Fungal Diversity (2011) 46:183–191
DOI 10.1007/s13225-010-0064-y

ORIGINAL PAPER

Practice towards DNA barcoding of the nectriaceous fungi

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Received: 18 April 2010 / Accepted: 26 September 2010 / Published online: 14 October 2010
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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-1α 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-1α 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

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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.

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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 *Cortinarius* 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 1α gene (EF-1α) 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-CO1 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-1α 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-1α 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-1α 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 μL reaction system consisting of 16 μL of double distilled water, 2.5 μL of 10× PCR buffer, 2 μL of $MgCl_2$ (25 mM), 1.25 μL of each primer (10 μM), 0.5 μL of dNTP (10 mM each), 1.25 μL of DNA template, 0.25 μL Taq DNA polymerase (5 U/μL). 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-1α
Albonectria rigidiuscula 6939	HMAS 183508	China	**HM054153**[b]	**HM042413**	**HM054098**	**HM054059**
A. rigidiuscula 6940	HMAS 183135	China	**HM054147**	**HM042411**	**HM054099**	**HM054061**
A. rigidiuscula 6941	HMAS 183509	China	**HM054148**	**HM042412**	**HM054100**	**HM054062**
A. rigidiuscula 7192	HMAS 183518	China	**HM054158**	**HM042403**		**HM054060**
Chaetopsinectria chaetopsinae H50	HMAS 76860	China	GU075858	**HM042404**	**HM054101**	**HM054063**
C. chaetopsinae-penicillatae	CBS 608.92	New Zealand	GU075859	GU075865	**HM054128**	**HM054064**
Cosmospora cupularis 6790-2	HMAS 97514	China	EF121864	EF121870	**HM054129**	**HM054065**
C. gigas 6598	HMAS 99592	China	EF121863	EF121869	**HM054102**	**HM054066**
C. henanensis 6724	HMAS 183528	China	GU075856	GU075863	**HM054103**	**HM054067**
C. meliopsicola 5186	HMAS 86473	China	**HM054159**	**HM042406**	**HM054104**	**HM054068**
C. vilior 7497	HMAS 183536	China	**HM054160**	**HM042407**	**HM054105**	**HM054069**
C. vilior 7093	HMAS 183535	China	**HM054144**	**HM042414**		
"Cosmospora" consors H17	HMAS 76861	China	EF121861	**HM042405**	**HM054106**	**HM054070**
Gibberella acuminata	P323	USA	AF132802			
	NRRL 6227			U85516	U85567	
	F30					EF531698
G. pulicaris	aurim1174	Lithuania	DQ093673			
	NRRL 22203			AF006326	AF006366	
	DDPP 0611p	Poland				EU128235
	MTFC12 (IRBV)		DQ132833			
	NRRL22187			U85523	U85574	
					AF484166	
	xsd08118	China	FJ481029			
G. zeae	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
Haematonectria haematococca 5133	HMAS 91771	China	**HM054145**	**HM042415**	**HM054107**	**HM054071**
H. haematococca 5804	HMAS 99199	China	**HM054152**	**HM042416**	**HM054108**	**HM054072**
H. haematococca	HMAS 83364	China		DQ119558		
Lanatonectria flavolanata 5622	HMAS 97516	China	EF121860	**HM042417**	**HM054109**	**HM054073**
L. flavolanata	DAOM 216608	Costa Rica		AY281098		
L. flocculenta H113	HMAS 76873	China	EF121858	DQ119567	**HM054110**	**HM054074**
L. flocculenta	HMAS 83374	China		DQ119566		
Leuconectria grandis 5630	HMAS 98302	China	EF121859	EU031441	EU984072	**HM054075**
Nectria australiensis H13	HMAS 83397	China	GU075855	**HM042418**	**HM054111**	**HM054076**
N. cinnabarina 5175	HMAS 91782	China	**HM054135**	**HM042420**	**HM054130**	**HM054077**
N. cinnabarina 5698	HMAS 98306	China	**HM054137**	**HM042419**		
N. cinnabarina 5179	HMAS 91783	China	**HM054155**	**HM042421**	**HM054112**	
N. cinnabarina 5744	HMAS 98311	China	**HM054156**	**HM042422**		
N. pseudotrichia 6722	HMAS 97518	China	EF121865	**HM042423**	**HM054113**	**HM054078**
N. pseudotrichia 7134	HMAS 183175	China	**HM054138**	**HM042424**	**HM054114**	**HM054079**
N. pseudotrichia 7107	HMAS 183559	China	**HM054154**	**HM042425**	**HM054115**	**HM054080**
N. pseudotrichia W7061a	HMAS 183172	China	GU232860	**HM042426**	**HM054116**	**HM054081**
N. sinensis 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-1α
N. sinensis H49	HMAS 83356	China	GU075854	**HM042427**	**HM054118**	**HM054083**
Neonectria castaneicola 6846	HMAS 183542	China	**HM054141**	**HM042430**	**HM054119**	**HM054084**
N. castaneicola H28	HMAS 76865	China	**HM054142**	**HM042429**	**HM054120**	**HM054085**
N. castaneicola H30	HMAS 83369	China	**HM054143**	**HM042431**	**HM054121**	**HM054086**
N. coccinea AR3700	CBS 119156	Slovakia		**HM042432**	**HM054122**	**HM054087**
N. coccinea	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	
N. discophora var. *discophora* 5542	HMAS 98333	China	**HM054136**	**HM042433**	**HM054131**	**HM054088**
N. discophora var. *discophora* W7104a	HMAS 183155	China	**HM054161**	**HM042408**	**HM054132**	**HM054089**
N. discophora var. *discophora* 5621	HMAS 98327	China	**HM054140**	**HM042434**	**HM054123**	
N. ditissima	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
N. hubeiensis 5620	HMAS 98331	China	FJ560439	FJ560434	FJ860056	**HM054090**
N. ramulariae	CBS 151.29	UK	**HM054150**	**HM042436**	**HM054124**	**HM054091**
N. ramulariae 188474	HMAS 188474	China	**HM054157**	**HM042435**	**HM054125**	**HM054092**
N. ramulariae	CBS 730.87	Germany	AJ279446			
	ATCC 16237	Germany			DQ789857	
N. ramulariae	CBS 182.36				DQ789864	
N. shennongjiana Z153	HMAS 183185	China	FJ560440	**HM042409**	FJ860057	**HM054093**
N. veuillotiana 5686	HMAS 98332	China	**HM054151**	**HM042437**	**HM054133**	**HM054094**
N. veuillotiana 5832	HMAS 99207	China	**HM054146**	**HM042438**	**HM054134**	**HM054095**
N. veuillotiana	GJS 91-116	USA	**HM054149**	**HM042439**	**HM054126**	**HM054096**
Bionectria wenpingii 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-1α, 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-1α 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

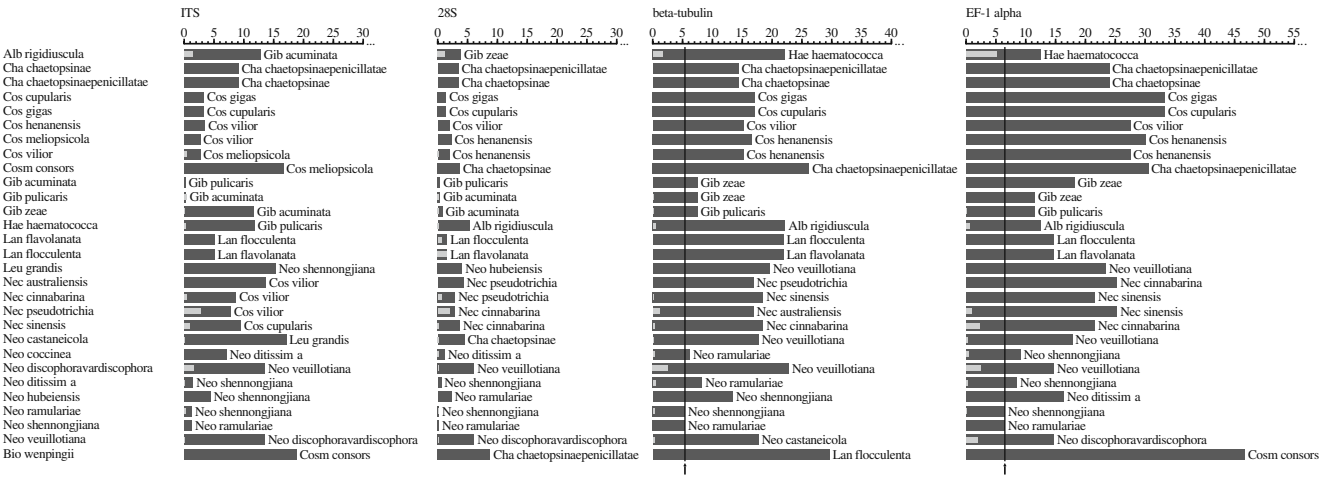

Fig. 1 Comparisons of intra- and inter-specific variations among ITS, 28S rDNA, β-tubulin and EF-1α genes of the nectriaceous fungi generated by the software TaxonGap. The grey and black bars represent the intra- and inter-specific variations respectively. The thin, black lines indicate the smallest interspecific variation. Names next to the dark bars indicate the closet species. *Bionectria wenpingii* was used as outgroup

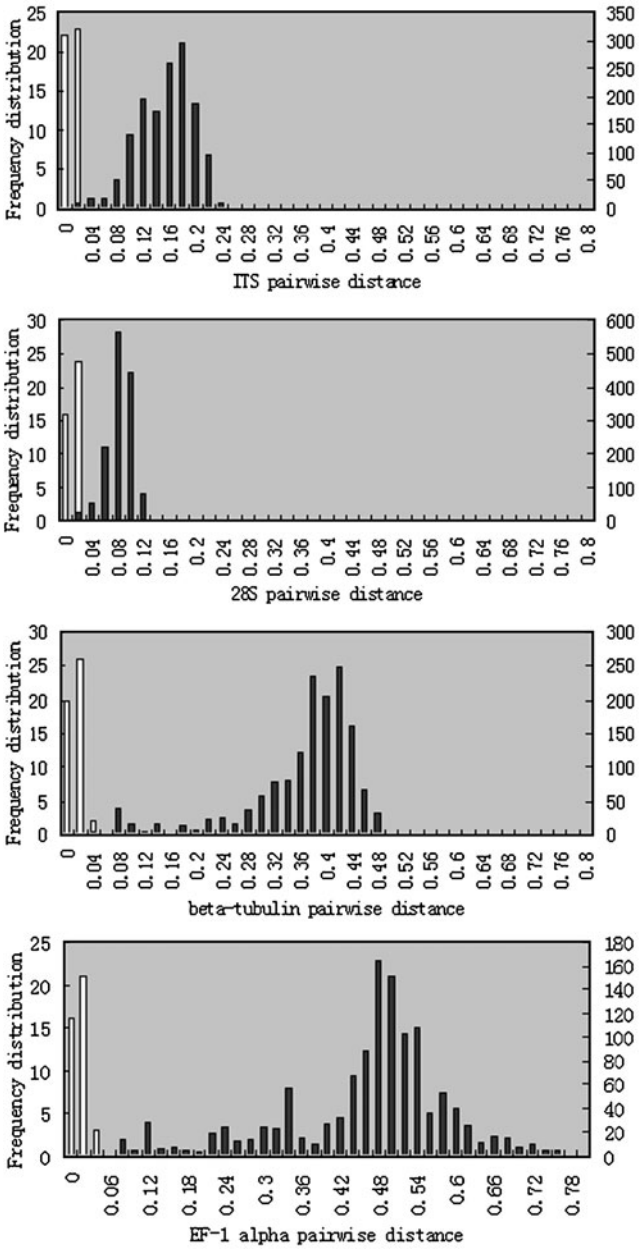

Fig. 2 Comparisons of frequency distribution of intra- and inter-specific pairwise distances among ITS, 28S rDNA, β-tubulin and EF-1α genes for the nectriaceous fungi generated with MEGA and Excel. The intra- and inter-specific distances are shown as white and black bars respectively

inter-specific variations shown by the β-tubulin and EF-1α 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-1α 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-1α) 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-1α 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 (≥ 92%) except for EF-1α 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-1α
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-1α 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-1α 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-1α genes of 28 species of *Nectriaceae*

Candidate barcode	ITS	28S rDNA	β-tubulin	EF-1α
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.

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