Identification of EeSt-genome species in 
Pseudoroegneria and Elytrigia (Poaceae: 
Triticeae) by using SCAR markers from ITS 
sequences

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ABSTRACT. To detect EeSt-genome species in 
Pseudoroegneria and Elytrigia, the primers ES45 (5'-GTAGCGACGTTTTCA-3') and 
ES261 (5'-TCGCTACGTTCTTCATC-3') were designed as sequence 
characterized amplified region markers based on the 6-base pair indel in 
internal transcribed spacer 1 (ITS1) regions and conserved sites in the 
5.8S regions, respectively. Polymerase chain reaction of ITS fragments 
in 27 Triticeae accessions was used for amplification with a touchdown 
thermocycling profile. Two amplicons were purified, sequenced, and 
aligned. The results indicated that: 1) primers ES45 and ES261 generated 
the expected products, 2) ITS sequences of EeSt-genome species are 
characterized by a 6-base pair indel, and 3) 13 taxa in Pseudoroegneria and 
Elytrigia should be included in Trichopyrum. The primers ES45 and ES261 
were useful for detecting ITS fragments with 6-bp indel and are helpful for 
clarifying taxonomic classifications of EeSt-genome species in Triticeae.

Key words: Indel; Lophopyrum; Perennial genera; Polyploid
INTRODUCTION

_Pseudoroegneria_, _Elytrigia_, and _Lophopyrum_ are 3 perennial genera in tribe Triticeae (Löve, 1984). Löve (1984) and Dewey (1984) suggested that the taxonomic classification of Triticeae species should be based on genomic constitutions. This view has been widely accepted (Hsiao et al., 1986; Wang et al., 1986; Jensen et al., 1992; Liu and Wang, 1993a,b; Lu, 1994; Zhou et al., 1999; Yen et al., 2005a; Yu et al., 2009). Species in the 3 genera contain the basic genome symbols designated as St, EsSt, and E (Ee and Eb), respectively (Löve, 1984; Dewey, 1984; Wang et al., 1994).

Morphologically, _Pseudoroegneria_ species are cespitose, long-anthered, cross-pollinating perennials; similar morphological characteristics are observed in _Elytrigia_ (Löve, 1980; Dewey, 1984). Cytological data indicate that EsSt-genome species are grouped in _Pseudoroegneria_ and _Elytrigia_ (Dewey, 1962; Dvořák, 1981; Löve, 1984, 1986; Liu and Wang, 1993a,b). Cytologically, genome analysis is powerful for determining the genome constitutions and origin of polyploid taxa in tribe Triticeae (Löve, 1984; Dewey, 1984). However, it is necessary to obtain artificial hybrids and their robust flowering plants. Moreover, multivalents in intergeneric hybrids suggest that chromosome pairing at high ploidy levels make interpreting genomic constitutions difficult (Liu and Wang, 1993b). Therefore, detecting EsSt genomes in polyploid species using genome analysis or based on morphology similarity is inadequate, and the taxonomic treatment of EsSt-genome species remains disordered. Previous internal transcribed spacer (ITS) analysis showed that a 6-base pair (bp) indel (TTTTCA) exits in EsSt-genome species, but not in species with St or E (Ee and Eb) genomes (Li et al., 2004; Yu et al., 2008). This provides insight into EsSt-genome diagnosis using sequence characterized amplified region (SCAR) markers on a molecular level.

Recently, reliable SCAR markers have been used in molecular analysis (Dai et al., 2005; Paxton et al., 2005; Rahman et al., 2007; Liao et al., 2009; Bandyopadhyay and Raychaudhuri, 2010; Duan et al., 2011; Lee et al., 2011; Yu et al., 2011a,b). In the current study, SCAR primers were designed based on ITS characterization to amplify ITS fragments of EsSt-genome species. The products amplified by polymerase chain reaction (PCR) were cloned and sequenced randomly. The objectives of this study were to develop SCAR primers for amplification of ITS fragments in the EsSt-genome, detect EsSt-genome species on a molecular level, and determine general taxonomic classification for these species.

MATERIAL AND METHODS

The study was conducted from June 2010-March 2013 at Luzhou city, Research Center for Preclinical Medicine of Luzhou Medical College.

Plant materials

A total of 27 Triticeae accessions were examined in this study, including 12 _Pseudoroegneria_ accessions with different genomic constitutions (i.e. St and EsSt genomes), 12 accessions of _Elytrigia_ with different genomic constitutions (i.e. EsSt, EeEsSt and EeEeEsStSt genomes), and 3 species of _Lophopyrum_ (Ee, Eb, and EeEeEb genomes). All seed materials were provided by the American National Plant Germplasm System (Pullman, WA, USA).
Identification of E'eSt-genome species

The names, accession numbers, genomic constitutions, geographic origins, and GenBank accession numbers are listed in Table 1. The nomenclature and genome symbols for most species used in this study were as described by Löve (1984), Wang et al. (1994), and Yen et al. (2005b). The voucher specimens were deposited at Luzhou Medical College.

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Accession No.</th>
<th>2n</th>
<th>Genome</th>
<th>Geographic origin</th>
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</table>

Table 1. Species and accessions used in this study.

*GenBank accession No. was deposited previously from the GenBank (http://www.ncbi.nlm.nih.gov); Elytrigia hybrid is lacking an acceptable binomial.

DNA extraction and purification

Seeds were germinated and grown in a growth chamber in the dark at 22°C. Leaf samples collected from each accession at the seedling stage were ground in liquid nitrogen in a 1.5-mL microfuge tube. DNA was extracted and purified using the cetyltrimethylammonium bromide procedure described by Doyle and Doyle (1990).

Primer design

SCAR primers were searched and designed to amplify specific ITS sites using DNAMAN (Lynnon Biosoft, version 5.2.9 Demo). ES45 (5'-GTAGGCGACGGTTTTCA-3') at site 45 was designated as the forward primer. A site mutation (C to A) was introduced at the third base from the 5' end to avoid self-complementarity. The reverse primer

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ES261 (5'-TCGCTACGTTCTTCATC-3') was designed to amplify the 5.8S conserved region at position 261 (Figure 1). The expected amplicon was 233 bp in size.

Figure 1. Primers ES45 and ES261 in ITS sequences. The boxed regions and numbers above show primer sequences and positions in ITS1 and 5.8S regions, respectively. Numbers after species refer to the accession numbers shown in Table 1. The dotted lines represent the incomplete sequences in 5.8S regions.

**PCR amplification, cloning, sequencing, and alignment**

Amplification of ITS fragments in 27 Triticeae accessions was conducted using SCAR primers. The PCR was carried out in a total volume of 25 μL containing 1X reaction buffer, 1.5 mM MgCl₂, 0.5 μM of each primer, 200 μM of each dNTP [TaKaRa Biotechnology (Dalian) Co., Ltd., Shiga, Japan], 0.5 U ExTaq Polymerase (TaKaRa), and sterile water to the final volume. The touchdown thermocycling profile consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, annealing temperatures starting at 60°C for 40 s (decreasing by 0.3°C/cycle), and 72°C for 1 min for extension. This step was followed by 15 cycles at 94°C for 30 s, 51°C for 40 s, 72°C for 1 min, and finally 72°C for 6 min. PCRs for each accession were carried out in a Mastercycler 5331 (Eppendorf, Hamburg, Germany). Amplification products were purified using the Gel Extraction Kit (50) (Omega Bio-Tek, Norcross, GA, USA) and ligated into a pMD18-T Easy Vector according to the manufacturer instruction (TaKaRa). Positive clones for each species were randomly selected and sequenced by Sunbiotech Co., Ltd. (Beijing, China). The amplified fragments were analyzed by alignment with the ITS sequence of *Pseudoroegneria libanotica* (Hackel) D.R. Dewey (GenBank accession No. AY740794) (Liu et al., 2006). Sequence alignment was executed using DNA-MAN (Lynnon Biosoft, version 5.2.9 Demo).

**RESULTS**

Primers ES45 and ES261 were effective for amplification of the target sequences (Figure 2). Amplification produced expected fragments in 13 taxa, including *Pseudoroegneria geniculata* ssp prunifera (Nevski) Á. Löve, *Pseudoroegneria geniculata* ssp scythica
Identification of E\textit{St}-genome species


\textbf{Figure 2.} PCR results of species used in the analysis with primer ES45 and ES261. Numbers refer to the accession numbers shown in Table 1. \textit{Lane M} = 500-bp DNA ladder. \textit{Lane 0} = the blank control. Arrow indicates the size of fragments.


Amplicons generated from \textit{E. caespitosa} and \textit{E. pontica} were cloned, sequenced, and aligned with ITS fragments of \textit{P. libanotica} (AY740794). These sequences showed 97.14\% identity with 6-bp indel and complete primers in \textit{E. caespitosa} and \textit{E. pontica} (Figure 3).

\textbf{Figure 3.} Sequence alignment of \textit{Elytrigia caespitosa}, \textit{E. pontica}, and \textit{Pseudoroegneria libanotica}. Numbers after species refer to the accession numbers shown in Table 1. Numbers on the right refer to sequence lengths. The boxed regions show bases of primers ES45 and ES261, respectively. Bar in the upper left indicates sequence identity.
DISCUSSION

Nuclear rDNA ITSs are present in multiple copies in Triticeae species, which is significant at high taxonomic levels for inferring phylogenetic relationships and ancestors of polyploid species with a fixed variation rate (Hsiao et al., 1995). Based on chloroplast and nuclear DNA data, Mason-Gamer (2004) found a complex pattern of reticulate evolution, introgression, and intertribal gene capture in E. repens. Yu et al. (2008) found that the 6-bp indel in the ITS1 regions of EeSt-genome species could be clearly distinguished as species with the St or Ee genomes. Arterburn et al. (2011) indicated that K-genome species of Crithopsis were genome donors of ITS fragments with the 6-bp indel in E. intermedia. These data suggest that there are reticulate and complicated phylogenetic relationships among polyploid species in Pseudoroegneria and Elytrigia.

Cytologically, P. geniculata ssp scythica, P. geniculata ssp prunifera, E. caespitosa, E. caespitosa ssp nodosa, E. intermedia, E. intermedia ssp intermedia, E. pontica, E. pungens, E. pycnantha, and E. scirpea possess EeSt-genome species could be clearly distinguished as species with the St or Ee genomes. Arterburn et al. (2011) indicated that K-genome species of Crithopsis were genome donors of ITS fragments with the 6-bp indel in E. intermedia. These data suggest that there are reticulate and complicated phylogenetic relationships among polyploid species in Pseudoroegneria and Elytrigia.

CONCLUSIONS

In this study, we developed SCAR markers to detect EeSt-genome species in Pseudoroegneria and Elytrigia based on 6-bp indel sequences. Among all 27 accessions, 10 taxa were found to contain EeSt genomes, which were identical to previously reported cytological data. Three species, E. hybrid, E. lolioides, and E. varnensis, were found to have EeSt genomes because of their similar amplicon sizes. Thus, primers ES45 and ES261 are useful for detecting the 6-bp indel of ITS fragments, and are helpful for guiding taxonomic treatment of polyploid Triticeae species with EeSt genomes.

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Identification of E'st-genome species

REFERENCES


