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Identification of amplified fragment length polymorphism markers associated with freezability of boar semen – a preliminary study*)

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Summary

In this study the amplified fragment length polymorphism (AFLP) technique, based on the analysis of genomic restriction fragments by polymerase chain reaction (PCR) amplifications, was utilized to identify genomic markers associated with differences in the freezability of boar semen. Collected from seven Polish Large White boars, semen was cooled in a lactose-lipoprotein fractions-glycerol extender (lac-LPFo-G), packaged into aluminum tubes and frozen using a programmable computer freezer. The DNA, isolated from each boar, was screened for AFLP markers using different primer pair combinations for selective amplifications. Sperm samples prior to and after freezing-thawing were analyzed for motility, mitochondrial function (Rhodamine 123), plasma membrane integrity (SYBR-14 positive) and DNA integrity using the single cell gel electrophoresis (neutral Comet assay). The authors' preliminary findings have indicated that there were consistent inter-boar variations in terms of post-thaw sperm characteristics. Distinct differences in AFLP DNA patterns in each boar were detected using different primer combinations. Furthermore, amplified DNA fragments, with similar base pairs, were detected only in the DNA profile of boars with good semen freezability. The AFLP technique can be used to select reproductive boars that produce semen with good quality characteristics for freezing-thawing procedure.

Keywords: boar, semen, AFLP technique, sperm characteristics, freezing-thawing

Despite recent methodological advances, cryopreservation exerts detrimental effects on spermatozoa, resulting in impaired motility and mitochondrial function, and a loss of plasma membrane integrity (4, 8). Cryopreservation of boar semen is not as efficient as that in other domestic species. Many methods have been sought to improve the quality of frozen-thawed boar semen, including the identification of genetic differences linked to genes controlling semen freezability (15).

A novel genetic technique, the amplified fragment length polymorphism (AFLP) developed by Vos et al. (17), is based on the amplification of short restriction endonuclease-digested genomic DNA fragments onto which adaptors have been ligated at both ends. Primers complementary to the adaptors and possessing 3' selective nucleotides of one to four bases are used in a selective amplification reaction. The AFLP technique produces bands (DNA fragments) that are separated in accordance to differences in length using polyacrylamide gel electrophoresis (PAGE). Each AFLP band corresponds to a specific position on the genome, and therefore it can be used as a genetic marker if it shows polymorphism, characterized by its size and the primers required for its amplification (1). The AFLP technique has been used to generate polymorphic markers on the DNA fingerprinting level in plants, fungi, animals and bacteria (1, 10). In this preliminary study an attempt was made to address the question of whether variations in the quality of frozen-thawed boar semen could be related to polymorphic AFLP markers. The evaluation of frozen-thawed semen included sperm motility, mitochondrial function (Rhodamine 123), plasma membrane integrity (SYBR-14) and DNA fragmentation (neutral Comet assay).

Material and methods

Animals and semen. In this study a total of 56 whole ejaculates were collected from 7 sexually mature boars (aged 2 years, Polish Large White breed), using the gloved-hand technique. The boars were fed with a commercial porcine

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ration and were kept in individual pens, under standard environmental conditions. Water was available *ad libitum*. The local ethics committee approval was obtained for this study. Fresh, unextended semen sample were analyzed for motility, mitochondrial function plasma membrane integrity and DNA fragmentation.

Cryopreservation procedure. Semen was frozen using a standard protocol (13) with modifications (4). Briefly, diluted semen was placed at 16°C for 3 h and later centrifuged at 800 × g for 10 min. The supernatant was discarded and the sperm pellet was re-suspended in an extender containing 11% lactose and lipoprotein fractions of ostrich egg yolk (lactose--LPFo Ext). Lipoprotein fractions isolated from ostrich egg yolk were lyophilized and have been shown to give acceptable results following semen preservation (14). The diluted semen samples were cooled at 5°C for 3 h and then mixed with the lactose-LPFo extender consisting of 9% glycerol and 1.5% Orvus Es Paste, OEP (lac-LPFo-G). The final concentrations of LPFo, glycerol, OEP were 5%, 3% and 0.5%, respectively. The cooled semen samples were packaged into 10-mL sterilized aluminum tubes (Medical Materials, Polfa, S.A, Boleslawiec, Poland) and frozen in a programmable computer freezing machine (Ice Cube 1810, SY-LAB, Austria). Aluminum tubes were thawed in a circulating water bath at 50°C for 60 s and immediately diluted with K-3 extender (13) at 37° prior to sperm assessments.

Sperm assessments. Total motility was evaluated prior to and after freezing-thawing. For the analysis, 6 µL aliquots of semen samples were placed on prewarmed microscopic slides, covered with coverslips and assessed under a light microscope (200 × magnification) equipped with an attached heated stage (37°C).

The percentage of live spermatozoa with functional mitochondria was assessed using a combination of fluorescent stains, rhodamine 123 (R123, Molecular Probes, Eugene, OR, USA) and propidium iodide (PI), as described in a previous study (3). A minimum of 200 cells per slide were examined in random fields of each aliquot, using an epifluorescence microscope (Olympus CH 30, Japan, Tokyo) at 600 × magnification.

Plasma membrane integrity was assessed with dual fluorescent probes, SYBR-14 and PI (Live/Dead Sperm Viability Kit; Molecular Probes, Leiden, The Netherlands) according to the method of Garner et al. (5). A minimum of 200 cells per slide was examined under an epifluorescence microscope.

The neutral Comet assay (NCA) procedure was used to assess sperm DNA fragmentation, as described in a previous study (4). Ethidium bromide-stained slides were examined at 400 × magnification under an epifluorescence microscope (Olympus BX 41, Japan, Tokyo). A minimum of 200 cells per slide were classified as spermatozoa with non-fragmented DNA (undamaged) and with fragmented DNA (damaged).

Isolation and purification of sperm DNA. A MasterPure Complete DNA and RNA Purification Kit (Epicentre, Madison, Wisconsin, USA) was used for the isolation and purification of sperm DNA from semen samples obtained from 7 boars. Briefly, semen samples were centrifuged (10 000 × g, 10 min) and the sperm pellets were mixed thoroughly with a lysis buffer. Following incubation for 15 min at 65°C, the samples were placed on ice for 3-5 min and subjected to DNA extraction according to the manual instruction provided by the kit. Subsequently, the isolated DNA samples were suspended in a buffer containing 10 mM Tris and 1 mM EDTA (pH 8.0) and were used for the AFLP technique.

AFLP technique. An AFLP analysis protocol (LI-COR, AFLP Template Kit, Lincoln, Nebraska, USA) was used to identify the AFLP markers in the DNA samples of each boar according to the manufacturer's instructions. The primer combinations used in the AFLP techniques are shown in tab. 1. Briefly, the AFLP protocol comprised the following steps: (a) Digestion of genomic DNA with two different restriction enzymes, a six-cutter (*Eco*RI; 5'-G↓AATTC-3') and a four-cutter (*Mse*I; 5'-T↓TAA-3'), to generate hundreds of thousands of anonymous DNA fragments. (b) Ligation of double-stranded (ds) adapters to the ends of the restriction enzymes (*Eco*RI/*Mse1*adapters) to yield templates for amplification. (c) Pre-amplification to increase the template available for selection, by performing 20 cycles of PCR amplification (Gene Amp PCR system 9700, PE Biosystems) at 94°C for 30 sec, 56°C for 1 min, and 72°C for 1 min, and soak at 4°C. (d) Selective amplification to amplify subsets of pre-amplified templates using *Eco*RI primer terminally labeled with infrared dye IRD 800 (LI-COR, USA), unlabelled *Mse1* primer with dNTPs. (e) Separation of selective amplification products by PAGE using 6% acrylamide, 0.25% methylene bisacryl, 6 M urea and TBE buffer containing 89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA (pH 8.3). Gel electrophoresis was performed on a LI-COR 4200 automated sequencer. (f) Visualization of the DNA fragments was attained by means of autoradiography. The AFLP fragment image data were collected and analyzed using SAGA-Mx software (version 4.0; LI-COR).

Tab. 1. Primer combinations used in AFLP technique

No.	Primers	
	*Eco*RI (E)	*Mse1* (M)
1.	AAC	CTT
2.	AAG	CTT
3.	ACA	CTT
4.	ACT	CTT
5.	AGG	CTT
6.	AAC	CAT
7.	AAG	CAT
8.	ACC	CAT
9.	ACG	CAT
10.	ACT	CAT
11.	AGC	CAT
12.	AGG	CAT

Statistical analysis. Data were examined by analysis of variance (ANOVA) to investigate the effects of boar and ejaculate on post-thaw sperm charactertics using the Statistica software package, version 5 (StatSoft Incorporation, Tulsa OK., USA). Significant main effects were compared using the Neuman-Keuls *post hoc* test at $P < 0.05$.

Results and discussion

In this study there was no significant ($P > 0.05$) variation in any of the sperm charactertics between boars or between ejaculates prior to freezing. In fresh semen, the overall percentage (mean ± S.E.M) of total sperm motility was 73.5 ± 0.7, viable spermatozoa with functional mitochondria was 87.1 ± 0.8, a membrane-intact spermatozoa was 86.2 ± 1.1 and DNA fragmentation was 3.7 ± 0.4. There were significant primary effects of boar ($P < 0.001$) on all analyzed sperm charactertics following freezing-thawing. However, no significant ($P > 0.05$) effect of ejaculate or boar × ejaculate interaction on post-thaw sperm charactertics was observed.

A summary of sperm charactertics of each boar after freezing-thawing in lactose-LPFo-extender is shown in fig. 1. In the current study boars were classified as

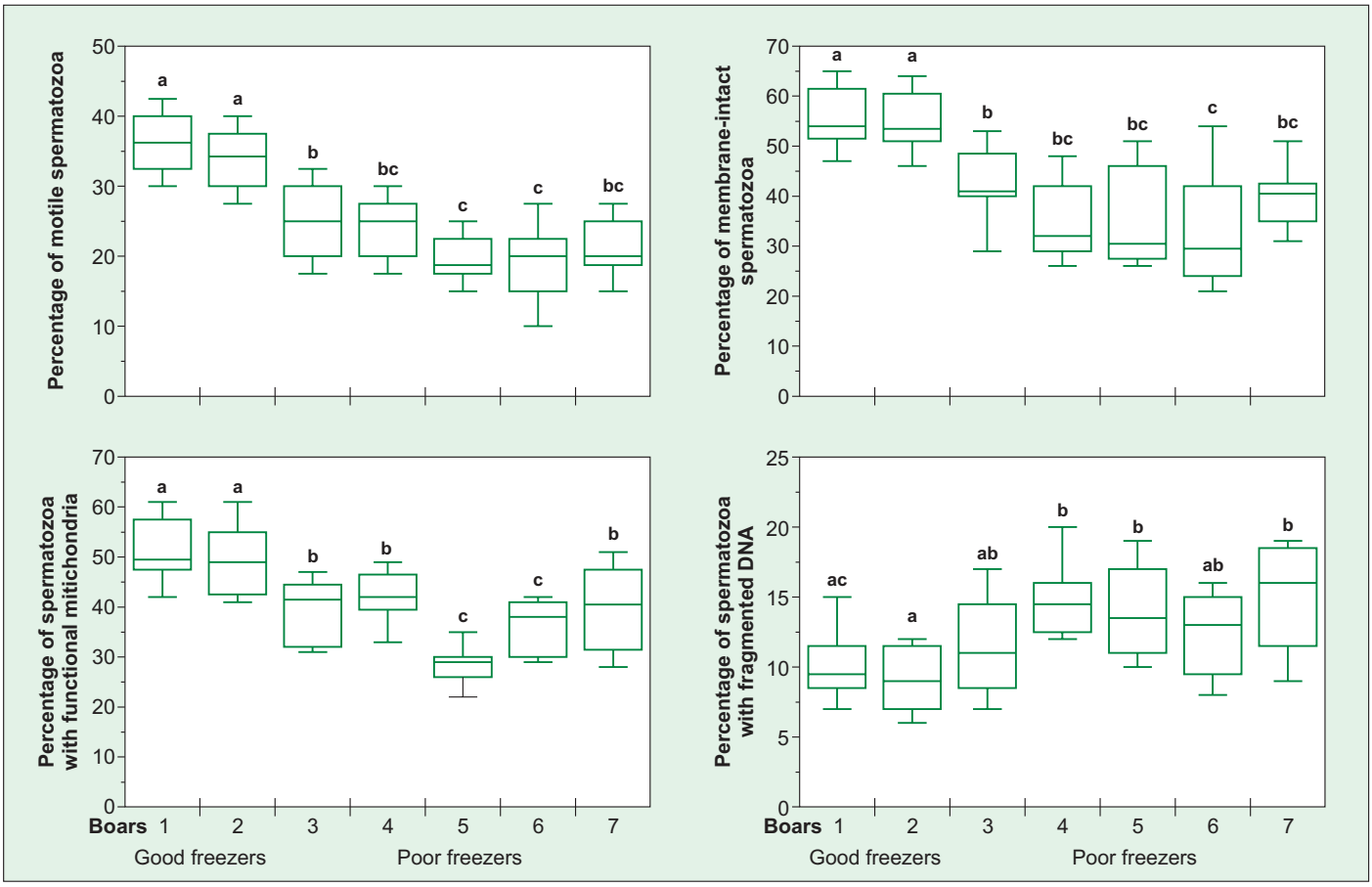

Fig. 1. Whisker-plots showing the charactertics of frozen-thawed boar spermatozoa. The horizontal lines (–) indicate the medians with 25th and 75th percentile (boxes) and minimum–maximum values (I). Values with different letters (a-c) indicate a significant difference (P < 0.05)

„good" or „poor" freezer, according to their post-thaw semen quality. Overall, post-thaw sperm motility varied between boars, being significantly ($P < 0.05$) higher in Boars no. 1 and 2, which were classified as „good" freezers. Furthermore, boars no. 1 and 2 had a significantly higher ($P < 0.05$) percentage of membrane-intact spermatozoa (SYBR-14-positive) and viable spermatozoa with functional mitochondria (R123-positive) compared with the other boars. Boars differed in terms of sperm DNA fragmentation, being markedly lower in boars no. 1 and 2 following freezing--thawing. Distinct differences in AFLP band patterns were detected in the DNA profile of boars with good and poor semen freezability. However, the use of four different primer combinations, E-ACT/M-CTT; E-ACC/M-CAT; E-ACT/M-CAT; and E-AGG/M-CAT, generated polymorphic AFLP bands, with similar base pairs, in the DNA profile of „good" freezers (fig. 2).

In this preliminary study, a comparison of AFLP DNA fingerprinting profiles of individual boars with good and poor semen freezability was performed to identify genetic differences that might be associated with variations in post-thaw sperm survival. Assessment of post-thaw sperm quality attributes in boars classified as „good" or „poor" freezers might detect genetic differences, which are linked to cryosurvival (7, 11). Recently, the use of AFLP analysis for genotyping has demonstrated that AFLP markers have the potential to identify genetic differences at the level of DNA fingerprinting of individual boars (15). Moreover, Thurston et al. (15) have suggested that AFLP markers are linked to genes controlling post-thaw sperm charactertics such as motility, plasma membrane integrity and acrosome integrity. It is noteworthy that the assessment of multiple sperm attributes provides a more accurate evaluation of post-thaw sperm survival and improves the chances of identifying individuals who are particularly resistant to cryoinjury (4, 11).

It has been suggested that the semen of certain individual boars will consistently freeze badly, resulting in poor post-thaw sperm quality, irrespective of the cryopreservation conditions (9, 16). Furthermore, these authors postulated that boars classified as „good" freezers had higher post-thaw semen quality than in the case of „poor" freezers, confirming that that the individuals were an important factor affecting the freezability of semen. Our study showed that boars classified as „good" freezers (boars no. 1 and 2) exhibited significantly higher post-thaw sperm survival and depicted AFLP DNA fragment patterns, with similar base pairs, when four different primer combinations were used. In contrast, AFLP DNA fragment patterns generated by different primer combinations were observed in boars with poor semen freezability. It is possible that these AFLP profile patterns detected differences in the genomic DNA profile, which were related to the sperm

susceptibility to cryoinjury. Our findings within a small cohort of animals indicate that genetic differences may be linked to post-thaw sperm survival and reaffirm the results of previous findings, indicating that variability in sperm susceptibility to cryoinjury might have a genetic basis (7, 15, 16). In a recent study it has been demonstrated that conventional semen parameters do not always accurately discriminate „good" and „poor" freezers and individual boars are a significant source of variation of *in vitro* fertility of post-thaw semen (6). Moreover, important variability among boars to sustain cryopreservation procedures could be due to the differences in seminal plasma components (12).

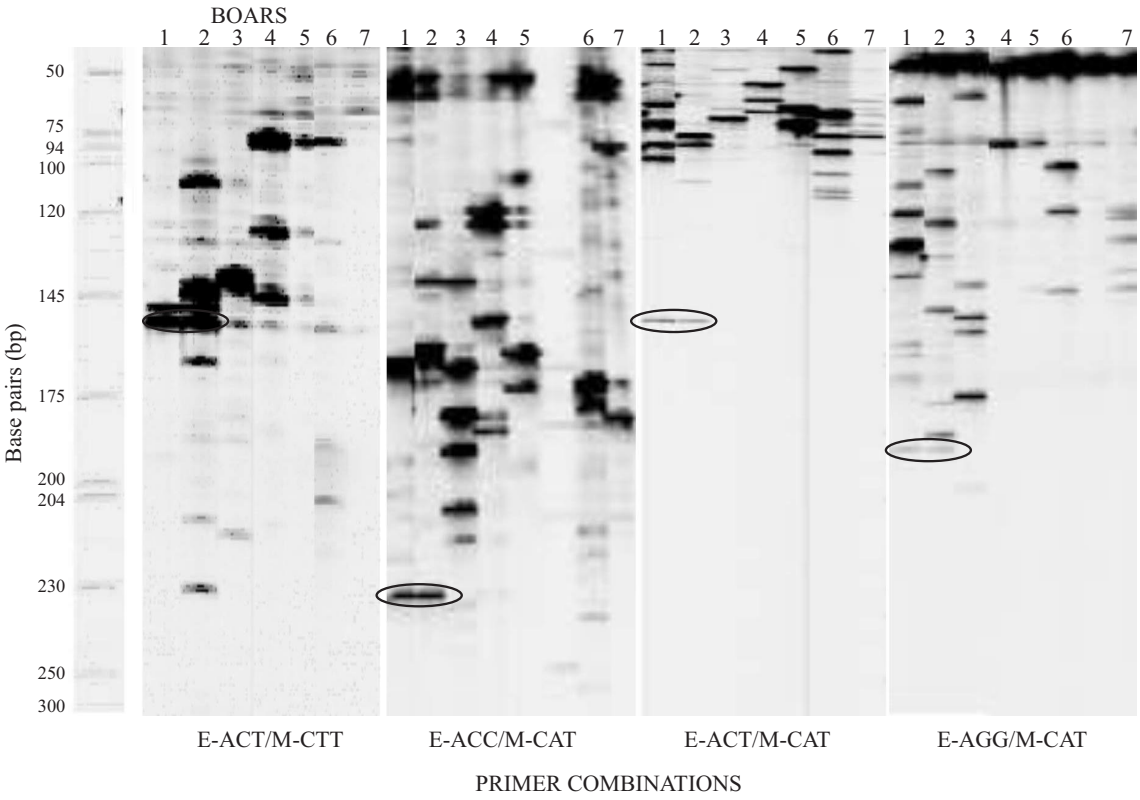

Fig. 2. AFLP patterns of boars with good (no. 1 and 2) and poor semen freezability (no. 3, 4, 5, 6 and 7)

It is important to emphasize that male-to-male differences in sperm cryotolerance have been a contributing factor to variations in post-thaw sperm DNA fragmentation (4). Sperm DNA integrity is essential for accurate transmission of genetic material and its evaluations has been demonstrated to be of paramount importance for fertilization and embryo development (2). In the current study, besides the use of conventional parameters, sperm DNA integrity was assessed to provide more information about the sperm susceptibility to cryo-induced DNA damage. Furthermore, the deterioration in post-thaw sperm DNA integrity was concomitant with impaired sperm charactertics, particularly in boars showing intrinsic poor semen freezability.

Since the boar is the primary factor influencing sperm cryosurvival it seems that AFLP markers can provide a better alternative for selection of boars with good semen freezability. However, a more comprehensive understanding of the genetic inheritance of selected AFLP markers linked to genes controlling post-thaw sperm survival may lead to an improvement in the cryopreservation of boar semen.

References

1. *Bensch S., Åkesson M.*: Ten years of AFLP in ecology and evolution: Why so few animals? Molecular Ecology 2005, 14, 2899-1914.
2. *Evenson D. P., Thompson L., Test L.*: Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. Theriogenology 1994, 41, 637-651.
3. *Fraser L., Lecewicz M., Strzeżek J.*: Fluorometric assessments of viability and mitochondrial status of boar spermatozoa following liquid storage. Pol. J. Vet. Sci. 2002, 5, 85-92.
4. *Fraser L., Strzeżek J.*: Effects of freezing-thawing on DNA integrity of boar spermatozoa assessed by the neutral comet assay. Reprod. Dom. Anim. 2005, 40, 530-536.
5. *Garner D. L., Johnson L. A.*: Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol. Reprod. 1995, 53, 276-2284.
6. *Gil M. A., Roca J., Cremades T., Hernández M., Vázquez J. M., Rodriguez--Martínez H., Martinez E. A.:* Does multivariate analysis of post-thaw sperm characteristics accurately estimate in vitro fertility of boar individual ejaculates? Theriogenology 2005, 64, 305-316.
7. *Holt W. V., Medrano A., Thurston L. M., Watson P. F.*: The significance of cooling rates and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. Theriogenology 2005, 63, 370-382.
8. *Johnson L. A., Weitze K. F., Fiser P., Maxwell W. M. C.*: Storage of boar semen. Anim. Reprod. Sci. 2000, 62, 143-172.
9. *Medrano A., Watson P. F., Holt W. V.*: Importance of cooling rate and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. Reproduction 2002, 123, 315-322.
10. *Mueller U. G., Wolfenbarger L. L.:* AFLP genotyping and fingerprinting. Trends in Ecology and Evolution 1999, 14, 389-394.
11. *Roca J., Hernández M., Carvajal G., Vázquez J. M., Martínez E. A.*: Factors influencing boar sperm cryosurvival. J. Anim. Sci. 2006, 84, 2692-2699.
12. *Strzeżek J.*: Secretory activity of boar seminal vesicle glands. Reprod. Biol. 2002, 3, 243-266.
13. *Strzeżek J., Glogowski J., Hopfer E. Wojtkiewicz K.*: The „Kortowska" method of freezing boar semen. Veterinary Medicine 1985, 41, 349-353.
14. *Strzeżek J., Lecewicz M., Dziekońska A., Fraser L.*: A simple method of extraction of lipoprotein fractions from avian egg yolk – protective effect on cooled boar semen. Theriogenology 2005, 6, 496-497.
15. *Thurston L. M., Siggins K., Mileham A. J., Watson P. F., Holt W. V.*: Identification of amplified restriction fragment polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. Biol. Reprod. 2002, 66, 545-554.
16. *Thurston L. M., Watson P. F., Holt W. V.*: Semen cryopreservation: a genetic explnantion for species and individual variation? CryoLetters 2002, 23, 255--262.
17. *Vos P., Hogers R., Bleeker M., Rijans M., Van de Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M.*: AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res.1995, 23, 4407-4414.

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