A Novel Nested Polymerase Chain Reaction (n-PCR) Assay for Identifying *Sorghum nitidum*

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Abstract

This work developed a novel nested polymerase chain reaction (n-PCR) assay to identify *Sorghum nitidum* (*S. nitidum*). It has been designed a set of specific n-PCR inner primers Snit5/Snit2 and outer primers Nout1/Nout2 based on a conserved nucleotide sequence of *adh1-like* gene of *S. nitidum*. Fourteen samples of sorghum were used to investigate the specificity of the primers and the n-PCR assay. The result showed that 9 samples of *S. nitidum* displayed a positive strong, specific amplified band at ~873 bp in gel spectra, while other relatives, including *Sorghum halepense*, *Sorghum almum*, *Sorghum bicolor*, *Sorghum propinum* and *Sorghum sudanse* exhibited negative amplifications. This assay was able to specifically identify *S. nitidum* fast and effectively, which could be applied widely in field inspection, agriculture production and plant protection.

Keywords: *Sorghum nitidum*, nested polymerase chain reaction (n-PCR), molecular detection

Introduction

Sorghum nitidum is a related species of the hard weed *Sorghum halepense* (Guo *et al.*, 1996), which originated from west of India and then spread widely into southeast Asia, Indonesia and Australia. *Sorghum* Moench could be classified into five groups, namely *Stiposorghum, Parasorghum, Eusorghum, Heterosorghum* and *Chaetosorghum* (Celarier, 1959; DeWet, 1978), while *S. nitidum* was recognized to be one of the most widely spreading species in the group of *Parasorghum* (Snowden, 1955). Like *S. halepense* and *S. almum*, *S. nitidum* is a perennial weed and the chromosome is as big as *S. halepense* and *S. bicolor*. Sun *et al.* (1994) believed that the *S. nitidum* should be cataloged into the *Eusorghum* group, while DeWet *et al.* (1978) thought *S. nitidum* should be classified into the *Parasorghum* group. The correct relationship of *S. nitidum* should be further studied. It is very difficult to distinguish the seed of different sorghum species morphologically from each other (Guo *et al.*, 1996). Because seeds of *S. nitidum* among farm products may be deformed and abraded during storage, loading and unloading, in addition, the morphology of the sorghum seed usually impacted by the environment, climate, condition of maturation. The method of cellular diagnostics would also be very tedious and time - consuming, which would not be very suitable for field detection (Harlan and De Wet, 1974).

ADH (Ethanol Dehydrogenase, EC 1.1.1.1), coded by the gene *adh*, which was widely distributed over various organs and tissues throughout the plant, catalyzes the conversion between the ethanol and acetaldehyde. Sivaraman *et al.* (1984) was the first to study the DNA polymorphism of the *adh* gene of *Arabidopsis*. The polymorphism of the *adh* gene could help discover the genetic relationship between different species and provide biological dates for identifying some specific species.

Nested PCR (n-PCR) originated from the typical PCR technique, which has been widely used in basic biological researches and clinical diagnostics (Khan *et al.*, 2004; Mayer and Palmer, 1996; Tanzer, 2000). The basic principle of n-PCR based on two pairs of specific primers designed to amplify one gene fragment by two round thermo-cycling. N-PCR technique could decrease the pseudo-positive amplification and improve the sensitivity of the assay. In this work, we designed a set of specific n-PCR primers based on a conserved nucleotide gene fragment of *adh1-like* gene of *S. nitidum* and developed a fast assay to identify the *S. nitidum* with high specificity and effectivity.

Materials and methods

Materials

Fourteen sorghum samples were obtained from Shanghai Entry-Exit Inspection and Quarantine Bereau (China) among which 9 samples were *S. nitidum*, others were *S. halepense*, *S. almum*, *S. bicolor*, *S. propinum* and *S. sudanse*, respectively (Tab. 1).

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Tab. 1 Samples of sorghum used in this work

Number	The latin name	Origin	Original number
Snit1	*S. nitidum*	Austrlia	AusTRCF302539
Snit2	*S. nitidum*	Austrlia	AusTRCF302540
Snit3	*S. nitidum*	Austrlia	AusTRCF302542
Snit4	*S. nitidum*	Austrlia	AusTRCF302543
Snit5	*S. nitidum*	Austrlia	AusTRCF302558
Snit6	*S. nitidum*	Austrlia	AusTRCF302559
Snit7	*S. nitidum*	Austrlia	AusTRCF316841
Snit8	*S. nitidum*	Austrlia	AusTRCF316922
Snit9	*S. nitidum*	Austrlia	AusTRCF316930
Shal1	*S. halepense*	America	-
Salm1	*S. almum*	Argentina	-
Sbic1	*S. bicolor*	Unknown	-
Spro1	*S. propinum*	China	-
Ssud1	*S. sudanse*	China	-

Tab. 2. The Genbank number of the sorghum for analyzing the conserved nucleotide sequence of the *S. nitidum*

Genbank Number	Latin Name	Genbank Number	Latin Name
DQ096188.1	*S. nitidum*	DQ096172.1	*S. macrospermum*
DQ096165.1	*S. bicolor*	DQ096174.1	*S. exstans*
AF050456.1	*S. bicolor*	DQ096175.1	*S. grande*
AF124045.1	*S. bicolor*	DQ096176.1	*S. plumosum*
DQ096167.1	*S. x almum*	DQ096177.1	*S. bulbosum*
FJ650402.1	*S. x almum*	DQ096178.1	*S. stipoideum*
FJ650403.1	*S. x almum*	DQ096179.1	*S. amplum*
FJ650405.1	*S. x almum*	DQ096180.1	*S. intrans*
FJ650407.1	*S. x almum*	DQ096181.1	*S. ecarinatum*
DQ096168.1	*S. halepense*	DQ096182.1	*S. angustum*
FJ650404.1	*S. halepense*	DQ096183.1	*S. matarankense*
FJ650406.1	*S. halepense*	DQ096184.1	*S. interjectum*
DQ096169.1	*S. x drummondii*	DQ096185.1	*S. timorense*
FJ650408.1	*S. x drummondii*	DQ096186.1	*S. brachypodum*
DQ096166.1	*S. arundinaceum*	DQ096187.1	*S. leiocladum*
DQ096170.1	*S. propinquum*	DQ096189.1	*S. purpureosericeum*
FJ650401.1	*S. sp. 'Silk'*	DQ096190.1	*S. versicolor*
DQ096171.1	*S. laxiflorum*		

Tab. 3. Outer primers of n-PCR for identification of the *S. nitidum*

Primer	Nucletide sequence
Nout1	5' CCGTGATTTCTCCTCTG 3'
Nout4	5' CGGGAGCTACATCAGTC 3'

Tab. 4. Typical PCR primers for identification of the *S. nitidum*

Primer	Nucletide sequence
Snit5	5' TTGCTATAGAACTTTCTAGC 3'
Snit2	5' ACAGCCCTAGTTGTACTG 3'

Extraction of genomic DNA form a seed of sorghum

Total Genomic DNA was extracted from a single seed of sorghum using an improved method developed in our laboratory (Chen *et al.*, 2009). After verified by the aga-rose gel electrophoresis and NanoDrop ND-1000 spectro-photometer (NanoDrop Technologies, Montchanin, DE, USA), the genomic DNA was stored at 4°C.

N-PCR primers design for identifying S. nitidum

The discrimination of the nucleotide sequence of the *adh 1-like* gene between the *S. nitidum* and other sorghum species were analyzed by the sorfware Cluster X. The typi-cal PCR primers were designed based on a conserved gene fragment of *S. nitidum* (Tab. 2). The outer primers of n-PCR for the *S. nitidum* were shown as Tab. 3, while the inner n-PCR primers were as the same as the primer of typical PCR (Tab. 4).

Typical PCR method

The typical PCR for identifying *S. nitidum* was per-formed in a volume of 20 µL system including ddH$_2$O 12.8 µl, PCR buffer 2 µl, Mg^{2+} 1.2 µl, dNTP (2.5 mmol/L) 0.5 µl, rTaq DNA polymerase (5 U/µl) 0.2 µl, Primer Snit5 (20 µmol/L) 1 µl, Primer Snit2 (20 µmol/L) 1µl and tem-plate DNA 1 µl. The PCR was carried out in a thermocy-cler, commenced with 3 min at 94°C, followed by the 35 cycles of 30 s at 94°C, 30 s at 50°C and 80 s at 72°C, finally ended with 6 min at 72°C. The amplified product was ana-lyzed by the agarose gel electrophoresis and the result was visualized and recorded by the gel documentation system.

Nested PCR method

The n-PCR was performed in a volume system of 20 µl, which include ddH$_2$O 12.8 µl, PCR buffer 2 µl, Mg^{2+} 1.2 µl, dNTP (2.5 mmol/L) 0.5 µl, rTaq DNA polymerase (5 U/µl) 0.2 µl, Primer Nout1 (20 µmol/L) 1 µl, Primer Nout4 (20 µmol/L) 1 µl and template DNA 1 µl. The am-plification was carried out with the commence of 3 min pre-denaturing at 94°C, followed by the 20 cycles of 30 s denaturing at 94°C, 30 s annealing at 50°C and elongation 80 s at 72°C. The second amplification system was as the same as the first round PCR, other than the primers and the template DNA, while the number of thermo-cycles was adjusted to 20 cyclers in the second round. The ampli-fied product was separated by the agarose gel electropho-resis and analyzed by gel documentation system.

Results and discussion

Establishment of typical-PCR assay for identifying sorghum nitidum

Primers of typical PCR Snit5/Snit2 were used to am-plify the genomic DNA extracted from a single seed of *S. nitidum*. The result was shown in Fig. 1, from which, a strong, specific band at ~873 bp appeared, according well with our predicting.

The application of the typical-PCR

The specificity of the typical PCR assay was verified us-ing 2 samples of *S. nitidum* and 1 sample of *S. halepense*.
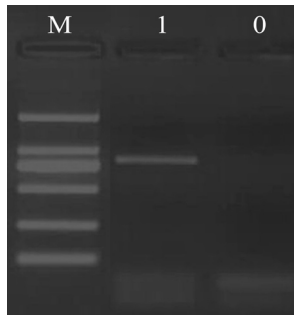
Fig. 1. Typical PCR for identification of the *S. nitidum*; M: Marker (DL 2000); 1: Snit1; 0: Blank

Fig. 3. Nested PCR for identification of the *S. nitidum*; M: Marker(DL 2000); 1: Snit1; 2: Snit2; 3: Blank

The result was as shown in Fig. 2, from which, we observed that a specific amplified band occurred to the sample of *S. nitidum* while there was no positive signal to the *S. halepense*, indicating the high specificity of the assay.

Fig. 2. The spcificity of the typical PCR; M: Marker(DL 2000); 1: Snit1; 2: Snit2; 3: Shal1; 0: Blank

Establishment of nested PCR assay for identifying S. nitidum

We made use of primers Nout1/Nout4 and Snit5/Snit2 to amplify the genomic DNA extracted from a single seed of *S. nitidum*. The result was as shown in Fig. 3, from which, we could observe a strong specific band at ~873 bp, which was well accorded with our predicting.

The application of n-PCR

We used the n-PCR assay established above to detect 14 sorghum samples. The result was as following (Fig. 4). Samples 1-9 displayed strong specific amplified band at ~873 bp while other samples exhibited negative amplification on the electrophoretic gel. As a matter of fact, these 9 samples were confirmed to be *S. nitidum* while others were *S. halepense*, *S. almum*, *S. bicolor*, *S. propinum* and *S. sudanse*. The reproducibility of the n-PCR assay was con-
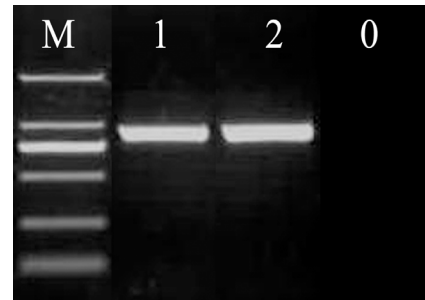
firmed to be higher than typical PCR after many tests and meet the requirement of clinical applications.

The specificity of the PCR primers is very critical to the PCR assay. In this study, we compared the nucleotide sequence of the *adh1-like* gene between the *S. nitidum* and other 28 sorghum species using the software Cluster X. The analysis showed the great discrimination of the *adh1-like* gene of the *S. nitidum* with other sorghum species. Although the high-similarity of the nucleotide sequence of *adh1* gene with the *S. leiocladum*, there is a great diversity within the gene fragment of 1035-1068 bp. Therefore, we designed a backward primer in the gene region of 1035-1068 bp, while the forward primer was designed in the low-similarity gene fragment, which would like to guarantee the high specificity of the primers for identifying the *S. nitidum*. After all, the high-specificity of the primers was the technical foundation of the nested-PCR assay.

The reason of the low-efficiency of the typical PCR when compared with the nested PCR is as follows: 1) the space/advanced structure of the *adh1-like* gene may be very complex; 2) the number of the loci of *adh1-like* gene is small throughout the genomic gene of the sorghum, which reduce the target number and the amplification efficiency. In order to proof the first suggestion, we designed series of primers targeting at the nucleotide fragment throughout ~2000 bp nucleotide acids near the *adh1-like* gene. However, all of the primers showed low-efficient amplification, which tells us that the first reason seems not like exit. In order to verify the second reason, we blast the *adh1-like* loci in the database of *S. bicolor* and observed that there is only one locus in the genome of the sorghum. Therefore, typical PCR would possibly displayed low-efficiency; especially the DNA concentration is low in the amplification system. Wen WG *et al.* (2008) applied the
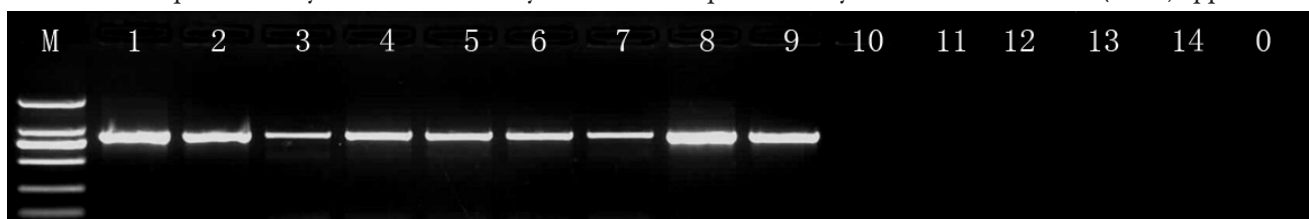

Fig. 4. The specificity of the n-PCR for identifying the *S. nitidum*; M, DL2000 (TAKARA); 1~9, Snit1~Snit9; 10, Shal1; 11, Salm1; 12, Sbic1; 13, Spro1; 14, Ssud1, 0, Blank

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semi-nested PCR assay to detect the genetically modified rice in the food and observed the high sensitivity of the assay when compared with the typical PCR. Nested PCR was much more specific and sensitive than typical PCR and the stability of the n-PCR could be very good in some cases. We herein established a novel n-PCR assay for fast and reproducible detection of the *S. nitidum*.

Conclusions

A set of specific n-PCR inner primers Snit5/Snit2 and outer primers Nout1/Nout2 based on a conserved nucleotide sequence of *adh1-like* gene of *S. nitidum* was designed. A novel nested polymerase chain reaction (n-PCR) assay to identify *Sorghum nitidum* (*S. nitidum*) was developed. This assay was able to specifically identify *S. nitidum* fast and effectively, which could be applied widely in field inspection, agriculture production and plant protection.

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