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DNA barcoding of the fungal genus *Neonectria* and the discovery of two new species

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To determine a suitable DNA barcode for the genus *Neonectria*, the internal transcribed spacer rDNA, β-tubulin, EF-1α, and RPB2 genes were selected as candidate markers. A total of 205 sequences from 19 species of the genus were analyzed. Intra- and inter-specific divergences and the ease of nucleotide sequence acquisition were treated as criteria to evaluate the feasibility of a DNA barcode. Our results indicated that any single gene among the candidate markers failed to serve as a successful barcode, while the combination of the partial EF-1α and RPB2 genes recognized all species tested. We tentatively propose the combined partial EF-1α and RPB2 genes as a DNA barcode for the genus. During this study, two cryptic species were discovered, based on the combined data of morphology and DNA barcode information. We described and named these two new species *N. ditissimopsis* and *N. microconidia*.

intra-specific variation, inter-specific variation, morphology, DNA barcode, sequence analysis

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Rapid and precise identification of species is the vital and critical first step in the fields of conservation, sustainable use of biodiversity [1], ecology [2], pathogen prevention and control [3], quarantine control of exotic species [4], forensic investigations [5], and human health [6,7]. Using purely morphological methods to identify fungal species poses some problems. The number of taxonomic mycologists is very limited, and for a specific fungal group fewer specialists can be consulted. Furthermore, morphological identification is somewhat time-consuming especially of species that lack sufficient diagnostic features, such as unicellular groups or those that fail to sporulate. In the past two decades, analysis of DNA sequences has been incorporated to establish phylogenetic relationships among groups and to aid species delimitation [8–12]. Nowadays, efforts have been made to seek a short, standardized, and universal gene marker for rapid species identification of diverse groups of fungi [5,13–19]. So far, a single universal DNA barcode for fungi has not been obtained [20]. Attempts have been made to develop a molecular identification system based on multilocus species identification, rather than a single locus [21].

Neonectria, a genus of the family Nectriaceae, was redefined, and three species were first accepted with the genus *Cylindrocarpon* as anamorphs by Rossman *et al*. [22]. Species belonging to the *Nectria mammoidea*-Group, *Nectria rugulosa*-Group, *Nectria radicicola*-Group and *Nectria veuillotiana*-Group were later assigned to the genus [23,24]. More recently, quite a few species were added to the genus [25–32]. As the number of species increased, it became necessary to investigate a DNA barcode for the genus to get a better understanding of the species diversity of the group. In this study, we used ITS (rDNA internal transcribed spacer), β-tubulin, EF-1α (elongation factor 1α), and RPB2 (RNA

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polymerase second largest subunit) genes, which have been widely used in the exploration of phylogeny among species, to select a suitable DNA barcode for *Neonectria*. The intra- and inter-specific divergences and ease of nucleotide sequence acquisition were treated as criteria to evaluate the feasibility of a DNA barcode.

During the screening of DNA barcodes for the genus *Neonectria*, two cryptic species were described as new species. One of them is closely related to *Nectria ditissima* and *Nectria major*, and the other is associated with *Nectria confusa* and its allies.

1 Materials and methods

1.1 Materials studied

A total of 82 strains representing 19 species of the genus *Neonectria* were sampled, including the type species *Nectria ramulariae* (Table 1).

1.2 Morphological study

The methods of Rossman *et al*. [22] and Luo and

Table 1 Materials used in this study

Species	Collection number or source[a)]	Geographical origin	GenBank accession number			
			ITS	β-tubulin	EF-1α	RPB2
Neonectria castaneicola (W. Yamam. & Oyasu) Tak. Kobay. & Hirooka	HMAS 183542	China	HM054141	HM054119	HM054084	**JF268693**[b)]
	HMAS 76865	China	HM054142	HM054120	HM054085	**JF268694**
	HMAS 83369	China	HM054143	HM054121	HM054086	**JF268695**
N. coccinea (Pers.) Rossman & Samuels	CBS 119158	Germany	**JF268759**	DQ789892	**JF268734**	DQ789818
	CBS 291.81	Austria	FJ474075		DQ789731	
	CBS 394.80	Netherlands		DQ789877	DQ789734	
	CBS 118914	France		DQ789831	DQ789688	DQ789760
	CBS 119156	Slovakia		HM054122		DQ789764
	CBS 119150	Slovakia				DQ789767
N. confusa J. Luo & W.Y. Zhuang	HMAS 99197	China	FJ560437	FJ860054	**JF268735**	**JF268696**
	HMAS 99198	China	**JF268760**	**JF268721**	**JF268736**	**JF268697**
N. discophora var. *discophora* (Mont.) Mantiri & Samuels	HMAS 98333	China	HM054136	HM054131	HM054088	**JF268702**
	HMAS 98327	China	HM054140	HM054123		
	HMAS 183155	China	HM054161	HM054132		
N. ditissima (Tul. & C. Tul.) Samuels & Rossman	CPC 12078	Netherlands	DQ178169			
	CBS 117752	Slovenia	DQ178168			
	CBS 316.34	Canada	AY677278			
	CBS 100472	USA	AJ228686			
	CBS 226.31	Germany		DQ789869		DQ789798
	CBS 100316	Ireland		DQ789858		DQ789787
	CBS 227.31	Norway		DQ789870		DQ789799
	CBS 100325	UK		DQ789861		DQ789790
	CBS 100319	Canada			DQ789716	DQ789788
	CBS 118927	USA			DQ789743	
	CBS 100320	Canada			DQ789717	
	HMAS 91784	China		**JF268726**	**JF268741**	
	HMAS 99205	China			**JF268742**	
N. ditissimopsis P. Zhao, J. Luo & W.Y. Zhuang	HMAS 98328	China	**JF268762**	**JF268727**	**JF268743**	**JF268703**
	HMAS 99206	China	**JF268763**	**JF268728**	**JF268744**	**JF268704**
	HMAS 98329	China	**JF268764**	**JF268729**	**JF268745**	**JF268705**
N. faginata (M.L. Lohman, A.M.J. Watson & Ayers) Castl. & Rossman	CBS 119160	USA	**HQ840384**	DQ789883	DQ789740	DQ789811
	CBS 217.67	Canada	**HQ840385**	**JF268730**	**JF268746**	DQ789797
	CBS 118938	USA		DQ789846		
	CBS 118918	USA		DQ789856	DQ789713	
	CBS 119155	USA			DQ789686	
	CBS 118917	USA				DQ789781
	CBS 119153	USA				DQ789755
N. fuckeliana (C. Booth) Castl. & Rossman	CBS 119200	Austria	**HQ840387**	**JF268731**	**JF268747**	**JF268706**
	CBS 239.29	UK	**HQ840386**	DQ789871	**JF268747**	**JF268707**
	GJS 90-31	Switzerland	AJ557579			

(*To be continued on the next page*)

(*Continued*)

Species	Collection number or source[a)]	Geographical origin	GenBank accession number			
			ITS	β-tubulin	EF-1α	RPB2
N. hubeiensis W.Y. Zhuang, Y. Nong & J. Luo	HMAS 98331	China	FJ560439	FJ860056	HM054090	**JF268708**
N. liriodendri Halleen, Rego & Crous	CBS 112607	South Africa		AY677241		
	CBS 112610	France	AY677270	AY677244	**JF268749**	**JF268709**
	STE-U6624	South Africa		GU183660		
	STE-U6625	South Africa		GU183661		
	CBS 117527	Portugal	DQ178165			
	CBS 110.81	USA	DQ178163			
	CBS 112602	South Africa	AY997532			
N. macrodidyma Halleen, Schroers & Crous	CBS 112605	South Africa	AY997549	AY677230		
	CBS 112594	South Africa		AY677231		
	CBS 112603	South Africa		AY677232		
	CBS 112615	South Africa	AY677290	AY677233	**JF268750**	**JF268710**
	CBS 112601	South Africa	**JF268765**		**JF268751**	**JF268711**
	KRP 51-1	Lithuania	HM036602			
N. major (Wollenw.) Castl. & Rossman	HMAS 183183	China	**JF268766**	**JF268732**	**JF268752**	**JF268712**
	HMAS 183184	China	**JF268767**	**JF268733**	**JF268753**	**JF268713**
	CBS 118981	France		DQ789833		
	CBS 119229	France		DQ789834		DQ789763
	CBS 240.29	Norway		DQ789872	DQ789729	DQ789800
	CBS 118982	USA				DQ789782
N. microconidia J. Luo, P. Zhao & W.Y. Zhuang	HMAS 98295	China	**JF268761**	**JF268722**	**JF268737**	**JF268698**
	HMAS 98293	China		**JF268723**	**JF268738**	**JF268699**
	HMAS 98294	China		**JF268724**	**JF268739**	**JF268700**
	HMAS 98296	China		**JF268725**	**JF268740**	**JF268701**
N. neomacrospora (C. Booth & Samuels) Mantiri & Samuels	CBS 118984	Canada	**HQ840388**	DQ789882	**JF268754**	DQ789810
	CBS 118985	Canada	**HQ840389**	DQ789890	**JF268755**	DQ789816
N. punicea (J.C. Schmidt) Castl. & Rossman	CBS 119724	Austria	**JF268768**	DQ789824	**JF268756**	DQ789753
	CBS 152.29	Germany	AY677260			
	CBS 242.29	Germany		DQ789873	DQ789730	DQ789801
N. radicicola (Gerlach & L. Nilsson) Mantiri & Samuels	CBS 153.37	France	**HQ840391**	AY677251	**JF268757**	**JF268714**
	CBS 156.47	Belgium	**HQ840390**	AY677252	**JF268758**	**JF268715**
	NTU 103	China	FJ205457			
	MH 337	Czech Republic	FJ430728			
N. ramulariae Wollenw.			DQ350126			
	CBS 182.36		HM054157	DQ789864	HM054092	DQ789793
	CBS 151.29	UK	AY677291	DQ789863	HM054091	DQ789792
	CBS 730.87	Germany	AJ279446	DQ789879		
	ATCC 16237	Germany		DQ789857		DQ789786
N. shennongjiana J. Luo & W.Y. Zhuang	HMAS 183185	China	FJ560440	FJ860057	HM054093	**JF268716**
N. veuillotiana (Sacc. & Roum.) Mantiri & Samuels	HMAS 98332	China	HM054151	HM054133	HM054094	**JF268717**
	HMAS 99207	China	HM054146	HM054134	HM054095	**JF268718**
	CBS 124740	USA	HM054149	HM054126	HM054096	**JF268719**
Nectria pseudotrichia (Schwein.) Berk. & M.A. Curtis	HMAS 183172	China	GU232860	HM054116	HM054081	**JF268720**

a) ATCC: American Type Culture Collection, Bethesda, MD, USA; CBS: Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CPC: Culture collection of P. W. Crous, housed at CBS; GJS: G. J. Samuels; HMAS: Mycological Herbarium, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; MH: M. Hujslová; NTU: National Taiwan University; STE: Departement van Plantkunde, Universiteit van Stellenbosch, Stellenbosch, South Africa; b) GenBank accession numbers in bold indicate the sequences generated in this study.

Zhuang [31,32] were generally followed for morphological characterization. Water was used as mounting fluid for microscopic examinations and measurements, and photographs were taken from water or lactic acid mounts with a Canon G5 (Tokyo, Japan) digital camera connected to a Zeiss Axioskop 2 plus microscope (Göttingen, Germany). The color names of colonies follow Ridgway's nomenclature [33]. Specimens are deposited in the Mycological Herbarium, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China.

1.3 DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from mycelia using the CTAB method [34]. Complete ITS rDNA was amplified and sequenced with ITS5 and ITS4 (or ITS1 and ITS4) primers [8]. A partial β-tubulin gene was PCR amplified with the primers T1 and T224 (or T22, T222) and sequenced with primers T1 and Bt2b [10,35]. The partial EF-1α gene was amplified by the primer pair 526F (or 728F) and 1567R. The amplicon was sequenced with the primers EFjR or 1567Ra in

addition to the amplification primers 526F or 728F [36,37] (http://www.aftol.org/pdfs/EF1primer.pdf), and the region between 728F and EFjR was analyzed. The taxon specific primers for EF-1α gene, nEF-1 (5′-CACGTCGATTCTGGCAAGTC-3′, forward), nEF-12 (5′-TTGGTGGTGTCCATCTTGTT-3′, reverse), and nEF-13 (5′-GCGTTGTAGCCGACCTTCTT-3′, reverse), were designed for PCR and sequencing when the commonly used primers did not perform well. The RPB2 gene was amplified with the primer pair fRPB2-5F and fRPB2-7cR [38]. The primers fRPB2-5F and RPB2intR [25] were used for sequencing. The following primers were designed for RPB2 amplification and sequencing, and used when necessary: nRPB2-147 (5′-TCGGCAARAAGCGWYTKGATC-3′, forward); nRPB2-764 (5′-AYTTBCGKCGRGTATCCA-3′, reverse); nRPB2-766 (5′-BGAYTTBCGKCGRGTATC-3′, reverse).

PCR was performed with the 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using a 25 μL reaction system. For ITS, 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 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. For RPB2, PCR conditions were an initial step of 5 min at 95°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, 1 min or 90 s at 72°C; followed by 10 min at 72°C. The obtained amplicons were sequenced in both directions with an ABI 3730 XL DNA Sequencer (SinoGenoMax Co. Ltd., Beijing, China).

1.4 Comparison of intra- and inter-specific divergences

Sequences were aligned using ClustalX 1.81 [39] and manually edited to adjust the aligned sequences by BioEdit 7.0 [40]. A partition homogeneity test (PHT) was performed with 1000 replicates in PAUP 4.0b10 [41] to evaluate statistical congruence between sequence data from EF-1α and RPB2 gene regions. The aligned sequences of each gene and the combined EF-1α and RPB2 genes were analyzed using DNAstar 7.1.0 (Lasergene, USA) to calculate the similarity matrices and then illustrate the intra- and inter-specific variations of the candidate barcode loci for each of the 19 species tested in this study, using a visualization analysis tool, TaxonGap 2.4.1 [42]. As suggested by Martens *et al*. [43], *Nectria pseudotrichia* was designated as the outgroup in the analysis.

The other distance-dependent method for evaluation the sequence variations within and between species of the potential barcode regions using Kimura's two-parameter (K2P) model, with clustering at a given threshold, was generated with TaxonDNA 1.6.3 [44].

1.5 Evaluation of the ease of test barcode sequence acquisition

Success rates of PCR amplification and sequencing of the considered DNA barcode markers for the genus were assessed. A single PCR band obtained was considered as successful amplification. A high quality chromatogram counted as successful sequencing. The success rate of PCR amplification multiplied by that of sequencing produced the overall success rate of PCR amplification and sequencing.

1.6 Neighbor-joining tree reconstruction

A neighbor-joining tree was constructed based on sequence analysis of the combined EF-1α and RPB2 genes using MEGA 4.0.2 [45] with the K2P substitution model to show the relationships among the *Neonectria* species. Branch support was calculated by a bootstrap analysis with 1000 replicates, and *N. pseudotrichia* was used as the outgroup.

2 Results

2.1 Selection of DNA barcode markers for *Neonectria*

A total of 205 sequences of the four candidate DNA barcode regions, ITS, β-tubulin, EF-1α and RPB2 genes, from 19 *Neonectria* species were analyzed (Table 1). To meet the requirements for a standard DNA barcode, the sequences of all the candidate markers must be short, i.e., 412–475 base pairs (bp) for ITS, 489–515 bp for β-tubulin, 461–478 bp for EF-1α and 473 bp for RPB2.

Intra- and inter-specific variation, clustering with a given threshold, and the success rate of PCR and sequencing were used to evaluate the feasibility of the candidate markers [46]. Among them, intra- and inter-specific variation was treated as a very important criterion. The successful species identification of a DNA barcode requires a clear distinction between intra- and inter-specific divergences [47]. The intra- and inter-specific variations of the candidate DNA barcode regions for each of the 19 *Neonectria* species generated using TaxonGap [42] indicated that the EF-1α gene provided a somewhat better resolution compared with the ITS, β-tubulin, and RPB2 genes (Figure 1). For the EF-1α gene, the smallest inter-specific variation was 1.7%, which is shown as a thin and black line in Figure 1. All species, except for *N. confusa* and *N. veuillotiana*, had intra-specific variations lower than 1.7%. When the β-tubulin gene was tested, the minimum inter-specific variation between *N. confusa* and *Nectria punicea* was only 0.7%, the intra-specific variations of four species (*Nectria discophora* var.

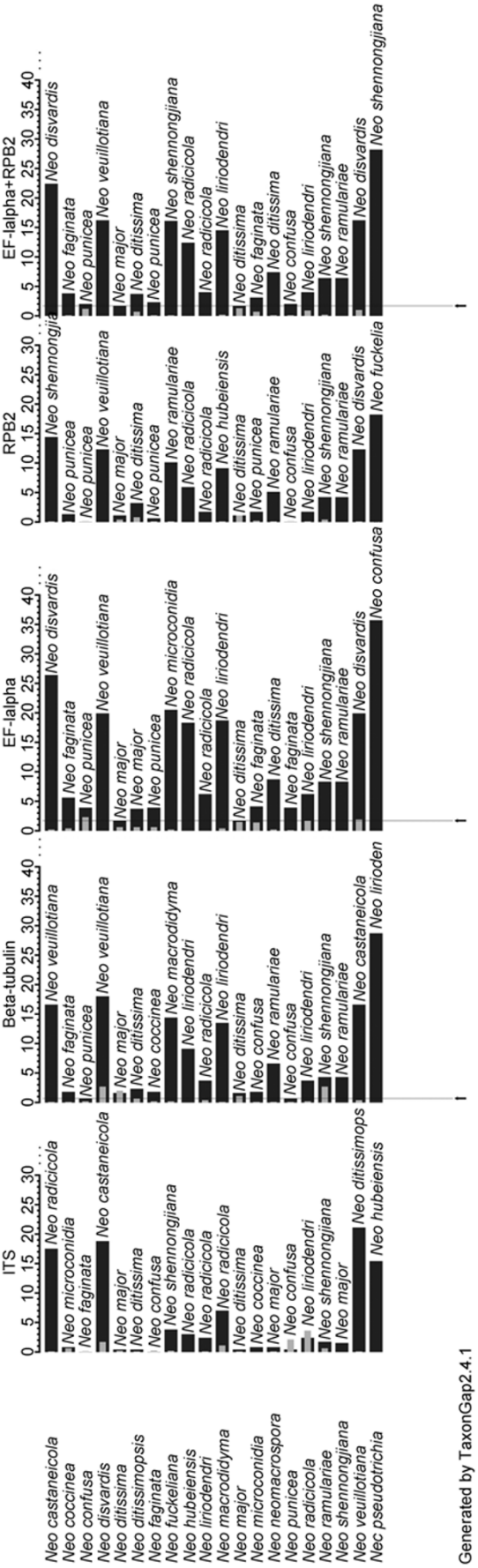

Figure 1 Comparisons of intra- and inter-specific variations among the candidate barcode genes ITS, β-tubulin, EF-1α, and RPB2 from 19 *Neonectria* species generated by the software TaxonGap. The gray and black bars represent the intra- and inter-specific variations respectively. The thin, black lines denote the smallest inter-specific variation for the candidate barcode. Taxon names next to the dark bars denote the closest species of the species listed on the left. *Nectria pseudotrichia* was used as the outgroup.

discophora, *N. ditissima*, *N. major* and *N. ramulariae*) exceeded the smallest inter-specific variation. The species identification abilities of ITS and RPB2 genes were even worse, and thus the thin and black line cannot be detected. Thus, no individual DNA marker could serve as a suitable barcode; therefore, gene combinations were considered. When the EF-1α and RPB2 genes were combined, the analysis provided a fairly good result in that all intra-specific variations were lower than the smallest inter-specific variation (Figure 1).

Clustering with a given threshold was calculated using TaxonDNA [44], which provided additional measurements of the intra- and inter-specific divergences for the candidate barcode markers (Table 2). The largest intra-specific distance was employed as the threshold of clustering to explore the coincidence of a cluster and its correlation with a single species. For the EF-1α gene, a total of 18 clusters were recognized, which means this gene separated 17 of the 19 species (89.5%); RPB2 and β-tubulin genes discriminated 16 and 12 species, respectively; and ITS was able to identify only 10 species. When RPB2 gene and EF-1α gene were combined, all examined species were identified successfully.

The success rate of PCR and sequencing was one of the criteria to estimate candidate barcode markers. EF-1α, RPB2, and β-tubulin genes were easily PCR amplified and sequenced, and the success rates reached 100%. New primer pairs were required for RPB2 and EF-1α genes from a few species, although the commonly used primers worked well in most species of the genus. Unexpectedly, ITS scored a relatively low success rate (83.7%).

The partition homogeneity test (P=0.018) suggested that the individual partitions were congruent [48,49]. EF-1α and RBP2 sequences were therefore combined for the subsequent analysis. In the neighbor-joining tree generated from the combined EF-1α and RPB2 genes, all species were well-separated from each other as independent terminal branches (Figure 2). Sequences from different strains of the

Table 2 Clustering at a given threshold of the candidate barcode genes ITS, β-tubulin, EF-1α and RPB2 of nineteen *Neonectria* species derived using TaxonDNA [44]

Candidate gene (s)	ITS	β-tubulin	EF-1α	RPB2	EF-1α+RPB2
Largest intra-specific distance	1.07%	2.41%	2.57%	1.07%	1.30%
Number of cluster	12	14	18	17	19
Corresponding to species taxa	10 (52.6%)	12 (63.2%)	17 (89.5%)	16 (84.2%)	19 (100%)

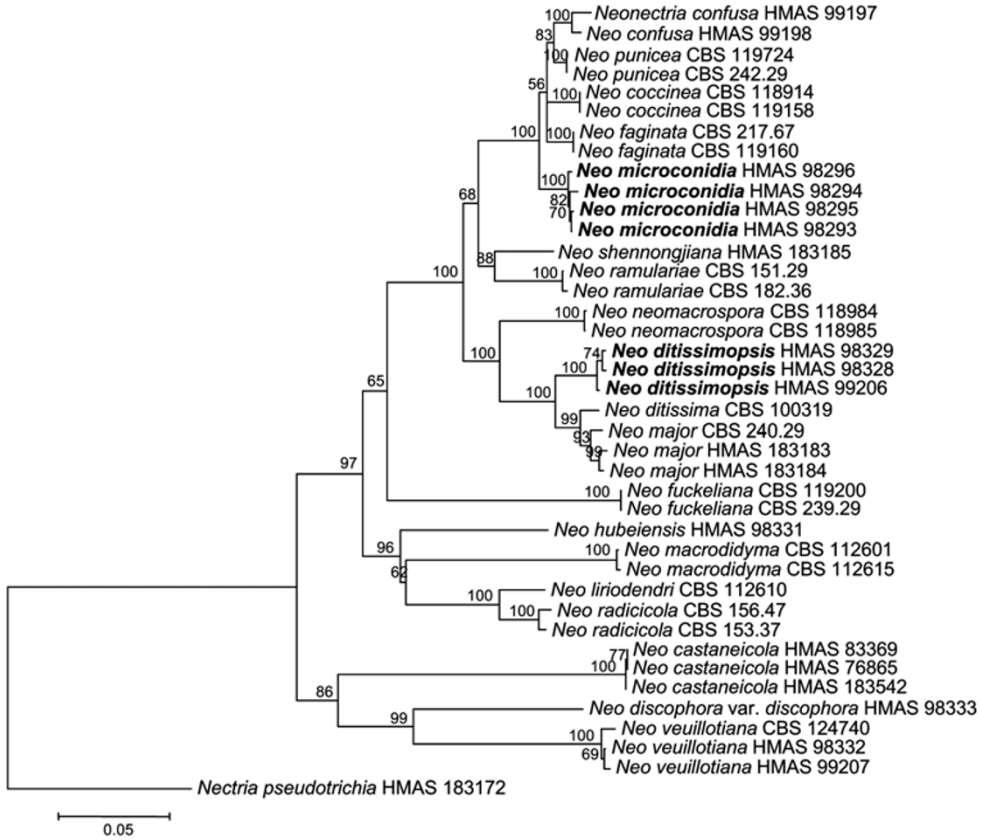

Figure 2 A neighbor-joining tree generated by analysis of the combined sequences of the EF-1α and RPB2 genes (TreeBase: S11186).

same species showed high cohesion. The three *Neonectria* strains (HMAS 98238, 98239, 99206) at the same terminal branch with 100% bootstrap support and closely related to and distinctive clearly from *N. ditissima*, and the four strains (HMAS 98293, 98294, 98295, 98296) at a same terminal branch with high bootstrap value (100%) associated *N. confusa* and its allies, were found to be cryptic species of the genus.

2.2 Taxonomy

Neonectria ditissimopsis P. Zhao, J. Luo & W.Y. Zhuang, **sp. nov.** (Figures 3A and 4)

MycoBank MB519626

Peritheciis subglobosis, papillatis, 380–450 μm diam; ascis clavatis, 8-sporis, 90–110×9.5–13 μm; ascosporis ellipsoideis, uniseptatis, spinulosis, 19–23.5×6.5–7.5 μm.

Ascomata perithecial, gregarious up to 20 in a group, with a well-developed stroma that is erumpent through bark, superficial, subglobose, 420–510 μm high, 380–450 μm diam, with a small papilla, not collapsing when dry, red when fresh and brown red when dry, turning dark red in 3% KOH and orange-red to orange in lactic acid, rough to slightly warted; warts red, 10–20 μm high, cells angular, 6.5–16×5–10 μm, cell wall 1.5–2.5 μm thick. Ascomatal wall 38–60 μm thick, of two layers; outer layer 27–44 μm thick, cells angular, 7.5–20.5×6–9 μm, cell wall 1.5–2.5 μm thick; inner layer 11–16.5 μm thick, cells flattened, 16–27×2–5 μm, cell wall 0.5–1.5 μm thick. Asci subcylindrical to clavate, 8-spored, with an apical ring, 90–110×9.5–13 μm (*n*=50). Ascospores fusoid-ellipsoid, uniseptate, not constricted at septum, hyaline, spinulose, irregularly biseriate, 19–23.5×6.5–7.5 μm (*n*=50). Anamorph unknown.

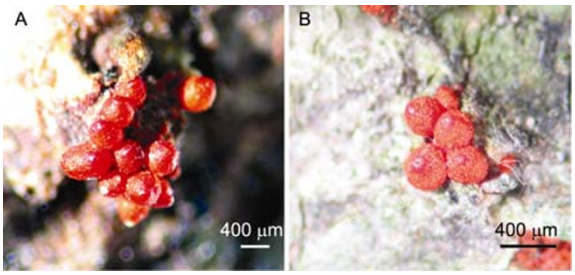

Figure 3 Ascomata on natural substrate. A, *Neonectria ditissimopsis* (HMAS 98329); B, *Neonectria microconidia* (HMAS 98294).

Colonies on PDA 2.8 cm in diam after 4 d in dark at 24°C, Capucine Yellow, surface floccus, aerial mycelium yellowish, reverse pigmented, Raw Sienna. Colonies on CMD reaching 2.2 cm after 4 d in dark at 24°C, Pale Yellow-Orange, aerial mycelium spare, reverse pigmented, Orange-Buff. Conidia not produced in culture.

Etymology: Specific epithet refers to the morphological similarity to *N. ditissima*.

Holotype: CHINA. HUBEI, Shennongjia, 1800 m, on twigs of a dicotyledon tree, 16 Sept 2004, W.Y. Zhuang and Y. Nong 5748, HMAS 98329.

Paratypes: HUBEI: Shennongjia, 1800 m, on twigs of a dicotyledon tree, 16 Sept 2004, W.Y. Zhuang and Y. Nong 5742, 5743, 5749, HMAS 98328, 99206, 98330.

Notes: Among the known species of the genus, *N. ditissimopsis* is most similar to *N. ditissima* in the subglobose

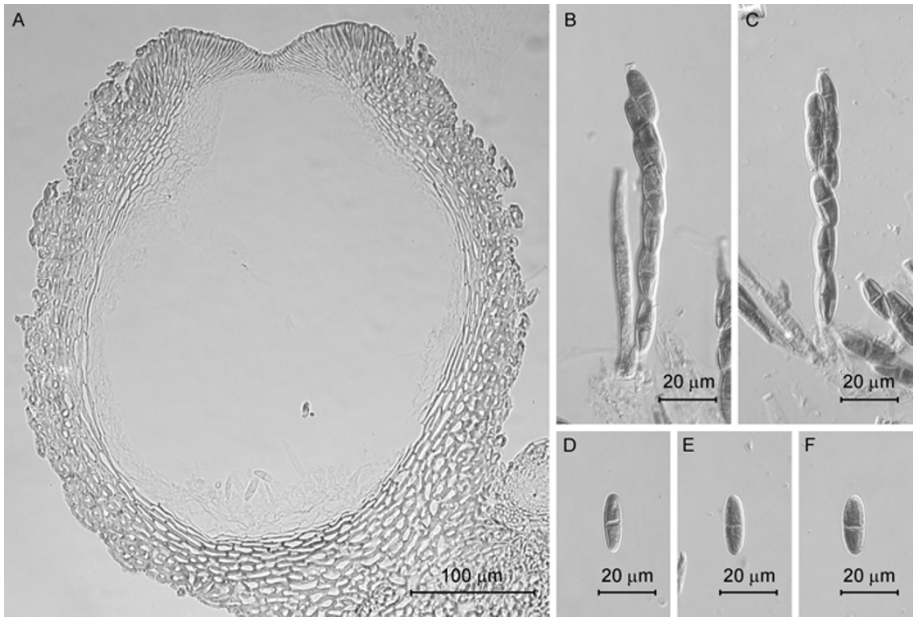

Figure 4 Morphology of *Neonectria ditissimopsis* (HMAS 98329). A, median section of an ascoma; B and C, asci; D–F, ascospores.

perithecia, which do not collapse when dry and have a rough surface, subcylindrical to clavate asci, and spinulose ascospores. However, *N. ditissima* differs from *N. ditissimopsis* in somewhat smaller perithecia (250–400 μm diam), wider asci [(77–)88–116(–130)×(11–)12–17(–20) μm], the lack of an apical ring, and shorter ascospores [(12.2–)14.9–18.9(–24.3)×(5.5–)6.5–8.3(–10.2) μm] [25]. The new species is also similar to *N. coccinea* in the subglobose perithecia, which do not collapse when dry and have a rough surface, the thickness of the perithecial wall, cylindrical to clavate asci with an apical ring, and spinulose ascospores. However, *N. coccinea* differs in smaller perithecia (200–350 μm diam), smaller asci [(71–)76–93(–98)×(7–)8–10 (–11) μm], and much smaller ascospores [(10.5–)12.4–14.4×5.1–6.5(–8.5) μm] [25,50,51].

In addition to the morphological characteristics, recognition of this new species and its close relation to *N. ditissima* were also supported by sequence analysis of the combined EF-1α and RPB2 genes (Figure 2), which will be discussed later.

Neonectria microconidia J. Luo, P. Zhao & W.Y. Zhuang, **sp. nov.** (Figures 3B and 5)

MycoBank MB561075

Peritheciis subglobosis, papillatis, 210–340 μm diam; ascis cylindricis, 8-sporis, 65–90×5–9 μm; ascosporis ellipsoideis, uniseptatis, 11–15×4–6 μm.

Ascomata perithecial, gregarious, up to 50 in a group, on a well-developed stroma, superficial, subglobose to obpyriform, 245–360 μm high, 210–340 μm diam, with a small papilla, not collapsing when dry, orange-red when fresh and red when dry, turning dark red in 3% KOH and orange red to orange in lactic acid, surface smooth. Ascomatal wall 25–42 μm thick, of two layers; outer layer 20–28 μm thick, cells angular, 7–17×5–10 μm, cell wall 0.5–2.5 μm thick; inner layer 4.5–15 μm thick, cells flattened, 9–29×2–5.5 μm, cell wall 0.5–1.5 μm thick. Asci cylindrical to broadly cylindrical, 8-spored, with an apical ring, 65–90×5–9 μm (*n*=50). Ascospores fusoid to ellipsoid, uniseptate, not constricted at septum, hyaline to yellowish, warted, irregularly biseriate, 11–15×4–6 μm (*n*=50).

Colonies on PDA 3.2 cm in diam after 4 d in dark at 24°C, Argus Brown, surface floccus, aerial mycelium yellowish, reverse pigmented, Antique Brown. Colonies on CMD reaching 2.5 cm after 4 d in dark at 24°C, Buckthorn Brown, aerial mycelium spare, reverse pigmented, Ochraceous Tawny. Conidiophores simple. Simple conidiophores unbranched to sparsely branched, septate, 20–55×1.5–2.5 μm, conidiogenous cells monophialidic, cylindrical, 15–27.5 μm long, 1–2.5 μm at base, 0.5–1 μm near aperture. Microconidia ellipsoid to cylindrical, not or slightly curved, hyaline, 0–1-septate; 0-septate: 3–12×1.5–2.5 μm (*n*=50), 1-septate: 6.5–13×1.5–3.5 μm (*n*=50). Macroconidia or Chlamydospores not observed.

Etymology: Specific epithet refers to producing purely microconidia in culture.

Holotype: CHINA. HUBEI, Wufeng, 800 m alt., on twigs, 13 Sept 2004, W.Y. Zhuang and Y. Nong 5639, HMAS 98294.

Paratypes: CHINA. HENAN, Jigongshan, 400 m alt., on twigs, 14 Nov 2003, W.Y. Zhuang and Y. Nong 5129, HMAS 91750. HUBEI, Wufeng, Houhe Nature Reserve, 800 m alt., on twigs, 12 Sept 2004, W.P. Wu, W.Y. Zhuang

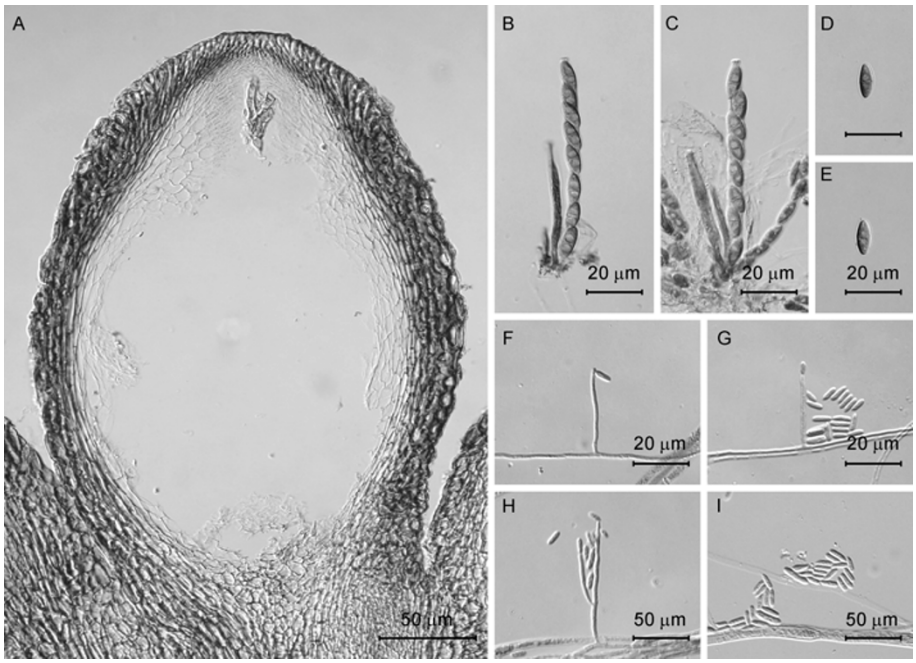

Figure 5 Morphology of *Neonectria microconidia* (HMAS 98294). A, median section of an ascoma; B and C, asci; D and E, ascospores; F–H, conidiophores bearing microconidia; I, microconidia.

and Y. Nong 5544, 5550, HMAS 99196, 98292; *ibid*., on twigs, 12 Sept 2004, W.Y. Zhuang and Y. Nong 5554, HMAS 98293; *ibid*., on twigs, 13 Sept 2004, W.Y. Zhuang and Y. Nong 5642, HMAS 98295; *ibid*., on bark, 13 Sept 2004, W.Y. Zhuang and Y. Nong 5640, HMAS 98296.

Notes: Among the existing species of the genus, *N. microconidia* is most similar to *N. confusa* in smooth and subglobose perithecia, which do not collapse when dry, cylindrical asci with an apical ring, fusoid ascospores with a warted surface, ellipsoid to cylindrical microconidia, and brown colony on PDA. *N. confusa*, however, differs in smaller ascomata (205–260×175–240 μm), thinner ascomatal wall (18–30 μm thick), slightly smaller ascospores (9–13×3.5–5 μm), and producing abundant macroconidia in culture [32]. The DNA sequence divergences support strongly the separation of *N. microconidia* from *N. confusa* (Figure 2). Different strains of any one of the two species share very similar sequences. We treat the above distinctions at the species level.

3 Discussion

As previously indicated, the two important criteria for estimating a DNA barcode are suitable intra- and inter-specific sequence variation and high success rate of PCR amplification and sequencing. In our study, the success rates of PCR amplification and sequencing for all tested markers were more or less the same because of the new primer pairs introduced. Therefore, a suitable intra- and inter-specific sequence variation becomes critical. Our results suggest that the combination of partial EF-1α and RPB2 genes may serve as the DNA barcode for the genus *Neonectria*.

As a qualified DNA barcode, a short, single DNA fragment should be distinct enough to separate a wide range of species. The fact that fungi display extremely high species diversity among living organisms may cause problems during determination of a universal barcode. In the case of *Neonectria*, when a single DNA fragment is considered as a barcode marker, the EF-1α and RPB2 genes were both better than the ITS and β-tubulin genes. The EF-1α gene possessed more or less adequate intra- and inter-specific variations, and therefore had the highest species identification power among the candidate markers. It is able to recognize 17 of the 19 species tested (89.5%, Table 2), with the exception of the two closely related species. RPB2 gene ranks the next (16/19, 84.2%, Table 2). This result conforms to the previous studies in certain other fungal groups [38,52–54]. The other two genes, β-tubulin and ITS, had undesirable intra- and inter-variations and exhibited relatively poor species resolution capacity (Table 2), which indicated that the partial β-tubulin gene and ITS gene are inadequate to identify closely related species. The low inter-specific variation in ITS failed to discriminate closely related species (Figure 1), which was also reported in other groups of fungi [55–57] and thus limits its application as a DNA barcode [20].

A two-locus DNA barcode, the combination of plasmid genes *rbcL* and *matK*, was adopted recently as the main barcode of land plants [58]. In our case, no single gene could serve as a powerful DNA barcode. We thus proposed to use two gene fragments instead one as the barcode for the group. The combination of the EF-1α and RPB2 genes recognized all species of the genus (Figure 1; 19/19, 100%, Table 2). This is because the three species (*N. confusa*, *Nectria faginata* and *N. punicea*) that the RPB2 gene failed to identify can easily be separated by the EF-1α gene. Similarly, the two species (*N. ditissima* and *N. major*) that the EF-1α gene was unable to distinguish are well-differentiated by the RPB2 gene. These two gene fragments appear to be complementary and work jointly as the DNA barcode of *Neonectria*.

During our screening of the DNA barcode, attention was also paid to the establishment of the correct species concepts for this group of fungi. The neighbor-joining tree generated by combined sequences of the EF-1α and RPB2 genes indicates that collections formerly treated as *N. ditissima* (as *Nectria galligena*) and *N. confusa* were not completely identical in DNA sequences, although their morphological distinctions were relatively few or negligible at the species level. The sequence analysis and inter- and intra-specific variations displayed by these collections led to the discovery of two cryptic species, *N. ditissimopsis* and *N. microconidia* (Figures 1–5). The former was previously merged with *N. ditissima* [30], and the latter was treated as an intra-specific variant of *N. confusa*, based on morphological and culture characteristics [32].Our sequence analysis also revealed that *N. ditissimopsis* is closely related to *N. ditissima*, *N. major*, and *Nectria neomacrospora*, with 100% bootstrap value, which is in accordance with their morphological similarity. *Neonectria microconidia* is closely related to *N. confusa*, *N. coccinea*, *N. punicea,* and *N. faginata*, also with 100% bootstrap support. Detailed morphological comparisons between *N. microconidia* and *N. confusa* have already been discussed.

DNA barcoding is becoming a helpful tool in the assessment of biodiversity. Discovery of cryptic species by DNA barcoding has been reported in butterfly [59] and yew [60]. The current work suggests that integrated studies on morphology and DNA sequence data have a bright future in the exploration of fungal diversity and the establishment of clear species concepts.

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1 Ardura A, Linde A R, Moreira J C, *et al*. DNA barcoding for conservation and management of Amazonian commercial fish. Biol Conserv, 2010, 143: 1438–1443
2 Chen X Y, Daniell T J, Neilson R, *et al*. A comparison of molecular methods for monitoring soil nematodes and their use as biological indicators. Eur J Soil Biol, 2010, 46: 319–324
3 Crous P W, Groenewald J Z, Risède J M, *et al*. *Calonectria* species and their *Cylindrocladium* anamorphs: species with clavate vesicles. Stud Mycol, 2006, 55: 213–226
4 Wingfield M J, Slippers B, Roux J, *et al*. Worldwide movement of exotic forest fungi, especially in the tropics and the southern hemisphere. Bioscience, 2001, 51: 134–140
5 Boehme P, Amendt J, Disney R H L, *et al.* Molecular identification of carrion-breeding scuttle flies (Diptera: Phoridae) using COI barcodes. Int J Legal Med, 2010, 124: 577–581
6 Schwarz P, Bretagne S, Gantier J C, *et al*. Molecular identification of zygomycetes from culture and experimentally infected tissues. J Clin Microbiol, 2006, 44: 340–349
7 Lowenstein J H, Burger J, Jeitner C W, *et al*. DNA barcodes reveal species-specific mercury levels in tuna sushi that pose a health risk to consumers. Biol Lett, 2010, 6: 692–695
8 White T J, Bruns T, Lee S, *et al.* Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M A, Gelfand D H, Sninsky J J, *et al.*, eds. PCR Protocols: A Guide to Methods and Applications. New York: Academic Press, 1990. 315–322
9 Bruns T D, White T J, Taylor J W. Fungal molecular systematics. Ann Rev Ecol Syst, 1991, 22: 525–564
10 O'Donnell K, Cigelnik E. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol Phylogenet Evol, 1997, 7: 103–116
11 James T Y, Kauff F, Schoch C L, *et al.* Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature, 2006, 443: 818–822
12 Hibbett D S, Binder M, Bischoff J F, *et al*. A higher-level phylogenetic classification of the Fungi. Mycol Res, 2007, 111: 509–547
13 Geiser D M, Klich M A, Frisvad J C, *et al*. The current status of species recognition and identification in *Aspergillus*. Stud Mycol, 2007, 59: 1–10
14 Min X J, Hickey D A. Assessing the effect of varying sequence length on DNA barcoding of fungi. Mol Ecol Notes, 2007, 7: 365–373
15 Seifert K A, Samson R A, de Waard J R, *et al*. Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case. Proc Natl Acad Sci USA, 2007, 104: 3901–3906
16 Tedersoo L, Jairus T, Horton B M, *et al.* Strong host preference of ectomycorrhizal fungi in a Tasmanian wet sclerophyll forest as revealed by DNA barcoding and taxon-specific primers. New Phytol, 2008, 180: 479–490
17 Feau N, Vialle A, Allaire M, *et al.* Fungal pathogen (mis-) identifications: A case study with DNA barcodes on *Melampsora* rusts of aspen and white poplar. Mycol Res, 2009, 113: 713–724
18 Eberhardt U. A constructive step towards selecting a DNA barcode for fungi. New Phytol, 2010, 187: 265–268
19 Stockinger H, Krüger M, Schüßler A. DNA barcoding of arbuscular mycorrhizal fungi. New Phytol, 2010, 187: 461–474
20 Seifert K A. Progress towards DNA barcoding of fungi. Mol Ecol Res, 2009, 9: 83–89
21 Roe A D, Rice A V, Bromilow S E, *et al.* Multilocus species identification and fungal DNA barcoding: insights from blue stain fungal symbionts of the mountain pine beetle. Mol Ecol Res, 2010, 10: 946–959
22 Rossman A Y, Samuels G J, Rogerson C T, *et al.* Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). Stud Mycol, 1999, 42: 1–248
23 Mantiri F R, Samuels G J, Rahe J E, *et al*. Phylogenetic relationships in *Neonectria* species having *Cylindrocarpon* anamorphs inferred from mitochondrial ribosomal DNA sequences. Can J Bot, 2001, 79: 334–340
24 Brayford D, Honda B M, Mantiri F R, *et al*. *Neonectria* and *Cylindrocarpon*: the *Nectria mammoidea* group and species lacking microconidia. Mycologia, 2004, 96: 572–597
25 Castlebury L A, Rossman A Y, Hyten A S. Phylogenetic relationships of *Neonectria*/*Cylindrocarpon* on *Fagus* in North America. Can J Bot, 2006, 84: 1417–1433
26 Halleen F, Schroers H, Groenewald J Z, *et al*. *Neonectria liriodendri* sp. nov., the main causal agent of black foot disease of grapevines. Stud Mycol, 2006, 55: 227–234
27 Hirooka Y, Kobayashi T, Natsuaki K T, *et al*. *Neonectria amamiensis* and *Cylindrocarpon amamiense*, a new nectrioid fungus and its sporodochial anamorph on *Pinus luchuensis* from Japan. Mycoscience, 2006, 47: 248–256
28 Samuels G J, Rossman A Y, Chaverri P, *et al*. Hypocreales of the Southeastern United States: An Identification Guide. CBS Biodiversity Series 4. Utrecht, the Netherlands: Cebtraalbureau voor Schimmelcultures, 2006. 1–145
29 Hirooka Y, Kobayashi T. Taxonomic studies of nectrioid fungi in Japan I: the genus *Neonectria*. Mycoscience, 2007, 48: 53–62
30 Zhuang W Y, Nong Y, Luo J. New species and new Chinese records of Bionectriaceae and Nectriaceae (Hypocreales, Ascomycetes) from Hubei, China. Fungal Divers, 2007, 24: 347–357
31 Luo J, Zhuang W Y. Four new species and a new Chinese record of the nectrioid fungi. Sci China Life Sci, 2010, 53: 909–915
32 Luo J, Zhuang W Y. Three new species of *Neonectria* (Nectriaceae, Hypocreales) with notes on their phylogenetic positions. Mycologia, 2010, 102: 142–152
33 Ridgway R. Color Standards and Color Nomenclature. Ridgway, Washington, DC., USA, 1912
34 Wang L, Zhuang W Y. Designing primer sets for amplification of partial calmodulin genes from penicillia. Mycosystema, 2004, 23: 466–473
35 Glass N L, Donaldson G C. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microb, 1995, 61: 1323–1330
36 Carbone I, Kohn L M. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia, 1999, 91: 553–556
37 Rehner S A, Buckley E. A *Beauveria* phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. Mycologia, 2005, 97: 84–98
38 Chaverri P, Castlebury L A, Overton B E, *et al. Hypocrea*/*Trichoderma*: species with conidiophore elongations and green conidia. Mycologia, 2003, 95: 1100–1140
39 Thompson J D, Higgins D G, Gibson T J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res, 1994, 22: 4673–4680
40 Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser, 1999, 41: 95–98
41 Swofford DL. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4b10. Sunderland, Massachusetts: Sinauer Associates, 2002
42 Slabbinck B, Dawyndt P, Martens M, *et al.* TaxonGap: a visualisation tool for intra- and inter-species variation among individual biomarkers. Bioinformatics, 2008, 24: 866–867
43 Martens M, Dawyndt P, Coopman R, *et al.* 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, 2008, 58: 200–214
44 Meier R, Shiyang K, Vaidya G, *et al.* DNA barcoding and taxonomy in *Diptera*: A tale of high intraspecific variability and low identification success. Syst Biol, 2006, 55: 715–728
45 Tamura K, Dudley J, Nei M, *et al.* MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol, 2007, 24: 1596–1599
46 Zhao P, Luo J, Zhuang W Y. Practice towards DNA barcoding of the nectriaceous fungi. Fungal Divers, 2011, 46: 183–191
47 Hebert P D, Cywinska A, Ball S L, *et al*. Biological identifications through DNA barcodes. Proc R Soc Lond, 2003, 270: 313–321

48 Farris J S, Kallersjo M, Kluge A G, *et al*. Testing significance of incongruence. Cladistics, 1995, 10: 315–319
49 Cunningham C W. Can three incongruence tests predict when data should be combined? Mol Biol Evol, 1997, 14: 733–740
50 Booth C. Studies of Pyrenomycetes. IV. *Nectria* (Part 1). Mycol Pap, 1959, 73: 1–117
51 Booth C. The genus *Cylindrocarpon*. Mycol Pap, 1966, 104: 1–56
52 Zhong Z, Pfister D H. Phylogenetic relationships among species of *Leotia* (Leotiales) based on ITS and RPB2 sequences. Mycol Res, 2004, 3: 237–246
53 Frøslev T G, Matheny P B, Hibbett D S. Lower level relationships in the mushroom genus *Cortinarius* (Basidiomycota, Agaricales): A comparison of RPB1, RPB2, and ITS phylogenies. Mol Phylogenet Evol, 2005, 37: 602–618
54 Hansen K, LoBuglio K F, Pfister D H. Evolutionary relationships of the cup-fungus genus *Peziza* and Pezizaceae inferred from multiple nuclear genes: RPB2, β-tubulin and LSU rDNA. Mol Phylogenet Evol, 2005, 36: 1–23
55 Lieckfeldt E, Seifert K A. An evaluation of the use of ITS sequences in the taxonomy of the Hypocreales. Stud Mycol, 2000, 45: 35–44
56 Varga J, Frisvad J C, Samson R A. Polyphasic taxonomy of *Aspergillus* section Candidi based on molecular, morphological and physiological data. Stud Mycol, 2007, 59: 75–88
57 Crouch J A, Clarke B B, Hillman B I. What is the value of ITS sequence data in Colletotrichum systematics and species diagnosis? A case study using the falcate-spored graminicolous *Colletotrichum* group. Mycologia, 2009, 101: 648–656
58 Hollingsworth P M, Forrest L L, Spouge J L, *et al*. A DNA barcode for land plants. Proc Natl Acad Sci USA, 2009, 106: 12794–12797
59 Hebert P D, Penton E H, Burns J M, *et al*. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proc Natl Acad Sci USA, 2004, 101: 14812–14817
60 Liu J, MÖller M, Gao L M, *et al*. DNA barcoding for the discrimination of Eurasian yews (*Taxus* L. Taxaceae) and the discovery of cryptic species. Mol Ecol Res, 2011, 11: 89–100