

Barcoding and microcoding using “identiprimers” with *Leptographium* species

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Abstract: *Leptographium* species provide an ideal model to test the applications of a PCR microcoding system for differentiating species of other genera of ascomycetes. *Leptographium* species are closely related and share similar gross morphology. Probes designed for a PhyloChip for *Leptographium* have been transferred and tested as primers for PCR diagnostic against *Leptographium* species. The primers were combined with complementary universal primers to identify known and suspected undescribed species of *Leptographium*. The primer set was optimized for 56 species, including the three varieties of *L. wageneri*, then blind-tested against 10 random DNA samples. The protocols established in this study successfully identified species from the blind test as well as eight previously undescribed isolates of *Leptographium*. The undescribed isolates were identified as new species of *Leptographium* with the aid of the microcoding PCR identification system established in this study. The primers that were positive for each undescribed isolate were used to determine close relatives of these species and some of their biological characteristics. The transfer of oligonucleotides from a micro-array platform to a PCR diagnostic was successful, and the identification system is robust for both known and unknown species of *Leptographium*.

Key words: microcoding, PCR, phenogram, species identification

INTRODUCTION

Amplification of nuclear DNA with different primer sequences and the subsequent analysis of resulting band patterns have been used to differentiate morphologically similar species of fungi (Chen et al. 2001, Fujita et al. 2001, Hamelin et al. 1996). A common approach to identification is to use universal primers to amplify a gene region, sequence this region and then perform a phylogenetic analysis on the sequence data. This approach has been used to identify new species of *Leptographium* or to confirm the identities of previously described species (Jacobs et al. 2000, 2005). The reverse approach is to use available sequence data to design specific primers that amplify a unique and characterized sequence of DNA, thus circumventing a sequencing step (Bäckmann et al. 1999, Hamelin et al. 1996). These primers can be present as a pair in a PCR mix or multiplexed with other specific primers (Fujita et al. 2001, Jackson et al. 2004, Redecker 2000). Primers for species identification in bacteria have been designed around unique polymorphisms that are species specific (Bäckmann et al. 1999, Easterday et al. 2005). In other cases universal primers are designed to amplify a single amplicon of a particular length that is definitive of a species (Chen et al. 2001, Fujita et al. 2001).

The genealogical concordance phylogenetic species recognition system uses several gene regions to fully delineate and separate clusters of species into single taxonomic units (Taylor 2000) and can be used to provide the framework for an identification system. Species are delineated through shared and unshared sequence characteristics or polymorphisms across several gene regions (Jacobs et al. 2006, O'Donnell et al. 2000, Taylor et al. 2000). It is possible to design primers around these polymorphisms so that a single amplicon of a known size will be amplified from a DNA sample only if the primer sequence is present in the genome, thereby identifying a species (Bäckmann et al. 1999, Hamelin et al. 1996, Tran and Rudney 1996). This approach has been used for microcoding species of fungi and can be equally as diagnostic as PCR amplification followed by sequencing (Summerbell et al. 2005).

Microcoding has been defined as a specific type of DNA barcoding that allows for the identification of genus or species (Summerbell et al. 2005). DNA barcoding traditionally uses highly conserved genes, such as 18S rDNA and the large ribosomal

subunit, to assign fungi to higher taxonomic classifications such as family and order (Summerbell et al. 2005). Generic and species gene regions include the internal transcribed spacer region (ITS), β -tubulin (β T) and translational elongation factor (EF1 α) as well as the mitochondrial CO1 gene that are less conserved (Seifert et al. 2007, Summerbell et al. 2005). The primers used for microcoding are 20-mer primers that are designed based on variable regions of these genus and species genes and serve to differentiate organisms at either rank. A set of 20-mer primers were designed for 56 species of *Leptographium* (van Zuydam 2009) to be used on a micro-array platform as a PhyloChip.

The term PhyloChip is used to describe a species diagnostic micro-array that has an intrinsic probe hierarchy (Metfies and Medlin 2007). The hierarchy is based on the phylogeny of a group of taxa where certain probes will identify nodes of a phylogram. The progression of probes eventually leads to the identification of a known species or a new species (Anderson et al. 2006, Loy et al. 2002).

PhyloChip for *Leptographium* was designed with a hierarchical set of probes designed from the ITS2, β T and EF1 α gene regions available for 56 species. The design consisted of a mixture of common and unique 20-mer probes that identified individual species in different combinations. The ITS2 probes included a single generic probe ITSP1 in combination with specific probes, which identified particular nodes on a phenogram and delineated species. The ITS2 probes split the genus into five clades that approximated phylogenetic and morphological groups within the genus (van Zuydam 2009). The large clades, defined by the ITS2 probes, were divided into smaller clades and individual species based on β T and EF1 α probes.

In the current study we modeled a PCR diagnostic system on a PhyloChip design concept with the probes designed for the *Leptographium* PhyloChip. The system uses the phenograms constructed from the probes for PhyloChip to define the sequence of diagnostic PCRs that led to species identification (van Zuydam 2009). If a primer is common to a group of species it will define a node, and if a primer is species specific it will define a branch (van Zuydam 2009). Therefore amplifications using primers for nodes will be conducted before those defining a species. This is similar in organization to PhyloChips, but the primers are combined with either a forward or a reverse universal primer that allows a dynamic system that can identify known as well as new species. This approach thus is potentially more powerful than micro-arrays, and it is much cheaper because it

requires less costly equipment and reagents. We chose to validate our primers on the fungal genus *Leptographium*.

Leptographium is the anamorph genus of *Grosmanina* and is relatively small when compared to other genera within Ophiostomatoid fungi (Zipfel et al. 2006). *Leptographium* species are characterized by mononematous, branched conidiophores that produce aseptate, hyaline conidia in a slimy matrix (Jacobs 1999, Kendrick 1962). *Leptographium* species are differentiated based on culture color, optimal growth temperature, cyclohexamide tolerance, size and branching pattern of the conidiophores and morphology of the conidia (Jacobs et al. 2001). *Leptographium* species are difficult to identify based on morphological characters because the morphology is so similar among species. It is possible to identify *Leptographium* species accurately by employing molecular techniques; this is achieved through constructing phylogenies from available sequence data (Jacobs et al. 2000, Zhou et al. 2000). Molecular characters are combined with morphological characters to describe new species (Jacobs et al. 2000, 2001). As a result there is a comprehensive sequence dataset for 56 species across regions of the ITS2, β T and EF1 α genes (Jacobs et al. 2006).

The sequence data available for genus *Leptographium* have been used to design a probe set for PhyloChip based on shared and unshared sequence polymorphisms. The phenograms constructed from the probe set approximate the phylogenies and morphological groups presented by Jacobs (2006) and van Zuydam (2009). Thus our aim was to microcode 56 known species of *Leptographium* and eight previously undescribed isolates with probes from PhyloChip as “identiprimers” combined with a complementary universal primer.

MATERIALS AND METHODS

DNA isolation and isolates.—Isolates in this study were identified according to morphological characters. Species identification of all isolates had been confirmed by DNA sequence comparisons (Jacobs et al. 2006, van Zuydam 2009). DNA was extracted with the soil microbe DNA isolation kit (Fermentas, USA) according to the manufacturer’s instructions.

Primers.—Those used in this study were designed previously as probes for a species diagnostic micro-array (van Zuydam 2009). They were combined with a universal primer that was designed from the opposite strand and, as the name implies, was identical in DNA sequence for all species in *Leptographium*. Identiprimers for the ITS2 region were combined with either ITS3 (+) or LR3 (–) (White et al. 1990), identiprimers for β T were combined with either Bt2a (+) or BT2b (–) (Glass and Donaldson 1995) and the

TABLE I. Primer sequences, amplicon size and optimized conditions for the identification of 56 described *Leptographium* species

Species	Primer	Sequence	Sense	T _A (C)	Size (bp)	MgCl ₂ (mM)	2-pyrrolidone
Node primer	ITSP1 ^a	AATGCTGCTCAAAATGGGAGG	+	55	370		
Node primer	ITSP7 ^a	CAGACCGCAGACGCAAGT	+		700		
Node primer	ITSP8 ^a	CCAGCCTTTGTGAAGCTCC	+		400		
Node primer	ITSP9 ^a	CCCTAAAGACGGCAGACG	+		800		
<i>L. abieticolens</i>	BTP4	CCGTCCTTGTGGATCTCG	+	68	130		
<i>L. peucophilum</i>	BTP17	ATATGGCGGATTAGATAACCACC	−	68	200		
	BTP18	CTAACAGATGTCACAGGCAG	+	68	250		
	EF1 α P13	AAGGTCCCACAAGGCAGA	−	65	200		1/1000
<i>L. euphyes</i>	EF1 α P12	TCGCCGCTAATACCCAATAC	+	65	250		1/1000
<i>L. neomexicanum</i>	EF1 α P2	AAACAGGGAATGAAGAATTGCC	−	58	900	2	
	EF1 α P3	AAAGGCAGGGAATGAAGAATTG	−	58	900	2	
<i>L. reconditum</i>	EF1 α P3	AAAGGCAGGGAATGAAGAATTG	−	58	900		
<i>L. douglassi</i>	ITSP9	CCCTAAAGACGGCAGACG	+	55	800		
<i>L. pineti</i>	ITSP25	AAGGAAAGGAGACTTGCGT	−	54	380		
Node primer	ITSP1 ^a	AATGCTGCTCAAAATGGGAGG	+	55	390		
<i>L. abietinum</i>	ITSP3	ATTGGTTGCTGCAAGCGT	−	51	200		
	EF1 α P42	AATGAAAAGAGGGGCGAGG	−	68	900		
	ITSP3	ATTGGTTGCTGCAAGCGT	−	51	200		
<i>L. americanum</i>	EF1 α P37	AATGCAGGTCACACAGG	−	68	490		
	ITSP2	GGAGCTTCGCAAAGGCCA	−	55	450		
	EF1 α P8	AAAGAGCCCTTGCCGAGC	−	68	550		
<i>L. brachiatum</i>	ITSP2	GGAGCTTCGCAAAGGCCA	−	55	450		
	EF1 α P27	AACAACCAATACAGGAGGCTG	+	68	200		
<i>L. rubrum</i>	ITSP2	GGAGCTTCGCAAAGGCCA	−	60	900		
	EF1 α P28	AAACGAGGATGATTTGGGCAA	−	68	200		1/10
	EF1 α P38	AAACACACACGCCACAACC	+	65	400	2	
<i>L. bistatum</i>	ITSP4	CAAAGCGAGGGCTAATGCT	−	62	150		
	BTP22	ACACGCCATTGCTGTCCA	−	58	150		
<i>L. eucalyptophilum</i>	ITSP4	CAAAGCGAGGGCTAATGCT	−	62	150		
	BTP5 ^b	CGTCTTCGCCAGGTACAACG	+		250		
	BTP3 ^b	ACAGCATCCATCGTGCCG	−		150		
<i>L. calophylli</i>	ITSP17	TGTAATTTGGAGAGGATGCTTT	+	55	200		
	EF1 α P21	AAACGGGCTTTATCTCAGGAC	−	68	250		
<i>L. dryocoetidis</i>	ITSP17	TGTAATTTGGAGAGGATGCTTT	+	55	400		
	BTP24	CCTCGTTGAAGTAGACGCTC	−	68	100		1/10
<i>L. chlamydatum</i>	BTP20	CCAGGCAGCAGATTTCCG	+	68	490		
	BTP25	CTGGAGATCAGAGTTGCCAT	−	60	150	1.5	
	BTP21	CATGGATGCCGTCCGTGC	+	65		1.5	
<i>L. costaricense</i>	BTP21	CATGGATGCCGTCCGTGC	+	65		1.5	
<i>L. leptographioides</i>	EF4lep	CGA[C]ATTGCTCTGTGGAAGTT ^c	+	65		1.5	
<i>L. pruni</i>	BTP24	CCTCGTTGAAGTAGACGCTC	−	67	400	1.5	
<i>L. curvisporum</i>	BTP20	CCAGGCAGCAGATTTCCG	+	68	490		
<i>L. brevicollis</i>	BTP24	CCTCGTTGAAGTAGACGCTC	−	68	100		
<i>L. crassivaginatatum</i>	BTP3 ^b	ACAGCATCCATCGTGCCG	−		150		
	ITSP6	CACAAGGTTGACCTCGGAT	+	58	550	2.0	
<i>L. francke-grosmanniae</i>	ITSP24	AACCTTTGAGATAGACTTGCG	−	58	400		
	ITSP1 ^a	AATGCTGCTCAAAATGGGAGG		55	370		
	ITSP12 ^a	CGGTTGGACGCCTAGCCTTT	+				
<i>L. bhutannense</i>	ITSP22	AAATGACCGGCAGACGCAA	+				
Node primer	ITSP15 ^a	GAGCTTCACAAAGGCTAGGC	−	60			
<i>L. aenigmaticum</i>	BTP11	GACCGTGCTCGCTGGAGATC	−	55	159		
	BTP12	CAGACGTGCCGTTGTACC	−	60	250		
<i>L. albofini</i>	BTP16	AATGGCGTGTAGGTTTCCG	+	68	300		
<i>L. clavigerum</i>	EF1 α P36	AAGCAGGTGGGGATGAGATG	−	55	260		
<i>L. koreanum</i>	BTP8	CAACAAGTACGTGCCTCGC	+	68	150	1.5	

TABLE I. Continued

Species	Primer	Sequence	Sense	T _A (C)	Size (bp)	MgCl ₂ (mM)	2-pyrrolidone
<i>L. aureum</i>	BTP3 ^b	ACAGCATCCATCGTGCCG	–	64	200		
	BTP1 ^b	ACAGCAATGGAGTGTAGGT	+				
<i>L. guttulatum</i>	BTP37	CGGAAGAGCTGCCCAAAG	–	68	380		
<i>L. laricis</i>	EF1 α P5	TTAAAACCTGACCGCCCAAAA	–				
<i>L. longiclavatum</i>	EF1 α P32 ^b	AGGCAGAAAGACAGGGAAAGAGA	–	66	250	2	
<i>L. lundbergii</i>	BTP37	CGGAAGAGCTGCCCAAAG	–	68	480		
<i>L. pyrinum</i>	BTP31b	AAGAGCGTCTATTGTGGTGT	–		150		
<i>L. robustum</i>	EF1 α P39	AAAGACAGGGAGGAGGATTTG	–	60	150		
<i>L. trinacriforme</i>	BTP30 ^b	AGAATTTGTCACTTCAAGCAGA	–				
<i>L. truncatum</i>	BTP13	CACGGCATCCATCGTACC	–	57	450		
	EF1 α P20	TGGGCAAGGGCTCTTTCAA	+	68	250		
	BTP8	CAACAAGTACGTGCCTCGC	+	55	200		
<i>L. wingfieldii</i>	EF1 α P36	AAGCAGGTGGGGATGAGATG	–	64	280	1.5	
<i>L. yunnanensis</i>	BTP13	CACGGCATCCATCGTACC	–	60	195		
	BTP15	AATGGCGTGTAGGTTCCG	+	60			
<i>L. sibiricum</i>	ITSP23	AAATGACCGGGAAGACGCA	+	65	550		
<i>L. piceaperdum</i>	ITSP27	CCAAAATAAGGGCAGGGCG	–	65	700		
<i>L. huntii</i>	ITSP11	CGAGTCTGTCTCCTTCTCAA	+	65	650		
	BTP13	CACGGCATCCATCGTACC	–	58	350	1.5	
<i>L. pityophilum</i>	ITSP11	CGAGTCTGTCTCCTTCTCAA	+	65	650		
	EF1 α P11	AAGACTTCTCCAACAGGTGG	–	58	700		
Node primer	ITSP22 ^a	AAATGACCGGCAGACGCAA	+	45	790		
<i>L. penicillatum</i>	BTP18	CTAACAGATGTCACAGGCAG	+	69	250	2.0	
	BTP10	AGATTTCTAGCGAGCATGGC	+	69	900	1.5	
<i>L. profanum</i>	BTP18	CTAACAGATGTCACAGGCAG	+	69	300	2.0	
<i>L. pini-densiflorae</i>	ITSP18	AAAGGAGGGACAGACTTGC	–	65	900		
<i>L. procerum</i>	ITSP15	GAGCTTACAAAGGCTAGGC	–	64	500		
<i>L. serpens</i>	BTP4	CCGTCCTTGTGGATCTCG	+	60	250		
<i>L. terebrantis</i>	BTP3	ACAGCATCCATCGTGCCG	–	63	350		
<i>L. wageneri</i> var. <i>wageneri</i>	EF1 α P22	AAAGGAAACACGGAGAGCATCG	+		600		
	Node primer	ITSP10 ^a	CTCCGAGCGTAGTAAGCA	+			
<i>L. fruticetum</i>	ITSP10 ^a		+	55	600		
<i>L. wageneri</i> var. <i>pseudotsugae</i>	ITSP10 ^a			55	500		
Node primer	ITSP9 ^a	CCCTAAAGACGGCAGACG	+	57	750		
<i>L. elegans</i>	ITSP1 ^a	AATGCTGCTCAAAATGGGAGG	+	55	370		
	EF5ele	CGGTGCCTATTCTCGTGGT	+	60	400		
<i>L. wageneri</i> var. <i>ponderosa</i>	BTP28	AATCATGCACAGAGACTAACA	+	55	290	1.5	
	BTP29	ATCGCAGCTCGGGTAGATC	–		300		
	EF1 α P22	AAAGGAAACACGGAGAGCATCG	+		600		
<i>L. grandifoliae</i>	BTP33	AAACCTCCGAGATGTCCAC	+		150		

^aNode primers are common to different *Leptographium* species that are found within the same clade of the phenograms.

^bIndicates primer failures where primers need to be redesigned.

^cSquare brackets indicate a locked nucleic acid at a SNP site within a primer.

identiprimers for EF1 α were combined with either EF1F (+) or EF2R (–) (Jacobs et al. 2005).

PCR optimization.—*Multiplex PCR.* The identiprimers for Clade 1 were combined into a multiplex PCR that consisted of 2.5 mM MgCl₂, 1 \times Buffer, 0.4 mM dNTPs, and 1 U SuperTherm *Taq* polymerase (Southern Cross), 0.4 mM of

ITSP1, ITSP7, ITSP8 and ITSP9, 1.6 mM of LR3, 0.8 \times V DNA in a 5 μ L reaction. These primers were optimized against all species in clade 1 to amplify the correct regions. The reaction conditions of PCR using clade-specific probes were optimized so that amplicons were produced only when DNA from isolates within a clade were used in the reaction. The probes were optimized for DNA from isolates within

clades along the subbranches. The amplifications were optimized according to temperature, magnesium chloride concentration and 2-pyrrolidone concentration on each species in this study (TABLE I). A negative control containing no DNA was included in every optimization step. The stock solution of 2-pyrrolidone was diluted 1:10, and further dilutions were made from this working solution. The standard PCR mixture consisted of 2.5 mM MgCl₂, 1× buffer, 1 U SuperTherm *Taq* polymerase (Southern Cross, South Africa), 0.4 mM dNTP mix, 0.4 mM of each primer and 0.08 × reaction volume of DNA. Five microliter reactions were used and the entire volume was used to determine amplicon presence and size. Amplicons were separated by gel electrophoresis through a 3% agarose gel at 80V 40 min and stained with GelRed (Anatech, USA) and viewed under UV light.

Blind test.—Ten DNA samples representing 10 species were chosen independently at random from DNA isolated from the 56 species in this study and relabeled 1–10. These samples were analyzed and identified to species with the protocols established in this study. Positive controls with the DNA from amplicon positive species and a negative control containing no DNA were included in every PCR identification step. The identification process was repeated in triplicate to measure reproducibility.

Identification of new isolates.—Isolates representing eight previously undescribed (TABLE II) *Leptographium* species were included. These species were tested with established protocols from this study, and the same positive and negative controls included in the blind test were included in PCR identification steps.

Phenogram construction.—NTSYSpc21 2.11 (Applied Biostatistics) was used to construct phenograms. A matrix was built for each gene region based on in silico alignments of probes with partial gene sequences from each species of *Leptographium*. The matrix was scored such that 1 represents a positive amplification of a DNA fragment of the expected size and 0 represents no amplification. This matrix was amended with the banding patterns obtained for the blind test isolates as well as for the undescribed *Leptographium* species. The file was formatted according to the software developers' instructions and used as the primary input for subsequent analysis. The variables were standardized with STAND and a distance matrix was constructed with SIMINT. The OTUs were clustered with NJOIN, and the trees were visualized with TREE. Default settings were used for all analyses. Phenograms were drawn individually for each gene region. Smaller phenograms were constructed for the eight new species, *L. bhutannense*, *L. yunnanensis*, *L. procerum* and *L. koreanum*, using the same method.

RESULTS

Primers and PCR optimization.—Individual diagnostic PCRs were optimized for 56 species included in this study. (Details of the optimized conditions are

summarized in TABLE I and results for βTP20 are shown in FIG. 1.) Nonspecific binding was encountered for BTP1, EF1αP32, BTP30 and BTP31, resulting in multiple bands; thus these are not useful as “identiprimers” and must be redesigned.

Blind test.—DNA isolations 3, 4, 6 and 8 from the blind test were identified accurately as *L. procerum*, *L. pineti*, *L. pini-densiflorae* and *L. fruticetum* with ITSP2 “identiprimers” (FIG. 2). Blind tests 1, 5, 7 and 9 were identified as *L. profanum*, *L. lundbergii*/*L. guttulatum*, *L. wagneri* var. *ponderosa* and *L. chlamydatum* with ITSP2 and βT “identiprimers” (FIGS. 2, 3). Blind test 2 was identified as *L. euphyes* based on the banding patterns produced by amplification with “identiprimers” from all three gene regions (FIGS. 2–4). Blind test 10 could not be identified to species due to the failure of BTP30 and BTP31 and is grouped in a large group by the ITSP2 “identiprimers” (FIG. 2). (Matrices can be found in APPENDIX 1, the online data supplement.)

Undescribed isolates.—(Isolates are listed in TABLE II, and PCR results are listed in TABLE III.) All previously undescribed isolates included were recognized as new *Leptographium* species by the diagnostic technique developed in this study. The species all were positive for the generic ITSP1 primer diagnostic for genus *Leptographium*. *Leptographium* isolates 1, 2, 3, 4, 5 and 8 grouped with *L. elegans* and *L. huntii* (FIG. 2). *Leptographium* isolates 6 and 7 grouped closely with *L. abieticolens* and *L. peucophilum* in the comprehensive ITS2 tree (FIG. 2). The comprehensive βT tree showed that *Leptographium* isolates 1 and 4 grouped into a clade with *L. huntii*, *L. piceaperdum*, *L. truncatum*, *L. albopini*, *L. koreanum*, *L. yunnanensis*, *L. guttulatum* and *L. lundbergii* (FIG. 3). *Leptographium* isolates 2, 3 and 5 grouped with *L. brevicollis*, *L. dryocoetidis* and *L. pruni*, and *Leptographium* isolates 6, 7 and 8 grouped with another large clade that included *L. calophylli*, *L. clavigerum*, *L. leptographioides*, *L. francke-grosmanniae*, *L. pityophilum*, *L. wagneri* var. *wagneri* and *L. sibiricum* (FIG. 3). The comprehensive EF1α tree showed that *Leptographium* isolates 1 and 2 grouped with *L. neomexicanum* and *L. reconditum*; *Leptographium* isolates 4 and 8 grouped with *L. reconditum*; *Leptographium* isolates 3 and 5 grouped with *L. pruni*, *L. crassivaginatam*, *L. douglassi*, *L. francke-grosmanniae*, *L. leptographioides*, *L. sibiricum*, *L. peucophilum* and *L. grandifoliae*; and *Leptographium* isolate 6 and 7 grouped with *L. brachiatum* and *L. rubrum* (FIG. 4).

Three smaller phenograms were constructed from subsets of the ITS2, βT and EF1α matrices to include the eight undescribed *Leptographium* isolates, *L.*

TABLE II. Eight previously undescribed isolates of *Leptographium* were included in this study that came from different geographical regions

CMW no.	Country ID	Host ID
12346	Seychelles	<i>Calophyllum</i>
12398	Tanzania	<i>Eucalyptus</i> spp.
12326	Chile	<i>Pinus radiata</i>
12422	Chile	<i>Araucaria araucana</i>
12319	Chile	<i>Eucalyptus globulus</i>
12425	China	Unknown
12471	China	<i>Picea koraiensis</i>
12473	USA	<i>Pinus thunbergii</i>

yunnanensis, *L. bhutannense*, *L. procerum* and *L. koreanum*. The ITS2 phenogram represents *Leptographium* isolates 6 and 7 as a single taxon that is closely related to *L. procerum* (FIG. 5). The ITS2 phenogram also shows that *Leptographium* isolates 1 and 2 are closely related as are *Leptographium* isolates 3 and 4 (FIG. 5). *Leptographium* isolates 8 and 5 occupy separate branches and show no close associations with other *Leptographium* species (FIG. 5). The β T phenogram showed that *Leptographium* isolates 1 and 4 are closely related to *L. yunnanensis* and *L. koreanum*; *Leptographium* isolates 2 and 3 formed a single taxon that is related to *Leptographium* isolates 5, *L. bhutannense* and *L. procerum*; and *Leptographium* isolates 6, 7 and 8 formed a single taxon that was related to *Leptographium* isolates 2, 3, 5, *L. bhutannense* and *L. procerum* (FIG. 6). The EF1 α phenogram showed that *Leptographium* isolates 1, 4 and 8 were grouped distantly from the other taxa but were more closely related to each other; *Leptographium* isolates 2, 3, 5, 7, *L. yunnanensis*, *L. koreanum*, *L. bhutannense* and *L. procerum* grouped together with *Leptographium* isolates 3 and 5 collapsed into a single taxon with *L. yunnanensis*, *L. koreanum*, *L. bhutannense* and *L. procerum* (FIG. 7).

DISCUSSION

This is the first study to apply a microcoding system to differentiate species in an ascomyceteous genus. *Leptographium* species typically are difficult to identify on the basis of morphological characters alone, necessitating the use of both morphological and molecular characters for identification (Jacobs 1999). Phylogenies were constructed from partial sequences of the β T, ITS2 and EF1 α regions and showed that the *Leptographium* species concept is phylogenetically valid (Jacobs et al. 2001, 2006). Probes were designed for PhyloChip from these gene regions to have at least a 10% difference between the primer and similar, but

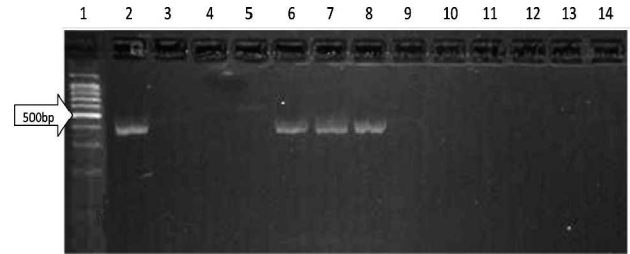


FIG. 1. A 3% agarose gel resolved the amplicon produced by β TP20 from *L. chlamydatum* (lane 2), *L. costaricense* (lanes 3–5), *L. curvisporum* (lanes 6–8), *L. leptographioides* (lanes 9–11) and *L. pruni* (lanes 12–14). The product for β TP20 is 400 bp and is positive for *L. chlamydatum* and *L. curvisporum*. The species share ITSPI, and are differentiated according to different β -tubulin identiprimers.

incorrect, target sequences (van Zuydam 2009). These probes were applied to this study as “identiprimers” for species identification. In this study we have achieved species differentiation with “identiprimers” in PCRs comparable to the differentiation achieved through phylogenetic analysis.

The identification system established in this study is unconventional because primers were designed from multiple gene regions and used in a hierarchical sequence. Identification began with “identiprimers” from the ITS2 region and then higher order “identiprimers” from the β T and EF1 α regions were used to achieve a more complete delineation of species. This hierarchical system has been adopted for PhyloChip studies (Loy et al. 2002, Metfies et al. 2008) but has not been transferred to a PCR diagnostic. More commonly in the case of fungi PCR diagnostics have been designed from a single gene region that only differentiates among a few species (Chen et al. 2001, Fujita et al. 2001, Hamelin et al. 1996). In Fujita et al. (2001) ITS1, ITS3 and White et al. (1990) ITS4 primers were optimized in a multiplex to amplify ITS1 and ITS2 regions to type 120 fungal strains consisting of 30 species of yeast. The differences in lengths of ITS1 and ITS2 regions among species were used to differentiate species (Fujita et al. 2001). Our study used a combination of selective “identiprimers” and amplicon size to identify *Leptographium* species. With closely related taxa, as is the case within genus *Leptographium*, a single gene region is insufficient to differentiate species. We therefore suggest that if this identification technique is applied generally to ascomycetes it is essential to use multiple gene regions and associated primers.

This study showed that it is possible to transfer 20-mer probes from a micro-array study to a PCR

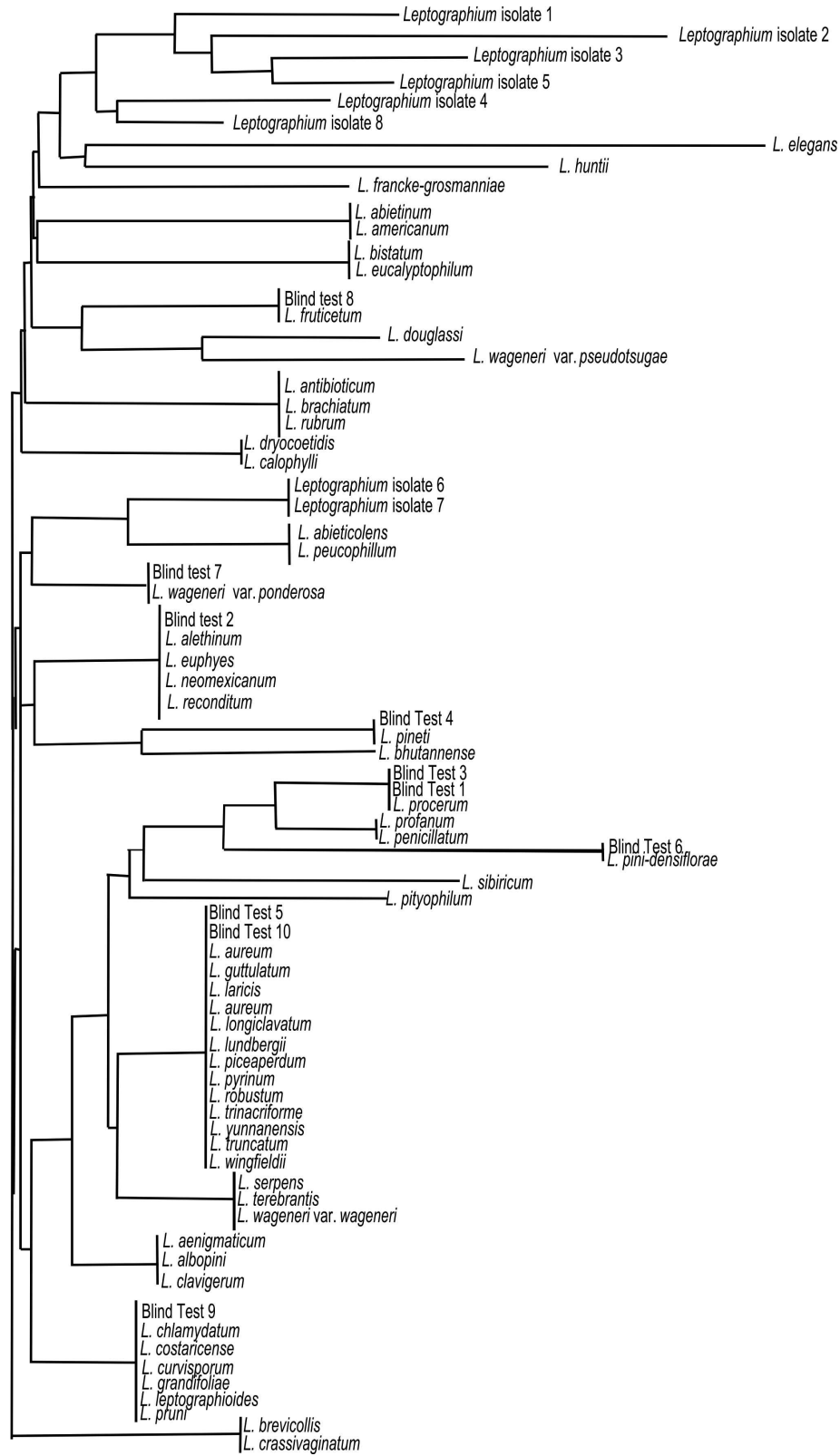


FIG. 2. ITS2 identiprimers phenogram constructed for the blind test species and undescribed species of *Leptographium*.

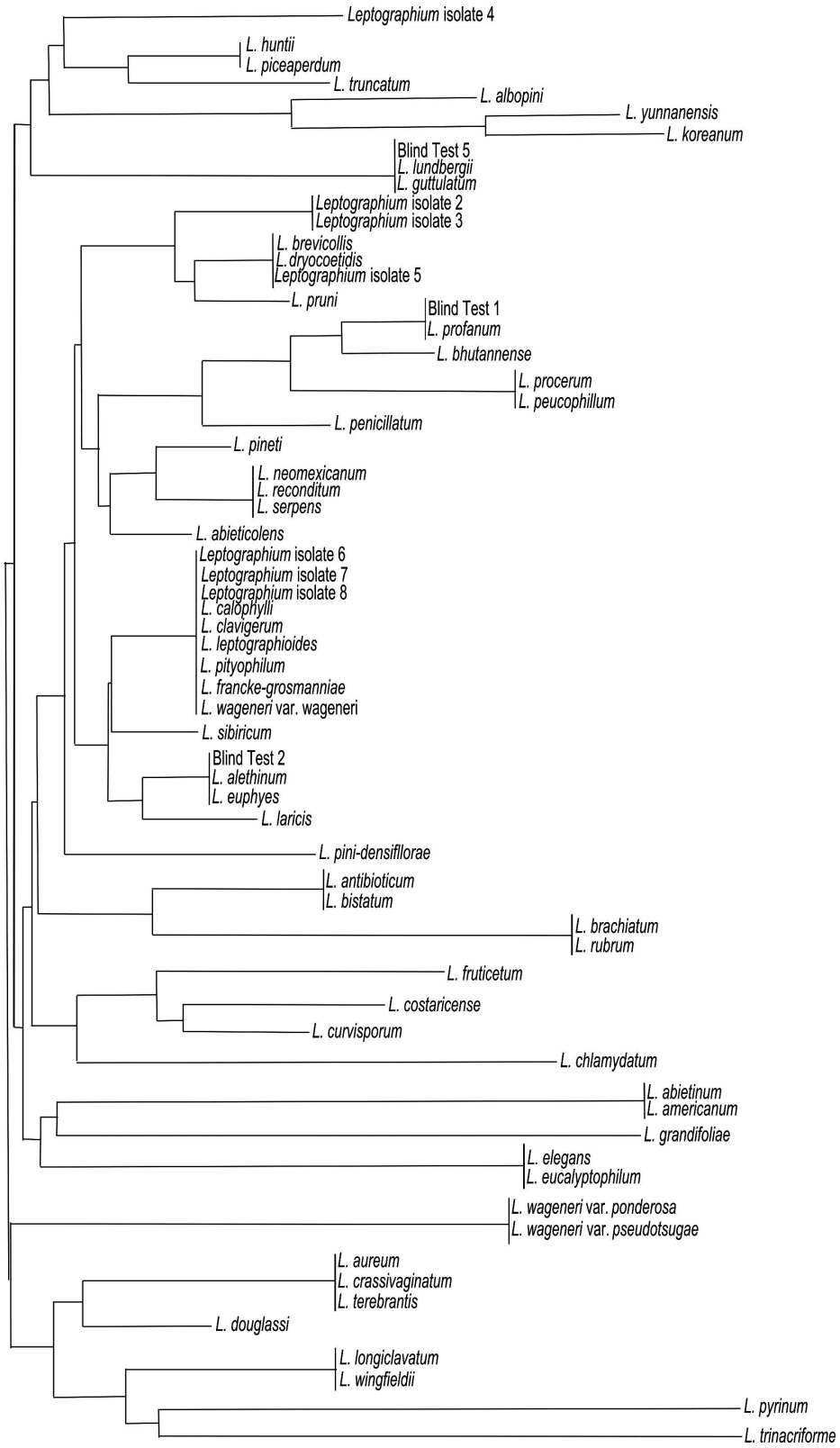


FIG. 3. β -tubulin identiprimer phenogram constructed for the blind test species and undescribed species of *Leptographium*.

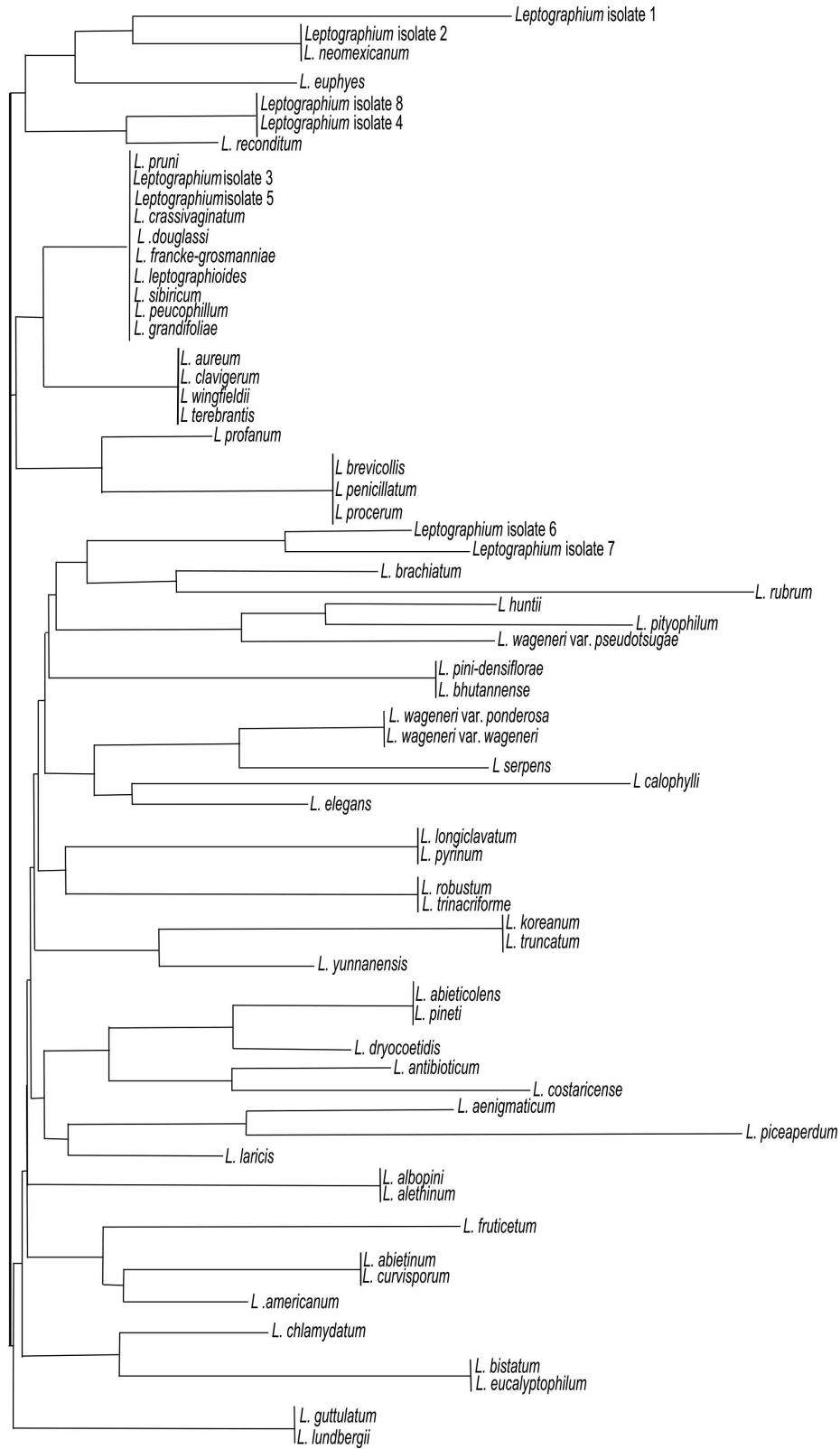


FIG. 4. Elongation factor 1 α identiprimer phenogram constructed for the blind test species and undescribed species of *Leptographium*.

TABLE III. Eight previously undescribed isolates of *Leptographium* were tested against the primer set

Species Probes ^a	CMW12346 <i>Leptographium</i> isolate 1	CMW12398 <i>Leptographium</i> isolate 2	CMW12326 <i>Leptographium</i> isolate 3	CMW12422 <i>Leptographium</i> isolate 4	CMW12319 <i>Leptographium</i> isolate 5	CMW12425 <i>Leptographium</i> isolate 6	CMW12471 <i>Leptographium</i> isolate 7	CMW12473 <i>Leptographium</i> isolate 8
ITSP1	1	1	1	1	1	1	1	1
ITSP6	1	1	1	1	1	0	0	1
ITSP7	0	0	0	0	0	1	1	0
ITSP8	1	0	0	0	0	0	0	0
ITSP11	1	1	1	0	1	0	0	0
ITSP12	1	1	1	1	1	0	0	1
ITSP15	0	0	0	0	0	1	1	0
ITSP17	0	0	1	1	0	0	0	0
ITSP18	0	1	0	0	0	0	0	0
ITSP23	0	1	1	0	1	0	0	0
ITSP24	0	1	0	0	0	0	0	0
ITSP25	1	1	0	0	0	0	0	0
EF1 α P3	1	0	0	1	0	0	1	1
EF1 α P12	1	0	0	0	0	0	0	0
EF1 α P36	1	0	0	1	0	0	0	1
EF1 α P38	0	0	0	0	0	1	1	0
EF1 α P42	0	0	0	0	0	1	1	0
BTP4	0	1	1	0	0	0	0	0
BTP13	1	0	0	1	0	0	0	0
BTP15	1	0	0	1	0	0	0	0
BTP24	0	1	1	0	1	0	0	0

^aTable includes the primers that showed positive amplification, for all the other primers the value is indicated as zero, no amplification.

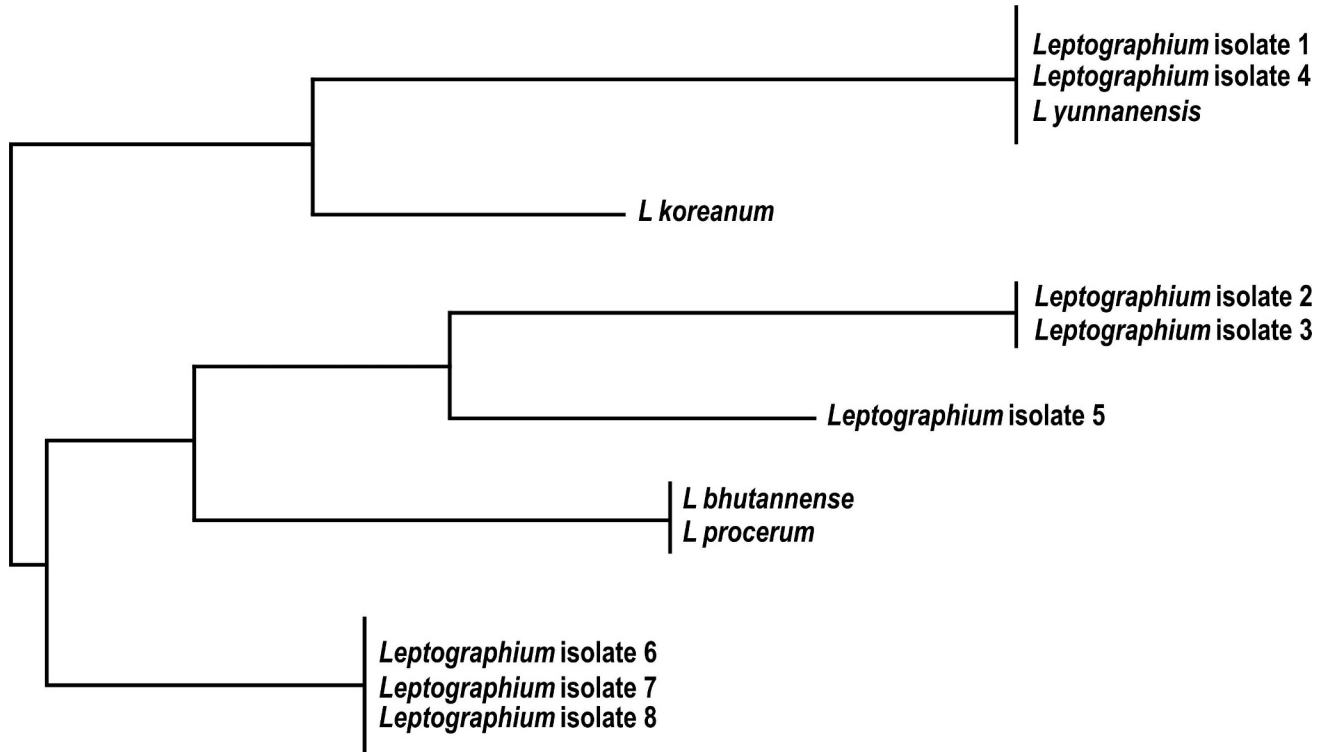


FIG. 5. A phenogram constructed from a small matrix of ITS2 identiprimers for eight undescribed species of *Leptographium* and related, described *Leptographium* species.

diagnostic application. The design for the micro-array was suited to the PCR diagnostic application because the probes were similar in length to PCR primers and multiple probes were designed. It was not possible to design a unique probe for each species within genus *Leptographium*. Therefore multiple probes from multiple gene regions were designed (van Zuydam 2009). These probes were transferred to the PCR diagnostic as “identiprimers”.

“Identiprimers” were incorporated with complementary universal primers, and this allowed for a dynamic identification system instead of a static PCR diagnostic based on a pair of species-specific primers. ITS2 primers were multiplexed to categorize DNA samples according to shared sequence characteristics in the ITS2 region with a single reaction. This approach was successful for one subset but not for all ITS2 primers. However identification by means of single primer amplifications was deemed successful because only four primer failures were encountered despite the large primer set and number of species tested in this study. Primers were determined to have failed if they produced random amplification or failed to yield an amplification product. When

primers had been optimized and interrogated for known species, they were tested on undescribed isolates of *Leptographium* and revealed intriguing results.

The “identiprimers” developed in this study support a phenogram that can be compared to an amplification profile to identify described and new species. Our design also allowed for inferences about phylogenetic relationships to be drawn because the phenograms approximate the phylogenies constructed by Jacobs et al. (2006). The undescribed isolates all were identified as representing new species of *Leptographium* and showed interesting cladistic associations indicated by “identiprimers”. A dichotomy was observed within the new species according to their primer amplification profiles when they were compared to phenograms constructed by van Zuydam (2009). *Leptographium* isolates 1, 2, 4 and 5 associated more closely with species that colonize coniferous hosts, and 6, 7 and 8 associated more closely with species that colonize non-coniferous hosts according to the ITS2 primers. These results are supported by the collection data and phylogenies for these species (Paciura 2009). Higher order ITS2 primers showed

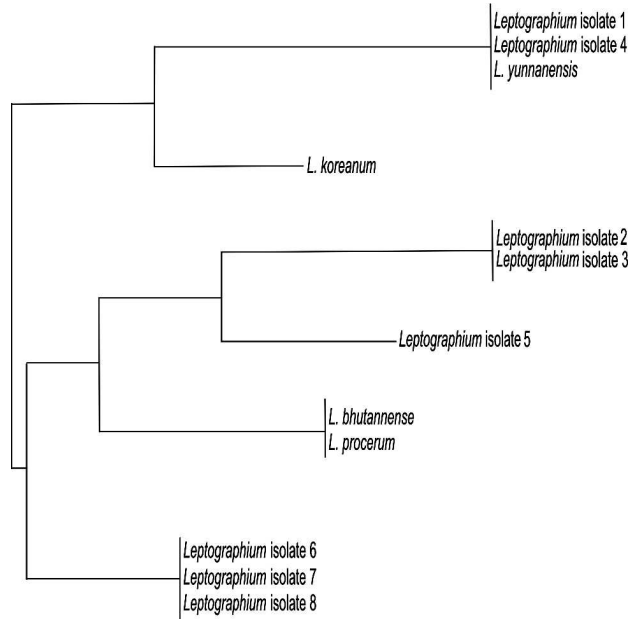


FIG. 6. A phenogram constructed from a subset of β -tubulin identiprimers for eight undescribed species of *Leptographium* and related, described *Leptographium* species.

that *Leptographium* isolates 1, 2, 3, 4 and 5 are related to *L. bhutannense* and that 6, 7 are more closely related to *L. abieticolens* but also share sequence homology with *L. yunnanensis*.

The β T and EF1 α primer associations of the undescribed isolates revealed more about their associations with each other and with known species. The phylogeny constructed by Paciura (2009) suggests that *Leptographium* isolates 2 and 3 are closely related, which is supported by the primer profiles generated in this study for those isolates. The same is true for *Leptographium* isolates 6 and 7 that have similar profiles but are dissimilar to the other new species; they are phylogenetically close to each other and more distantly related to the other new species (Paciura 2009). The difference can be attributed to the different hosts that they colonize. *Leptographium* isolates 6 and 7 were obtained from non-coniferous hosts, while the other new species were isolated from coniferous hosts. β T primers indicate that *Leptographium* isolates 1 and 4 are closely related to *L. yunnanensis*. This result differs from the other species identifications reported here in that the identification of *L. yunnanensis* was based on two specific primers instead of a specific primer and a universal primer. This confirms that *Leptographium* isolates 1 and 4 share two polymorphic regions common to the specific primers with *L. yunnanensis*. This also is

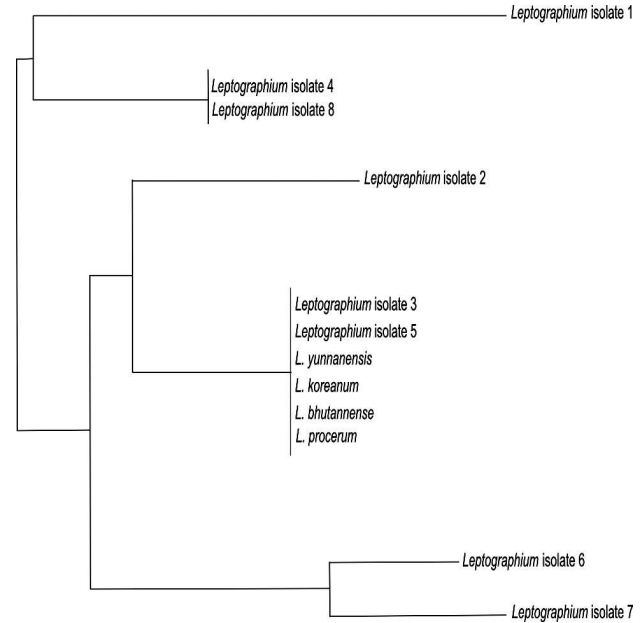


FIG. 7. A phenogram constructed from a subset of translation elongation factor 1 α identiprimers for eight undescribed species of *Leptographium* and related, described *Leptographium* species.

reflected in the phylogenetic relationships of these two species (Paciura 2009).

This study demonstrated that it is possible to detect undescribed species of *Leptographium* by microcoding and demonstrated the utility of this approach for fungal taxonomy. Microcoding was proposed as the next step to barcoding by Summerbell et al. (2005). Here the suggestion was that 20-mer oligonucleotides could be used to identify an isolate to genus or species. Likewise this study supported the use of short oligonucleotides in microcoding applications. We found that the relationships between the species based on primer sequence homology roughly resembled biological and phylogenetic relationships. This was true for the known species and the undescribed species of *Leptographium*. It indicated that DNA microcoding would be successful in identifying known and new species as well as indicating biological and phylogenetic relationships.

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