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Identification of E^{e}St-genome species in *Pseudoroegneria* and *Elytrigia* (Poaceae: Triticeae) by using SCAR markers from ITS sequences

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ABSTRACT. To detect E^{e}St-genome species in *Pseudoroegneria* and *Elytrigia*, the primers ES45 (5'-GTAGGCGACGGTTTTCA-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 E^{e}St-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 E^{e}St-genome species in Triticeae.

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

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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, E^eSt, and E (E^e and E^b), 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 E^eSt-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 E^eSt genomes in polyploid species using genome analysis or based on morphology similarity is inadequate, and the taxonomic treatment of E^eSt-genome species remains disordered. Previous internal transcribed spacer (ITS) analysis showed that a 6-base pair (bp) indel (TTTTCA) exits in E^eSt-genome species, but not in species with St or E (E^e and E^b) genomes (Li et al., 2004; Yu et al., 2008). This provides insight into E^eSt-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 E^eSt-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 E^eSt-genome, detect E^eSt-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 *Pseudorogeneria* accessions with different genomic constitutions (i.e. St and E^eSt genomes), 12 accessions of *Elytrigia* with different genomic constitutions (i.e. E^eSt, E^eE^bSt and $E^eE^bE^xStSt$ genomes), and 3 species of *Lophopyrum* (E^e, E^b, and $E^eE^bE^b$ genomes). All seed materials were provided by the American National Plant Germplasm System (Pullman, WA, USA).

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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 1. Species and accessions used in this study.

No.	Species	Accession. No.	2n	Genome	Geographic origin	GenBank accession No.
***Pseudoroegneria* (Nevski) Á. Löve**						
1	*P. cognata* (Hack.) Á. Löve	PI 531720	14	St	Estonia, Russian Federation	EF014226
2	*P. geniculata* (Trin.) Á. Löve	PI 565009	28	StSt	Russian Federation	EF014228
3	*P. geniculata* ssp *prunifera* (Nevski) Á. Löve	PI 547374	42	E^eESt	Ural, Russian Federation	EF014230
4	*P. geniculata* ssp *scythica* (Nevski) Á. Löve	PI 502271	28	E^eSt	Russian Federation	EF014232
5	*P. gracillima* (Nevski) Á. Löve	PI 440000	14	St	Stavropol, Russian Federation	EF014233
6	*P. kosaninii* (Nabelek) Á. Löve	PI 237636	56	St-	Turkey	EF014235
7	*P. libanotica* (Hackel) D.R. Dewey	PI 228389	14	St	Iran	AY740794
8	*P. spicata* (Pursh) Á. Löve	PI 547161	14	St	Oregon, United States	AY740793
9	*P. stipifolia* (Czern. ex Nevski) Á. Löve	PI 325181	14	St	Stavropol, Russian Federation	EF014240
10	*P. strigosa* (M. Bieb.) Á. Löve	PI 499637	14	St	Urumqi, Xinjiang, China	AY740795
11	*P. strigosa* ssp *aegilopoides* (Drobow) Á. Löve	PI 595164	14	St	Xinjiang, China	EF014243
12	*P. tauri* (Boiss. & Balansa) Á. Löve	PI 401323	14	St	Iran	EF014244
***Elytrigia* Desvaux**						
13	*E. caespitosa* (C. Koch) Nevski	PI 547311	28	E^eSt	Russian Federation	EF014246
14	*E. caespitosa* ssp *nodosa* (Nevski) Tzvelev	PI 547345	28	E^eSt	Ukraine	EF014248
15	*E. hybrid*	PI 276708	56	-	Leningrad, Russian Federation	
16	*E. intermedia* (Host) Nevski	PI 619581	42	E^eE^bSt	Russian Federation	EU883127
17	*E. intermedia* ssp *intermedia* (Host) Nevski	PI 469214	42	E^eE^bSt	Maryland, USA	AF507809
18	*E. lolioides* (Kar. & Kir.) Nevski	PI 440059	42	-	Russian Federation	
19	*E. pontica* (Podp.) Holub	PI 547313	70	$E^eE^bE^xStSt$	Leningrad, Russian Federation	AY090768
20	*E. pungens* (Pers.) Tutin	PI 531740	56	E^eStStP	France	
21	*E. pycnantha* (Godron.) Á. Löve	PI 531744	42	E^eStP	Netherlands	
22	*E. repens* (L.) Nevski	PI 634252	42	St-	Krym, Ukraine	DQ859051
23	*E. scirpea* (C. Presl) Holub	PI 531750	28	E^eSt	Greece	
24	*E. varnensis* (Velen.) Holub	PI 281863	84	-	Germany	
***Lophopyrum* Á. Löve**						
25	*L. bessarabicum* (Savul & Rayss) C. Yen, J.L. Yang & Y. Yen	PI 531712	14	E^b	Estonia, Russian Federation	L36506
26	*L. elongatum* (Host) Á. Löve	PI 547326	14	E^e	France	L36495
27	*L. junceum* (L.) C. Yen, J.L. Yang & Y. Yen	PI 414667	42	$E^eE^bE^b$	Greece	EU883124

*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

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.

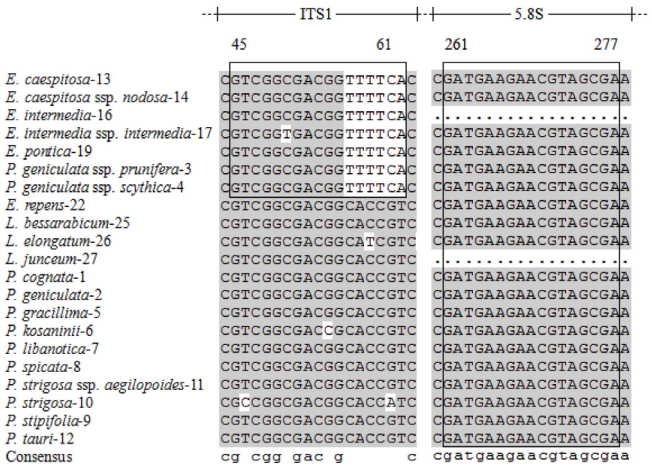

Figure 1. Primers ES45 and ES261 in ITS sequences. The boxed regions and numbers above show primer sequences and positions in ITS1 and 5.8S regions, respectively. Numbers after species refer to the accession numbers shown in Table 1. The dotted lines represent the incomplete sequences in 5.8S regions.

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 $MgCl_2$, 0.5 μM of each primer, 200 μM of each dNTP [TaKaRa Biotechnology (Dalian) Co., Ltd., Shiga, Japan], 0.5 U Ex*Taq* 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 DNAMAN (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*

(Nevski) Á. Löve, *Elytrigia caespitosa* (C. Koch) Nevski, *Elytrigia caespitosa* ssp *nodosa* (Nevski) Tzvelev, *Elytrigia hybrid* (no acceptable binomial), *Elytrigia intermedia* (Host) Nevski, *Elytrigia intermedia* ssp *intermedia* (Host) Nevski, *Elytrigia lolioides* (Kar. & Kir.) Nevski, *Elytrigia pontica* (Podp.) Holub, *Elytrigia pungens* (Pers.) Tutin, *Elytrigia pycnantha* (Godron.) Á. Löve, *Elytrigia scirpea* (C. Presl) Holub, and *Elytrigia varnensis* (Velen.) Holub. (Figure 2).

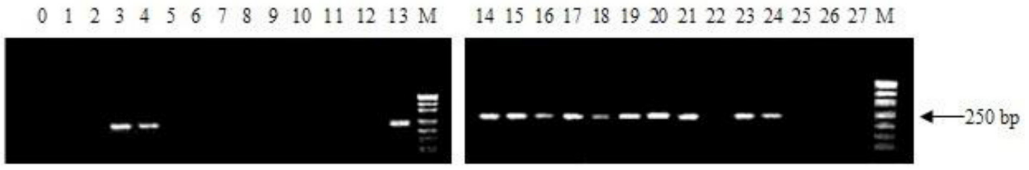

Figure 2. PCR results of species used in the analysis with primer ES45 and ES261. Numbers refer to the accession numbers shown in Table 1. *Lane M* = 500-bp DNA ladder. *Lane 0* = the blank control. Arrow indicates the size of fragments.

Fourteen taxa showed no amplification products, including *Pseudoroegneria cognata* (Hack.) Á. Löve, *Pseudoroegneria geniculata* (Trin.) Á. Löve, *Pseudoroegneria gracillima* (Nevski) Á. Löve, *Pseudoroegneria kosaninii* (Nabelek) Á. Löve, *P. libanotica*, *Pseudoroegneria spicata* (Pursh) Á. Löve, *Pseudoroegneria stipifolia* (Czern. ex Nevski) Á. Löve, *Pseudoroegneria strigosa* (M. Bieb.) Á. Löve, *Pseudoroegneria strigosa* ssp *aegilopoides* (Drobow) Á. Löve, *Pseudoroegneria tauri* (Boiss. & Balansa) Á. Löve, *Elytrigia repens* (L.) Nevski, *Lophopyrum bessarabicum* (Savul & Rayss) C. Yen, J.L. Yang & Y. Yen, *Lophopyrum elongatum* (Host) Á. Löve, and *Lophopyrum junceum* (L.) C. Yen, J.L. Yang & Y. Yen.

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

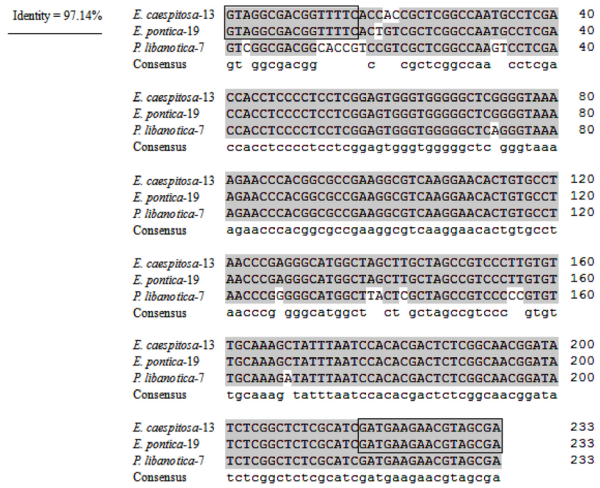

Figure 3. Sequence alignment of *Elytrigia caespitosa*, *E. pontica*, and *Pseudoroegneria libanotica*. Numbers after species refer to the accession numbers shown in Table 1. Numbers on the right refer to sequence lengths. The boxed regions show bases of primers ES45 and ES261, respectively. Bar in the 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 E^eSt-genome species could be clearly distinguished as species with the St or E^e 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 E^eSt genomes (Liu and Wang, 1993a,b; Xu and Conner, 1994; Zhang et al., 1996; Refoufi et al., 2001; Ellneskog-Staam et al., 2003; Yu et al., 2010). SCAR primers generated right fragments in these E^eSt-genome taxa, but not in St-genome and E-genome species. The 6-bp indel was detected in amplicons from *E. caespitosa* and *E. pontica*. This suggests that E^eSt-genome species are characterized by the 6-bp indel. Since the amplicons were identical in size, the *E. hybrid*, *E. lolioides*, and *E. varnensis* were considered to contain the E^eSt genomes.

The 6-bp indel in ITS fragments of *P. geniculata* ssp *scythica* and *P. geniculata* ssp *prunifera* suggest that it is unreasonable to treat the 2 taxa as subspecies of *P. geniculata*, which contains StSt genomes (Dewey, 1984). Based on genomic *in situ* hybridization analysis, *E. repens* are hexaploid with 1 set of St-genome, while the other 2 sets of genomes remain unknown (Orgaard and Anamthawat-Jónsson, 2001). The 6-bp indel was not detected in the reported ITSs of *E. repens* (Mahelka et al., 2007), and there were no amplicons in this study. This demonstrates that *E. repens* contains no E^eSt genomes. Based on Löve's conspectus, the E^eSt-genome species should be grouped in a new genus designated as *Trichopyrum* (Yen et al., 2005b).

CONCLUSIONS

In this study, we developed SCAR markers to detect E^eSt-genome species in *Pseudoroegneria* and *Elytrigia* based on 6-bp indel sequences. Among all 27 accessions, 10 taxa were found to contain E^eSt genomes, which were identical to previously reported cytological data. Three species, *E. hybrid*, *E. lolioides*, and *E. varnensis*, were found to have E^eSt 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 E^eSt genomes.

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