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Effects of Genotypes on *In Vitro* Maturation and Fertilization of Frozen-Thawed Porcine Oocytes

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ABSTRACT

In the present study, we investigated the effects of genotypes on *in vitro* maturation and fertilization in porcine fresh/frozen-thawed oocytes. The porcine cumulus-oocyte complexes (COCs) were divided into four groups according to whether they were: (1) *in vitro* matured; (2) cryopreserved and *in vitro* matured; (3) *in vitro* fertilized and (4) cryopreserved, and *in vitro* fertilized. Maturation of porcine COCs was accomplished by incubation in NCSU23 medium. Immature oocytes were cryopreserved by Open Pulled Straws (OPS) method according to Vajta *et al.*, (1998). Oocytes stained by Acetic-Orcein method were observed under the microscope. DNA extracted from the ovaries was analyzed by RAPD (random amplified polymorphic DNA) and SSCP (single strand conformational polymorphism) method. The rates of oocytes maturation and fertilization were significantly high in AA genotype. The results indicated that *in vitro* maturation and fertilization in porcine fresh/frozen-thawed oocytes may be affected by genotypes in pigs.

(Key words : Porcine oocytes, Cryopreservation, Genotype, IVM-IVF)

INTRODUCTION

DNA is a vast chemical information database that carries the complete set of instructions for making all the proteins a cell will ever need. Each gene contains a particular set of instructions, usually coding for a particular protein. The DNA in each chromosome constitutes many genes (as well as vast stretches of non-coding DNA, the function of which is unknown). A gene is any given segment along the DNA that encodes instructions that allow a cell to produce a specific product - typically, a protein such as an enzyme - that initiates one specific action. There are between 50,000 and 100,000 genes, and every gene is made up of thousands, even hundreds of thousands, of chemical bases. Genes have been known as effects on reproductive ability in pigs. Recent studies on growth phase porcine oocytes have revealed a deeper molecular and biological insight into the complex regulation of rRNA transcription at different stages of follicular development (Bjerregaard and Maddox-Hyttel, 2004). Genetic polymorphisms are also playing an increasingly important role as genetic markers in many fields of animal breeding.

The use of new reproductive technologies is progressing rapidly in many farm animal species. In 1974 Motlik and Fulka reported *in vivo* fertilization of porcine oocytes that had been matured *in vitro*. Two years later Baker and Polge (1976) summarized experiments on *in vitro* and *in vivo* fertilization of porcine and predicted that if *in vitro* maturation and *in vitro* fertilization techniques can be developed in domestic animals. It should be possible to utilize at least a significant portion of the vast number of follicular oocytes that are normally lost through atresia. Important steps towards realizing this prediction in the porcine were made by Iritani *et al.*, (1978) who reported the first successful *in vitro* maturation and fertilization in porcine using spermatozoa incubated in isolated female genital tracks, Cheng (1985) who obtained piglet from oocytes matured *in vivo* and then fertilized *in vitro* and Mattioli *et al.*, (1989) who generated piglets from *in vitro* maturaturation and fertilization oocytes.

The present study was carried out to investigate 1) effect of genes on *in vitro* maturation in porcine fresh oocytes, 2) effect of genes on *in vitro* maturation in porcine frozen-

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thawed oocytes, 3) effect of genes on *in vitro* fertilization in porcine fresh oocytes and 4) effect of genes on *in vitro* fertilization in porcine frozen-thawed oocytes.

MATERIALS AND METHODS

In Vitro Maturation and Fertilization

Porcine ovaries were collected at a local slaughterhouse and transported within 1~2 h to the laboratory in 0.85% physiological saline at 37℃. Cumulus-oocyte complexes (COCs) were obtained from the ovaries by aspiration of 2~6 mm follicles in diameter. After washing 2 times in PBS (PBS supplemented with 36 μg/mL pyruvate) and 3 times in North Carolina State University (NCSU) 23, groups of 30 COCs were matured in NCSU23 medium supplemented with 10% pig follicular fluid (PFF), 0.1% luteinizing hormone (LH), 0.1% follicle stimulating hormone (FSH), 0.1% epidermal growth factor (EGF), 0.1% human chorionic gonadotropin (hCG) and 0.1% β-mercaptoethanol in 35-mm dishes under mineral oil at 38.5℃ in the incubator of 5% CO_2 with high humidity. The oocytes were transferred into 98-μL NCSU23 drop with 10% PFF after washing 2 times and matured under mineral oil at 38.5℃, 5% CO_2 for 22~24 h in the incubator.

For *in vitro* fertilization, COCs were separated from the enclosed cumulus by mouth pipet in maturation medium containing 0.1% hyaluronidase. After being washed 3 times with pre-equilibrated mTBM medium containing caffeine and BSA, batches of 10~15 cumulus-free oocytes were placed in 50 μL drops of the same medium that had been covered with warm mineral oil in a 35×10 mm petri dish. Frozen ejaculated spermatozoa straws (0.5 mL) obtained from a boar were thawed in 37℃ water bath for 20~30 sec. Thawed spermatozoa was immediately diluted with 8 mL Dulbecco's Phosphate Buffered Saline (DPBS) supplemented with 0.1% BSA and washed 2 times by centrifugation at 1,500 rpm for 15 min in 38.5℃. After washing, the sperm pellet was resuspended in mTBM without caffeine medium to give a concentration of 2×10^6 spermatozoa/mL. Just before fertilization, motility was assessed by placing a drop of sperm suspension on a warm glass slide and examining it subjectively at 100 × magnification. A 50 μL drop of the sperm suspension was introduced into about 50 μL of the mTBM with caffeine medium containing cumulus-free oocytes. Oocytes were coincubated with spermatozoa for 6~8 h at 38.5 ℃ in an atmosphere of 5% CO_2 and 95% air with high humidity.

After coincubation for 6 h, the inseminated oocytes were transferred to NCSU23 medium supplemented with 4 mg /mL BSA, and the cumulus cells surrounding the oocytes were removed by pipetting. The denuded oocytes were incubated in the same medium at 38.5℃ in 5% CO_2 in air. At 22 h of post-insemination (6 h co-incubation + 16 h culture), oocytes were fixed and stained for assessment of spermatozoa penetration.

Vitrification of Immature Oocytes

The vitrification procedure was essentially as described by Vajta *et al.*, (1998). All manipulations were performed on a 38℃ hot plate in a room at 25~27℃ in order to keep media at 38℃.

Cumulus-oocyte complexes (COCs) at germinal vesicle (GV) stage were initially equilibrated in a holding medium (TCM199-Hepes supplemented with 20% fetal bovine serum) for 3 min. Subsequently, oocytes were incubated in vitrification solution 1 (holding medium supplemented with 10% ethylene glycol and 10% dimethylsulfoxide) for 3 min and then transferred into vitrification solution 2 (holding medium supplemented with 20% ethylene glycol and 20% dimethylsulfoxide) for 25~30 sec. Loading of oocytes (5~10/straw) by the capillary effect was performed by placing the narrow end of the effect pulled straw into the medium. The loaded straw was then directly plunged into liquid nitrogen within 25 sec. Thawing was performed by placing the end of the straw directly into TCM199-Hepes supplemented with 20% fetal bovine serum and 0.25 M sucrose. The vitrified medium became liquid within 1~2 sec, and then the other medium entered the straw. Immediately afterward, by means of sedimentation, the oocytes floated out of the straw and into the medium. They were kept for 5 min in the same medium and then transferred into TCM199-Hepes supplemented with 20% fetal bovine serum and 0.15 M sucrose for another 5 min. After two subsequent washes in the holding medium for 5 min each, the oocytes were transferred back into the maturation medium and matured.

Oocyte Fixing and Staining

After IVM culture (nuclear maturation) or 16 h after IVF (fertilization), oocytes were mounted on slides, fixed with acetic acid:ethanol (1:3 v/v) for 48~72 h at room temperature. The fixed oocytes were stained with acetic-orcein (1% orcein in 45% acetic acid) and examined under a phase-contrast microscope. The precise maturation stage of each oocytes were determined, based on the changes in chromosome configuration and nuclear membrane. Oocytes were considered to be fertilized when they had one or more enlarged sperm head(s) and/or both pronuclei and polyspermy.

Oocytes in which diffused or slightly condensed chromatin could be identified were classified as being in the GV stage. Oocytes that possessed clumped or strongly condensed chromatin which formed an irregular network of individual bivalents (prometaphase) or a metaphase plate but no polar body, were classified as being in the MI stage. Oocytes with either a polar body or two bright chromatin spots were classified as being in the MII stage.

With respect to fertilization, oocytes were considered

penetrated when they had one or more enlarged sperm heads and/or both pronuclei and their corresponding sperm tails. The fertilization parameters evaluated were penetration (percentage of the number of oocytes penetrated/total inseminated), enlarged sperm heads (percentage of the number of oocytes of enlarged sperm heads/total inseminated), both pronuclei (percentage of the number of oocytes of both pronuclei/total inseminated). Oocytes with two pronuclei or more than one decondensed sperm head were considered as polyspermy. Degenerated oocytes or oocytes with a broken oolemma or abnormal appearance of the cytoplasm were not counted.

DNA Extraction

1 g ovary tissue and 600 μL PBS were homogenized and centrifuged at 12,000 rpm for 5 min, then removed the upper layer. 500 μL Buffer B (lysis buffer) and 50 μL SDS were added to the tube and mixed. 2 μL RNase were added to the tube. The tube was put in the 37.5℃ incubator for 1 h, up-down every 20 min. 2 μL Proteinase K (20 μg /mL) was added, the tube was put in the 65℃ water bath for 3 h, up-down every 20 min. Before entire mixing, 500 μL NaCl (6 M, buffer C) were added, then up-down gently. After centrifuging at 12,000 rpm for 5 min, 400 μL upper layer was transferred to another new tube and mixed with 800 μL 100% cool ethanol and 80 μL 7.5 M/10 M ammonium acetate, up-down slowly, centrifuged at 12,000 rpm for 5 min in 4℃. DNA made insoluble, and removed the upper layer. DNA was rinsed by centrifugation in 70% alcohol at 12,000 rpm for 5 min in a cold environment two to three times until clear. Subsequently DNA was dried up in the clear bench for about 2 h and made soluble in 100 μL TE buffer of pH 8.0 (10 mM Tris, 1 mM EDTA).

For confirmation of DNA stocks, 3 μL loading buffer was mixed with 3 μL DNA stock, and run on 1% agarose gel stained with ethidium bromide for 30 min at 150 V using 1 × TBE buffer. After confirming DNA stocks, to make working solutions, some of the DNA stocks were diluted in mqH_2O. Generally DNA concentration was 50 ×.

RAPD-PCR Analysis

Random Amplified Polymorphic DNA (RAPD) analysis was performed as outlined by Williams *et al.*, (1990). A standard RAPD-PCR reaction was performed in volumes of 17 μL distilled water, 1 μL primer (Operon Technologies), and 2 μL DNA template. The sequences of random primer showing DNA band polymorphism are listed in Table 1.

The thermal cycle of RAPD-PCR was programmed for 3 min initial denaturation at 94℃, followed by 35 cycles of 30 sec at 94℃ (denaturation), 60 sec at 40℃ (primer annealing), and 60 sec at 72℃ (elongation). The program ended with 5 min at 72℃ for extra elongation.

RAPD-PCR products were stored in the thermal cycle at 4℃ after the program was ended. The amplified products were analyzed by electrophoresis in 1.5% agarose gel and detected by ethidium bromide staining. The results were visualized under UV transilluminator and recorded by photograph.

Table 1. Sequences of random primer showing polymorphism

Primer UBC No.	Sequences (5'-3')
5	CCT GGG TTC C
9	CCT GCG CTT A
24	ACA GGG GTG A
60	TTG GCC GAG C

UBC: The University of British Columbia.

PCR-SSCP Analysis

The single strand conformational polymorphism (SSCP) method was modified from the report of Orita *et al.*, (1989) as a means of detecting mutated sequences. For SSCP analysis, 8 μL PCR products were mixed with 8 μL loading buffer containing 95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol and 10 mM EDTA pH 8. With denaturing solution, then the samples were subjected to 95 ℃ temperature for 8 min and immediately cooled at 4 ℃ for 10 min. Then the sample was electrophoresed onto 30% MDE gel in an automatic system. The SSCP fingerprints were visualized on Polarold 667 film (Polarold Co. Ltd., Herfordshire, UK) under ultraviolet light after the gel was stained in ethidium bromide (1.5 μg /mL) for 10 min.

RESULTS

As shown in Table 2, the effect of genotypes on *in vitro* maturation in porcine fresh oocytes was examined. The genotypes were identified by RAPD-SSCP method. The rates of MII stage oocytes were significantly lower ($P<0.05$) for AB genotype or BB genotype, compared to AA genotype (49% versus 79%, or 16% versus 79%). Likewise, there was significant difference ($P<0.05$) in the AB genotype (49%) and BB genotype (16%).

As shown in Table 3, the effect of genotypes on *in vitro* maturation in porcine frozen-thawed oocytes was examined. After freezing and thawing, the oocytes were matured *in vitro*. After cryopreservation, the rates of MII stage oocytes were lower. The rates of MII stage oocytes were significantly lower ($P<0.05$) for AB genotype or BB genotype, compared to AA genotype (18% versus 28%, or 8% versus 28%).

Table 2. Effect of genotypes on *in vitro* maturation in porcine fresh oocytes

Genotype (UBC No. 24)	No. of oocytes examined	No. of oocytes at stage of (%)					
		GV	PI	MI	AI	TI	MII
AA	90	14(16)	3(3)	2(2)	0(0)	0(0)	71(79)[a]
AB	170	26(15)	30(18)	23(14)	5(3)	2(1)	84(49)[b]
BB	43	11(26)	11(26)	12(28)	2(5)	0(0)	7(16)[c]

[a,b,c]: Values are significantly different ($P<0.05$).
GV: germinal vesicle, PI: prophase I, MI: metaphase I, AI: anaphase I, TI: telophase I, MII: metaphase II.

Table 3. Effect of genotypes on *in vitro* maturation in porcine frozen-thawed oocytes

Genotype (UBC No. 60)	No. of oocytes examined	No. of oocytes at stage of (%)					
		GV	PI	MI	AI	TI	MII
AA	192	46(24)	39(20)	41(21)	8(4)	5(3)	53(28)[a]
AB	85	18(21)	17(20)	31(36)	5(6)	3(4)	15(18)[b]
BB	24	10(42)	6(25)	5(21)	1(4)	0(0)	2(8)[b]

[a,b]: Values are significantly different ($P<0.05$).

As shown in Table 4, the effect of genotypes on *in vitro* fertilization in porcine fresh oocytes was examined. The fertilization rates were significantly lower ($P<0.05$) for BB genotype, compared to AA genotype (43% versus 62%). There was significant difference ($P<0.05$) in the penetration rate between AB genotype and BB genotype (57% and 43%).

As shown in Table 5, the effect of genotypes on *in vitro* fertilization in porcine frozen-thawed oocytes was examined. After thawing, the oocytes were matured and fertilized *in vitro*. After cryopreservation, the fertilization rates were lower. The fertilization rates were significantly lower ($P<0.05$) for BB genotype, compared to AA genotype (17 versus 34%).

Table 4. Effect of genoypes on *in vitro* fertilization in porcine fresh oocytes

Genotype (UBC No. 60)	No. of oocytes inseminated	No. of oocytes penetrated with			
		Total	ESH (%)	BPN (%)	Polyspermy (%)
AA	108	67(62)[a]	24(36)	20(19)	23(21)
AB	126	72(57)[a]	13(10)	28(22)	31(25)
BB	51	2(43)[b]	13(25)	0(0)	9(18)

[a,b]: Values are significantly different ($P<0.05$).
ESH: enlarged sperm head; BPN: both pronuclei.

Table 5. Effect of genotypes on *in vitro* fertilization in porcine frozen-thawed oocytes

Genotype (UBC No. 9)	No. of oocytes inseminated	No. of oocytes penetrated with			
		Total	ESH (%)	BPN (%)	Polyspermy (%)
AA	121	41(34)[a]	16(13)	13(11)	12(10)
AB	67	17(25)[ab]	6(9)	6(9)	5(7)
BB	23	4(17)[b]	4(17)	0(0)	0(0)

[a,b]: Values are significantly different ($P<0.05$).

DISCUSSION

In the present study the effects of genotypes on *in vitro* maturation and fertilization in porcine fresh/frozen-thawed oocytes were evaluated. DNA extracted from the ovaries was analyzed by RAPD-PCR and PCR-SSCP method to identify the genotype of gene.

The rates of maturation and fertilization achieved by the porcine fresh oocytes were significantly higher than the rates recorded for frozen-thawed oocytes. Several studies have demonstrated that the major problem in the derivation of successful protocols to cryopreserve mammalian oocytes is to maintain the integrity of the meiotic

spindle when oocytes are cooled (Eroglu *et al.*, 1998; Martino *et al.*, 1996). The main consequence of cooling is a pronounced depolymerization and disappearance of microtubule organizing centers (Webb *et al.*, 1986). Data on the sensitivity of porcine oocytes to low temperatures are limited. Didion *et al.*, (1990) examined the viability of pig GV oocytes. Following cooling and freezing by conventional methods, it was found that GV oocytes do not survive cooling to temperatures at or below 15℃. The meiotic spindle becomes partially or completely disassembled when *in vitro* matured porcine oocytes are maintained for 5 min at 4℃ and limited recovery was observed by incubating the oocytes after they had been cooled (Liu *et al.*, 2003). In freezing of immature oocytes at the GV stage up until now, very low survival rates have been reported after cryopreservation of GV oocytes in cattle (Lim *et al.*, 1992; Otoi *et al.*, 1995; Vieira *et al.*, 2002) and pig (Didion *et al.*, 1990; Isachenko *et al.*, 1998). Examination of the ultrastructure of immature bovine (Fuku *et al.*, 1995) and equine (Hochi *et al.*, 1995) oocytes after vitrification suggested that freezing damage is associated with the destruction of these intercellular contacts between cumulus cells and the oocyte. The presence of this intercellular contact via gap-junctions plays an important role in the metabolic cooperation between oocyte and cumulus cells during the growth phase and final maturation of the oocytes.

In the present experiment for *in vitro* maturation of fresh oocytes, the rates of oocytes maturation were significantly lower for AB genotype or BB genotype, compared to AA genotype. There were significant differences ($P<0.05$) in the rates of MII stage oocytes among AA genotype (79%), AB genotype (49%) and BB genotype (16%). In the experiment for *in vitro* maturation of frozen-thawed oocytes, the rate of MII stage oocytes were decreased because of effect of cryopreservation. The rates of MII stage oocytes were significantly lower ($P<0.05$) for AB genotype or BB genotype, compared to AA genotype (18% versus 28%, or 8% versus 28%).

In the experiment for *in vitro* fertilization of fresh oocytes, the fertilization rates were significantly lower for BB genotype, compared to AA genotype and AB genotype. There were significant differences ($P<0.05$) in the fertilization rates between AA genotype (62%) and BB genotype (43%) or between AB genotype (57%) and BB genotype (43%). And there were similar fertilization rates between AA genotype and AB genotype (62% and 57%). In the experiment for *in vitro* fertilization of frozen-thawed oocytes, the fertilization rates were decreased because of effect of cryopreservation. The fertilization rates were significantly lower ($P<0.05$) for BB genotype, compared to AA genotype (17% versus 34%).

These data demonstrated that SSCP analysis can be used as a tool for porcine genotyping since it allows the detection of sequence variation not residing in the endonuclease recognition sites, and, in addition, it can detect DNA polymorphisms and point mutations at a variety of positions in DNA fragments (Orita *et al.*, 1989; Lin *et al.*, 1993; Palacio and Duran-Vila, 1999; Arens, 1999; Binder *et al.*, 1999).

The data indicate that by analyzing genotype of the SSCP band using RAPD-PCR products for the pig, it was found that different genotypes affect *in vitro* maturation and fertilization in porcine fresh/frozen-thawed oocytes.

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REFERENCES

1. Arens M (1999): Methods for subtyping and molecular comparison of human viral genomes. Clinical Microbiology Reviews 124:612-626.
2. Baker RD, Polge C (1976): Fertilization in swine and cattle. Canadian J Anim Sci 56: 105-109.
3. Binder T, Siegert W, Kruse A, Oettle H, Wilborn F, Peng R, Timm H, Neuhaus P, Schmidt CA (1999): Identification of human cytomegalovirus variants by analysis of single-strand conformation polymorphism and DNA sequencing of the envelope glycoprotein B gene region -distribution frequency in liver transplant recipients. Journal of Virological Methods 78:153-162.
4. Bjerregaard B, Maddox-Hyttel P (2004): Regulation of ribosomal RNA gene expression in porcine oocytes. Animal Reproduction Science 83:605-616.
5. Cheng WTK (1985): *In vitro* fertilization of farm animal oocytes. PhD Thesis. Council of National Academic Awards Cambridge. UK.
6. Didion BA, Pomp D, Martin MJ, Homanics GE, Markert CL (1990): Observations on the cooling and cryopreservation of pig oocytes at the germinal vesicle stage. J Anim Sci 68:2803-2810.
7. Eroglu A, Toth TL, Toner M (1998): Alterations of the cytoskeleton and polyploidy induced by cryopreservation of metaphase II mouse oocytes. Fertil Steril 69:944-957.
8. Fuku E, Xia L, Downey BR (1995): Ultrastructural changes in bovine oocytes cryopreserved by vitrification. Cryobiology 32:139-156.
9. Hochi S, Fujimoto T, Oguri N (1995): Viability of immature horse oocytes cryopreserved by vitrification. Theriogenology 43:236.

10. Iritani A, Niwa K, Imai H (1978): Sperm penetration *in vitro* of pig follicular oocytes matured in culture. J Reprod Fert 54:379-383.
11. Isachenko V, Soler C, Isachenko E, Perez-Sanchez F, Grishchenko V (1998): Vitrification of immature porcine oocytes: effects of lipid droplets, temperature, cytoskeleton, and addition and removal of cryoprotectant. Cryobiology 36:250-253.
12. Lim JM, Fukui Y, Ono H (1992): Developmental competence of bovine oocytes frozen at various maturation stages followed by *in vitro* maturation and fertilization. Theriogenology 37:351-361.
13. Lin JC, Kumar B, Lin SC (1993): Rapid and sensitive genotyping of Epstein-Barr virus using single-strand conformation polymorphism analysis of polymerase chain reaction products. Journal of Virological Methods 43:233-246.
14. Liu RH, Sun QY, Li YH, Jiao LH, Wang WH (2003): Effects of cooling on meiotic spindle structure and chromosome alignment within *in vitro* matured porcine oocytes. Mol Reprod 65:212-218.
15. Martino A, Songsasen N, Leibo SP (1996): Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod 54: 1059-1069.
16. Mattioli M, Bacci ML, Galeati G, Seren E (1989): Developmental competence of pig oocytes matured and fertilized *in vitro*. Theriogenology 31: 1201-1207.
17. Motlik J, Fulka J (1974): Fertilization of pig oocytes cultivated *in vitro*. J Reprod Fertil 36:235-237.
18. Orita M, Suzuki Y, Sekiya T, Hayashi K (1989): Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5:874-879.
19. Otoi T, Yamamoto K, Koyama N, Suzuki T (1995): *In vitro* fertilization and development of immature and mature bovine oocytes cryopreserved by ethylene glycol with sucrose. Cryobiology 32:455-460.
20. Palacio A, Duran-Vila N (1999): Single-strand conformation polymorphism (SSCP) analysis as a tool for viroid characterisation. Journal of Virological Methods 77:27-36.
21. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T. (1998): Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod 12:53-58.
22. Vieira AD, Mezzalira A, Barbieri DP, Lehmkuhl RC, Rubin MI, Vajta G (2002): Calves born after open pulled straw vitrification of immature bovine oocytes. Cryobiology 45:91-94.
23. Webb M, Howlett SK, Maro B (1986): Parthenogenesis and cytoskeletal organization in ageing mouse eggs. J Embryol Exp Morphol 95: 131-145.
24. Williams JGK, Kubelik AR, Livak KJ, Rafalski A, Tingey SV (1990): DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18:531-535.

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