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Using DNA Barcoding to Identify the Genus *Lolium*

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Abstract

Seeds of the genus *Lolium* are difficult to identify based on morphology for morphological likeness and some physical deformation such as friction and flattening during storage and transport. DNA barcoding, a newly-established method, has been used to discriminate a variety of agricultural crops with its own advantages. In present study, DNA barcodes for the genus *Lolium* were investigated for the first time. DNA sequences of *psbA-trnH*, *rbcL*, *atpF-atpH*, and the *ITS2* region were evaluated for their ability to differentiate *Lolium* from the related genus *Festuca*. As confirmed by inter-intraspecific divergence and Kimura 2 parameter analysis, the greatest divergence existed in *ITS2*, followed by psbA-trnH. On the contrary, *rbcL* and *atpF-atpH* possessed poor genetic variation of 0-0.0115, and was relatively difficult in discrimination of genus *Lolium*. For *ITS2* sequence, no inter-intraspecific distance overlaps were observed and each species has a distinct barcoding gap. *ITS2* could effectively discriminate all species based on a neighbor-joining tree. Thus, the *ITS2* region is a candidate for DNA barcoding of *Lolium*.

Keywords: *atpF-atpH,* DNA barcodes, *Festuca*, *ITS2*, *Lolium*, *psbA-trnH*, *rbcl*

Introduction

Lolium, a genus of *Poaceae*, consists of six species and one variety: *Lolium perenne*, *L. persicum*, *L. remotum*, *L.multiflorum*, *L. rigidum*, *L. temulentum*, and *L. temulentum* var. *arvense* (Raven and Zhang, 2013). *Lolium perenne* and *L. multiflorum* are widely used as cool-season forage and have high economic value. However, *L. temulentum,* when infected by fungus, can produce the toxic alkaloid temulin, which can poison livestock and humans, occasionally resulting in death (Hurst, 1942). *Lolium temulentum* and its variety are morphologically similar to *L. perenne*, *L. multiflorum*, and *L. rigidum* and cannot be distinguished routinely. In addition, the genus *Festuca* is closely related to *Lolium*, and their gene structures indicate they shared a common ancestor about 2.8 million years ago (Charmet *et al.*, 1997; Torrecilla and Catalan, 2002).

Traditional taxonomy mainly relies on morphology, including seed shape, size, and other characteristics. But seed morphology is severely damaged during import, export, and transport processes. Accordingly, an accurate, sensitive, and simple alternative method is urgently needed to practically differentiate these species (Liu *et al.*, 2012; Hebert *et al.*, 2003).

The term "DNA barcode" for global species identification was first proposed by Hebert *et al.* (2003). Compared with the conventional morphology, DNA barcodes are not affected by the part of the plant tested and individual developmental stages. The key to identification is to find an appropriate DNA barcode. In recent years, *rbcL, atpF-atpH, psbA-trnH*, and *ITS2* have been commonly used as DNA barcodes for plants (CBOL Plant Working Group, 2009; Chen *et al.*, 2010).

The DNA barcodes *rbcL* and *atpF-atpH* are universal and easy to amplify in plants, and are widely used for phylogenetic reconstructions at the genus levels. The *psbA-trnH* region has several advantages, including easy amplification across a broad range of land plants, conserved coding regions that simplify design, and high variability owing to the presence of huge insertions or deletions (Bhargava and Sharma, 2013).The use of the *ITS2* region in phylogenetic studies has increased in recent years, so a large number of *ITS2* sequence data for *Poaceae* are available in GenBank. It's neighboring regions, 5.8S and 26S rDNA, are conserved and can be used for designing primers (Gao *et al.*, 2010; Selvaraj *et al.*, 2012; Kitthawee *et al.*, 2013).

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The objective of this study was to evaluate four potential DNA barcodes suggested by the CBOL Plant Working Group (2009) and other authors (Kress *et al.*, 2005; Lahaye *et al.*, 2008a) and applied them to *Lolium* and *Festuca* species for phylogenetic reconstructions.

Materials and Methods

Plant materials

Lolium, *Festuca*, and *Cynodon* samples were collected from America, Canada, and China in 2012 (Table 1). The samples from France were bought from B&T World Seeds. The species were identified at the Shanghai Entry-Exit Inspection and Quarantine Bureau, Shanghai, China (*L. multiflorum*, *L. perenne* and *F. arundinacea* were cultivar, other samples were spontaneous).

Table 1. Plant materials and sources

No	Species	Location	Latitude/longitude
WLM1	*L. multiflorum*	America, Oregon	N42.6/E-124.1
WLM2	*L. multiflorum*	America, Arizona	N34.1/E-110.7
WLM3	*L. multiflorum*	France	-
WLM4	*L. multiflorum*	France	-
WLM5	*L. multiflorum*	France	-
WLP1	*L. perenne*	Canada, Manitoba	N49.6/E-95.6
WLP2	*L. perenne*	Canada, Ontario	N49.7/E-94.3
WLP3	*L. perenne*	America, Oregon	N42.6/E-124.1
WLP4	*L. perenne*	America, Arizona	N34.1/E-110.7
WLP5	*L. perenne*	China, Guangdong	N23.6/E116.8
WLP6	*L. perenne wild*	France	-
WLR1	*L. rigidum*	France	-
WLT1	*L. temulentum* var. *arvense*	France	-
WLT2	*L. temulentum* var. *arvense*	China, Guangdong	N23.7/E116.8
WLT3	*L. temulentum*	China, Shanghai	N30.9/E121.8
WFA1	*F. arundinacea*	America, Oregon	N42.3/E-124.1
WFA2	*F. arundinacea*	America, Arizona	N34.1/E-110.6
WFA3	*F. arundinacea*	Canada, Manitoba	N49.6/E-95.6
WFA4	*F. arundinacea*	Canada, Ontario	N49.7/E-94.4
WFA5	*F. arundinacea*	France	-
WFA6	*F. arundinacea*	China, Jiangsu	N32.1/E120.4
WCD1	*Cynodon dactylon*	America Arizona	N34.1/E-110.7

Table 2. Primer sequences and annealing temperatures

DNA region	Primer	Annealing temperature	Sequence (5'-3')
rbcl	rbcl A-F rbcl A-R	55°C	ATGTCACCACAAACAGAGACTAAAGC GTYAAATCAAGTCCACCYCG
psbA-trnH	psbA-F trnH-R	60°C	GTTATGCATGAACGTAATGCTC CGCGCATGGTGGATTCACAATCC
ITS2	S1-F S2-R	55°C	ATGCGATACTTGGTGTGAAT GACGCTTCTCCAGACTACAAT
atpF-atpH	atp-F atp-H	55°C	ACTCGCACACACTCCCTTTCC GCTTTTATGGAAGCTTTAACAAT

DNA extraction, amplification, and sequencing

Total DNA was extracted from dried seeds using Plant Genomic DNA Kit (DP305-02, Tiangen Biotech, Beijing, China). PCR was performed in 25-μL reaction volumes containing approximately 30 ng of genomic DNA, 2 μL dNTP (2.5 mM), 1.5 mM $MgCl_2$, 1 μL of each primer, and 0.2 μL Taq DNA polymerase (5 U/μL; Tiangen). The PCR program was as follows: initial denaturation at 94 °C for 5 min; 35 cycles of 0.5 min at 94 °C, 0.5 min at the annealing temperature shown in Table 2, and 0.5 min at 72 °C; followed by 10 min at 72 °C, then 4 °C. The primer sequences were selected from CBOL (Table 2). PCR products were resolved by 1.5% agarose gel electrophoresis, purified, and subjected to sequencing (Sangon Biotech, Shanghai, China).

Sequence alignment and phylogenetic analysis

The DNA sequences were assembled and aligned using the programs ContigExpress (Invitrogen, Carlsbad, CA) and MEGA 5.0 (Tamura *et al.*, 2011). The Kimura 2-parameter (K2P) distances of *rbcL*, *trnH-psbA*, *atpF-atpH*, and *ITS2* were calculated using MEGA 5.0 to evaluate intra-specific and inter-specific divergences (Tamura *et al.*, 2011). Based on the K2P model, neighbor joining (NJ) trees were constructed using MEGA 5.0 with *Cynodon dactylon* as an outgroup. Node support for the NJ tree was inferred with bootstrap analysis (1000 replicates). Species were considered discriminated if all individuals of one species formed a monophyletic group (Hebert *et al.*, 2003). The DNA barcoding gap was calculated by TAXON DNA (Slabbinck *et al.*, 2008).

Results

PCR amplification and sequencing

PCR amplification was performed using four pairs of primers, and the results of gel electrophoresis are shown in Fig. 1. Amplifications were successful, and the same results were obtained every time for all primer pairs. Clear individual target bands of about 500 bp (*ITS2*) and 600 bp (*psbA-trnH, rbcL, atpF-atpH*) were consistent with the predicted results. All PCR products of *rbcL, atpF-atpH*, and *ITS2* and 93% of the PCR products of *psbA-trnH* could be sequenced successfully (Supplemental data 1-4).

Inter-intraspecific K2P distances for four individual DNA barcodes

The pairwise genetic distance values (K2P) calculated using MEGA 5.0 are listed in Table 4. The highest interspecies genetic distance (0.03) was between *L. perenne* and *F. arundinacea* for *psbA-trnH*, and the lowest genetic distance (0) was between *L. perenne* and *L. multiflorum* for *atpF-atpH*. The maximum intraspecific distance for *ITS2* was 0.0012 while the minimum intraspecific distance obtained for *atpF-atpH* was 0. The mean of the inter-specific divergences was significantly higher than those of intra-specific variations in *psbA-trnH* and *ITS2* (Table 3).

Wilcoxon signed rank tests were calculated using SPSS to analyze the interspecific variability of four DNA barcodes (Table 4). The results showed that *psbA-trnH* and *ITS2* had the highest inter-specific variability, followed by *rbcL* and *atpF-atpH*. The maximum interspecies distances of *ITS2* and *psbA-trnH* were greater than the maximum intraspecific distances, in agreement with the requirement for ideal DNA barcodes.

Barcoding gap assessment

Based on the distribution of inter-intraspecific K2P distances, the barcoding gap and accuracy of each DNA region was assessed (Fig. 2). Intra-interspecific distance overlaps existed in *rbcL* and *atpF-atpH*, and two distinct barcoding gap existed in each of *ITS2* and *psbA-trnH* between intraspecific and interspecific distances, corresponding to the pairwise genetic

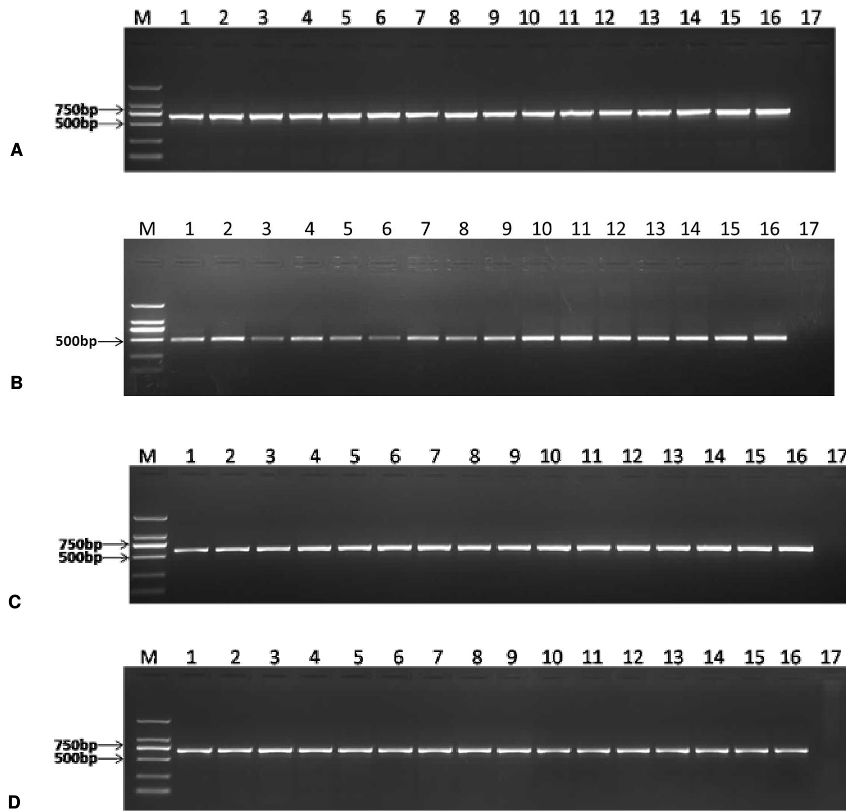

Fig. 1. PCR amplification of *ITS2*, *psbA-trnH*, *rbcL* and *atpF-atpH* regions

M: DM2000 DNA maker, 1-4: *Lolium perenne*; 5-6: *L. multiflorum*; 7-8: *L. temulentum* var. *arvense*; 9: *L. rigidum*; 10: *L. temulentum*; 11-16: *Festuca arundinacea*; 17: negative control A: *psbA-trnH; B: ITS2; C: rbcl; D: atpF-atpH*

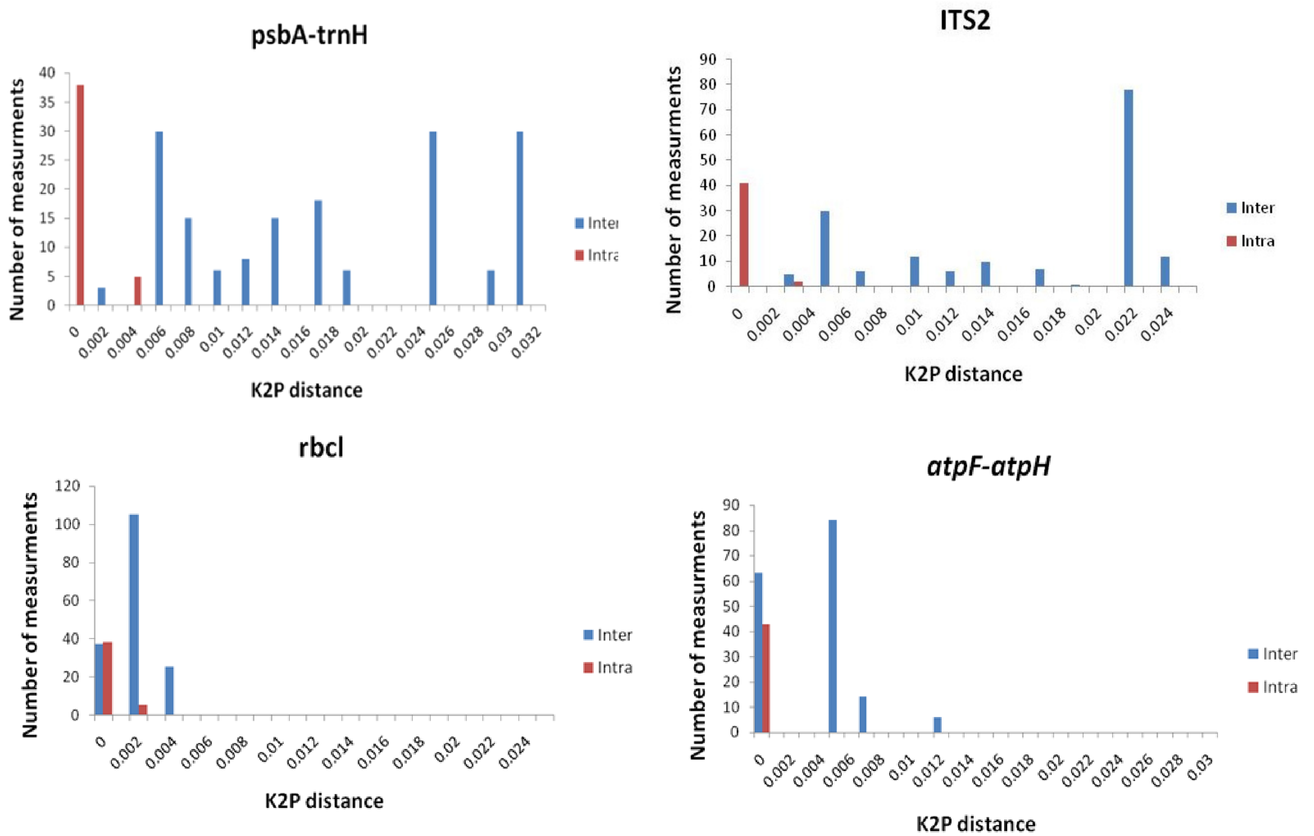

Fig. 2. Relative distributions of DNA barcoding gap between inter and intra-specific K2P distances

Table 3. The inter-intraspecific K2P distances for four individual DNA barcodes

Parameters	*psbA-trnH*	*rbcl*	*atpF-atpH*	*ITS2*
Number of aligned nucleotide sites	488	564	631	437
% amplification success	100	100	100	100
% sequencing success	90	100	100	100
% Variable nucleotide sites	16	2	7	14
Lolium multiflorum mean intraspecific distance	0	0	0	0
Lolium perenne mean intraspecific distance	0.0012	0.0006	0	0
Lolium rigidum mean intraspecific distance	n/c	n/c	n/c	n/c
Lolium temulentum mean intraspecific distance	0	0	0	0.0015
Festuca arundinacea mean intraspecific distance	0	0	0	0
Mean interspecific distance between *Lolium multiflorum* and *Lolium perenne*	0.0062	0.0033	0.0000	0.0046
Mean interspecific distance between *Lolium multiflorum* between *Lolium rigidum*	0.0055	0.0018	0.0066	0.0023
Mean interspecific distance between *Lolium multiflorum* between *Lolium temulentum*	0.0074	0.0018	0.0000	0.0148
Mean interspecific distance between *Lolium multiflorum* between *Festuca arundinacea*	0.0243	0.0018	0.0049	0.0211
Mean interspecific distance between *Lolium perenne* between *Lolium temulentum*	0.0127	0.0015	0.0000	0.0101
Mean interspecific distance between *Lolium perenne* between *Lolium rigidum*	0.0108	0.0015	0.0066	0.0070
Mean interspecific distance between *Lolium perenne* between *Festuca arundinacea*	0.0298	0.0015	0.0049	0.0211
Mean interspecific distance between *Lolium rigidum* between *Lolium temulentum*	0.0018	0.0000	0.0066	0.0171
Mean interspecific distance between *Lolium rigidum* between *Festuca arundinacea*	0.0187	0.0000	0.0115	0.0235
Mean interspecific distance between *Lolium temulentum* between *Festuca arundinacea*	0.0168	0.0000	0.0049	0.0219

Table 4. Wilcoxon signed rank test of the inter-specific divergences among the four loci

W+	W-		n	P value	Result
psbA-trnH	ITS2	W+=24.0 W-=31.0	n=10	P≤0.376	*psbA-trnH*=ITS2
psbA-trnH	*rbcl*	W+=55.0 W-=0	n=10	P≤0.001	*psbA-trnH*>*rbcl*
psbA-trnH	*atpF-atpH*	W+=51.0 W-=4.0	n=10	P≤0.002	*psbA-trnH*>*atpF-atpH*
ITS2	*rbcl*	W+=55.0 W-=0	n=10	P≤0.001	ITS2>*rbcl*
ITS2	*atpF-atpH*	W+=53.0 W-=2.0	n=10	P≤0.003	IT2>*atpF-atpH*
atpF-atpH	*rbcl*	W+=47.0 W-=8.0	n=10	P≤0.004	*atpF-atpH*>*rbcl*

distances.

Species identification based on phylogenetic tree

The Neighbor Joining trees (Fig. 3) were used to evaluate the ability of these DNA barcodes to identify species. All authenticate species clades were clearly monophyletic and distinct from other clades in the *ITS2* and *psbA-trnH* trees. However, *rbcL* had 47.6% resolving ability, and *atpF-atpH* could not identify any species in *Lolium*, because there was no genetic variation among individuals. However, the Neighbor Joining trees for *ITS2* and *psbA-trnH* did not correctly identify all of the relationships in *Lolium* and *Festuca*; for example, the *ITS2* and *psbA-trnH* trees identified different relationships for *L. temulentum* and *F. arundinacea.*

Discussion

DNA barcoding has been extensively used for species identification and for diversity and ecological studies. In this study, it has been examined four potential DNA barcodes suggested by the CBOL Plant Working Group (2009) and other authors (Kress *et al*., 2005; Lahaye *et al*., 2008a). DNA barcodes *rbcL* and *atpF-atpH* are universal and easy to amplify. Fazekas *et al*. (2008) and Newmaster *et al*. (2008) found that *rbcL* could not identify all species, but it generally performs quite well in differentiating genera. Sass *et al*. (2007) obtained similar results. Although *atpF-atpH* have been used successfully in combination with *matK* (Lahaye *et al*., 2008b), the present results revealed that *atpF-atpH* possessed less sequence variation and could not be used for species of *Lolium*. This finding was in accordance with a previous report by Newmaster *et al*. (2006), where *atpF-atpH* did not vary among closely-related species . Thus, these markers should be used only in combination with other barcodes. Several studies have shown *psbA-trnH* could be potential barcodes. Kress *et al*. (2007) used *psbA-trnH* successfully to discriminate algae, mosses, ferns, gymnosperms, and angiosperms. The current study showed that *psbA-trnH* was highly variable at the species level, but we encountered severe obstacle in sequencing (perhaps because of a long poly (A) domain), preventing it from being useful in experiment. Chen *et al*. (2010) used *ITS2* to discriminate more than 6600 plant samples from 480 species in 753 genera with a 92.7% success rate and Gao *et al*. (2010) obtained 96.2% identification success rates at the species level within *Fabaceae*. In the current study, comparing the five barcode markers in the discrimination of *Lolium*, *ITS2* was the best option.

Neighbor Joining data of *ITS2* indicated that three cross-pollinated species: *L. perenne*, *L.multiflorum*, *L. rigidum* and two self-pollinated species: *L. temulentum*, *L. temulentum* var. *arvense* grouped together respectively. These findings were in agreement with previous studies (Terrell, 1968; Catalan *et al*., 1997, 2004; Torrecilla *et al*., 2004). However, some unusual groupings have occurred in the NJ dendrogram of *psbA-trnH*. For example, *L. temulentum* (WLT1-WLT3) and *L. rigidum* (WLR1) was positioned outside all other *Lolium* samples, which formed a single clade with *F. arundinacea*. These unusual groupings could be explained by close relationship between two genuses (Charmet *et al*., 1997).

There are different opinions on relationship of *Lolium* and *Festuca*. Some researchers thought that cytological and morphological data do not support separate genera (Stebbins, 1956; Crowder, 1953; Terrell, 1966). Other authors have assumed that *Lolium* had a common ancestor with *Festuca* based on its genetic structure (Xu and Sleper, 1994; Torrecilla and Catalan, 2002; Inda *et al*., 2008). As the present results show, *Festuca* have shared high homoplasy with *Lolium* in the *psbA-trnH* region caused by rapid molecular evolution at the loci studied. In the recent studies, Inda (2013) performed a Fluorescent *in situ* hybridization analysis of genus *Lolium* based on ribosomal RNA genes, and found that some species could be intermediate between *Festuca* and *Lolium*. The current results offered an added data for the taxonomy phylogenetics.

The goals of DNA barcoding have been discussed for a long

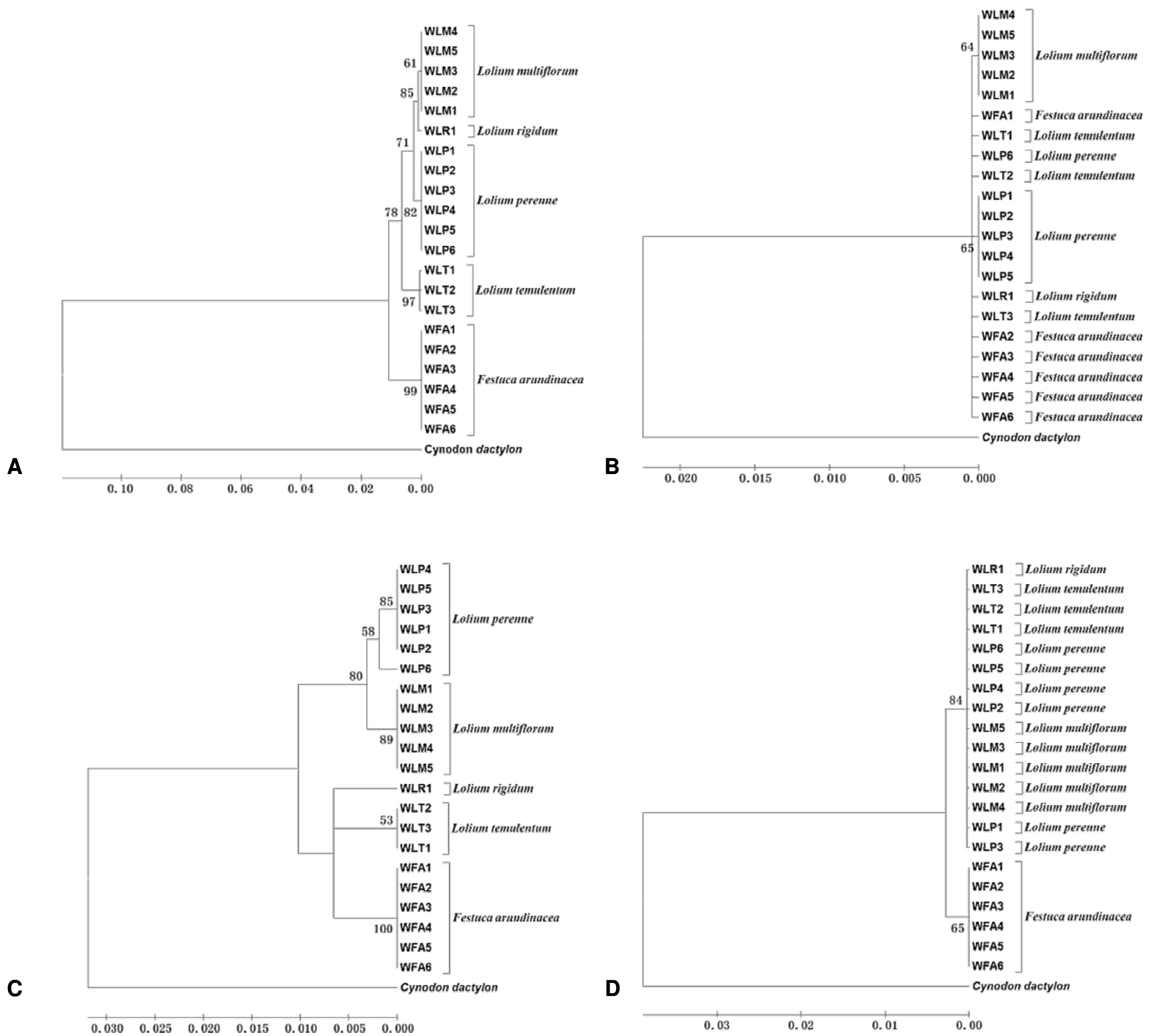

Fig. 3. The Neighbor Joining trees of four DNA barcoding regions. A: *ITS2; B: rbcl; C: psbA-trnH; D: atpF-atpH*

time; species "discovery" and "identification" are two important and controversial aspects (Desalle, 2006; Hollingsworth, 2011; Zhang *et al.*, 2014; Diana *et al.*, 2012). This method has been extensively applied in animals, but no available DNA barcodes have thus far been able to perfectly identify species in plants (Hollingsworth *et al.*, 2011; China Plant BOL Group, 2011). Although DNA barcodes cannot yet replace traditional taxonomic techniques, its accuracy, richness, and reproducibility will make this digital technology a useful complement for taxonomists and facilitate work on entry-and-exit inspections and quarantine.

Conclusions

All tested species of *Lolium* and *Festuca* were well differentiated and monophyletic using the *ITS2* region. The data from NJ trees provided a new understanding for the origin of two genuses. *ITS2* is a reliable DNA barcode for distinguishing genus *Lolium.*

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