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 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 DNA extraction, amplification, and sequencing

| 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 | China, Guangdong | N23.6/E-116.8 |
| 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/E-116.8 |
| WLP6 | L. perenne | France | - |
| WLR1 | L. rigidum | France | - |
| WLT1 | L. temulentum var. arvense | France | - |
| WLT2 | L. temulentum var. arvense | China, Guangdong | N23.7/E-116.8 |
| WFA1 | F. arundinacea | China, Shanghai | N30.9/E-121.8 |
| WFA2 | F. arundinacea | America, Oregon | N42.3/E-124.1 |
| WFA3 | F. arundinacea | America, Arizona | N41.4/E-110.6 |
| WFA4 | F. arundinacea | Canada, Manitoba | N49.6/E-95.6 |
| WFA5 | F. arundinacea | Canada, Ontario | N49.7/E-94.4 |
| WFA6 | F. arundinacea | China, Jiangsu | N32.1/E-120.4 |
| WCD1 | *Cynodon dactylon* | America, Arizona | N34.1/E-110.7 |

sequences of the PCR 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 ContingExpress (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. Mean of the inter-specific divergences was significantly higher than that of intra-specific variations in *psbA-trnH* and *ITS2* (Table 3).

**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-specific 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: L. 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 interspecific distance 0 0 0 0
Lolium perenne mean interspecific distance 0.0012 0.0006 0.00 0
Lolium rigidum mean interspecific distance n/c n/c n/c n/c
Lolium temulentum mean interspecific distance 0 0 0 0.0015
Festuca arundinacea mean interspecific 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 0.0095 0.0018 0.0066 0.0023
Mean interspecific distance between Lolium multiflorum between Lolium temulentum 0.0074 0.0018 0.0000 0.0048
Mean interspecific distance between Lolium multiflorum between Festuca arundinacea 0.0243 0.0018 0.0049 0.0111
Mean interspecific distance between Lolium perenne between Lolium temulentum 0.0027 0.0015 0.0000 0.0101
Mean interspecific distance between Lolium perenne between Festuca arundinacea 0.0108 0.0015 0.0066 0.0070
Mean interspecific distance between Lolium perenne between Festuca arundinacea 0.0138 0.0000 0.0000 0.0171
Mean interspecific distance between Lolium rigidum between Lolium temulentum 0.0018 0.0000 0.0066 0.0017
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 0.0009 0.0219

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

|    |    | n | P value | Result |
|----|----|---|---------|--------|
| psbA-trnH | ITS2 | 10 | 0.376   | psbA-trnH>ITS2 |
| psbA-trnH | rbcl | 10 | 0.001   | psbA-trnH>rbcl |
| psbA-trnH | atpF-atpH | 10 | 0.002 | psbA-trnH>atpF-atpH |
| ITS2 | rbcl | 10 | 0.001 | ITS2>rbcl |
| ITS2 | atpF-atpH | 10 | 0.003 | ITS2>atpF-atpH |
| atpF-atpH | rbcl | 10 | 0.004 | atpF-atpH>rbcl |

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 authenticated 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
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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