveria bassiana (52) | mycelium | - | - | - | - | - | - |
|  | broth | 9 (>40) | $9(>40)$ | - (40) | 8.5 ( $>40$ ) | - | - |
| Paecilomyces sp. (15) | mycelium | 9 (15) | 9 (15) | 11 (5) | 10 (10) | - | - |
|  | broth | 8.5 (>40) | 8.5 (>40) | 8.5 (30) | 8.5 (40) | - | - |
| Paecilomyces sp. (46) | mycelium | 6.5 (20) | 7 (20) | 8.5 (5) | 6.5 (10) | - | - |
|  | broth | 8.5 (>40) | $9(>40)$ | 8.5 (40) | $8(>40)$ | - | - |
| Paecilomyces sp. (48) | mycelium | - | - | - |  | - | - |
|  | broth | - | - | - | - | - | - |
| Paecilomyces sp. (55) | mycelium | - | - | - | - | - | - |
|  | broth | - | - | - | - | - | - |
| Lecanicillium muscarium (20) | mycelium | - | - | - | - | - | - |
|  | broth | - | - | - | - | - | - |
|  |  |  |  |  |  |  |  |
| Metarhizium anisopliae (41) | mycelium | 9 (12.5) | 9.5 (20) | 7.5 (20) | 10 (3.75) | - | - |
|  | broth | $7.5(>40)$ | $7.5(>40)$ | - $-(40)$ | $7.5(>40)$ | - | - |
| Clade III |  |  |  |  |  |  |  |
| Tolypocladium cylindrosporum (4) | mycelium | 8 (2.5) | 8.5 (5) | 9.5 (30) | $11(>40)$ | - | - |
|  | broth | 15* (0.63) | 18* | 14* | 19* | - | - |
| Gentamicin (1 mg/ml) |  | 20.8 (0.138) | $21.2(0.234)$ | 22.6 (0.713) | $7.9(>40)$ | - | - |

* an asterisk designates that only a single replicate was performed

Table 3.2: Fungal isolates, culture fraction, zones of inhibition, and MIC when grown on liquid SD medium; first number indicates diameter of zone of inhibition ( mm ) and the second number (in parentheses) indicates MIC ( $\mu \mathrm{l}$ extract $/ \mathrm{ml}$ media $/ 10^{5} \mathrm{CFU}$ ).

| Fungal species (isolate \#) | Fraction | Diameter of zone of inhibition (mm) (MIC ( $\mu \mathrm{l}$ extract/ml media/ $10^{5} \mathrm{CFU}$ )) |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | S. aureus | S. aureus MR | B. subtilis | E. faecalis | E. coli | S. typhimurium |
| Clade I |  |  |  |  |  |  |  |
| Beauveria bassiana (52) | mycelium | - | - | , |  | - | - |
|  | broth | 9.5 (>40) | $9.5(>40)$ | - (>40*) | $10\left(>40^{*}\right)$ | - | - |
| Paecilomyces sp. (15) | mycelium | 9.25 (15) | 10.5 (10) | 11 (5*) | 10 (20*) | - | - |
|  | broth | 6.5 (>40) | 7 ( $>40$ ) | 8.5 (40*) | 6.5 ( $>40^{*}$ ) | - | - |
| Paecilomyces sp. (46) | mycelium | 7.5 (20) | 8 (20) | 7.5 (10*) | 7.25 (20*) | - | - |
|  | broth | 6.5 (>40) | 6.5 (>40) | 7.5 ( $>40^{*}$ ) | 6.25 (>40*) | - | - |
| Paecilomyces sp. (48) | mycelium | 6.75 (20) | 7 (40) | 7 (10*) | 6.5 (20*) | - | - |
|  | broth | -* | -* | 7* | -* | - | - |
| Paecilomyces sp. (55) | mycelium | 7 (40) | 6.75 (30) | 6.5 (10*) | 6.5 (20*) | - | - |
|  | broth | - | - | - | - | - | - |
| Lecanicillium muscarium (20) | mycelium | - | - | - | - | - | - |
|  | broth | - | - | - | - | - | - |
| Clade II |  |  |  |  |  |  |  |
| Metarhizium anisopliae (41) | mycelium | $8(40)$ | $8(30)$ | 7 (20*) | $8.5\left(20^{*}\right)$ | - | - |
|  | broth | - | $-(60)$ | - (40*) | $-\left(>40^{*}\right)$ | - | - |
| Clade III |  |  |  |  |  |  |  |
| Tolypocladium cylindrosporum (4) | mycelium | - | - | - | - | - | - |
|  | broth | - | - | - | - | - | - |
| Gentamicin ( $1 \mathrm{mg} / \mathrm{ml}$ ) |  | 20.8 (0.138) | $21.2(0.234)$ | 22.6 (0.713) | $7.9(>40)$ | - | - |

[^1]inhibition in the disc assay, but no inhibition in the 96 well MIC assay or vice versa. Production of antibacterial compounds was dependent on the growth medium for some species, but not for others. Some fungi released the active compounds into the culture broth (such as B. bassiana, and T. cylindrosporum), while other species kept the compounds more within the mycelium (Paecilomyces sp. and M. anisopliae). Variation was observed among the four isolates of Paecilomyces sp. Bacteria showed different susceptibility to extracts from different fungi. S. aureus and $S$. aureus MR were most susceptible to the extract from T. cylindrosporum, B. subtilis was most susceptible to the extract of Paecilomyces sp., and E. faecalis was most susceptable to the M. anisopliae extract. TLC combined with the phenol-red overlay bioassay showed that the active compounds produced when grown on MYP medium correlated with different $\mathrm{R}_{\mathrm{f}}$ values among those three species (Fig. 3.4).


Figure 3.4: Comparison of extracts by TLC and phenol-red overlay bioassay; mobile phase - 3 CH2Cl2 : 2 acetone : $0.5 \mathrm{MeOH}+2$ drops $90 \%$ formic acid. TLC plate on left developed in phosphomolybdic acid hydrate. TLC plate on right visualized using phenol-red overlay assay and devloped with MTT spray.

### 3.3.2 Active compounds

Three active compounds were isolated at about $80 \%$ purity from MeOH extracts of cultures of T. cylindrosporum. The majority of the activity was in the EtOAc and BuOH fractions (Fig. 3.5). The compounds reacted with ninhydrin, suggesting they are peptides. MS and NMR analysis showed that compound 1 has a molecular weight of 1634 and is likely one of the previously reported efrapeptins (efrapeptin E or F ) or a mixture of several related compounds. Compounds 2 and 3 have molecular weights of 1196 and 1182 respectively (plus $\mathrm{Na}^{+}$), and likely differ by a single methyl group (Fig 3.6, Appendix IV). A mixture of compounds 2 and 3 was active against $S$. aureus at $2 \mu \mathrm{~g}$ / disc, although this needs confirmed using the 96 well dilution assay. These are possibly novel compounds, and an invention disclosure (No. 06-140) has been filed with the University of British Columbia University Industry Liaison Office. Precise structural elucidation is currently underway with the help of Dr. Dongsheng Ming.

### 3.4 Discussion

Clavicipitalean entomogenous fungi represent a rich source of biologically active secondary metabolites (Isaka et al. 2005). A number of antibacterial compounds have been described from the group, including akanthomycin (Wagenaar, Gibson \& Clardy 2002), beauvericin (Ovchinnikov, Ivanov \& Mikhaleva 1971), cicadapeptins (Krasnoff et al. 2005), cordycepin (Ahn et al. 2000), efrapeptins (Bandani et al. 2000), oosporein (Vining, Kelleher \& Schwarting 1962; Wainwright, Betts \& Teale 1986), paecilosetin (Lang et al. 2005), saintopin (Yamashita et al. 1990), and a cerebroside (Jovel 2002). A recent study of Japanese isolates of entomogenous fungi found that over $80 \%$ of screened


Figure 3.5: Comparison of fractions of MeOH extract of T. cylindrosporum grown on MYP using TLC and phenol-red overlay bioassay; mobile phase - $\mathbf{3} \mathbf{C H} 2 \mathrm{Cl} 2: 2$ acetone. TLC plate on left developed in phosphomolybdic acid hydrate. TLC plate on right visualized using phenol-red overlay assay and devloped with MTT spray.


Figure 3.6: Possible structures of compounds 2 and 3 as inferred from MS-MS and 1H-NMR spectra. Compounds are small peptides, likely differ by a methyl group, and are associated with $\mathrm{Na}^{+}$. Structure created using ChemDraw (CaimbridgeSoft).
fungi produced compounds inhibitory to $B$. subtilis or $S$. aureus, and that media composition played an important role (Lee et al. 2005). Here I report similar findings in this study of Pacific northwestern North American isolates. Four of five species of clavicipitalean entomogenous fungi screened produced compounds inhibitory to bacterial growth. Extracts were inhibitory to gram-positive bacteria, but showed no inhibition of gram-negative bacteria.

No activity was seen in the extracts from $L$. muscarium. The extract from $B$. bassiana showed weak inhibition (MIC $>40 \mu \mathrm{l} / \mathrm{ml} / 10^{5} \mathrm{CFU}$ ) of $S$. aureus, $S$. aureus MR, and $E$. faecalis, but no inhibition of $B$. subtilis or the gram-negative bacteria. No activity was observed in the TLC overlay bioassay, possibly due to low concentration or weak activity. Activity was observed when grown on both MYP and SD media, and this activity was mostly in the broth and may be from oosporein or beauvericin. Both of these compounds are produced by a number of fungi including $B$. bassiana and have a variety of biological activities, including antibacterial (Vey, Hoagland \& Butt 2001). Extracts from M. anisopliae were inhibitory to all four gram-positive bacteria, but activity was strongest against $E$. faecalis when grown on MYP (MIC $3.75 \mu \mathrm{l} / \mathrm{ml} / 10^{5} \mathrm{CFU}$ ). Compounds were produced on both media, but there was higher production on MYP. Antibacterial activity was stronger in the mycelium extract. A number of bioactive metabolites have previously been reported from M. anisopliae such as the well characterized destruxins (Pedras, Zaharia \& Ward 2002), however there are no reports of antibacterial activity from these compounds.

Variation in antibacterial activity was observed among extracts from the four isolates of Paecilomyces sp. screened. Compounds inhibitory to all four gram-positive
bacteria were produced. All active extracts inhibited B. subtilis stronger than the other three bacteria, and strain 15 had the highest activity on both MYP and SD media (MIC 5 $\mu 1 / \mathrm{ml} / 10^{5} \mathrm{CFU}$ ). Only two isolates produced active compounds when grown on MYP, but all four produced active compounds on SD. Activity was mostly in the mycelium extract. These isolates match other isolates labeled as Paecilomyces farinosus. However, they are distinct from the recent epitype of Isaria farinosa on the basis of morphology and ITS sequences (Obornik, Jirku \& Dolezel 2001; Gams et al. 2005; Hodge et al. 2005). One active compound may be paecilosetin, recently isolated from P. farinosus (Lang et al. 2005). Activity may also be from saintopin or related compounds, isolated from Paecilomyces sp. (Yamashita et al. 1990).
T. cylindrosporum consistently produced the strongest activity of all fungi screened. Active compounds were only produced when grown on MYP media, and were inhibitory to all four gram-positive bacteria. Nearly four times the activity was seen in the broth extract compared to the mycelium extract. TLC of the broth extract developed in phosphomolybdic acid hydrate showed a large yellow spot which is a mixture of several compounds. The mycelium extract had a smaller spot with a smaller $\mathrm{R}_{\mathrm{f}}$ value and likely contains a smaller number of compounds. Inhibition was strongest against $S$. aureus (MIC $0.63 \mu \mathrm{l} / \mathrm{ml} / 10^{5} \mathrm{CFU}$ ). Roughly $4.5 \mu \mathrm{l}$ of crude extract would show inhibition comparable to 1 mg of gentamicin. Unfortunately, due to small extract quantity only a single replicate of the broth extract could be performed on the disc-diffusion bioassay. Two replicates of the MIC were performed for $S$. aureus, but no MIC were determined for the other three bacteria. Although the efrapeptins are known to be produced by T. cylindrosporum, they are only known to be active against Micrococcus
luteus and were inactive against B. cerreus at a concentration of $20 \mu \mathrm{~g} /$ disc (Krasnoff et al. 1991; Krasnoff \& Gupta 1992; Bandani et al. 2000). Efrapeptins are also antifungal and insecticidal (Krasnoff et al. 1991). Bandani et al. (2000) concluded that death of infected insects was due to toxicosis, but in vivo production of efrapeptins was less than the amount required to cause paralysis and death. This suggests the presence of other toxic metabolites. Bioassay-guided fractionation of extracts from T. cylindrosporum cultured on MYP led to the isolation of three active compounds. Compound 1 was a previously reported efrapeptin. Partial structural charactarization suggests compounds 2 and 3 may be novel peptides. These compounds show strong inhibition of $S$. aureus activity (MIC $2 \mu \mathrm{~g} / \mathrm{disc}$ ). Further chemical and biological characterization of these compounds is currently underway.

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## Chapter 4 CONCLUSION

### 4.1 Species diversity

Through investigation of diversity in Pacific Northwest entomogenous fungi, I addressed some fundamental issues regarding the taxonomy, nomenclature, and phylogeny of these organisms. Overall, my data supports the conclusions of others who have addressed the phylogenetic relationships among Cordyceps spp. and their anamorphs (Nikoh \& Fukatsu 2000; Zare, Gams \& Culham 2000; Gams \& Zare 2001; Nikoh \& Fukatsu 2001; Obornik, Jirku \& Dolezel 2001; Sung et al. 2001; Zare \& Gams 2001a, 2001b; Zare, Gams \& Evans 2001; Gams \& Zare 2002; Luangsa-Ard, HywelJones \& Samson 2004; Gams et al. 2005; Hodge et al. 2005; Luangsa-Ard et al. 2005; Rehner \& Buckley 2005; Stensrud, Hywel-Jones \& Schumacher 2005). Most of these studies have focused on members of the clade referred to in this study as clade I. While many of the relationships have been satisfactorily addressed, one taxon stands out as needing further attention. The fungus referred to in this study as Paecilomyces sp . has historically often been called P. farinosus. However, P. farinosus has been lectotypified as Isaria farinosa, and I. farinsoa and Paecilomyces sp. are clearly different based on molecular evidence. It appears that Paecilomyces sp. is actually closer related to Lecanicillium than to Isaria. Because of the historical significance of $P$. farinosus, it is crucial to clarify the identity of Paecilomyces sp. and re-evaluate the past 100 years of references to this taxon. Furthermore, clade III is of particular interest since that is where the host shift from insects to hypogeous fungi occurred. Some molecular attention was given to this group in 2001, but little has followed (Nikoh \& Fukatsu 2000). In addition, there seems to be considerable sequence variation in the two Elaphomyces parasitic
species C. capitata and C. ophioglossoides. Since these mycopathogenic species are possibly of entomopathogenic origin, they should be further investigated in terms of insect-fungus host shift.

### 4.2 Chemical ecology

Genetic diversity and natural product chemical diversity are intimately connected. Genes are the foundation for all life, and the interplay of genes and the environment results in the ultimate phenotype. Entomogenous fungi are so interesting because their environment is so unique; they are dependent on and spend most of their life cycle in insects. Although a few members of the Clavicipitaceae are symbionts of insects, the majority of insect-associated members of this family are pathogens (Suh, Noda \& Blackwell 2001). Symbionts and pathogens share a common environment (the insect host), however symbionts act in a mutualistic way that benefits both host and fungus. Pathogens on the other hand take advantage of their host, and ultimately lead to host death or lowered fitness. The insect host will attempt to eliminate the pathogen. The pathogen responds by evading, suppressing, or overcoming that host response (Goettel \& Inglis 1997; Gillespie et al. 2000; Goettel, Inglis \& Wraight 2000; Inglis et al. 2001).

Despite obstacles to their growth entomogenous fungi are surprisingly successful. They are represented in all four divisions of true fungi, and are found worldwide (Hawksworth et al. 1995; Goettel, Inglis \& Wraight 2000). An array of chemicals diverse in structure and function appear to facilitate infection, although direct evidence for this is lacking (Vey, Hoagland \& Butt 2001; Isaka et al. 2005). It is tempting to propose sophisticated roles of these metabolites in the infection process - a "chemical
symphony" with each metabolite being synthesized when it is needed to fulfill a specific role in the infection process.

For example, first the fungus penetrates and proliferates cryptically within the insect host, growing as a yeast-like or hyphal body that may mimic insect haemocytes (Inglis et al. 2001). The fungus may produce compounds that inhibit an immune response from the insect, but the pathogen is eventually recognized, and the insect attempts to isolate and destroy the fungal bodies (Hassan \& Al-Yahya 1987; Fujita et al. 1996; Tribe 1998; Vilcinskas et al. 1999; Gillespie et al. 2000; Vey, Hoagland \& Butt 2001; Pedras, Zaharia \& Ward 2002). The fungus responds by producing more chemicals, some of which inhibit the vacuolar-type ATPase, responsible for maintaining acidity in vacuolar organelles (Pedras, Zaharia \& Ward 2002). This presumably would render phagocytosis ineffective. Some have suggested a behavioral change in infected insects, known as "summit syndrome" (Evans 1982). While such behavior is largely speculative, there are several compounds produced by insect pathogenic fungi which affect nerve cells (Schmidt et al. 2002; Cheng et al. 2004; Kikuchi et al. 2004). The role of these compounds in insect behavior has yet to be studied. A number of metabolites produced by entomopathogenic fungi are insect toxins, and eventually the host insect dies through depletion of nutrients or toxicosis (Krasnoff et al. 1991; Mazet \& Vey 1995; Bandani et al. 2000; Vey, Hoagland \& Butt 2001; Kim et al. 2002). Entomopathogenic fungi also produce antibacterial, antifungal, and antiviral compounds (Vining, Kelleher \& Schwarting 1962; Wainwright, Betts \& Teale 1986; Yamashita et al. 1990; Krasnoff et al. 1991; Terry et al. 1992; Bandani et al. 2000; Vey, Hoagland \& Butt 2001; Wagenaar, Gibson \& Clardy 2002; Lang et al. 2005). These may be a substitute for the weakened
insect immune system, or a defense of the fungal substrate - the dead insect cadaver. But as previously mentioned, while it is tempting to propose such a scheme, direct evidence for these roles is often lacking.

### 4.3 Chemical discovery

The fields of natural product chemistry, secondary metabolism, and chemical ecology are being incorporated by an emerging field, chemical biology. Chemical biology seeks to integrate chemical tools and biological processes. Or vice versa, it seeks to use molecular tools to investigate biochemical systems. While initially the idea of "chemical prospecting" was envisioned as a way to discover new and useful compounds, treat human disease, empower developing nations, and conserve natural resources, the current status of the discipline is unclear. Patent issues and intellectual property rights have drawn opposition from industry and government in developed nations, while an indifference of many chemists to natural product chemistry has relegated it to "secondary metabolism." This is a shame, because technical advances are making it possible to efficiently separate and characterize chemical structures and biological activity from complex mixtures with only minute quantities of material (Eisner 2003). Furthermore, natural product chemistry is essential to the understanding of biological and ecological systems, and the interactions among living organisms. For funding and support to be withdrawn from such studies is truly a great loss.

In order to bring natural product chemistry back to center stage, it would help to affiliate more closely with molecular biology (Eisner 2003). High throughput bioassays to detect novel compounds have begun to look at biological activity on a molecular level
(Ireland et al. 2003). To advance beyond the random "grind and find" mentality of traditional natural product chemists, it is important to approach chemical prospecting with a more rational approach. For example, recent studies have screened entomopathogenic fungi for the presence of genes involved in key secondary metabolic pathways (Lee et al. 2001; Ireland et al. 2003). Such studies have found that many insect pathogens possess the metabolic pathways required for production of secondary metabolites. Now, it is a matter of applying the appropriate culture conditions to elicit production of the final metabolic products of those pathways (Knight et al. 2003; Radman et al. 2003).

### 4.4 Summary

This study explored the genetic and antimicrobial natural product diversity of clavicipitalean entomogenous fungi collected in the Pacific Northwest. I have strengthened the knowledge of entomogenous fungal diversity in the Pacific Northwest, and supported recent phylogenetic revisions of those taxa. This study has identified areas of uncertainty that need to be addressed in current and future studies and taxonomic revisions. I report new distributions of one or more species, and the isolation of a potentially novel antibacterial metabolite. These studies show that there is value in studying things that are, quite literally, in your own back yard. They provide another example of the richness and diversity of British Columbian and Pacific Northwestern natural resources.

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## Appendix I IMAGES OF COLLECTED FUNGI

## Cordyceps capitata (Holmsk.) Link (1833)



Figure I.1: C. capitata a) stroma emerging from Elaphomyces sp. (false truffle) collected in Detroit, OR, October, 2004 and b) anamorph isolated from teleomorphic tissue.

Cordyceps heteropoda Kobayasi (1939)


Figure I.2: C. heteropoda a) stromata emerging from buried Orthopteran insects, collected near Trout Lake, WA, April 30-May1, 2005 and b) conidia from anamorph isolated from teleomorphic tissue.

## Cordyceps militaris (L.) Link (1833)



Figure I.3: C. militaris a) anamorphic Lecanicillium isolated from teleomorphic ascospores and b) anamorph culture after 11 days at $25^{\circ} \mathrm{C}$ on malt extract agar (Becton Dickenson) reverse and c) top.

Cordyceps ophioglossoides (Ehrh.) Link (1833)


Figure I.4: C. ophioglossoides a) dried stromata and Elaphomyces sp. (false truffle) collected near Campbell River, BC, October 2004, b) conidia and conidiophores of anamorphic culture isolated from teleomorphic ascospores, and c) two cultures after 11 days at $25^{\circ} \mathrm{C}$ on malt extract agar (Becton Dickenson) reverse and d) top.

Beauveria bassiana (Bals.-Criv.) Vuill. (1912)


Figure I.5: B. bassiana a) growing on adult Coleopteran host collected in the Pacific Spirit Regional Park, Vancouver, BC, August 28, 2004, b) conidia produced in culture, and c) culture after 11 days at $25^{\circ} \mathrm{C}$ on malt extract agar (Becton Dickenson) reverse and d) top.


Figure I.6: B. brongniartii a) producing synnemata from buried adult Coleopteran host collected in the Pacific Spirit Regional Park, Vancouver, BC, December 9, 2003, b) growing on unidentified insect wrapped in leaf material collected in Renton, WA March 15, 2005, and c) conidia produced in culture, and d) culture after 11 days at $25^{\circ} \mathrm{C}$ on malt extract agar (Becton Dickenson) reverse and e) top.


Figure I.7: I. cicadae a) growing on unidentified host collected in the Queen Charlotte Islands, BC, 2004, b) microscopic features, and c) culture after 11 days at $25^{\circ} \mathrm{C}$ on malt extract agar (Becton Dickenson) reverse and d) top.

Isaria farinosa (Dicks.) Fr. (1832)


Figure I.8: I. farinosa growing on unidentified host collected in the Pacific Spirit Regional Park, Vancouver, BC a) October 26, 2003, b, c) October 27, 2003, d) March 10, 2004, e) conidia produced in culture, and culture after 11 days at $25^{\circ} \mathrm{C}$ on malt extract agar (Becton Dickenson) f) reverse and g) top.


Figure I.9: L. muscarium a) growing on unidentified substrate collected in the Pacific Spirit Regional Park, Vancouver, BC, October 28, 2003, b) conidia and conidiophores produced in culture, and culture after 11 days at $25^{\circ} \mathrm{C}$ on malt extract agar (Becton Dickenson) c) reverse and d) top.


Figure I.10: M. anisopliae a) growing on Orthopteran host collected in the Pacific Spirit Regional Park, Vancouver, November 5, 2003, b) conidia and conidiophores produced in culture, and c) culture after 11 days at $5^{\circ} \mathrm{C}$ on malt extract agar (Becton Dickenson) reverse and d) top.

Paecilomyces sp.



Figure I.11: Paecilomyces sp. a) growing on buried Lepidopteran pupa host collected in the Pacific Spirit Regional Park, Vancouver, BC, October 21, 2003, b) October 23, 2003, c) Lepidopteran pupa host not buried, October 27, 2003, d) unidentified host, October 28, 2003, e) unidentified host wrapped in leaf, October 27, 2003, f) unidentifed buried host, October 30, 2003, g) Lepidopteran larvae, October 30, 2003, h) unidentified host wrapped in leaf, Roberts Creek, BC, December 9, 2004, i) growing on spider, near Squamish, BC, j) unidentified host inside 1 cm diameter twig, Pacific Spirit Regional Park, Vancouver, BC, August 28, 2004, j) on buried Hymenopteran adult, March 8, $\mathbf{2 0 0 5}$, l) conidia and conidiophores produced in culture, and m) culture after 11 days at $25^{\circ} \mathrm{C}$ on malt extract agar (Becton Dickenson) reverse and $n$ ) top.

Tolypocladium cylindrosporum W. Gams (1971)


Figure I.12: T. cylindrosporum a) growing on Hymenopteran adult host collected in the Pacific Spirit Regional Park, Vancouver, October 26, 2003, b) conidia and conidiophores produced in culture, and c) culture after 11 days at $25^{\circ} \mathrm{C}$ on malt extract agar (Becton Dickenson) reverse and d) top.

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## Appendix II TAXA, GENBANK ACCESSION NUMBERS, CULTURE NUMBERS, AND REFERENCES FOR INCLUDED SEQUENCES

Table II.1: Taxa, GenBank accession numbers, culture numbers, and references for included sequences.

| Taxon | Culture No. | Source of Sequence | GenBank accession number |  |
| :---: | :---: | :---: | :---: | :---: |
|  |  |  | ITS | LSU |
| Hypocrea rufa |  | GenBank (Hagedorn \& Gams unpubl.) | AJ301991 | AJ301991 |
| Hypocrea lutea | IFO 9061 | GenBank (Nikoh \& Fukatsu 2000) | AB027384 |  |
| Hypocrea lutea | ATCC 208838 | GenBank (Currie et al. 2003) |  | AF543791 |
| "Paecilomyces farinosus" | CBS 262.58 | GenBank (Luangsa-Ard et al. 2005) | AY624179 |  |
| "Paecilomyces farinosus" | PFA 2179 | GenBank (Obornik, Jirku \& Dolezel 2001) |  | AF172342 |
| Syspastospora parasitica | IMI 255607 | GenBank (Zhang \& Blackwell 2002) |  | AY015634 |
| Paecilomyces sp. | BK 18 | This study |  |  |
| Cordyceps militaris | 3856.H | GenBank (Stensrud, Hywel-Jones \& Schumacher 2005) | AJ786573 |  |
| Cordyceps militaris |  | GenBank (Nikoh \& Fukatsu 2000) |  | AB027379 |
| Cordyceps militaris | CME | This study |  |  |
| Torrubiella confragosa | IMI 304817 | GenBank (Zare, Gams \& Culham 2000) | AJ292383 |  |
| Torrubiella confragosa | IMI 304807 | GenBank (Sung et al. 2001) |  | AF339555 |
| Lecanicillium muscarium | IMI 068689 | GenBank (Zare, Gams \& Culham 2000) | AJ292388 |  |
| "Torrubiella confragosa" |  | GenBank (Calmin, Belbahri \& Lefort unpubl.) | AY833600 |  |
| "Torrubiella confragosa" |  | GenBank (Seifert, Louis-Seize \& Sampson 2003) |  | AY283556 |
| Lecanicillium muscarium | BK 20 | This study |  |  |
| Isaria tenuipes | BCMU IJ25 | GenBank (Yokoyama, Yamagishi \& Hara 2004) | AB086224 |  |
| Isaria tenuipes |  | GenBank (Nikoh \& Fukatsu 2000) |  | AB027380 |
| Cordyceps pruinosa | ARSEF 5413 | GenBank (Sung \& Spatafora 2004) |  | AY184968 |
| Isaria cicadae | IFO 33259 | GenBank (Yokoyama, Yamagishi \& Hara 2004) | AB086631 |  |
| Isaria cicadae | BK 53 | This study |  |  |
| Isaria farinosa | CBS 111113 | GenBank (Luangsa-Ard et al. 2005) | AY624181 |  |
| Isaria farinosa | BK 10 | This study |  |  |
| Beauveria bassiana | ARSEF 344 | GenBank (Rehner \& Buckley 2005) | AY532023 |  |
| Beauveria bassiana | IFO 4848 | GenBank (Nikoh \& Fukatsu 2000) |  | AB027382 |
| Beauveria bassiana | BK 52 | This study |  |  |
| Beauveria brongniartii | ARSEF 4362 | GenBank (Rehner \& Buckley 2005) | AY532025 |  |


|  | Beauveria brongniartii | IFO 5299 | GenBank (Nikoh \& Fukatsu 2000) | AB027381 | AB027381 |
| :---: | :---: | :---: | :---: | :---: | :---: |
|  | Beauveria brongniartii | BK 42 | This study |  |  |
|  | Cordyceps brittlebankisoides | 473 | GenBank (Bidochka \& Small unpubl.) | AY387580 |  |
|  | Cordyceps brittlebankisoides | ARSEF | GenBank (Sung et al. 2001) |  | AF339530 |
|  | Metarhizium anisopliae | IFO 5940 | GenBank (Nikoh \& Fukatsu 2000) | AB027383 | AB027383 |
|  | Metarhizium anisopliae | BK 41 | This study |  |  |
|  | Cordyceps subsessilis | 2747.S | GenBank (Stensrud, Hywel-Jones \& Schumacher 2005) | AJ786592 |  |
|  | Tolypocladium inflatum | NBRC 31668 | GenBank (Yokoyama, Yamagishi \& Hara 2004) | AB103381 |  |
|  | Tolypocladium inflatum | IFO 31669 | GenBank (Nikoh \& Fukatsu 2001) |  | AB044645 |
|  | Tolypocladium cylindrosporum | NBRC 100548 | GenBank (Yokoyama \& Hara unpubl.) | AB208110 |  |
|  | Tolypocladium cylindrosporum | NRRL 28025 | GenBank (Cigelnik unpubl.) |  | AF049173 |
|  | Tolypocladium sp. | BK 4 | This study |  |  |
|  | Cordyceps longisegmentis | 2731.S | GenBank (Stensrud, Hywel-Jones \& Schumacher 2005) | AJ786568 |  |
|  | Cordyceps heteropoda |  | GenBank (Nikoh \& Fukatsu 2000) | AB027373 | AB027373 |
|  | Cordyceps gracilis | 2684.S | GenBank (Stensrud, Hywel-Jones \& Schumacher 2005) | AJ786563 |  |
|  | Cordyceps gracilis | Cg | This study |  |  |
|  | Cordyceps capitata |  | GenBank (Nikoh \& Fukatsu 2000) | AB027364 | AB027364 |
|  | Cordyceps capitata | 3087.P | GenBank (Stensrud, Hywel-Jones \& Schumacher 2005) | AJ786557 |  |
| $\stackrel{\infty}{+}$ | Cordyceps capitata 2 | OSC 71233 | GenBank (Castlebury et al. 2004) |  | AY489721 |
|  | Cordyceps capitata | Cclp | This study |  |  |
|  | Cordyceps ophioglossoides |  | GenBank (Nikoh \& Fukatsu 2000) | AB027367 | AB027367 |
|  | Cordyceps ophioglossoides |  | GenBank (Liu 2001) | AJ309360 |  |
|  | Cordyceps ophioglossoides 2 | Col | This study |  |  |

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# Appendix III ITS AND LSU SEQUENCE ALIGNMENTS 

Figure III.1: Sequence alignment for ITS data matrix containing 37 taxa, 590 characters, 329 constant characters, 49 parsimony-uninformative variable characters, and $\mathbf{2 1 2}$ parsimony-informative characters.

HRU301991 CCGAGTT--TACAACTCCCAAA-CCCAATGTGAACGTTACCA--AACTGTTGCCTCGGCG
AB027384 CCGAGTT--TACAACTCCCAAA-CCCAATGTGAACGTTACCA--AACTGTTGCCTCGGCG
AY624179 CCGAG--TTTTCAACTCCCAAACCCTTTTGTGAAC-ATACCT---ATCGTTGCTTCGGCG
T18-p-ITS5 CCGAG--TTTTCAACTCCCAAACCCTTTTGTGAAC-ATACCT---ATCGTTGCTTCGGCG
AJ786573 ACGAG-TTTTCCAACTCCCAA--CCCTTTGTGAAC-ATACCT---ATCGTTGCTTCGGCG
TCmE-p-ITS ACGAG-TTTTCCAACTCCCAA--CCCTTTGTGAAC-ATACCT---ATCGTTGCTTCGGCG
VLE292383 TCGAG--TTTACAACTCCCAAACCCTTCTGTGAAC-ATACCT---ACCGTTGCTTCGGCG
CAP292388 TCGAG--TTTACAACTCCCAAACCCTTATGTGAAC-ATACCT---ACTGTTGCTTCGGCG
AY833600 TCGAG--TTTACAACTCCCAAACCCTTATGTGAAC-ATACCT---ACTGTTGCTTCGGCG
T20-p-ITS5 TCGAG--TTTACAACTCCCAAACCCTTATGTGAAC-ATACCT---ACTGTTGCTTCGGCG
AB086224 CCAGAGTTTTACAACTCCCAA-CCCTTCTGTGAAC-CTACCC---ATAGTTGCTTCGGCG
AB086631 CCAGAGTTTTACAACTCCCAA-CCCTTCTGTGAAC-CTACCC---ATCGTTGCTTCGGCG
T53-p-ITS5 CCAGAGTTTTACAACTCCCAA-CCCTTCTGTGAAC-CTACCC---ATCGTTGCTTCGGCG
AY624181 CCAGAGTTTTACAACTCCCAA-CCCTTCTGTGAAC-CTACCT---ATCGTTGCTTCGGCG
T10-p-ITS5 CCAGAGTTTTACAACTCCCAA-CCCTTCTGTGAAC-CTACCT---CTCGTTGCTTCGGCG
AY532023 CCGAG--TTTTCAACTCCCTAACCCTTCTGTGAAC-CTACCT---ATCGTTGCTTCGGCG
T52-beta CCGAG--TTTTCAACTCCCTAACCCTTCTGTGAAC-CTACCT---ATCGTTGCTTCGGCG
AY532025 CCGAG--TTTTCAACTCCCTAACCCTTATGTGAAC-CTACCT---ATTGTTGCTTCGGCG
AB027381 CCGAG--TTTTCAACTCCCTAACCCTTATGTGAAC-CTACCT---ATTGTTGCTTCGGCG
T42-p-ITS5 CCGAG--TTTTCAACTCCCTAACCCTTATGTGAAC-CTACCT---ATTGTTGCTTCGGCG
AY387580 CCGAGTTA-TCCAACTCCCAAC-CCC--TGTGAATTATACCTTTAATTGTTGCTTCGGCG
AB027383 CCGAGTTA-TCCAACTCCCAAC-CCC--TGTGAATTATACCTTTAATTGTTGCTTCGGCG
T41-p-ITS5 CCGAGTTA-TCCAACTCCCAAC-CCC--TGTGAATTATACCTTTAATTGTTGCTTCGGCG
AJ786592 CCGAGTT--ATCAACTCCCAAA--CCCCTGTGAAC-ATACCC---AACGTTGCTTCGGCG
AB103381 CCGAGTT--ATCAACTCCCAAA--CCCCTGTGAAC-ATACCC---AACGTTGCTTCGGCG
AB208110 CCGAGTT--ATCAACTCCCAAA--CCCCTGTGAAC-ATACCC---AACGTTGCTTCGGCG
T4-p-ITS5 CCGAGTT--ATCAACTCCCAAA--CCCCTGTGAAC-ATACCC---AACGTTGCTTCGGCG
AJ786568 CCGATTTT-ATCATCTCCCAAA--CCCCTGTGAAC-ATACCC--GAACGTTGCCTCGGCG
AB027373 TAGAGTTGCCC-GACTCCAAAAACCCCCTGTGAACCGTACCC--AGGCGTTGCCTCGGCG
AJ786563 CCGAGTTGCCCCAACTCCCAAAACCCACTGCGAACCGTACCC--AGCCGTTGCCTCGGCG
$5 \mathrm{E}-\mathrm{p}-\mathrm{ITS} 3$ ????????????????????????????????????????????????????????????
AB027364 CCGAGTC--ATCAACTCCCAAA--CCCCTGTGAAC-GTACCC--GAACGTTGCTTCGGCG
AJ786557 CCGAGTT--CTCAACTCCCAAA--CCCCTGTGAAC-ATACCC--GAACGTTGCCTCGGCG
TCc1p-p-IT CCGAGTT--TTCAACTCCCAAA--CCCCTGTGAAC-ATACCT--GAACGTTGCCTCGGCG
AB027367 CCGAGTT--ATCAACTCCCAAA--CCCCTGTGAAC-ATACCT--GAACGTTGCTTCGGCG
COP309360 CCGAGTT--ATCAACTCCCAAA--CCCCTGTGAAC-ATACCT--GAACGTTGCTTCGGCG
TCO1-p-ITS CCGAGTT--ATCAACTCCCAAA--CCCCTGTGAAC-ATACCT--GAACGTTGCTTCGGCG

HRU301991 GGGTCAC-GCCCCGGGCG----------------CGTCGCAGCCCCGGAA--CCAGGCGCC
AB027384 GGATTTCTGCCCCGGGCG----------------CGTCGCAGCCCCGGAC--CAAGGCGCC
AY624179 GACTCGCCCCAGCGTCCGGCCGGCCCCGCGCCG-GCCGCGGCCTGGAT---CCAGGCGGC
T18-p-ITS5 GACTCGCCCCAGCGTCCGGCCGGCCCCGCGCCG-GCCGCGGCCTGGAT---CCAGGCGGC
AJ786573 GACTCGCCC-AGCGCCTGGACG---------CG-------GGCCTGGGC------GGGGGC
TCmE-p-ITS GACTCGCCC-AGCGCCTGGACG----------CG-------GGCCTGGGC------GGCGGC
VLE292383 GACTCGCCCCGGCGTCCGGACGGCCTCGCGCCG-CCCGCGGCCCGGAC---CCAGGCGGC
CAP292388 GACTCGCCCCGGCGTCCGGACGGCCTCGCGCCG-CCCGCGGCCCGGAC---CCAGGCGGC
AY833600 GACTCGCCCCGGCGTCCGGACGGCCTCGCGCCG-CCCGCGGCCCGGAC---CCAGGCGGC
T20-p-ITS5 GACTCGCCCCGGCGTCCGGACGGCCTCGCGCCG-CCCGCGGCCCGGAC---CCAGGCGGC
AB086224 GACCCGCCCCAGCGTCCGGACGGCCCAGCGCCGGCCCGCGACCTGGAC---CCAGGCGGC
AB086631 GACTCGCCCCAGCGTCCGGACGGCCCCGCGCCGGCCCGCGACCTGGAC---CCAGGCGGC
T53-p-ITS5 GACTCGCCCCAGCGTCCGGACGGCCCCGCGCCGGCCCGCGACCTGGAC---CCAGGCGGC
AY624181 GACTCGCCCCAGCGTCCGGACGGCCCCGCGCCGGCCCGCGACCTGGAC---CCAGGCGGC
T10-p-ITS5 GACTCGCCCCAGCGTCCGGACGGCCCCGCGCCGGCCCGCGACCTGGAC---CCAGGCGGC
AY532023 GACTCGCCCCAGC--CCGGACG----------------CGGACTGGAC---C--AGCGGC
T52-beta GACTCGCCCCAGC--CCGGACG-------------------CGGACTGGAC---C--AGCGGC

AB027381 GACTCGCCCCAGC--C-GGACG-----------------------AGGACTGGAC----AGGC
T42-p-ITS5 GACTCGCCCCAGC--C-GGACG------------------CGGACTGGAC---C--AGCGGC



AJ786592 GGACCGCCCCGG-GCCC--------------------TCGGCGTCCCGGAA---CCAGGCGCC
AB103381 GGACCGCCCCGGCGCC------------------------CCGGCGTCCCGGAA---CCGCC
AB208110 GGACCGCCCCGGCGCCC-------------------TCGGCGTCCCGGAA---CCAGGCGCC
T4-p-ITS5 GGACCGCCCCGGCGCCC-------------------TCGGCGTCCCGGAA---CCAGGCGCC
AJ786568 GGACCGCCC-GGCGCCC--------------------ATGGCGGCCCGGAA---CCAGGCGCC
AB027373 GTGCCGCCCTGGGGGGCGGGGG---------TGCCTGACGGCCACCCCCCCCCAGGCGCC
AJ786563 GTGCCGCCC-GGGGGGC-----------------CCCAAGAGCCCCGC---------GGCGCC
5Е-p-ITS3 ????????????????????????????????????????????????????????????
AB027364 GGACCGCCCCGGCGCCC-------------------TCG-CGGCCCGGAA---CCAGGCGCC
AJ786557 G-ACCGCCCCGGCGCCC-------------------ACG-CGGCCGGAA----CCAGGCGCC
TCc1p-p-IT GGACCGCCCCGGCGCCC----------------------ACG-CGGCCCGGA----CCAGGCGCC
AB027367 GGACCGCCCCGGCGCCC--------------------ACGGCGGCCCGGAA---CCAGGCGCC
COP309360 GGACCGCCCCGGCGCCC-------------------ACGGCGGCCCGGAA---CCAGGCGCC
TCo1-p-ITS GGACCGCCCCGGCGCCC-------------------ACGGCGGCCCGGAA---CCAGGCGCC

HRU301991 CGCCGGAGGGACCAAC---CAAACTCTTTCTGTAGTCCCCTCGCGGACGTTATTTCTCAC AB027384 CGCCGGA-GGACCAATTTACAAACTCTTTGTATGTCCCTTTGCGGATTTTTATTATACAT AY624179 CGCCGGA--GACCC----CCAAACTCT-GTA-------------------TCTCAG------









T53-p-ITS 5 CGCCGGA--GACCA----CGCAACCCT-GTA-------------------------







AY387580 CGCCGG--GGACC------CAAACCTTCTGAAT-----------------TTTTTAATAAGTAT
AB027383 CGCCGG--GGACC------CAAACCTTCTGAAT-----------------TTTTTAATAAGTAT
T41-p-ITS5 CGCCGG--GGACC------CAAACCTTCTGAAT---------------TTTTTAATAAGTAT
AJ786592 CGCCGGA-GGACCC------AAACTCTTGT---------------TTAA-CCATAGTGG----


T4-p-ITS5 CGCCGGA-GGACCC------AAACTCTTGT----------------TTAACCATAGTGG----

AB027373 CGCCGGA-GGACAC-----CCCAAACTCTTGCAATCCGC---TCCCCGCCCCGGGGGGGGAG
AJ786563 CGCCGGA-GGACACA--CCCAAACTCTTGCAAACCG------CCCGCCCGCCGCGGGGGG
5Е-p-ITS3 ????????????????????????????????????????????????????????????

AJ786557 CGCCGGA-GGACCC------AAACTCTTGC-----------------
TCclp-p-IT CGCCGGA-GGACCC------AAACTCTTGC----------------TTGCACCATAGCGG----

COP309360 CGCCGGA-GGACCC------AAACTCTTGT--------------TTAATTTATAGCGG-----


HRU301991 AG-------CTCTGAGCA--------AAAAAAATTCAAAATGAATCAAAACTTTCAACAA AB027384 TCTG-AGCTTTCTCGGCGCTCCTAGCGAGCGTTTCGAAAATGAATCAAAACTTTCAACAA AY624179 - - --TATCTTCTGAATCCGCC---GCAAGGCAAAACAAATGAATCAAAACTTTCAACAA T18-p-ITS5 -----TATCTTCTGAATCCGCC---GCAAGGCAAAACAAATGAATCAAAACTTTCAACAA
AJ786573 -----TTTTT-CTGAATCCGCC---GCAAGGCAAAACAAATGAATCAAAACTTTCAACAA
TCmE-p-ITS -----TTTTT-CTGAATCCGCC---GCAAGGCAAAACAAATNAATCAAAACTTTCAACAA
VLE292383 -----CATCTTCTGAATTCGCC---GCAAGGCAAAACAAATGAATCAAAACTTTCAACAA
CAP292388 -----CATTTTCTGAATCCGCC---GCAAGGCAAAACAAATGAATCAAAACTTTCAACAA
AY833600 -----CATTTTCTGAATCCGCC---GCAAGGCAAAACAAATGAATCAAAACTTTCAACAA
T20-p-ITS5 -----CATTTTCTGAATCCGCC---GCAAGGCAAAACAAATGAATCAAAACTTTCAACAA
AB086224 -----CCTCT-CTGAATCCGCC---GCAAGGCAACACAAACGAATCAAAACTTTCAACAA
AB086631 -----TCTCT-CTGAATCCGCC---GCAAGGCAACACAAATGAATCAAAACTTTCAACAA
T53-p-ITS5 -----TCTCT-CTGAATCCGCC---GCAAGGCAACACAAATGAATCAAAACTTTCAACAA
AY624181 -----TCTCT-CTGAATCCGCC---GCAAGGCAAAACAAACGAATCAAAACTTTCAACAA
T10-p-ITS5 - ----TCTCT-CTGAATCCGCC---GCAAGGCAAAACAAACGAATCAAAACTTTCAACAA
AY532023 -----CATCTTCTGAATACGCC---GCAAGGCAAAACAAATGAATCAAAACTTTCAACAA
T52-beta -----CATCTTCTGAATACGCC---GCAAGGCAAAACAAATGAATCAAAACTTTCAACAA
AY532025 - - ---CATCTTCTGAATACGCC---GCAAGGCAAAACAAATAAATCAAAACTTTCAACAA
AB027381 -----CATCTTCTGAATACGCC---GCAAGGCAAAACAAATAAATCAAAACTTTCAACAA
T42-p-ITS5 -----CATCTTCTGAATACGCC---GCAAGGCAAAACAAATAAATCAAAACTTTCAACAA
AY387580 C--------TTCTGAGTG------------GTTAAAAAAATGATCAAAACTTTCAACAA
AB027383 C--------TTCTGAGTG------------GTTAAAAAAATGAATCAAAACTTTCAACAA
T41-p-ITS5 C--------TTCTGAGTG-----------GTTAAAAAAATGAATCAAAACTTTCAACAA
AJ786592 -----CATATTCTGAGTCTC---------ACAAG-AAAAATGAATCAAAACTTTCAACAA
AB103381 -----CATATTCTGAGTCTC---------ACAAG-AAAAATGAATCAAAACTTTCAACAA
AB208110 -----CATATTCTGAGTCTC---------ACAAG-AAAAATGAATCAAAACTTTCAACAA
T4-p-ITS5 -----CATATTCTGAGTCTC---------ACAAG-AAAAATGAATCAAAACTTTCAACAA
AJ786568 -----CATCTTCTGAGTCTC---------GCGAGGAAAAATGAATCAAAACTTTCAACAA
AB027373 CGCGTCGTCCTCTGAGTCCC---------CTCAAAGAAAACGAGTTAAAACTTTCAACAA
AJ786563 CGCGTCGTCCTCTGAGTCCC---------CCCAA-GAAAACGAATCAAAACTTTCAACAA
5E-p-ITS3 ????????????????????????????????????????????????????????????
AB027364 -----CATCTTCTGAGTCTC---------ACGAG--AAAATGAATCAAAACTTTCAACAA
AJ786557 -----CGTCTTCTGAGTCTA------------AA--CGAATGAATCAAAACTTTCAACAA
TCc1p-p-IT -----CATCTTCTGAGTCTC----…--T--AA--AAAATGAATCAAAACTTTCAACAA
AB027367 -----CATATTCTGAGTCTC---------ACAAG-AAAAATGAATCAAAACTTTCAACAA
COP309360 -----CATATTCTGAGTCTC--------ACAAA-AAAAATGAATCAAAACTTTCAACAA
TCO1-p-ITS -----CATATTCTGAGTCTC---------ACAAA-AAAAATGAATCAAAACTTTCAACAA

HRU301991 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AB027384 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AY624179 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT T18-p-ITS5 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AJ786573 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT TCmE-p-ITS CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT VLE292383 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT CAP292388 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AY833600 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT T20-p-ITS5 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AB086224 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAAT AB086631 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAAT T53-p-ITS5 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAAT AY624181 TGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAAT T10-p-ITS5 TGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAAT AY532023 CGGATCTCTTGGGCTCTGGCATCGATGAAGAACGCAGCGAAACGCGATAAGTAATGTGAAT T52-beta CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAACGCGATAAGTAATGTGAAT AY532025 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AB027381 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT T42-p-ITS5 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AY387580 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AB027383 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT T41-p-ITS5 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AJ786592 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AB103381 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AB208110 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT T4-p-ITS5 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AJ786568 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AB027373 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATATGTAATGTGAAT AJ786563 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT 5E-p-ITS3 ??????????????????????????????????????????????????????GTGAAT AB027364 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AJ786557 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT TCc1p-p-IT CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT AB027367 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT COP309360 CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT

[^2]HRU301991 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC AB027384 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC AY624179 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC T18-p-ITS5 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC AJ786573 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC TCmE-p-ITS TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC VLE292383 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC CAP292388 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC AY833600 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC T20-p-ITS5 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC AB086224 TGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC AB086631 TGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC T53-p-ITS5 TGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC

## AY624181

 T10-p-ITS5 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC AY532023T52-beta
AY532025 TGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC
AB027381 TGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC
T42-p-ITS5 TGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC
AY387580 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGTCAGTATTCTGGC
AB027383 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGTCAGTATTCTGGC
T41-p-ITS5 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGTCAGTATTCTGGC
AJ786592 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC
AB103381 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC
AB208110 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC
T4-p-ITS5 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC
AJ786568 TGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCACTCTGGC
AB027373 TGCAGATTTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCACTCTGGC
AJ786563 TGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCACTCTGGC
5E-p-ITS 3 TGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCACTCTGGC
AB027364 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCACTCTGGC
AJ786557 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCACTCTGGC
TCc1p-p-IT TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCACTCTGGC
AB027367 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC
COP309360 TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC
TCo1-p-ITS TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC

HRU301991 GGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCCCCC---GGGGGGTCCGGCG AB027384 GGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCC-----GGGGGGTCGGCG AY624179 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACTTCCCTTT---GGGGAAAT-GGCG T18-p-ITS5 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACTTCCCTTT---GGGGAAATCGGCG AJ786573 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACGTCCCCTG---GGGGATGTCGGCG
TCmE-p-ITS GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACGTCCCCTG---GGGGATGTCGGCG
VLE292383 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCCTC---GGGGAAGTCGGCG
CAP292388 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACTTCCCTTT---GGGGAAATCGGCG
AY833600 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACTTCCCTTT---GGGGAAATCGGCG
T20-p-ITS5 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACTTCCCTTT---GGGGAAATCGGCG
AB086224 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACGTCCCCC-----GGGACGTCGGCC
AB086631 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACGTCCCCT-----GGGACGTCGGCC
T53-p-ITS5 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACGTCCCCC-----GGGACGTCGGCC
AY624181 GGGCATGCCTGTTCGAGCGTCATTGCAACCCTCGACGTCCCCT-----GGGACGTCGGCC
T10-p-ITS5 GGGCATGCCTGTTCGAGCGTCATTGCAACCCTCGACGTCCCCC-----GGGACGTCGGCC
AY532023 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCCTT---GGGGAGGTCGGCG
T52-beta GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCCTT---GGGGAGGTCGGCG
AY532025 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCTTT---GGGGAAGTCGGCG
AB027381 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCTTT---GGGGAAGTCGGCG
T42-p-ITS5 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCTTT---GGGGAAGTCGGCG
AY387580 GGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGTCCC-CT-----GTGGACTTGGTG
AB027383 GGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGTCCC-CT-----GTGGACTTGGTG
T41-p-ITS5 GGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGTCCC-CT-----GTGGACTTGGTG
AJ786592 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCC-----AGC---GGCTTGGTG
AB103381 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCC-----AGC---GGCTTGGTG
AB208110 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCC-----AGCC--GGCTTGGTG
T4-p-ITS5 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCC-----AGCC--GGCTTGGTG
AJ786568 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGAGCCCCGCCC-AGCG--GGCTCGGTG
AB027373 GGGCATGCCTGTTCGAGCGTCGTTTCAACCCTCGGGCCCCCCCCCCTCGGGGGCCCGGTG
AJ786563 GGGCATGCCTGTCCGAGCGTCATTTCGACCCTCGAGCCCCCCA-----GGGGGCTCGGTG
5E-p-ITS3 GGGCATGCCTGTCCGAGCGTCGTTTCAACCCTCGAGCCCCCCCCCG--GGGGGCTCGGTG
AB027364 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGGGCC----CCCCGCG--GC-CCGGTG
AJ786557 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAGGCCCG--CCCGGCG--GGACTGGTG
TCc1p-p-IT GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAGGCCCG--CCCAGCG--GGACTGGTG
AB027367 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAG-CCCCCCCTTAGCGGGGGACTGGTG
COP309360 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAGGCCCCCCCCTAGCGGGGGACTGGTG
TCo1-p-ITS GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAGGCCCCCCCCTAGCGGGGGACTGGTG

HRU301991 TTGGGGATCGGGGAC-----CCCTCAGACGGGATCCCGGCCCCGAAATACAGTGGCGGTC AB027384 TTGGGGATCGGC---------CTTCTACCG--G---CCGGCCCCGAAATACAGTGGCGGTC
AY624179 TTGGGGACCGGCC------GTATACC--CG--------GCCCCGAAATGAAGTGGCGGCC
T18-p-ITS5 TTGGGGACCGGCC------GTATACCGCCG--------GCCCCGAAATGAAGTGGCGGCC
AJ786573 TTGGGGACCGGCA------GCACACCGCCG--------CCCCCGAAATGAAGTGGCGGCC
TCmE-p-ITS TTGGGGACCGGCA------GCACACCGCCG---------CCCCCGAAATGAAGTGGCGGCC
VLE292383 TTGGGGAACGGCA------GCACACCGCCG--------GCCCCGAAATGGAGTGGCGGCC
CAP292388 TTGGGGAACGGCA------GCATACCGCCG--------GCCCCGAAATGGAGTGGCGGCC
AY833600 TTGGGGAACGGCA------GCATACCGCCG--------GCCCCGAAATGGAGTGGCGGCC
T20-p-ITS5 TTGGGGAACGGCA-------GCATACCGCCG--------GCCCCGAAATGGAGTGGCGGCC
AB086224 TTGGGGACCGGCA------GCACCCCGCCG--------GCCCTGAAATGGAGTGGCGGCC
AB086631 TTGGGGACCGGCA------GCACCCCGCCG--------GCCCTGAAATGGAGTGGCGGCC
T53-p-ITS5 TTGGGGACCGGCA------GCACCCCGCCG--------GCCCTGAAATGGAGTGGCGGCC
AY624181 TTGGGGACCGGCA------GCACACCGCCG--------GCCCTGAAATGGAGTGGCGGCC
T10-p-ITS5 TTGGGGACCGGCA------GCACACCGCCG--------GCCCTGAAATGGAGTGGCGGCC
AY532023 TTGGGGACCGGCA------GCACACCGCCG--------GCCCTGAAATGGAGTGGCGGCC
T52-beta TTGGGGACCGGCA------GCACACCGCCG--------GCCCTGAAATGGAGTGGCGGCC
AY532025 TTGGGGACCGGCA------GCACACCGCCG--------GCCCTGAAATGGAGTGGCGGCC
AB027381 TTGGGGACCGGCA------GCACACCGCCG--------GCCCTGAAATGGAGTGGCGGCC
T42-p-ITS 5 TTGGGGACCGGCA------GCACACCGCCG--------GCCCTGAAATGGAGTGGCGGCC
AY387580 TTGGGGATCGGCGAGGCTGGTTTTCCAGCACAG--CCGTCCCTTAAATTAATTGGCGGTC
AB027383 TTGGGGATCGGCGAGGCTGGTTTTCCAGCGCAG--CCGTCCCTCAAATCAATTGGCGGTC
T41-p-ITS5 TTGGGGATCGGCGAGGCTGGTTTTCCAGCGCAG--CCGTCCCTCAAATCAATTGGCGGTC
AJ786592 TTGGGGACCGGCC-------CCGGCCGCC------------CCCCAAATGCAGTGGCGACC
AB103381 TTGGGGACCGGCC-------CCGGCCGCC-------------CCCAAATGCAGTGGCGACC
AB208110 TTGGGGACCGGCC-------CCGGCCGCC-------------CCCCAAATGCAGTGGCGACC
T4-p-ITS5 TTGGGGACCGGCC-------CCGGCCGCC-------------CCCCAAATGCAGTGGCGACC
AJ786568 TTGGGGGCCGGCC-------CAGGCCGCC------------CCCGAAATGCAGTGGCGACC
AB027373 TTGGGGGGCGCGG-------CCCGCTACAGGGT-CGCCCCCCCTAAATACAGTGGCGACC
AJ786563 TTGGGGGGCGCGG-------CCC-CCACA------CCCCCCCCCAAATGCAGTGGCGACC
5E-p-ITS3 TTGGGGGGCGCGG-------CCCGCCACAGGGC-CGCCCCCCCTAAATGCAGTGGCGACC
AB027364 TTGGGGACCGGCC-------CCGGCCGCC------------CCCGAAATGCAGTGGCGACC
AJ786557 TTGGGGACCGGCC-------CCGGCCGCC-------------CCCGAAATGCAGTGGCGACC
TCc1p-p-IT TTGGGGACCGGCC-------CTGGCCGCC------------CCCGAAATGCAGTGGCGACC
AB027367 TTGGGGACCGGCC-------CCGGCCGCC-------------CCCGAAATGCAGTGGCGACC
COP309360 TTGGGGACCGGCC-------CCGGCCGCC------------CCCTAAATGCAGTGGCGACC
TCo1-p-ITS TTGGGGACCGGCC-------CCGGCCGCC------------CCCTAAATGCAGTGGCGACC

HRU301991 -TCGCC-GCAGCC-TCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCG
AB027384 -TCGCC-GCAGCC-TCTCCTGCGCAGTAGTTTGCACA-CTCGCACCGGGAGCGCGGCGCG
AY624179 --CGTCCGCGGCG-ACCTCTGCGTAGTAATCCA-AC---TCGCACCGGAACCCCGACG-T
T18-p-ITS5
AJ786573
TCmE-p-ITS --CGTCCGCGGCG-ACCTCTGCGTAGTAATCCA-AC---TCGCACCGGAACCCCGACG-T --CGTCCGCGGCG-ACCTCTGCGTAGTACCCCA-AC---TCGCACCGGGAACCCGACG-T --CGTCCGCGGCG-ACCTCTGCGTAGTACCCCA-AC---TCGCACCGGGAACCCGACG-T
VLE292383
CAP292388 --CGTCCGCGGCG-ACCTCTGCGTAGTAATCCA-AC---TCGCACCGGAAACCCGACG-C
--CGTCCGCGGCG-ACCTCTGCGTAGTAATCCA-AC---TCGCACCGGAACCCCGACG-T
AY833600 --CGTCCGCGGCG-ACCTCTGCGTAGTAATCCA-AC---TCGCACCGGAACCCCGACG-T
T20-p-ITS5 --CGTCCGCGGCG-ACCTCTGCGTAGTAATCCA-AC---TCGCACCGGAACCCCGACG-T
AB086224 --CGTCCGCGGCG-ACCTCTGCGCAGTACAAGC-AC---TCGCACCGGGAACCCGACG-C
AB086631 --CGTCCGCGGCG-ACCTCTGCGCAGTACAACC-AC---TCGCACCGGGAACCCGACG-C
T53-p-ITS5 --CGTCCGCGGCG-ACCTCTGCGCAGTACAACC-AC---TCGCACCGGGAACCCGACG-C
AY624181
--CGTCCGCGGCG-ACCTCTGCGCAGTAATCCA-AC---TCGCACCGGGAACCCGACG-C
T10-p-ITS5 --CGTCCGCGGCG-ACCTCTGCGCAGTAATCCA-AC---TCGCACCGGGAACCCGACG-C
AY532023 --CGTCCGCGGCG-ACCTCTGCGCAGTAATACA-GC---TCGCACCGGAACCCCGACG-C
T52-beta --CGTCCGCGGCG-ACCTCTGCGCAGTAATACA-GC---TCGCACCGGAACCCCGACG-C
AY532025 --CGTCCGCGGCG-ACCTCTGCGTAGTAATCCA-AC---TCGCACCGGAACCCCGACG-C
AB027381 --CGTCCGCGGCG-ACCTCTGCGTAGTAATCCA-AC---TCGCACCGGAACCCCGACG-C
T42-p-ITS5 --CGTCCGCGGCG-ACCTCTGCGTAGTAATCCA-AC---TCGCACCGGAACCCCGACG-C
AY387580 -TCGCC-GTGGCCCTCCTCTGCGCAGTAGTAAA-ACA-CTCGCAACAGGAGCCCGGCGCG
AB027383 -TCGCC-GTGGCCCTCCTCTGCGCAGTAGTAAA-ACA-CTCGCAACAGGAGCCCGGCGCG
T41-p-ITS5 -TCGCC-GTGGCCCTCCTCTGCGCAGTAGTAAA-ACA-CTCGCAACAGGAGCCCGGCGCG
AJ786592 -TCGCC-GCAGCC-TCCCCTGCGTAGTAGCACA-AC---TCGCACC-GGAGCGCGGAGAC
AB103381 -TCGCC-GCAGCC-TCCCCTGCGTAGTAGCACA-AC---TCGCACC-GGAGCGCGGAGAC
AB208110 -TCGCC-GCAGCC-TCCCCTGCGTAGTAGCACA-ACC--TCGCACC-GGAGCGCGGAGAC
T4-p-ITS5 -TCGCC-GCAGCC-TCCCCTGCGTAGTAGCACA-ACC--TCGCACC-GGAGCGCGGAGAC
AJ786568 -TCGCCCGCAGCC-TCCCCTGCGCAGTAGCACA-ACC--TCGCACC-GGAGCGCGGAGAC
AB027373 ACCGCC-GCGGCC-TCCCCTGCGAAGTAGCACA-GCG--ACGCACT-GGAGCGCGGTGGC
AJ786563 ACCGCC-GCGGCC-TCCCCTGCGAAGTAGCACA-GCG--ACGCACT-GGAGCGCGGTGGC
5E-p-ITS3 ACCGCC-GCGGCC-TCCCCTGCGAAGTAGCACA-GCG--ACGCACT-GGAGCGCGGTGGC
AB027364 CTCGCC-GCGGCC-TCCCCTGCGCAGTAGCACA-ACC--TCGCACC-GGAGCGCGGCGGC
AJ786557 -TCGCC-GCAGCC-TCCCCTGCGCAGTAGCACA-ACC--TCGCACC-GGAGCGCGTCGAC
TCC1p-p-IT -TCGCC-GCAGCC-TCCCCTGCGCAGTAGCACA-ACC--TCGCACC-GGAGCGCGCCGAC
AB027367 -TCGCC-GCAGCC-TCCCCTGCGTAGTAGCACA-ACC--TCGCACC-GGAGCGCGGAGAC
COP309360 -TCGCC-GCAGCC-TCCCCTGCGTAGTAGCACA-ACC--TCGCACC-GGAGCGCGGAGAC
TCO1-p-ITS -TCGCC-GCAGCC-TCCCCTGCGTAGTAGCACA-ACC--TCGCACC-GGAGCGCGGAGAC

HRU301991 -TCCACGTCCGTAAAACACCCA---ACTCTCTGAA----ATGTTGACCTC
AB027384 -TCCACGTCCGTAAAACACCCA---ACTTTCTGAA----ATGTTGACCTC
AY624179 GGCCAC-GCCGTAAAACCCCC----GACTTCTGAA-----CGTTGACCTC
T18-p-ITS5 GGCCAC-GCCGTAAAACCCCC----GACTTCTGAA-----CGTTGACCTC
AJ786573 GGCCAC-GCCGTAAAACGCCC----AAC-TCTGAA-----CGTTGACCTC
TCmE-p-ITS GGCCAC-GCCGTAAAACGCCC----AAC-TCTGAA-----CGTTGACCTC
VLE292383 GGCCAC-GCCGTAAAACACCC----AACTTCTGAA-----CGTTGACCTC
CAP292388 GGCCAC-GCCGTAAAACACCC----AACTTCTGAA-----CGTTGACCTC
AY833600 GGCCAC-GCCGTAAAACACCC----AACTTCTGAA-----CGTTGACCTC
T20-p-ITS5 GGCCAC-GCCGTAAAACACCC----AACTTCTGAA-----CGTTGACCTC
AB086224 GGCC-C-GCCGTGAAACCCCC----AACCTCTGAA-----CGTTGACCTC
AB086631 GGCC-C-GCCGTGAAACCCCC----AACCTCTGAA-----CGTTGACCTC
T53-p-ITS5 GGCC-C-GCCGTGAAACCCCC----AACCTCTGAA-----CGTTGACCTC
AY624181 GGCCAC-GCCGTAAAACACCC----AAC-TCTGAA-----CGTTGACCTC
T10-p-ITS5 GGCCAC-GCCGTAAAACACCC----AAC-TCTGAA-----CGTTGACCTC
AY532023 GGCCAC-GCCGTAAAACACCC----AACTTCTGAA-----CGTTGACCTC
T52-beta GGCCAC-GCCGTAAAACACCC----AACTTCTGAA------CGTTGACCTC
AY532025 GGCCAC-GCCGTAAAACACCC----AACTTCTGAA-----CGTTGACCTC
AB027381 GGCCAC-GCCGTAAAACACCC----AACTTCTGAA-----CGTTGACCTC
T42-p-ITS5 GGCCAC-GCCGTAAAACACCC----AACTTCTGAA-----CGTTGACCTC
AY387580 GTCCACTGCCGTAAAACCCCCC---AACTTTTTAT----A-GTTGACCTC
AB027383 GTCCACTGCCGTAAAACCCCCC---AACTTTTTAT----A-GTTGACCTC
T41-p-ITS5 GTCCACTGCCGTAAAACCCCCC---AACTTTTTAT----A-GTTGACCTC
AJ786592 GGTCAC-GCCGTAAAACGCCC----AACTTC-TCA----GAGTTGACCTC
AB103381 GGTCAC-GCCGTAAAACGCCC----AACTTC-TCA----GAGTTGACCTC
AB208110 GGTCAC-GCCGTAAAACGCCC----AACTTC-TCA----GAGTTGACCTC
T4-p-ITS5 GGTCAC-GCCGTAAAACGCCC----AACTTC-TCA----GAGTTGACCTC
AJ786568 GGTCAC-GCCGTAAAACGCCC----AACTTCCTCA----GAGTTGACCTC
AB027373 GGTCAC-GCCGTAAAACCCCCCCCGAAACCCCCCCCGTGGAGTTGACCTC
AJ786563 GGCCAC-GCCGTAAAACCCCCGT-GAACCCCCCCCCGTGGAGTTGACCTC
5E-p-ITS3 GGTCGC-GCCGTAAAACCCCC---GAAACCCCCCCCGTGGAGTTGACCTC
AB027364 GGTCAC-GCCGTAAAACGCCC----AACTTT-CGC----GAGTTGACCTC
AJ786557 GGTCAC-GCCGTAAAACGCCC----GACCCT-CA-----GAGTTGACCTC
TCc1p-p-IT GGTCAC-GCCGTAAAACGCCC----AACTCT-CA-----GAGTTGACCTC
AB027367 GGTCAC-GCCGTTAAACGCCC----AACTTC-TCA----GAGTTGACCTC
COP309360 GGTCAC-GCCGTAAAACGCCC----AACTTC-TCA----GAGTTGACCTC
TCo1-p-ITS GGTCAC-GCCGTAAAACGCCC----AACTTC-TCA----GAGTTGACCTC

Figure III.2: Sequence alignment for LSU data matrix containing 30 taxa, 523 characters, 410 constant characters, 23 parsimony-uninformative variable characters, and 90 parsimony-informative characters.

|  |  |
| :---: | :---: |
| AF543791 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCCC--------GGGTCCGAGTTGTAA |
| AY015634 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCTCC---------GGGTCCGAGTTGTAA |
| AF172342 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCTCC---------GGGTCCGAGTTGTAA |
| L18-p-LROR | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCTCC----------GGGTCCGAGTTGTAA |
| AB027379 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCC----------GGGTCCGAGTTGTAA |
| LCmE-p-LRO | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCC---------GGGTCCGAGTTGTAA |
| AF339555 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCCC---------GGGTCCGAGTTGTAA |
| AY283556 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCTT---------GGGTCCGAGTTGTAA |
| L20-p-LROR | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCTT-----------GGGTCCGAGTTGTAA |
| AY184968 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCCA--------GGGCCCGAGTTGTAA |
| AB027380 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCC----------GGGTCCGAGTTGTAA |
| L53-p-LROR | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCC----------GGGTCCGAGTTGTAA |
| L10-p-LROR | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCC----------GGGTCCGAGTTGTAA |
| AB027382 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCA--------GGGCCCGAGTTGTAA |
| L52-p-LROR | ??????? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? |
| AB027381 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCTC---------GGGTCCGAGTTGTAA |
| L42-p-LROR | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCTC-----------GGGTCCGAGTTGTAA |
| AF339530 | AAGCGGCAACAGCTCAAATTTGAAATCTGGTCCCCA---------GGGCCCGAGTTGTAA |
| AB027383 | AAGCGGCAACAGCTCAAATTTGAAATCTGGTCCCCA--------GGGCCCGAGTTGTAA |
| L41-p-LROR | AAGCGGCAACAGCTCAAATTTGAAATCTGGTCCCCA----------GGGCCCGAGTTGTAA |
| AB044645 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCCA--------GGGCCCGAGTTGTAA |
| AF049173 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCCA--------GGGCCCGAGTTGTAA |
| L4-p-LROR | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCCA---------GGGCCCGAGTTGTAA |
| AB027373 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCCCCGGCCGCGGGGGCCCGAGTTGTAA |
| L-cg-p-LRO | AAGCGGGCAACAGCTCAAATTTGAAATCTGGCCCCCCGGCCCGCGGGGGCCCGAGTTGTAA |
| AY489721 | AAGCGGCAGCAGCTCAGATTTGAAATCTGGCCCCCA--------GGGCCCGAGTTGTAA |
| LCclp-p-LR | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCCA--------GGGCCCGAGTTGTAA |
| AB027367 | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCTA--------GGGCCCGAGTTGTAA |
| LCO1-p-LRO | AAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCCA--------GGGCCCGAGTTGTAA |

HRU301991 TTTGTAGAGGATGCTTTTGGTGAGGTGCCGCCCGAGTTCCCTGGAACGGGACGCCGCAGA

AF543791
AY015634
AF172342
L18-p-LROR
AB027379
LCmE-p-LRO
AF339555
AY283556
L20-p-LROR
AY184968
AB027380
L53-p-LROR L10-p-LROR TTTGCAGAGGATGCTTTGGGCGAGGTGCCTTCCAAGTTCCCTAGAACGGGACGCCACAGA AB027382 TTTGTAGAGGATGCTTTTGGCGAGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCACAGA L52-p-LROR ??????????????????????????????????????????????????????????GA AB027381 TTTGTAGAGGATGCTTTTGGCGAGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCACAGA L42-p-LROR TTTGTAGAGGATGCTTTTGGCGAGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCACAGA AF339530 TTTGCAGAGGATGCTTTTGGTGAGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGA AB027383 TTTGCAGAGGATGCTTTTGGTGAGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGA L41-p-LROR TTTGCAGAGGATGCTTTTGGTGAGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGA AB044645 TTTGCAGAGGATGCTTTTGGCGCGGCGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGA AF049173 TTTGCAGAGGATGCTTTTGGCGCGGCGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGA L4-p-LROR TTTGCAGAGGATGCTTTTGGCGCGGCGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGA AB027373 TTTACAGAGGATGCTTTCGGCGCAGCGCCTTCCGAGTTCCCTGGAACGGGACGCCACAGA L-cg-p-LRO TTTGCAGAGGATGCTTTCGGCGCGGCGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGA AY489721 TTTGCAGAGGATGCTTTTGGCGCGGCGCCTTCCGAGTGCCCTGGAACGGGACGCCATAGA LCc1p-p-LR TTTGCAGAGGATGCTTTTGGCGCGGCGCCTTCCGAGTGCCCTGGAACGGGACGCCATAGA AB027367 TTTGCAGAGGATGCTTTTGGCGCGGCGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGA LCo1-p-LRO TTTGCAGAGGATGCTTTTGGCGCGGCGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGA

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L4-p-LROR GGGTGAGAGCCCCGTCTGGTCGGACGCCAAGCCAGTGTAAAGCTCCTTCGACGAGTCGAG
AB027373 GGGTGAGAGCCCCGTACGGTCGGACGCCCGGCCACTGTAAAGCTCCCTCAATGAGTCGAG
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AY489721 GGGTGAGAGCCCCGTCTGGTCGGACGCCAAGCCAGTGTAAAGCTCCTTCGACGAGTCGAG
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LCo1-p-LRO GGGTGAGAGCCCCGTCTGGTCGGACGCCAAGCCAGTGTAAAGCTCCTTCGACGAGTCGAG

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HRU301991 TACCCTGCGC-TGGACTGAGGACCGCGCATC-TGCAAGGATGC AF543791 TACCCTGCGG-TGGACTGAGGACCGCGCATC-TGCAAGGATGC AY015634 TACCCTGCGC-CGGACTGAGGTACGCGCAT--CGCAAGGATGC AF172342 TACCCTGCGC-CGGACTGAGGT-CGCGCAT--CGCAGGGATGC L18-p-LROR TACCCTGCGC-CGGACTGAGGTACGCGCAT--CGCAAGGATGC AB027379 TACCCTGCGC-TGGACTGAGGTACGCGCATT-CGCAAGGATGC LCmE-p-LRO TACCCTGCGC-TGGACTGAGGTACGCGCATT-CGCAAGGATGC AF339555 TACCCTGCGC-CGGACTGAGGCACGCGCAT--CGCAAGGATGC AY283556 TACCCTGCGC-CGGACTGAGGTACGCGCAT--TGCAAGGATGC L20-p-LROR TACCCTGCGC-CGGACTGAGGTACGCGCAT--TGCAAGGATGC AY184968 TACCCTGCGC-CGGACTGAGGTACGCGCAT--CGCAAGGATGC AB027380 TACCCTGCGC-CGGACTGAGGTACGCGCAT--CGCAAGGATGC L53-p-LROR TACCCTGCGC-CGGACTGAGGTACGCGCAT--CGCAAGGATGC L10-p-LROR TACCCTGCGC-CGGACTGAGGTACGCGCAT--CGCAAGGATGC AB027382 TGCCCTGCGC-CGGACTGAGGTACGCGCAT--TGCAAGGATGC L52-p-LROR TGCCCTGCGC-CGGACTGAGGTACGCGCAT--TGCAAGGATGC AB027381 TACCCTGCGC-CGGACTGAGGTACGCGCAT---TGCAAGGATGC L42-p-LROR TACCCTGCGC-CGGACTGAGGTACGCGCAT--TGCAAGGATGC AF339530 TACCTTGTGG-CGGGCTGAGGTTCGCGCTTTATGCAAGGATGC AB027383 TACCCTGTGG-CGGGCTGAGGTTCGCGCTTTATGCAAGGATGC L41-p-LROR. TACCCTGTGG-CGGGCTGAGGTTCGCGCTTTATGCAAGGATGC AB044645 TACCCTGCGG-TGGACTGAGGTTCGCGCTC--CGCAAGGATGC AF049173 TACCCTGCGG-TGGACTGAGGTTCGCGCTC--CGCAAGGATGC L4-p-LROR TACCCTGCGG-TGGACTGAGGTTCGCGCTC--CGCAAGGATGC AB027373 TGCCCCGCGCGCGGACTGAGGCACGCGCTC--CGCAAGGATGC L-cg-p-LRO TGCCCCGCGCGCGGACTGAGGCACGCGCTC--CGCAAGGATGC AY489721 TGCCCTGGGG-CGGACTGAGGTTCGCGCTC--CGCAAGGATGC LCc1p-p-LR TGCCCTGGGG-CGGACTGAGGTTCGCGCTC--CGCAAGGATGC AB027367 TACCCTGCGG-TGGACTGAGGTTCGCGCTC--CGCAAGGATGC LCo1-p-LRO TACCCTGCGG-TGGACTGAGGTTCGCGCTC--CGCAAGGATGC

## Appendix IV MS-MS AND 1H-NMR SPECTRA FOR COMPOUND 3 AND POSSIBLE STRUCTURES FOR COMPOUNDS 2 AND 3



Figure IV.1: Possible structures of compounds 2 and 3 as inferred from MS-MS and 1H-NMR spectra. Compounds are small peptides, likely differ by a methyl group, and are associated with $\mathrm{Na}^{+}$. Structure created using ChemDraw (CaimbridgeSoft).





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Sample No: 8960 Brian King BE3 / 20
とing8960 $111 \mathrm{H} / \mathrm{CD} 30 \mathrm{D}$


| Current Data Parameters |  |
| :--- | ---: |
| NAME | king8960 |
| EXPNO | 1 |
| PROCNO | 1 |

F2 - Acquisition Parameter
Date_ 20050922
Time 9.06
INSTRUM av400
$\begin{array}{llll} \\ \text { PROBHD } \\ 5 \mathrm{~mm} & \text { BBI } & \text { av4co } \\ \text { 1H-BB }\end{array}$
2930
TO 32768
SOLVEN
NS
NS
DS
SWH
FIDRES $\quad 4401.409 \mathrm{~Hz}$
$\begin{array}{lr}0.134320 \mathrm{~Hz} \\ A Q & 3.7224948 \mathrm{se}\end{array}$
456.1
113.600 us 6.00 us 300.0 K 1.00000000 se

| $=======$ CHANNEL $f 1 \quad======$ |  |
| :--- | ---: |
| NUC1 | 1 H |
| P1 | 7.25 us |
| PL. | 0.00 dB |
| SFO1 | 400.1319254 MH |
|  |  |
| F2 - Processing parameters |  |
| SI | 32768 |
| SF | 400.1300078 MH |
| WOW | EM |
| SSB | 0 |
| LB | 0.10 Hz. |
| GB | 0 |
| PC | 0.50 |

10 NMR plot parameters
CX $\quad 20.00$ 20.00 cm 0.00 cm 10.292 pp 4118.29 Hz -0.708 pp . $-283.12 \mathrm{~Hz}$ 0.55000 p 20.07042 Hz
Current Data Parameters
NAME
EXPNO
PROCNO King8960
king8960
Sample No: 8960 Brian King BE3 / 20 king8960 $1111 \mathrm{H} / \mathrm{CD} 30 \mathrm{D}$

| F2 - ACquisition Parameter |  |
| :--- | ---: |
| Date_ | 20050922 |
| Time | 9.06 |
| INSTRUM | av 400 |
| PROBHD | 5 mm BeI $1 \mathrm{H}-\mathrm{BE}$ |
| PULPROG | 2930 |
| TD | 32768 |
| SOLVENT | MeOH |
| NS | 192 |
| DS | 2 |
| SWH | 4401.409 Hz |
| FIDRES | 0.134320 Hz |
| AQ | 3.7224948 se |
| RG | 456.1 |
| DW | 113.600 us |
| DE | 6.00 us |
| IE | 300.0 K |
| DI | 1.00000000 se |


| NUC1 | 1 |
| :---: | :---: |
| P1 | 7.25 |
| PL. 1 | 0.00 |

SFO1 400.1319254 MH

| F2 - ProcessingDarameters <br> SI <br> SF | 32768 |
| :--- | :---: |
| WDW | 400.130007 MH |
| SSB | EM |
| LB | 0 |
| GB | 0.10 Hz |
| PC | 0 |
|  | 0.50 |

10 NMA plot parameters

| CX | 20.00 cm |
| :--- | ---: |
| CY | 0.00 cm |
| FiP | 10.292 pD |
| Fi | 4918.29 Hz |
| F2P | -0.70 pp |
| F2 | -283.12 Hz |
| PPMCM | 0.55000 pp |
| HZCM | 220.07042 Hz |

Sample No: 8950 Brian King BE3 / ZD
king8960 $111 \mathrm{H} / \mathrm{CO} 30 \mathrm{D}$
NAME
EXPNO
EXPNO
king8960
(i. in in



[^0]:    ${ }^{1}$ A version of this chapter is in preparation for submission to a peer reviewed journal.

[^1]:    * an asterisk designates that only a single replicate was performed

[^2]:    TCol-p-ITS CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT

