

Practice towards DNA barcoding of the nectriaceous fungi

Peng Zhao · Jing Luo · Wen-Ying Zhuang

Received: 18 April 2010 / Accepted: 26 September 2010
© Kevin D. Hyde 2010

Abstract Fungi of *Nectriaceae* are economically important and of high species diversity. For the purpose of accurate and rapid species identification, ITS, 28S rDNA, β -tubulin gene and EF-1 α gene were selected as the candidate DNA barcode markers to investigate their feasibility in identification of 28 well-circumscribed species belonging to 9 genera of the nectriaceous fungi. A total of 216 sequences of the candidate genes were analyzed. Intra- and inter-specific variations and success rate of PCR amplification and sequencing were considered as important criteria to estimate the candidate genes. The partial β -tubulin gene met the requirements for an ideal DNA barcode and functions well for correct species delimitation. No overlapping between the intra- and inter-specific pairwise distances was found. The smallest inter-specific distance of β -tubulin gene was 3.45%, while the largest intra-specific distance was 2.77%; which appeared to possess the appropriate intra- and inter-specific variations. Twenty-eight clusters were recognized in accordance with the 28 morphological species tested. In addition, it had a high PCR and sequencing success rate. As to the other candidates, EF-1 α gene showed fairly good sequence variations among

species, but the PCR and sequencing success rate reached only 75.3%. ITS had a high PCR and sequencing success rate (93.5%) and recognizes 92.9% of the total number of species, nevertheless, overlapping occurred between the intra- and inter-specific distances, which may lead to incorrect species identification. 28S rDNA is most conservative compared with any other candidate markers and able to recognize merely 60.7% of the total species. We propose β -tubulin gene as the possible barcode for the nectriaceous fungi.

Keywords DNA barcode · Barcoding gap · Intra-specific variation · Inter-specific variation · PCR and sequencing success rate

Introduction

The nectriaceous fungi (*Hypocreales*, *Sordariomycetes*, *Ascomycota*) are world-wide distributed, with a wide range of habitation; economically important as plant pathogens; mycoparasites; producers of antibiotics, new bioactive compounds and mycotoxins; and rarely are a source of food (Booth 1971; Rossman 1996; Rossman et al. 1999; Kirk et al. 2008; Li et al. 2009). This group shows a very high species diversity and broad range of lifestyles. There are increasing requirements of rapid species identification of the nectriaceous fungi for the purpose of plant disease diagnoses, discovery of new bioactive compounds, exploration of potential biocontrol agents, as well as protection from harmful mycotoxins.

For species identification of *Nectriaceae*, detailed morphology, anatomy, teleomorph–anamorph connection, perithecial reactions to KOH and lactic acid, and selected DNA sequence data are commonly required (Rehner and Samuels

Peng Zhao and Jing Luo contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s13225-010-0064-y) contains supplementary material, which is available to authorized users.

P. Zhao · J. Luo · W.-Y. Zhuang (✉)
Key Laboratory of Systematic Mycology and Lichenology,
Institute of Microbiology, Chinese Academy of Sciences,
Beijing 100101, China
e-mail: zhuangwy@im.ac.cn

P. Zhao
Graduate University of Chinese Academy of Sciences,
Beijing 100049, China

1995; Rossman et al. 1999; Zhang and Zhuang 2006; Luo and Zhuang 2010), which takes a lot of effort and is time-consuming. Sometimes, two different species may share the same sequence data of the internal transcribed spacers of ribosomal RNA (ITS) and 28S rDNA (unpublished information), while morphology distinguishes them clearly. Screening an appropriate DNA barcode for efficient species identification of the nectriaceous fungi is essential.

DNA barcoding is powerful in rapid species identification for biodiversity assessment, detection of pest species invasion, food and feedstuff safety, and human health (Hebert et al. 2003a; Armstrong and Ball 2005; Ball and Armstrong 2006; Stoeckle and Hebert 2008; Valentini et al. 2009). A DNA barcode is a short, universal DNA sequence which displays greater divergences among species than those within a species (Hebert et al. 2003a, b). It has been suggested that mitochondrial cytochrome *c* oxidase 1 gene (COI) delivers a good species resolution and may serve as a universal barcode for major groups of animals (Hebert et al. 2003b; 2004; 2009). The combination of plastid genes *rbcL* and *matK* was adopted recently as the main barcode for land plants (Hollingsworth et al. 2009). For the Kingdom Fungi, a universal barcode has not been formally established but potential DNA barcode markers for selected taxonomic groups has been refurbished (Seifert 2009). ITS has been successfully used for species discrimination of six genera in *Zygomycetes* (Schwarz et al. 2006), *Trichoderma* and *Hypocrea* in *Ascomycota* (Druzhinina et al. 2005), and *Cortinari* section *Calochroi* (Frøslev et al. 2007) and *Melampsora* (Feau et al. 2009) in *Basidiomycota*. But ITS appeared to be problematic in the identification of the blue stain fungi (Roe et al. 2010). For *Aspergillus* species diagnosis, beta (β)-tubulin gene is suggested as the potential DNA barcode locus against COI, ITS and the intergenic spacer of the ribosomal genes (IGS) (Geiser et al. 2007). Nuclear 28S rDNA and β -tubulin genes provide a perfect capability of species separation in *Fusarium* (O'Donnell and Cigelnik 1997). Translation elongation factor 1 α gene (EF-1 α) is a reliable barcode for *Fusarium* (Geiser et al. 2004), *Trichoderma* and *Hypocrea* (Druzhinina et al. 2005). However, our knowledge on exploration of DNA barcoding for fungi is still limited and clearly much more work is required.

Several criteria have been subjected to determination of an ideal DNA barcode, such as a short fragment, standard, universally used, having adequate variations among species and conservative within a species, exhibiting a high species resolving power (Taberlet et al. 2007; Hollingsworth et al. 2009; www.barcoding.si.edu/PDF/Guidelines for non-COI selection FINAL.pdf). We propose that the appropriate intra- and inter-specific sequence variations, i.e. divergent among species but relatively stable within a species (Hebert et al. 2004), and easiness of nucleotide sequence acquisition

(Hollingsworth et al. 2009) are the two important aspects in evaluating feasibility of a DNA fragment as a barcode marker. Accordingly, intra- and inter-specific variations of candidate DNA barcodes for species tested, frequency distribution of intra- and inter-specific pairwise distances, clustering at a given threshold for species identification are analyzed. Undoubtedly, success rate of PCR amplification and sequencing of each gene must be taken into account for assessing the ease of nucleotide sequence acquisition.

In this study, we selected ITS, 28S rDNA, β -tubulin gene and EF-1 α gene as candidate markers, which have been commonly used in fungal species identification or phylogenetic studies to investigate the possible DNA barcode for some nectriaceous fungi.

Materials and methods

Materials

A total of 216 sequences of the four candidate markers, ITS, 28S rDNA, β -tubulin gene and EF-1 α gene, from 28 clearly documented and generally accepted species belonging to 9 genera of *Nectriaceae* were analyzed. *Bionectria wenpingii* was used as outgroup. Most sequences were newly provided by our research group incorporated with some retrieved from the GenBank (Table 1).

DNA amplification and sequencing

Genomic DNA of each strain was isolated from mycelium grown on potato dextrose agar (PDA) at room temperature for about 2 weeks (Wang and Zhuang 2004). The two nuclear rDNA fragments, ITS and domains D1, D2 and D3 of 28S rDNA, were amplified and sequenced with two primer pairs, ITS5 and ITS4 (or ITS1 and ITS4) (White et al. 1990), and LROR and LR5 (Vilgalys and Hester 1990; Rehner and Samuels 1994), respectively. Partial β -tubulin gene was amplified with the primers T1, T22, T222 and T224; and sequenced with T1 and Bt2b (O'Donnell and Cigelnik 1997; Glass and Donaldson 1995). Primers 526 F, 728F and 1567R were employed to amplify partial EF-1 α gene segments; and the primers 526 F, 728F, EFjR and 1567Ra were used to sequence the amplicons (Carbone and Kohn 1999; Rehner and Buckley 2005; <http://www.aftol.org/pdfs/EF1primer.pdf>). The region between 728F and EFjR was analyzed.

PCR was performed with the Perkin Elmer GeneAmp PCR System 2400 using a 25 μ L reaction system consisting of 16 μ L of double distilled water, 2.5 μ L of 10 \times PCR buffer, 2 μ L of MgCl₂ (25 mM), 1.25 μ L of each primer (10 μ M), 0.5 μ L of dNTP (10 mM each), 1.25 μ L of DNA template, 0.25 μ L Taq DNA polymerase (5 U/ μ L). For ITS,

Table 1 Materials used in this study

Strain	Collection number or source ^a	Geographical origin	GenBank accession number			
			ITS	28S rDNA	β -tubulin	EF-1 α
<i>Albonectria rigidiuscula</i> 6939	HMAS 183508	China	HM054153^b	HM042413	HM054098	HM054059
<i>A. rigidiuscula</i> 6940	HMAS 183135	China	HM054147	HM042411	HM054099	HM054061
<i>A. rigidiuscula</i> 6941	HMAS 183509	China	HM054148	HM042412	HM054100	HM054062
<i>A. rigidiuscula</i> 7192	HMAS 183518	China	HM054158	HM042403		HM054060
<i>Chaetopsinectria chaetopsinae</i> H50	HMAS 76860	China	GU075858	HM042404	HM054101	HM054063
<i>C. chaetopsinae-penicillatae</i>	CBS 608.92	New Zealand	GU075859	GU075865	HM054128	HM054064
<i>Cosmospora cupularis</i> 6790-2	HMAS 97514	China	EF121864	EF121870	HM054129	HM054065
<i>C. gigas</i> 6598	HMAS 99592	China	EF121863	EF121869	HM054102	HM054066
<i>C. henanensis</i> 6724	HMAS 183528	China	GU075856	GU075863	HM054103	HM054067
<i>C. meliopsicola</i> 5186	HMAS 86473	China	HM054159	HM042406	HM054104	HM054068
<i>C. vilior</i> 7497	HMAS 183536	China	HM054160	HM042407	HM054105	HM054069
<i>C. vilior</i> 7093	HMAS 183535	China	HM054144	HM042414		
" <i>Cosmospora</i> " <i>consors</i> H17	HMAS 76861	China	EF121861	HM042405	HM054106	HM054070
<i>Gibberella acuminata</i>	P323	USA	AF132802			
	NRRL 6227			U85516	U85567	
	F30					EF531698
	aurim1174	Lithuania	DQ093673			
	NRRL 22203			AF006326	AF006366	
<i>G. pulicaris</i>	DDPP 0611p	Poland				EU128235
	MTFC12 (IRBV)		DQ132833			
	NRRL22187			U85523	U85574	
					AF484166	
	xsd08118	China	FJ481029			
<i>G. zeae</i>	NRRL 38381	USA	DQ459826		DQ459631	DQ459733
	F15			AB084297		
	NRRL 38395	USA	DQ459827		DQ459632	DQ459734
	NRRL 5883	USA		U34549		
	NRRL 38383	USA	DQ459828		DQ459633	DQ459735
	NRRL 38393	USA	DQ459829		DQ459634	DQ459736
<i>Haematonectria haematococca</i> 5133	HMAS 91771	China	HM054145	HM042415	HM054107	HM054071
<i>H. haematococca</i> 5804	HMAS 99199	China	HM054152	HM042416	HM054108	HM054072
<i>H. haematococca</i>	HMAS 83364	China		DQ119558		
<i>Lanatonectria flavolanata</i> 5622	HMAS 97516	China	EF121860	HM042417	HM054109	HM054073
<i>L. flavolanata</i>	DAOM 216608	Costa Rica		AY281098		
<i>L. flocculenta</i> H113	HMAS 76873	China	EF121858	DQ119567	HM054110	HM054074
<i>L. flocculenta</i>	HMAS 83374	China		DQ119566		
<i>Leuconectria grandis</i> 5630	HMAS 98302	China	EF121859	EU031441	EU984072	HM054075
<i>Nectria australiensis</i> H13	HMAS 83397	China	GU075855	HM042418	HM054111	HM054076
<i>N. cinnabarina</i> 5175	HMAS 91782	China	HM054135	HM042420	HM054130	HM054077
<i>N. cinnabarina</i> 5698	HMAS 98306	China	HM054137	HM042419		
<i>N. cinnabarina</i> 5179	HMAS 91783	China	HM054155	HM042421	HM054112	
<i>N. cinnabarina</i> 5744	HMAS 98311	China	HM054156	HM042422		
<i>N. pseudotrichia</i> 6722	HMAS 97518	China	EF121865	HM042423	HM054113	HM054078
<i>N. pseudotrichia</i> 7134	HMAS 183175	China	HM054138	HM042424	HM054114	HM054079
<i>N. pseudotrichia</i> 7107	HMAS 183559	China	HM054154	HM042425	HM054115	HM054080
<i>N. pseudotrichia</i> W7061a	HMAS 183172	China	GU232860	HM042426	HM054116	HM054081
<i>N. sinensis</i> 7145	HMAS 183561	China	HM054139	HM042428	HM054117	HM054082

Table 1 (continued)

Strain	Collection number or source ^a	Geographical origin	GenBank accession number			
			ITS	28S rDNA	β -tubulin	EF-1 α
<i>N. sinensis</i> H49	HMAS 83356	China	GU075854	HM042427	HM054118	HM054083
<i>Neonectria castaneicola</i> 6846	HMAS 183542	China	HM054141	HM042430	HM054119	HM054084
<i>N. castaneicola</i> H28	HMAS 76865	China	HM054142	HM042429	HM054120	HM054085
<i>N. castaneicola</i> H30	HMAS 83369	China	HM054143	HM042431	HM054121	HM054086
<i>N. coccinea</i> AR3700	CBS 119156	Slovakia		HM042432	HM054122	HM054087
<i>N. coccinea</i>	CBS 291.81	Austria	FJ474075		DQ789874	DQ789731
	CBS 237.29	Norway		AY677327		
	CBS 118914	France				DQ789688
	NRRL 20485			U88124		
	CBS 394.80	Netherlands			DQ789877	DQ789734
	CBS 119159	Germany			DQ789893	
	<i>N. discophora</i> var. <i>discophora</i> 5542	HMAS 98333	China	HM054136	HM042433	HM054131
<i>N. discophora</i> var. <i>discophora</i> W7104a	HMAS 183155	China	HM054161	HM042408	HM054132	HM054089
<i>N. discophora</i> var. <i>discophora</i> 5621	HMAS 98327	China	HM054140	HM042434	HM054123	
<i>N. ditissima</i>	CBS 117751	Slovenia	DQ178167			
	CBS 226.31	Germany		AY677330		
	CBS 835.97	Belgium			DQ789880	
	CBS 100319	Canada				DQ789716
	CBS 117752	Slovenia	DQ178168			
	CBS 118926	USA			DQ789881	
	CBS 100320	Canada				DQ789717
	CPC 12078	Netherlands	DQ178169			
	CBS 118919	USA			DQ789884	
	CBS 379.50	USA				DQ789733
CBS 118927	USA			DQ789886	DQ789743	
<i>N. hubeiensis</i> 5620	HMAS 98331	China	FJ560439	FJ560434	FJ860056	HM054090
<i>N. ramulariae</i>	CBS 151.29	UK	HM054150	HM042436	HM054124	HM054091
<i>N. ramulariae</i> 188474	HMAS 188474	China	HM054157	HM042435	HM054125	HM054092
<i>N. ramulariae</i>	CBS 730.87	Germany	AJ279446			
	ATCC 16237	Germany			DQ789857	
<i>N. ramulariae</i>	CBS 182.36				DQ789864	
<i>N. shennongjiana</i> Z153	HMAS 183185	China	FJ560440	HM042409	FJ860057	HM054093
<i>N. veuillotiana</i> 5686	HMAS 98332	China	HM054151	HM042437	HM054133	HM054094
<i>N. veuillotiana</i> 5832	HMAS 99207	China	HM054146	HM042438	HM054134	HM054095
<i>N. veuillotiana</i>	GJS 91-116	USA	HM054149	HM042439	HM054126	HM054096
<i>Bionectria wenpingii</i> W2792a	HMAS 172156	China	EF612465	HM042410	HM054127	HM054097

^a ATCC American Type Culture Collection, Bethesda, MD, U.S.A., CBS Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, CPC Culture collection of Pedro Crous, housed at CBS, DAOM Canadian Collection of Fungus Cultures, Agriculture & Agri-Food Canada, Ottawa, Canada, DDPP Department of Diagnostics & Plant Pathophysiology, University of Warmia & Mazury in Olsztyn, Plac Lodzki, Olsztyn, Poland, G.J.S. G.J. Samuels, HMAS Herbarium of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China, IRBV Institut de recherche en biologie végétale (Plant biology research institute) Montreal, Canada, NRRL National Center for Agricultural Utilization Research, U.S. Dept. of Agriculture, Peoria, IL, USA

^b GenBank accession numbers in boldface indicating the newly submitted sequences, others retrieved from GenBank

PCR conditions were an initial step of 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C, followed by 10 min at 72°C. For 28 S rDNA, PCR conditions were an initial step of 5 min at 94°C, 10 cycles of 30 s at 94°C, 30 s at 62°C (decreasing 1°C per cycle),

55 s at 72°C, plus 25 cycles of 30 s at 94°C, 30 s at 52°C, 55 s at 72°C, followed by 10 min at 72°C. For β -tubulin, PCR conditions were an initial step of 5 min at 94°C, 30 cycles of 35 s at 94°C, 55 s at 53°C, 1 min or 2 min at 72°C, followed by 10 min at 72°C. For EF-1 α , PCR

conditions were an initial step of 5 min at 94°C, 10 cycles of 30 s at 94°C, 55 s at 63°C or 66°C (decreasing 1°C per cycle), 90 s at 72°C, plus 36 cycles of 30 s at 94°C, 55 s at 53°C or 56°C, 90 s at 72°C, followed by 7 min at 72°C. The obtained amplicons were purified by PCR Product Purification Kit (Biocolor BioScience & Technology Company) and sequencing was carried out in both directions with an ABI 3730 XL DNA Sequencer (SinoGenoMax Co. Ltd.).

Estimation of the candidate barcode markers

Comparison of intra- and inter-specific divergences

Sequences were aligned using ClustalX 1.81 (Thompson et al. 1994) and manually edited to adjust the aligned sequences by BioEdit 7.0 (Hall 1999). The aligned sequences were input into DNASTAR 7.1.0 (Lasergene, WI, USA) to calculate the similarity matrices and then illustrate the intra- and inter-specific variations of the candidate barcode loci for each of the 28 investigated species in a visualization analysis tool, TaxonGap 2.4.1 (Slabbinck et al. 2008). As suggested by Martens et al. (2008), an outgroup, *Bionectria wenpingii*, was designated in the analyses.

The intra- and inter-specific pairwise distances were calculated using Kimura's two-parameter (K2P) model with MEGA 4.0.2 (Tamura et al. 2007). The frequency distribution of the intra- and inter-specific distances were analyzed to check the barcoding gap, i.e. space between intra- and inter-specific distances (Meyer and Paulay 2005), with Microsoft Office Excel (Windows XP).

Another distance-dependent method using K2P model, clustering at a given threshold, for evaluation of the potential barcode regions was generated with TaxonDNA 1.6.3-dev4 (Meier et al. 2006).

Neighbor-Joining tree reconstruction

Neighbor-joining (NJ) trees were constructed using K2P model with MEGA 4.0.2 (Tamura et al., 2007) for individual candidate barcode markers to provide a graphic representation of species divergence. *Bionectria wenpingii* was used as outgroup.

Assessment of the easiness of test barcode sequence acquisition

The success rates of PCR amplification and sequencing of the considered DNA barcode markers for the family were assessed. A single PCR band obtained was considered as success of amplification. High quality chromatogram counted for success of sequencing. The success rate of PCR amplification multiplied by that of sequencing made the success rate of PCR amplification and sequencing.

Results

To meet the requirements for a standard DNA barcode, the sequence lengths of all the candidate markers are short. The fragments obtained are 446–502 base pairs (bp) for ITS, 479–487 bp for 28S rDNA, 472–566 bp for β -tubulin gene (including 3 introns) and 328–472 bp for EF-1 α gene (including 2 introns).

To select an appropriate or ideal DNA barcode marker, comparison of intra- and inter-specific variations is treated as a very important criterion. The comparisons of the four candidate gene markers for each of the 28 *Nectriaceae* species tested are performed by TaxonGap (Slabbinck et al. 2008) and the results are illustrated in Fig. 1. In general, the

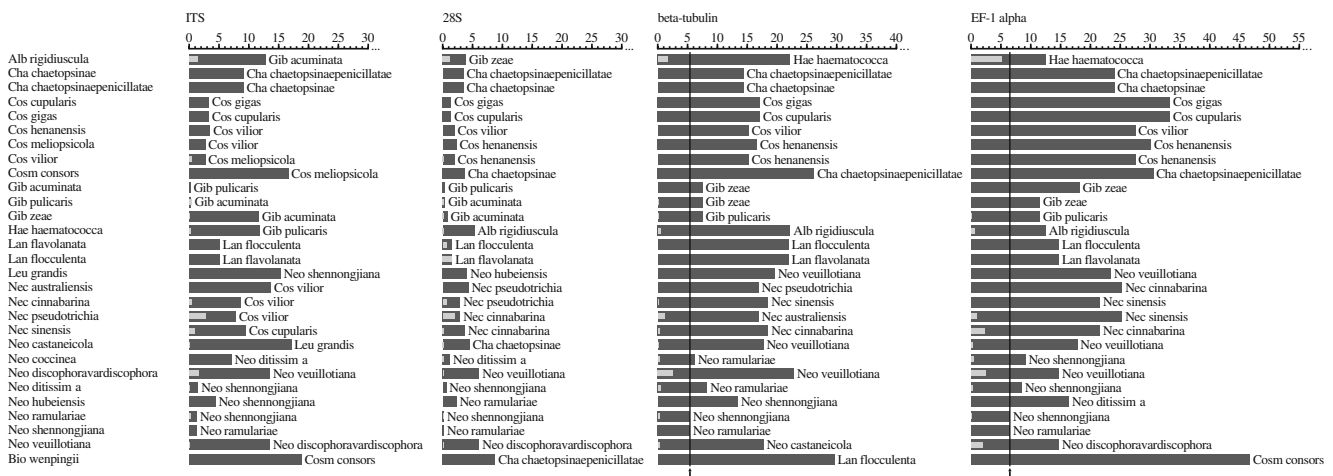


Fig. 1 Comparisons of intra- and inter-specific variations among ITS, 28S rDNA, β -tubulin and EF-1 α genes of the nectriaceous fungi generated by the software TaxonGap. The grey and black bars represent

the intra- and inter-specific variations respectively. The thin, black lines indicate the smallest interspecific variation. Names next to the dark bars indicate the closet species. *Bionectria wenpingii* was used as outgroup

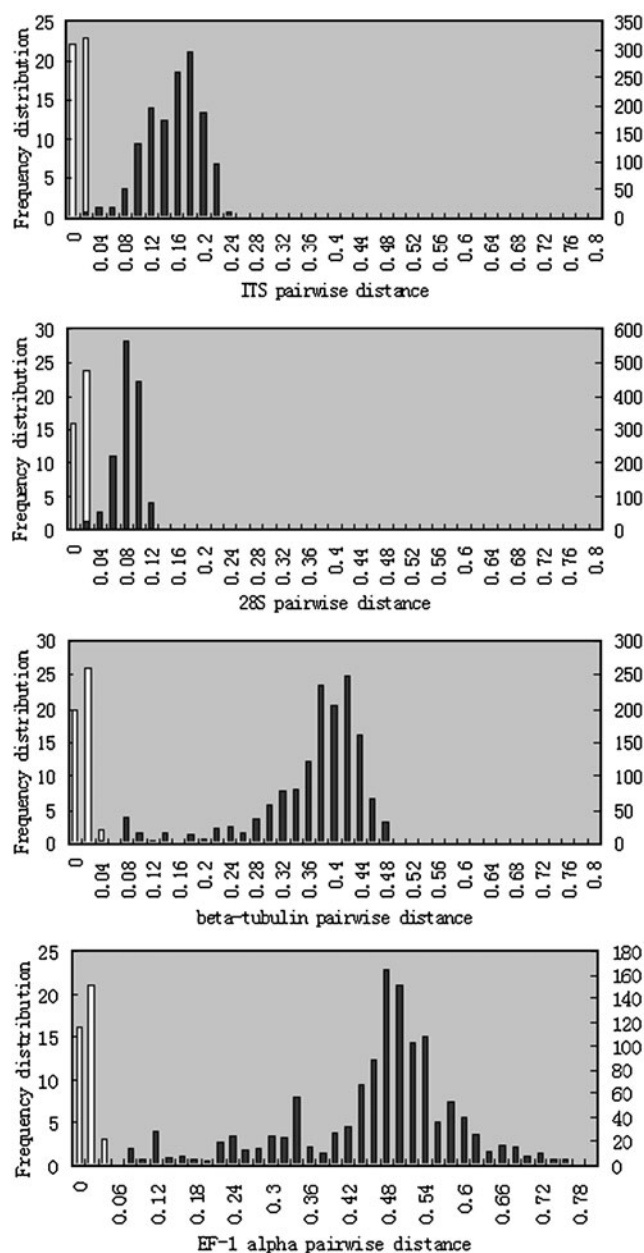


Fig. 2 Comparisons of frequency distribution of intra- and inter-specific pairwise distances among ITS, 28S rDNA, β -tubulin and EF-1 α genes for the nectriaceous fungi generated with MEGA and Excel. The intra- and inter-specific distances are shown as white and black bars respectively

inter-specific variations shown by the β -tubulin and EF-1 α genes were very similar and larger apparently than those of ITS and 28S rDNA. It is clear that the maximum intra-specific variations were smaller than the minimum inter-specific variations for all the species tested when the β -tubulin and EF-1 α genes were applied. In contrast, the intra-specific variations of an individual species exceeded the inter-specific variations of some others in the ITS and

28S rDNA datasets, which may lead to misidentifications. In the case that ITS acted as the candidate marker, the intra-specific variations for *Albnectria rigidiuscula* was larger than the inter-specific variations between *Gibberella acuminata* and *G. pulicaris*. The three species may not be identified correctly. As expected, the inter-specific variations for *Bionectria wenpingii*, outgroup of the nectriaceous fungi, to any nectriaceous fungi tested were the largest for all the candidate genes.

The frequency distribution of intra- and inter-specific pairwise distances of the four candidate genes analyzed by MEGA and Excel is shown in Fig. 2. The inter-specific distances were much larger than the intra-specific distances for the partial sequences of the protein-coding genes (β -tubulin and EF-1 α) and a distinct gap was present between intra- and inter-specific distances. Nevertheless, overlapping occurred in the datasets of ITS and 28S rDNA, which indicates that neither of them are qualified as a barcode marker.

Clustering at a given threshold calculated by TaxonDNA (Meier et al. 2006) provides additional measurements of species identification success of a barcode marker. The 28 clusters produced by the β -tubulin and EF-1 α genes are corresponding to the 28 well-circumscribed species of the family when the largest intra-specific distances were used as the threshold of clustering (Table 2). ITS recognized as many as 26 species, and 28S rDNA distinguished merely 17 species as a whole.

As another important criterion, success rate of PCR amplification and sequencing influences directly the efficiency of DNA barcode application. In this study, three of the four candidate genes were readily amplified and sequenced, and gained fairly high success rates ($\geq 92\%$) except for EF-1 α gene which received a relatively low rate (75.3%) (Table 3).

Almost all species were separated from each other in the four NJ trees generated from the candidate genes (Supplementary Figs. 1, 2, 3 and 4). Exception was found occasionally since *Gibberella acuminata* was highly cohesive with *G. pulicaris* in the ITS-based NJ tree. In most cases, sequences of the same species showed high cohesion, whereas, sequence divergence of the same species resulted in somewhat different tree topologies. For example, the terminal branches formed by the strains of *Neonectria discophora* var. *discophora* were not the same in the four NJ trees.

Discussion

Judged by the two important criteria for evaluating a DNA barcode marker, i.e. the suitable intra- and inter-specific variations and high success rate of PCR amplification and

Table 2 Clustering at a given threshold of the four candidate barcode genes of 28 species of *Nectriaceae* derived from the software TaxonDNA

Candidate barcode	ITS	28S rDNA	β -tubulin	EF-1 α
Number of sequences	55	54	56	51
Largest intraspecific distance	1.30%	1.89%	2.77%	2.87%
Number of cluster	27	21	28	28
Corresponding to species taxa	26 (92.9%)	17 (60.7%)	28 (100%)	28 (100%)

sequencing, our study suggests the adoption of the partial β -tubulin gene as a DNA barcode of the nectriaceous fungi.

The partial β -tubulin gene functions excellently as a barcode for the *Nectriaceae* species tested. Firstly, it possesses the appropriate intra- and inter-specific variations and distinguishes precisely these two types of variations. No overlapping occurred between the intra- and inter-specific pairwise distances (Fig. 2). This is substantiated by using the method TaxonGap (Slabbinck et al. 2008) where all the inter-specific variations were greater than the intra-specific ones, and where a gap existed (Fig. 1). To further confirm our conclusion, TaxonDNA was also applied (Meier et al. 2006) and the following example explains. Among *Neonectria coccinea*, *N. ramulariae*, *N. shennongjiana* and *N. ditissima*, the representatives of the genus *Neonectria*, the smallest inter-specific distance was 3.45% as happened between *N. shennongjiana* and *N. ramulariae*, while the largest intra-specific distance was 2.77% (Table 2). The 28 clusters match exactly the 28 species involved when the largest intra-specific distance was set up as the threshold of clustering (Table 2). This gene shows a high species identification power. Secondly, the PCR amplification and sequencing success rate of β -tubulin gene is high (92%) which is slightly lower than that of 28S rDNA (100%) (Table 3). Therefore, we recommend using the β -tubulin gene as a DNA barcode for *Nectriaceae*. As shown in the previous studies by other authors, it might also have the potential to become the barcode marker for other fungal groups (Glass and Donaldson 1995; Schroers et al. 2005).

EF-1 α gene has shown a fairly good sequence divergence which is equally high as that observed in β -tubulin gene. All 28 species were clustered and corresponding to the 28 species tested (Table 2). This result supports the previous statements by Druzhinina et al. (2005) and Geiser et al. (2004) that EF-1 α gene offers an excellent phylogenetic identification of species in the genera *Trichoderma*, *Hypocrea* and *Fusarium*. Unfortunately, it had a relatively poor PCR amplification and sequencing success rate (75.3%) (Table 3). It would be

possible to work as a barcode marker for the family if a higher success rate of PCR and sequencing can be reached. The optimization of primer designing might be the critical point of future research.

ITS has been one of the genes routinely employed to explore the phylogenetic relationships among species of different fungal groups. ITS has also been proposed tentatively as the universal DNA barcode for the Kingdom Fungi (Seifert 2009). In this study, we found that ITS has a high PCR and sequencing success rate (93.5%) and recognizes 92.9% of the total number of species involved (Tables 2 and 3). Nevertheless, overlapping occurred between the intra- and inter-specific distances. As calculated by TaxonDNA (Meier et al. 2006), the largest intra-specific pairwise distance was up to 1.30%, whereas the inter-specific pairwise distances between strains of *Gibberella acuminata* and that of *G. pulicaris* were only 0% and 0.21%. In another case, the inter-specific pairwise distance between *Neonectria ramulariae* and *N. shennongjiana* were 1.36% which is almost the same as the highest intra-specific distance (Figs. 1 and 2) (Table 2). The overlapping and failure of accurate identification restrict the use of ITS as a barcode marker for *Nectriaceae* even though it provides a relatively good species discrimination.

The nuclear large subunit ribosomal RNA gene (LSU) was commonly employed for investigations of phylogenetic relationships among certain groups of fungi at generic or a higher taxonomic level (Johnson and Vilgalys, 1998). It was reported to be suitable for species recognition in yeasts (Kurtzman and Robnett 1998; Fell et al. 2000; Ninet et al. 2003) and thus being considered as a barcode of yeasts (Seifert 2009). Our study shows that 28S rDNA (LSU) is the most conservative marker compared with any other candidate markers and that its sequence divergences are not sufficient to distinguish species accurately. In the case of *Neonectria* species, *N. coccinea*, *N. shennongjiana*, *N. ramulariae* and *N. ditissima* are grouped together as a single cluster when performed with TaxonDNA (Meier et al.

Table 3 Success rates of PCR and sequencing of ITS, 28 S rDNA, β -tubulin and EF-1 α genes of 28 species of *Nectriaceae*

Candidate barcode	ITS	28S rDNA	β -tubulin	EF-1 α
PCR	100%	100%	92% (92/100)	89.9% (80/89)
Sequencing	93.5% (87/93)	100%	100%	83.8% (67/80)
PCR & Sequencing	93.5%	100%	92%	75.3%

2006). Seventeen clusters are corresponding to the 28 taxonomic species, i.e. recognizing merely 60.7% of the total number of species tested. The inadequate intra- and inter-species variations make 28S rDNA fail to be a barcode for *Nectriaceae*.

Acknowledgements The authors express their deep thanks to Prof. R. P. Korf for corrections of the language, and Dr. R. Meier for providing a useful reference. This project was supported by the State 863 Project (no. 2008AA02Z312) and Special Project for Fundamental Research (no. 2006FY120100) from the Ministry of Science and Technology of China, and the National Natural Science Foundation of China (no. 31070015) to WYZ.

References

- Armstrong KF, Ball SL (2005) DNA barcodes for biosecurity: invasive species identification. *Phil Trans R Soc B* 360:1813–1823
- Ball SL, Armstrong KF (2006) DNA barcodes for insect pest identification: a test case with tussock moths (*Lepidoptera: Lymantriidae*). *Can J For Res* 36:337–350
- Booth C (1971) The genus *Fusarium*. Commonwealth Mycological Institute, Kew, Surrey
- Carbone I, Kohn LM (1999) A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91:553–556
- Druzhinina IS, Kopchinskiy AG, Komoń M, Bissett J, Szakacs G, Kubicek CP (2005) An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *FungGenet Biol* 42:813–828
- Feau N, Vialle A, Allaire M, Tanguay P, Joly DL, Frey P, Callan BE, Hamelin RC (2009) Fungal pathogen (mis-) identifications: A case study with DNA barcodes on *Melampsora* rusts of aspen and white poplar. *Mycol Res* 113:713–724
- Fell JW, Boekhout T, Fonseca A, Scorzetti G, Statzell-Tallman A (2000) Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* 50:1351–1371
- Froslev TG, Jeppesen TS, Læssøe T, Kjølner R (2007) Molecular phylogenetics and delimitation of species in *Cortinari* section *Calochroi* (*Basidiomycota, Agaricales*) in Europe. *Mol Phylogenet Evol* 44:217–227
- Geiser DM, Jiménez-Gasco M, Kang S, Makalowska I, Veeraraghavan N, Ward TJ, Zhang N, Kuldau GA, O'Donnell K (2004) FUSARIUM-ID v.1.0: A DNA sequence database for identifying *Fusarium*. *Eur J Plant Pathol* 110:473–479
- Geiser DM, Klich MA, Frisvad JC, Peterson SW, Varga J, Samson RA (2007) The current status of species recognition and identification in *Aspergillus*. *Stud Mycol* 59:1–10
- Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microb* 61:1323–1330
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95–98
- Hebert PD, Cywinska A, Ball SL, de Waard JR (2003a) Biological identifications through DNA barcodes. *Proc R Soc Lond* 270:313–321
- Hebert PD, Ratnasingham S, de Waard JR (2003b) Barcoding animal life: cytochrome c oxidase subunit I divergences among closely related species. *Proc R Soc Lond B (Suppl)* 270:S96–S99
- Hebert PD, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of Brids through DNA Barcodes. *PLoS Biol* 2:e312
- Hebert PD, de Waard JR, Landry J-F (2009) DNA barcodes for 1/1000 of the animal kingdom. *Biol Lett*. doi:10.1098/rsbl.2009.0848
- Hollingsworth PM, Forrest LL, Spouge JL et al (2009) A DNA barcode for land plants. *Proc Natl Acad Sci USA* 106:12794–12797
- Johnson J, Vilgalys R (1998) Phylogenetic systematics of *Lepiota* sensu lato based on nuclear large subunit rDNA evidence. *Mycologia* 90:971–979
- Kirk PM, Cannon PF, Minter DW, Stalpers JA (eds) (2008) *Dictionary of the Fungi* (10th ed). CAB International, Wallingford, UK
- Kurtzman CP, Robnett CJ (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26 S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* 73:331–371
- Li J, Liu SC, Niu SB, Zhuang WY, Che YS (2009) Pyrrolidinones from the ascomycete fungus *Albonectria rigidiuscula*. *J Nat Prod* 73:2184–2187
- Luo J, Zhuang WY (2010) Three new species of *Neonectria* (*Nectriaceae, Hypocreales*) with notes on their phylogenetic positions. *Mycologia* 102:142–152
- Martens M, Dawyndt P, Coopman R, Gillis M, De Vos P, Willems A (2008) Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*). *Int J Syst Evol Microbiol* 58:200–214
- Meier R, Shiyang K, Vaidya G, Ng PK (2006) DNA barcoding and taxonomy in *Diptera*: A tale of high intraspecific variability and low identification success. *Syst Biol* 55:715–728
- Meyer CP, Paulay G (2005) DNA barcoding: Error rates based on comprehensive sampling. *PLoS Biol* 3:e422
- Ninet B, Jan I, Bontems O, Léchenne B, Jousson O, Panizzon R, Lew D, Monod M (2003) Identification of dermatophyte species by 28 S ribosomal DNA sequencing with a commercial kit. *J Clin Microbiol* 41:826–830
- O'Donnell K, Cigelnik E (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol Phylogenet Evol* 7:103–116
- Rehner SA, Samuels GJ (1994) Taxonomy and phylogeny of *Gliocladium* analyzed from nuclear large subunit ribosomal DNA sequences. *Mycol Res* 98:625–634
- Rehner SA, Samuels GJ (1995) Molecular systematics of the *Hypocreales*: A teleomorph gene phylogeny and the status of their anamorph. *Can J Bot* 73(suppl 1):S816–S823
- Rehner SA, Buckley E (2005) A *Beauveria* phylogeny inferred from nuclear ITS and EF1- α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* 97:84–98
- Roe AD, Rice AV, Bromilow SE, Cooke JEK, Sperling FAH (2010) Multilocus species identification and fungal DNA barcoding: insights from blue stain fungal symbionts of the mountain pine beetle. *Mol Ecol Res* doi:10.1111/j.1755-0998.2010.02844.x
- Rossman AY (1996) Morphological and molecular perspectives on systematics of the *Hypocreales*. *Mycologia* 88:1–19
- Rossman AY, Samuels GJ, Rogerson CT, Lowen R (1999) Genera of *Bionectriaceae, Hypocreaceae* and *Nectriaceae* (*Hypocreales, Ascomycetes*). *Stud Mycol* 42:1–248
- Schroers H-J, Geldenhuis MM, Wingfield MJ, Schoeman MH, Yen Y-F, Shen W-C, Wingfield BD (2005) Classification of the guava wilt fungus *Myxosporium psidii*, the palm pathogen *Gliocladium vermoeseni* and the persimmon wilt fungus *Acremonium diospyri* in *Nalanthamala*. *Mycologia* 97:375–395
- Schwarz P, Bretagne S, Gantier J-C, Garcia-Hermoso D, Lortholary O, Dromer F, Dannaoui E (2006) Molecular identification of zygomycetes from culture and experimentally infected tissues. *J Clin Microbiol* 44:340–349

- Seifert KA (2009) Progress towards DNA barcoding of fungi. *Mol Ecol Resour* 9(Suppl 1):83–89
- Slabbinck B, Dawyndt P, Martens M, De Vos P, De Baets B (2008) TaxonGap: a visualisation tool for intra- and inter-species variation among individual biomarkers. *Bioinformatics* 24:866–867
- Stoeckle MY, Hebert PD (2008) Barcode of life. *Sci Am* 299(82–86):88
- Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C, Valentini A, Vermet T, Corthier G, Brochmann C, Willerslev E (2007) Power and limitations of the chloroplast *trnL* (UAA) intron for plant DNA barcoding. *Nucleic Acids Res* 35:e14
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Valentini A, Pompanon F, Taberlet P (2009) DNA barcoding for ecologists. *Trends Ecol Evol* 24:110–117
- Vilgalys R, Hester M (1990) Rapid genetic identification and mapping enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J Bacteriol* 172:4238–4246
- Wang L, Zhuang WY (2004) Designing primer sets for amplification of partial calmodulin genes from penicillia. *Mycosystema* 23:466–473
- White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR Protocols: A Guide to Methods and Applications*. Academic, New York, pp 315–322
- Zhang XM, Zhuang WY (2006) Phylogeny of some genera in *Nectriaceae* (*Ascomycetes*, *Hypocreales*) inferred from rDNA 28 S partial sequences. *Mycosystema* 25:15–22