

Clone S-RNase cDNAs and Establish CSP-PCR-RFLP System for Cultivars S- genotyping in Chinese Sand Pear

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Pyrus pyrifolia 'Cangxixueli'

Problems?

Pears: Self-incompatibility

How to plant a pear orchard?
(planting design of different pears)

Breeding new pears?
(which are compatible?)

Molecular Tech

Self-incompatibility (SI):

To prevent inbreeding

To promote out-crossing (de Nettancourt, 1997).

Gametophytic SI (GSI) in Rosease (Figure 1)

Single multi-allelic *S*-locus

Two separate genes

one stylar **S-gene**

one pollen **S-gene**

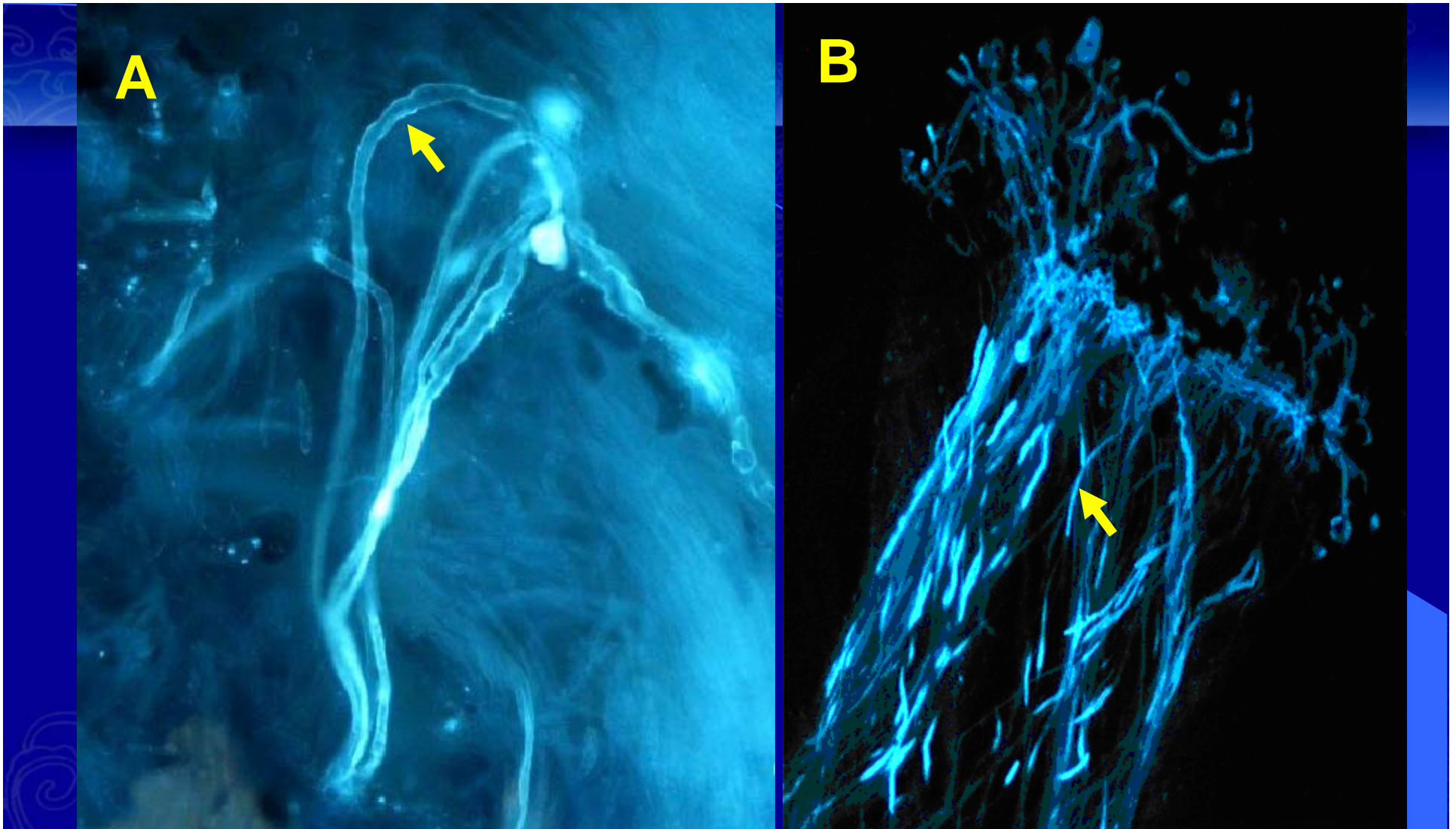


Figure 1: Fluorescence micrographs of squashed ‘Cangxixueli’ styles harvested after different pollination treatments and stained with aniline blue: (A) twisted pollen tubes after self-pollination; (B) straight pollen tubes after cross-pollination.

Literature Review

Japanese pear:

- **S-RNases identified** (Norioka *et al.*, 1995, 1996)
- **S1 to S7 cloned** (Sassa *et al.*, 1997)
- **Primary structure determined** (Ishimizu *et al.*, 1998)
C1, C2, HV(hypervariable region), C3, RC4, C5
- **PCR-RFLP system established** (Ishimizu *et al.*, 1999)
- **S8 to S10 and Sk discovered and cloned**
(Castillo *et al.*, 2002; Takasaki *et al.*, 2004; Kim *et al.*, 2007).

Literature Review (cont'd)

Why Chinese sand pear?

- **Many many cultivars** (Luo and Zhang, 2002)
- **Related to Japanese Pears** (Teng *et al.*, 2002)
- **Many new bred cultivars** (Tan *et al.*, 2005)
- **Good preliminary results** (Wuyun *et al.*, 2007)

Objectives

- **Establish CSP-PCR-RFLP system**
- **Clone all CSP S-genes**
- **Production & breeding application**

Materials and methods

Cultivars: 'Maogong', 'Guiguan', 'Cangxixueli', 'Hangqing', 'Sanhua', 'Huobali', 'Tianchengzi', 'Chubixiang', 'Chonghuadali', 'Huanghua', 'Qingxiang', 'Xizilv', 'Hongsucui', 'Mantianhong' and 'Qingkui'.

Young leaves collected & stored at -80°C

Flowers were collected at balloon stage.

remove styles and anthers

stored in liquid nitrogen -80°C until used.

Isolation of nucleic acids

- **Genomic DNAs isolated from young leaves**
Tan et al. (2007).
- **Total RNAs isolated from leaves, anthers and styles of 'Zhenghedaxueli' using Micro-to-Midi Total RNA Purification System (Invitrogen)**
- **The quality of DNAs and RNAs verified by electrophoresis in 0.8% TAE agarose gel.**

DNA Work

- **RT-PCR: 3'-Full RACE Core Set**
Primer PF1 and PR1
- **3' RACE: cDNA Amplification Kit.**
Primer S11FLF (S11)
Primer SF2 (S5a, S13, S16)
- **Cloning, sequencing, & sequence analysis**
- **PCR-RFLP analysis (for CSP)**
Before & after digestion

Table 1: S-genotypes of six Chinese sand pear cultivars revealed by RT-PCR.

Cultivar	S-genotype	Cultivar	S-genotype
Maogong	S11S12	Sanhua	S7S13
Guiguan	S2S14	Hangqing	S1S4
Cangxixueli	S5aS13	Huobali	S11S15S16

New S-genes:

After RACE, each cultivar produced one PCR product of approximately 1000 bp (Figure 2).

Seven full length cDNA sequences for S-RNases corresponding to S5a and S11 to S16 were obtained.

1000 bp

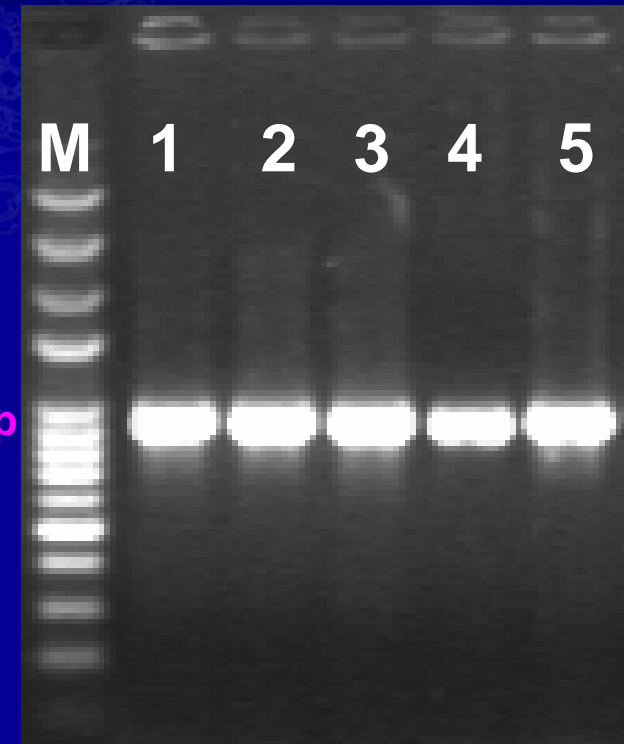


Fig. 2: 3' RACE from the RNAs of pistil
M: 100 bp plus DNA marker;
1: 'Maogong'; 2: 'Guiguan';
3: 'Cangxixueli'; 4: 'Sanhua';
5: 'Huobali'

PpS1 : MGVTGMITYMFMVFLIVLILSSSTVGYDYFQFTQOYC PAVCNSNPTPCNDPPDKLFTVHGLWFSNRNGPDPKCKTR : 78
 PpS2 : MRITGMIIYFMVFLIVLILSSSAARYDYFQFTQOYCGAECNSNPTPCKLPDPDKLFTVHGLWFSKRVCRDPEMCKTR : 78
 PpS4 : MGITGMITYMFMVFLIVLILSSSTVGYDYFQFTQOYC PAVCNSNPTPCNDPPDKLFTVHGLWFSNRNGPDPKCKTR : 78
 PpS5a : MGITGMVYVFMVFLIVLILSSSTVGYDYFQFTQOYCLAVCNSMRTPCKDPPDKLFTVHGLWFSMAGPDPKNCPIR : 78
 PpS7 : MGITGMIIYFMVFLIVLILSSSTVGYDYFQFTQOYC PAVCNSKPTPCKDPPDKLFTVHGLWFSNLNGPHPENCRNA : 78
 PpS11 : -MGITGMIIYMMVFLILLILCSSTVGYDYFQFTQOYC PAVCNSNPTPCKDPPDKLFTVHGLWFSNSNGNDPEYCKAP : 77
 PpS12 : MGITRMIYFMVFLLVILSSAVGYDYFQFTQOYC PAACNSNPTPCKDPPDKLFTVHGLWFSNMLPDPFKNT : 78
 PpS13 : MGITRMIYFMVFLIVLILSSSTMGYDYFQFTQOYCLAACNSMPTPCKLPPEKLFVHGLWFSNSMGPPVNCXPK : 78
 PpS14 : MGITRMIYFMVFLIVLILSSPTVGYDYFQFTQOYC PAVCHENPTPCKDPPDKLFTVHGLWFSNSTGNDPIYCKNT : 78
 PpS15 : MGITGMIIYFMVFLLVILSSSTVGYDYFQFTQOYC PAACNSMPTPCKLPDKLFTVHGLWFSNKIGGDPEYCKIR : 78
 PpS16 : MGIAGMIYFMVFLIVLILPERVGYDYFQFTQOYCLAVCHENPTPCKLPDPDKLFTVHGLWFSNSTGNDPEYCKNT : 78

Signal peptide

C1

C2

PpS1 : ALNSKIG----NMAADLBIIWPNVLRSDHYGFWRKBNIKHGTCSGYPTIKDDMHYLDQVIKMYITOKONVSEILSKA : 152
 PpS2 : RYRKLQRU----EPOLBIIWPNVSDRKRNGFWRKQNYKHGSCASPALPQKHYPEDQVIKMYIFLAERKONVSRILSMA : 150
 PpS4 : GNNSKIG----NMAADLBIIWPNVLRSDHYGFWRERENLKHGTCSGYPTIKDDMHYLDQVIKMYITOKONVSEILSKA : 152
 PpS5a : NIR-KREK----LLEPOLALIWPNVFDREKIKLEWQKENMKHGTCSGYPTIDENHYEEDQVIKMYISKKONVSRILSKA : 151
 PpS7 : FVYFHRIR----NMAADLBIIWPNVLDRENHVGFWKQWIKHGS CSGYPAIMNDTHYFDQVIKMYITOKONVSEILSKA : 152
 PpS11 : PHTLKL----EPOLVLIWPNVLRNDHEGFWRKQMDKHGSCASSPIONQKHYPEDQVIKMYITOKONVSEILSKA : 149
 PpS12 : HLPQIG----HMAADLBIIWPNVFNRRANHLVFWKQWIKHGS CSGYPTINDELQYEDQVIKMYITKONVSKILSKA : 152
 PpS13 : KRVPVYFPIDASLPEPOLBIIWPNVFNRRSNHGFWRKQMDKHGTCSGYPTIKDKNHYPEDQVIKMYITOKONVSEILSKA : 156
 PpS14 : GNNSKIA----NMAADLBIIWPNVLDRADHTEFWKQWIKHGS CSGHPAIONDMHYLDQVIKMYITOKONVSEILSRA : 152
 PpS15 : NPK-KRAK----KLEPOLBIIWPNVLDRENHGFWRQKQKHGACSGYPTIONENDYEDQVIKMYITEKONVSRILSNA : 151
 PpS16 : HLNSTKIA----NMAADLBIIWPNVLDREDHTEFWKQWIKHGS CGRPAIONDMHYLDQVIKMYITOKONVSEILSKA : 152

HV

C3

RC4

PpS1 : AIPFGTNRPLVDTIENAIRRGFNNTKPKPKCKKNTR--TTTELVEVTLCSDRDLKRFINCPHGPPAGSRFFSCPSSYQY- : 228
 PpS2 : HIEPFGKNRTELLEIENAIRAGFNNTKPKLKCCKVA--GTELVEVTLCHDSNLTQFINCPRPLPAGSPYFCPIDDIY : 226
 PpS4 : HIEPFGNRSFLVDIENAIRSGFNNTKPKPKCKKNTR--TTTELVEVTLCSNRDLTKRFINCPHGPPAGSRFYFCPANVYI- : 228
 PpS5a : KIEPFGKKRALLDIENAIRNGADNKKPKLKCCKKG--TTTELVEITLCSDKAGEFIDCPHPPEPISPHYCPENNKKY : 227
 PpS7 : KIEPFGIQRPLVHDIENAIRNSTNNKKPKPKCKKNE--GTELVEVGLCSGSLTQFRNCPHPPPGSPY----- : 218
 PpS11 : NIKPERKNERPLVDTIENAIRNVFNNTKPKPKCKKNTRTTTELVEVGLCSNSNLTQFINCPRPPEPAGSRFFSCPENN- : 226
 PpS12 : KIKPFGKNRTRABLIENAIRSISTNNMTPKLKCCKNN--GTELVEVTLCHDHTITRFINCRHPYDPSQFFCPRINILY : 228
 PpS13 : NINPFGIGRTRKLIENAIRNGFNDKPKLKCCKSN--GTELVEVTLCSNYLGRQFINCPKIPAGSRFYFCPIKDIY : 232
 PpS14 : KIEPFGKERTCKEIEAIRKGFNNTKPKLKCCKNT--KRETELVEVTLCSDRDLKRFIDCPRPILAGSRFYFCPENNILY : 228
 PpS15 : KIEPFGKSRALVDIENAIRNGFNNTKPKLKCCKKT--RVTELVEITLCSDKRANFIDCPHPPEPISPHYCPENNILY : 227
 PpS16 : KIEPFGRFWTCKEIEAIRKGFNNTKPKLKCCKNA--GTELVEVTLCSDRDLKRFIDCPRPILAGSRFYFCPENNILY : 228

C5

Fig. 3 Alignment of amino acid sequences of 11 S-RNases in Chinese sand pear

The 7 full-length cDNA sequences

open reading frame (654–696pb)

nucleotides encoding 218–232 aa (Fig. 3)

The primary structure

a putative signal peptide (25 or 27aa)

two histidine residues (T2/S type)

eight cysteine residues

five conserved regions

one HV region.

CSP-PCR-RFLP system

11 S-RNase alleles
two (S15 and S16) new S-alleles.

Cultivars S-genotyping by PCR-RFLP system

Genomic PCR with PF1 and PR1

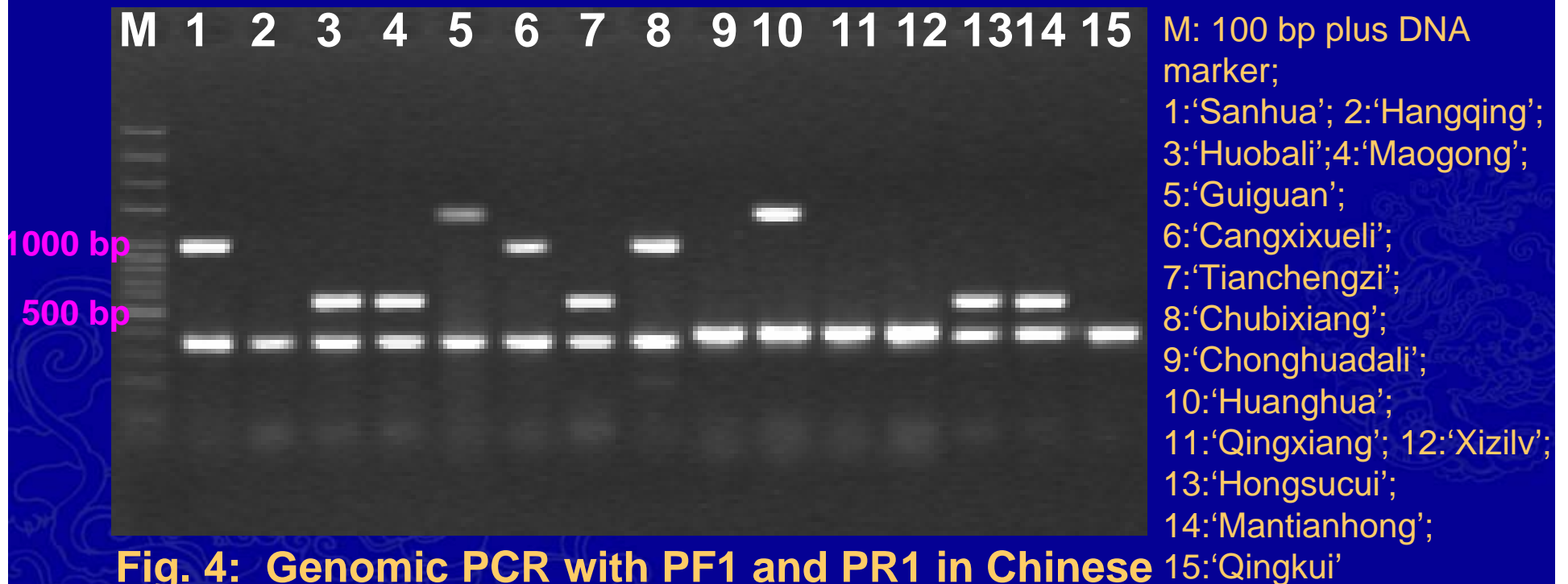


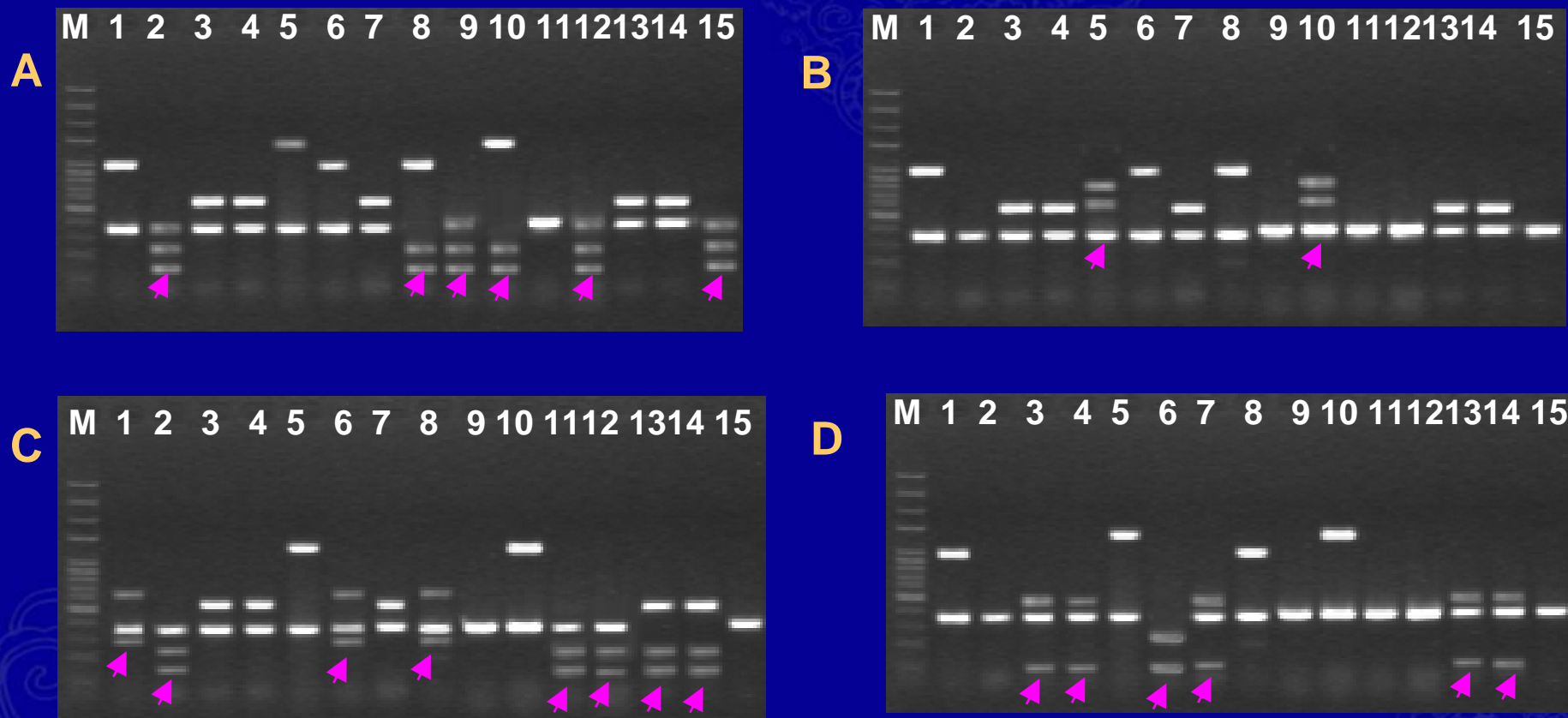
Fig. 4: Genomic PCR with PF1 and PR1 in Chinese sand pear cultivars

Tab. 2: Size of PCR products and fragments yielded by digestion with restriction endonuclease

S-allele	PCR Product (bp)	Restriction endonucleases									
		<i>Sfc</i> I	<i>Afl</i> II	<i>Nde</i> I	<i>Bsp</i> 1407 I	<i>Acc</i> II	<i>Mlu</i> I	<i>Eco</i> T22 I	<i>Hha</i> I	<i>Sph</i> I	
S1	367	135+232	—	—	—	—	—	—	—	—	
S2	1347	—	784+563	—	—	—	—	—	—	—	
S4	368	—	—	228+140	—	—	—	—	—	—	
S5a	374	—	—	—	265+109	—	—	—	—	—	
S7	351	—	—	—	—	250+101	—	—	—	—	
S11	537	—	—	—	424+113	—	—	—	—	—	
S12	350	—	—	—	—	—	232+118	—	—	—	
S13	989	—	—	676+313	—	—	—	—	—	—	
S14	345	—	—	—	—	—	—	233+112	—	—	
S15	345	—	—	—	—	—	—	—	213+132	—	
S16	344	—	—	—	—	—	—	—	—	234+110	

—: PCR products undigested with the restriction endonuclease

Fig. 5: Digestion pattern of the PCR products of 15 cultivars obtained with *Sfc* I (A), *Afl*II (B), *Nde* I (C) and *Bsp*1407 I



M: 100 bp plus DNA marker; 1:'Sanhua'; 2:'Hangqing'; 3:'Huobali';4:'Maogong'; 5:'Guiguan'; 6:'Cangxixueli'; 7:'Tianchengzi'; 8:'Chubixiang'; 9:'Chonghuadali'; 10:'Huanghua'; 11:'Qingxiang'; 12:'Xizilv'; 13:'Hongsucui'; 14:'Mantianhong'; 15:'Qingkui'. ◀ Indicates the digested PCR products.

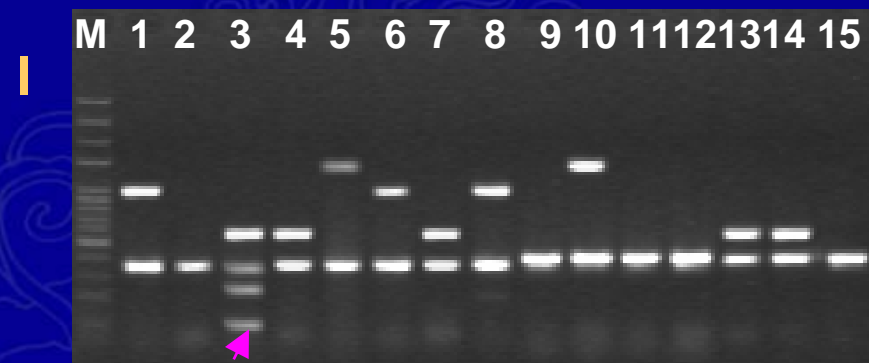
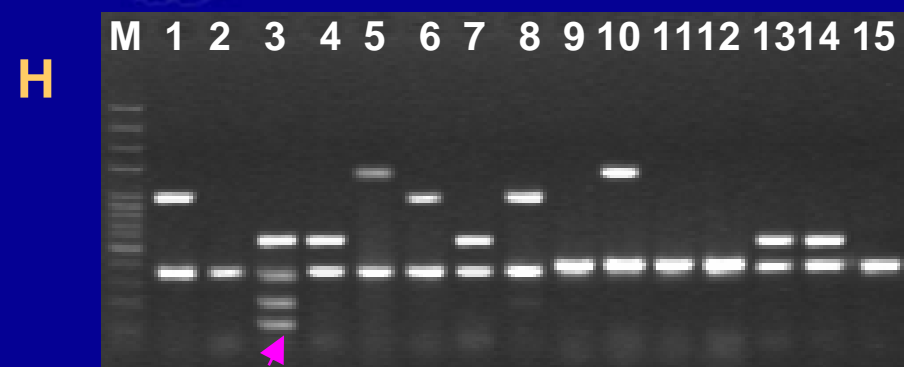
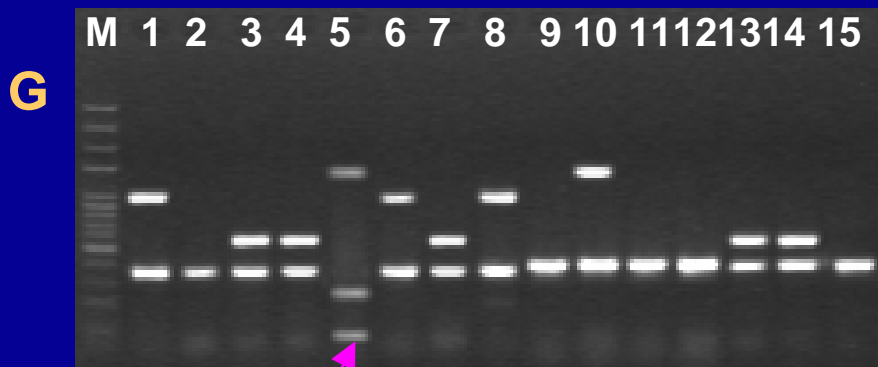
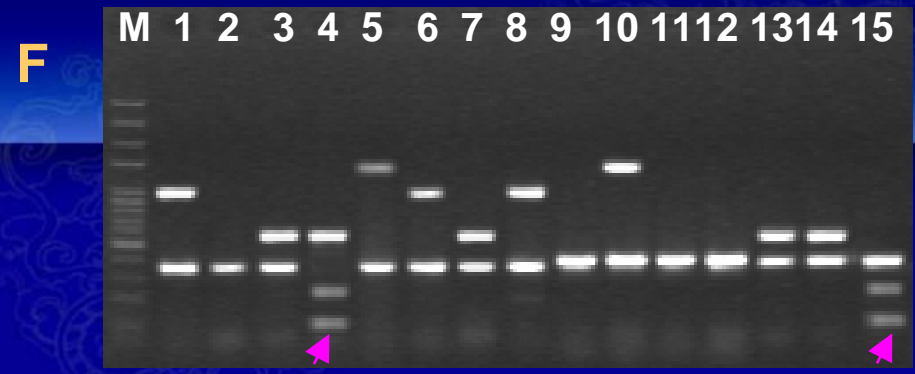
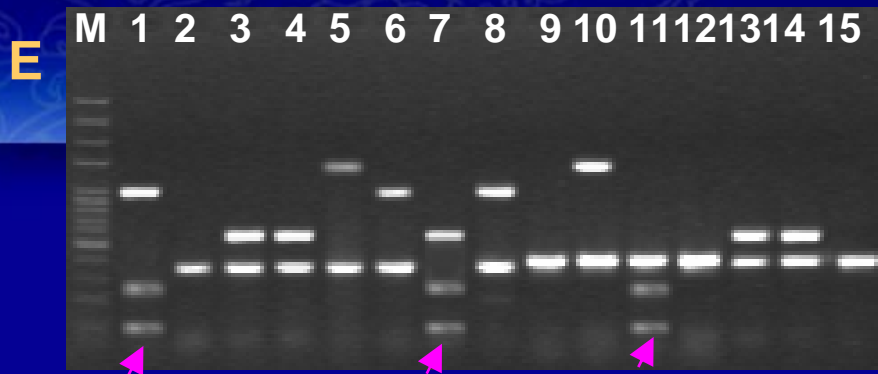


Fig. 5 Digestion pattern of the PCR products of 15 cultivars obtained with *Acc* II (E), *Mlu* I (F), *Eco*T22 I (G), *Hha* I (H) and *Sph* I (I)

M: 100 bp plus DNA marker; 1:'Sanhua'; 2:'Hangqing'; 3:'Huobali';4:'Maogong'; 5:'Guiguan'; 6:'Cangxixueli'; 7:'Tianchengzi'; 8:'Chubixiang'; 9:'Chonghuadali'; 10:'Huanghua'; 11:'Qingxiang'; 12:'Xizilv'; 13:'Hongsucui'; 14:'Mantianhong'; 15:'Qingkui'. ◀ Indicates the digested PCR products.

Table 3: Identification of S-genotypes of 15 Chinese sand pear cultivars with the PCR-RFLP system

Cultivar	S-allele-specific restriction endonucleases									S-genotype
	<i>Sfc</i> I	<i>AfI</i> II	<i>Nde</i> I	<i>Bsp</i> 1407 I	<i>Acc</i> II	<i>Mlu</i> I	<i>Eco</i> T22 I	<i>Hha</i> I	<i>Sph</i> I	
Sanhua	—	—	+	—	+	—	—	—	—	S7S13
Hangqing	+	—	+	—	—	—	—	—	—	S1S4
Huoba	—	—	—	+	—	—	—	+	+	S11S15S16
Maogong	—	—	—	+	—	+	—	—	—	S11S12
Guiguan	—	+	—	—	—	—	+	—	—	S2S14
Cangxixue	—	—	+	+	—	—	—	—	—	S5aS13
Tianchengzi	—	—	—	+	+	—	—	—	—	S7S11
Chubixiang	+	—	+	—	—	—	—	—	—	S1S13

+: S-RNase fragments digested with restriction endonucleases.

—: S-RNase fragments undigested with restriction endonucleases.

Table 3: (cont'd)

Cultivar	S-allele-specific restriction endonucleases									S-genotype
	<i>Sfc</i> I	<i>AfI</i> II	<i>Nde</i> I	<i>Bsp</i> 1407 I	<i>Acc</i> II	<i>Mlu</i> I	<i>Eco</i> T22 I	<i>Hha</i> I	<i>Sph</i> I	
Huanghua	+	+	—	—	—	—	—	—	—	S1S2
Qingxiang	—	—	+	—	+	—	—	—	—	S4S7
Xizilv	+	—	+	—	—	—	—	—	—	S1S4
Hongsucui	—	—	+	+	—	—	—	—	—	S4S11
Mantianhong	—	—	+	+	—	—	—	—	—	S4S11
Chonghuadali	+	—	—	—	—	—	—	+	—	S1S15
Qingkui	+	—	—	—	—	+	—	—	—	S1S12

+: S-RNase fragments digested with restriction endonucleases.

—: S-RNase fragments undigested with restriction endonucleases.

Conclusion

Two new S-RNase alleles

Seven full length cDNAs (S-RNases)

CSP-PCR-RFLP system

S-genotypes indentified 15.

Future application

New S-alleles identification

High-yield production

Successful breeding

Acknowledgement

Zhengzhou Fruit Research Institute

**Xingcheng Research Institute of
Pomology**

**Chinese Academy of Agricultural
Sciences (CAAS).**

Our colleagues

Any Question?

国家梨种质资源圃

NATIONAL PEAR GERMPLASM REPOSITORY

Chinese National Pear Germplasm Repository