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Assessment of Boar Semen Quality and Fertility in Relation to Variants of Reproductive Trait Associated Genes ESR2, COX2 and PLCz

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ABSTRACT

Background: The study was undertaken to assess the association of genetic polymorphisms in reproductive trait-related genes (ESR2, COX2 and PLCz) with semen quality and fertility in Large White Yorkshire (LWY) boars. Identifying favorable genotypes can enhance reproductive efficiency through targeted genetic selection.

Methods: Ten LWY boars were selected and ten ejaculates from each were collected. Semen quality was evaluated immediately after collection and after 24 hours of storage at 17°C in BTS extender. Preserved semen was used to inseminate 60 gilts/sows (6 per boar) and conception rate and litter size were recorded. Blood samples were collected for genomic DNA extraction using QIAamp Mini Kit. PCR amplification was performed using gene-specific primers for ESR2, COX2 and PLCz, followed by RFLP analysis with restriction enzymes *BsrBI, FatI* and *Tsp509fI*, respectively.

Result: Significant (P<0.01) variation was observed among boars for sperm concentration, mass activity, conception rate and litter size. Preservation significantly reduced motility, live sperm percentage, plasma membrane integrity and acrosome integrity. The AG genotype of ESR2 showed significantly higher motility, sperm concentration, normal sperm count, plasma membrane integrity, conception rate and litter size and lower abnormal sperm count. The AA genotype of COX2 was associated with significantly higher mass activity, live sperm count, conception rate and reduced abnormal sperm count. The PLCz locus was found to be monomorphic.

Key words: COX2, ESR2, Fertility, Large white yorkshire, PLCz.

INTRODUCTION

Fertility and sperm quality are critical traits for the selection of breeding boars in pig production systems, particularly in nucleus herds where genetic improvement is a primary objective. These traits not only influence reproductive efficiency but also significantly impact the success of artificial insemination (AI) programs. While genetic factors are important, the phenotypic expression of fertility traits is also strongly influenced by environmental conditions such as stress, nutrition and seasonality. Previous studies have shown that sperm quality is influenced by multifactorial genetic mechanisms Marques *et al*. (2017) and is sensitive to external factors including thermal stress and seasonal variation (Wettemann *et al.,* 1976). In pigs, semen quality and male fertility typically decline during the summer months due to heat stress, which alters transcript levels associated with spermatogenesis (Zasiadczyk *et al.,* 2015; Yang *et al*., 2010). Understanding the genetic basis of fertility traits is essential for improving selection programs. Gene association and expression studies provide a pathway for identifying genetic variants linked to superior reproductive performance. Despite its importance, the widespread use of frozen-thawed (FT) boar semen in AI remains limited due to poor post-thaw semen quality and reduced fertility compared to liquid-stored semen (Baishya *et al.,* 2016; Yeste *et al*., 2017). The selection of a high proportion of functionally viable spermatozoa from preserved semen represents a major challenge in swine AI practices (Roca *et al.,* 2015). Efficient use of preserved semen is necessary to reduce economic losses and support broader adoption of AI technologies (Didion *et al.,* 2013). Recent advances in molecular genetics have facilitated the identification of several single nucleotide polymorphisms (SNPs) associated with semen quality traits such as motility and morphology (Diniz *et al*., 2014). These polymorphisms can serve as molecular markers for boar fertility and improve selection efficiency (Marques *et al*., 2018; Luc *et al.,* 2022). Among the candidate genes, ESR2, COX2 and PLCz have

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garnered interest due to their roles in reproductive biology. Estrogens play a vital role in male fertility through their receptors, ESR1 and ESR2 (Gunawan *et al.,* 2012). While ESR1 deficiency has been linked to reduced sperm motility and fertility (Couse and Korach, 1999), overexpression of ESR2 may induce germ cell apoptosis and infertility (Selva *et al.,* 2004). In pigs, ESR2 is located on the q-arm of SSC1, a region associated with QTLs for sperm motility and total sperm count (Munoz *et al*., 2004; Xing *et al*., 2008). However, the precise function of ESR2 in boar spermatogenesis remains unclear and its expression in different reproductive tissues warrants further study. PLCz is a phospholipase involved in Ca^2z oscillations necessary for oocyte activation and fertilization (Kaewmala *et al.,* 2012). Expressed in testis and epididymal sperm (Yoshida *et al*., 2007; Yoneda *et al.,* 2006), PLCz has been proposed as a biomarker of fertility in stallions (Gradil *et al.,* 2006). Located on SSC5, it co-localizes with QTLs for seminiferous tubule diameter and testicular weight (Ren *et al.,* 2009). COX2 (also known as PTGH2) synthesizes prostaglandins from arachidonic acid and plays a key role in spermatogenesis (Walter, 2003; Sirianni *et al.,* 2009). Polymorphisms in COX2 have been associated with reproductive traits such as litter size in pigs (Sironen *et al*., 2010), while no study reports on association of PLCz in pigs. Functional male gametes are produced through complex processes in the thestis, epididymis and other male reproductive tract. Failure of any of these events may lead to male infertility. Moreover, there are still may uncertainties in the processes of spermatogenesis, sperm development, sperm maturation and fertilization. Since both PLCz and COX2 are involved in prostaglandin synthesis, therefore, we hypothesize that, they functionally interact in regulating male fertility. With the above information, the present study aimed to identify and evaluate genetic variants in ESR2, COX2 and PLCz genes in relation to semen quality and fertility in boars, thereby contributing to improved genetic selection strategies in pig breeding.

MATERIALS AND METHODS

The experimental plan of study was duly approved by the Institution Animal Ethics Committee (Regd. No.=1476/GO/Re/SL/11/CPCSEA) of College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Aizawl, Mizoram, India. The study was conducted on 10 numbers of Large White Yorkshire (LWY) boars at the age of 1.5-2 years, maintained at Livestock Farm Complex, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram. The semen was collected from each boar by gloved hand technique (Shipley, 1999) using dummy as mount for 2 times in a week. Semen collection was carried out in the morning time. The semen sample selected for further processing has a mass activity minimum of 3+ and a sperm motility percentage of 70 or higher. The fresh semen sample was evaluated for colour by visual observation, semen volume was measured by measuring cylinder, sperm concentration was evaluated by Neubauer haemocytometer counting chamber (Salisbury *et al.,* 1978), mass activity (Saxena, 2000), initial motility was evaluated by conventional method, percent live sperm (Bloom,1950) and sperm abnormality was evaluated by eosin-nigrosin stain, plasma membrane integrity by HOST (Jeyendran *et al*., 1984) and acrosomal integrity by Giemsa Stain (Watson, 1975). A total of 100 ejaculates, 10 from each boar were taken for the study. The fresh semen sample was extended using BTS extender and evaluated for the following parameters after adding extender at 0 (immediately after dilution) and 24 hours of preservation at 17°C in a BOD incubator.

A total of 6 gilts/sows were artificially inseminated with liquid-preserved semen collected from each boar, having total sperm concentration of 2.5 $\times 10^9$ with dose rate of 90 ml semen after 24 and 48 hours from the beginning of oestrus. The pregnancy was diagnosed after 30th day of AI by using ultrasonography. The conception rate and litter size at birth were calculated and reported. From ten numbers of LWY boar, 2 ml blood was collected from ear vein in EDTA vials maintaining all the aseptic measure and transported in a thermo flask maintaining the temperature and stored in deep freezer at -20°C for further processing. Genomic DNA was isolated using QIAamp blood minikit and then Genomic DNA was amplified using specific primers for ESR2, COX2 and PLCZ using25 µl PCR protocol maintaining specific PCR programme condition. The amplified PCR products were then subjected to RE digestion with specific restriction endonuclease enzyme *i.e. BsrBI*, *FatI* and *Tsp509fI,* respectively maintaining specific digestion temperature and time of RFLP markers and the RE digested products were subjected to electrophoresis in 2% (w/v) agarose gel. The bands were then visualized and recorded under UV trans-illuminator and photographs were taken in Gel Doc system. The different fragments obtained after proper digestion were observed and the different genotypes of the ESR2, COX2 locus were found out. The data collected from the study were subjected to statistical analysis using a suitable formula as per Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The presence of polymorphism in ESR2 gene of LWY boar were detected usingspecific primer sequences given in Table 1 in its 5′-3′ direction which yielded a PCR product of 458 bp.

The digestion of 458 bp PCR amplification product with the restriction endonuclease enzyme *FatI* was expected to produce the different restriction pattern producing three different genotypes AA (284, 146 and 28 bp), AG (284, 174,

Table 1: Primer sequences of ESR2.

Locus	Primer sequence 5′-3′ (F: Forward, R: Reverse)
ESR2F	CTTCCTTGGATTTAGCC
ESR2R	ATGCTCTCCTTCTTCGGTGA

146, 28 bp) and GG (284, 174 bp). The three genotypes AA, AG, GG recorded in the samples of LWY boar are shown in Table 2 and Fig 1.

The COX_2 gene was genotyped from the DNA isolated from whole blood employing PCR- RFLP analysis method using *BsrBI* restriction endonuclease enzyme in all the LWY boar samples included in the present study. The presence of polymorphism in COX2 gene of LWY boar was detected using specific primer sequences given in Table 3 in its 5'-3' direction which yielded a PCR product of 278 bp.

The digestion of 278 bp PCR amplification product with the restriction endonuclease enzyme *BsrBI* was expected to produce the different restriction pattern producing three different genotypes AA (284 bp), AG (278,165, 113 bp) and GG (135,113 bp). The three genotypes AA, AG, GG recorded in the samples of LWY boar are shown in Table 4 and Fig 2.

The PLCZ gene were genotyped from the DNA isolated from whole blood employing PCR- RFLP analysis method using *Tsp509fI* restriction endonuclease enzyme in all the LWY boar samples included in the present study. The PLCZ locus was monomorphic for all the blood samples from LWY boar investigated. No polymorphism in PLCZ gene of LWY boar were detected using specific primer sequences given in Table 5 in its 5′-3′ direction which yielded a PCR product of 488 bp.

The digestion of 488 bp PCR amplification product with the restriction endonuclease enzyme *Tsp509fI* didn't show any polymorphism as expected. The monomorphic fragment sizes of PLCZ recorded in the samples of LWY boar are shown in Table 6 and Fig. 3.

The effect of ESR2 Genotype on different semen characteristics of LWY boar are depicted in Table 8. The genotypes of ESR2 locus showed no significant effect on mean semen volume (gel and sperm rich fraction). The genotypes of ESR2 locus showed significant ($P<0.01$) differences on mean percentage of initial motility in fresh and preserved semen. The genotypes of ESR2 locus showed no significant effect on mass activity of sperm. The genotypes of ESR2 locus showed no significant effect on concentration of sperm. The genotypes of ESR2 locus showed significant ($P<0.01$) differences on mean percentage of live sperm in fresh and preserved semen.The genotypes of ESR2 locus showed significant ($P<0.01$) differences on mean percentage of normal sperm in fresh and preserved semen.The genotypes of ESR2 locus showed significant ($P<0.01$) differences on mean percentage of abnormal sperm in fresh and preserved semen. The genotypes of ESR2 locus showed no significant effect on percentage of intact acrosome in fresh boar semen but showed significant ($P<0.01$) differences on percentage of intact acrosome in preserved semen. The genotypes of ESR2 locus showed significant ($P<0.01$) differences on percentage of intact plasma membrane in fresh and preserved semen.The conception rate of gilts/ sows with respect to genotypes of ESR2 was 94.44±1.45,

Table 2: Fragment sizes corresponding to ESR2 genotypes after digestion with restriction enzyme *FatI.*

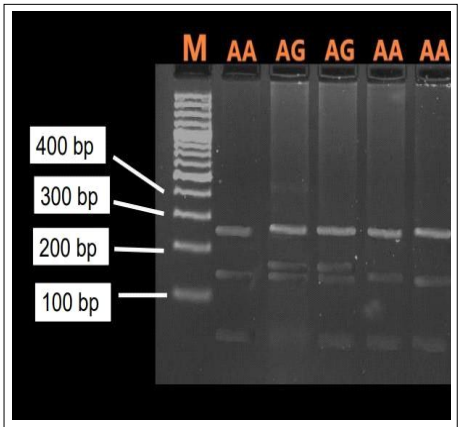
Locus	Genotypes	Amplicon size (bp)	Fragment sizes (bp)
ESR2	A A	458	284, 146, 28
	AG		284, 174, 146, 28
	GG		284, 174

Table 3: Primer sequences of COX2.

Gene	Prime rsequence 5′-3′ (F: Forward, R: Reverse)
COX2F	TCGACCAGAGCAGAGAGATGAGAT
COX2R	ACCATAGAGCGCTTCTAACTCTGC

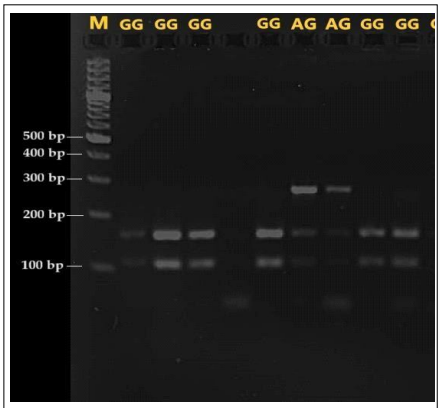
Table 4: Fragment sizes corresponding to COX_2 genotypes after digestion with restriction enzyme *BsrBI.*

Locus	Genotypes	Amplicon size (bp)	Fragmentsizes (bp)
COX2	A A	278	278
	AG		278, 165, 113
	GG		165, 113



(M-100 bp gene ladder; AA, AG, GG genotypes).

Fig 1: *Fat I* digested product of ESR2 in 2% agarose gel.



(M-100 bp gene ladder; AA, AG, GG genotypes).

Fig 2: *BsrB*I digested product of COX2 in 2% agarose gel.

91.66±1.08 and 66.66±0.00, respectively. The genotypes of ESR2 locus showed significant (P<0.01) differences on conception rate in gilts/sows against the boars. The average litter size at birth of gilts/sows with respect to genotypes of ESR2 was 9.11±0.17, 8.61±0.18 and 8.16±0.30, respectively. The genotypes of ESR2 locus showed no significant effect on average litter size at birth in gilts/sows against the boars. The genotypes of ESR2 locus showed significant (P<0.01) differences on mean percentage of initial motility, live sperm, normal and abnormal sperm, plasma membrane integrity and no significant effect on mean semen volume, intact acrosome and litter size at birth. The mean values of all the semen characteristics with respect to genotypes of the experimental LWY boar were given in Table 7. In the present study it was observed that AG genotype of ESR2 locus was comparatively superior to AA and GG genotype and contributed significantly higher initial motility, sperm concentration, normal sperm percentage, plasma membrane integrity, conception rate and litter size and a smaller number of abnormal sperms.

The effect of COX2 Genotype on different semen characteristics of LWY boar was depicted in Table 8. The genotypes of COX2 locus showed no significant effect on mean semen volume (gel and sperm rich fraction). The genotypes of COX2 locus showed no significant effect on mean percentage of initial motility and concentration. The genotypes of COX2 locus showed significant (P<0.01) differences on mass activity of sperm. The genotypes of COX2 locus showed significant (P<0.01) differences on mean percentage of live sperm in fresh and preserved semen. The genotypes of COX2 locus showed no significant effect on mean percentage of abnormal sperm, intact acrosome intact plasma membrane in fresh and preserved semen. The genotypes of COX2 locus showed significant (P<0.01) differences on conception rate in gilts/sows against the boars. The genotypes of COX2 locus showed no significant effect on average litter size at birth in gilts/sows against the boars. In the present study it was observed that AA genotype of COX2 locus was comparatively superior to AG and GG genotype and contributed significantly higher initial motility, mass activity, sperm concentration, live sperm count, conception rate and a smaller number of abnormal sperms.

The polymorphism in ESR2 locus were detected by specific primer sequence and yielded a PCR amplified products of 458 bp. This PCR amplified product then subjected to digestion with restriction endonuclease enzyme *FatI* and resulted in different restriction patterns producing genotypes AA (284, 146 and 28 bp), AG (284, 174, 146 and 28 bp) and GG (284 and 174 bp) in LWY boar samples taken under this study. In case of ESR_2, similar to the present findings *i.e.* the ESR2 locus was polymorphic (AA, AG, GG genotype) in different pig breeds *viz.* PI and PIHA population was reported by Gunawan *et al.* (2012), Large White Yorkshire (Rothschild *et al.*, 1996), Brazilian Large White, Landrace and Pietrain breeds (Selva *et al.*, 2004). In the present study, it was revealed an association of ESR2 with sperm quality and fertility traits in LWY boars. The genotypes of ESR2 locus showed significant effect on mean percentage of initial motility, live sperm, normal and abnormal sperm, plasma membrane integrity and no significant effect on mean semen volume, intact acrosome and litter size at birth. In case of ESR2, association has been described in sows by Munoz *et al.* (2004), but they did not find any statistically significant association. Polymorphism in ESR2 had effect on sperm quality traits in this present study. Aschim *et al.* (2005) reported a significantly increased frequency of the ESR2 AG genotype among infertile man, compared with fertile control. Munoz *et al.* (2004) reported that SNP in ESR2 are not associated with litter size in Iberian and Chinese European sows. Our study revealed that AG genotype of ESR2 gene contributed significantly higher initial motility, sperm concentration, normal sperm percentage, plasma membrane integrity, conception rate and litter size and a smaller number of abnormal sperm. Similar findings also reported by Gunawan *et al.* (2012) in Prestice Black- Pied boars in which genotype AG appeared to be superior for conception rate.

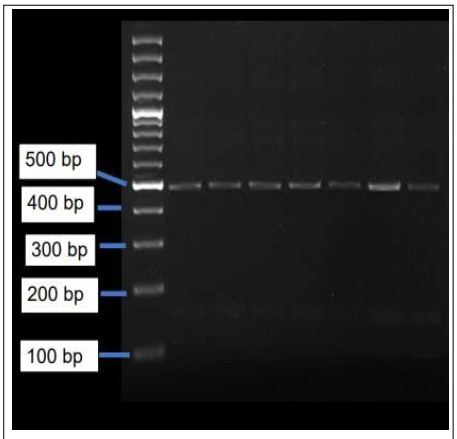
The polymorphism in COX2 locus were detected by specific primer sequence and yielded a PCR amplified

Table 5: Primer sequences of PLCZ.

Gene	Primersequence 5′-3′ (F: Forward, R: Reverse)
PLCZF	GGTGTTCAGACCGAAAGGAA
PLCZR	AACAGAAAGGACTTCTGAGTGGA

Table 6: Fragment sizes corresponding to PLCZ locus after digestion with restriction enzyme *Tsp509fI.*

Locus	Genotypes	Amplicon size (bp)	Fragment sizes (bp)
PLCz	Nil	488	488



(M-100 bp gene ladder).

Fig 3: *Tsp509fI* digested product of PLCz in 2% agarose gel.

products of 278 bp. This PCR amplified product then subjected to digestion with restriction endonuclease enzyme *BsrBI* and resulted in different restriction patterns producing genotypes AA (278 bp), AG (278, 165 and 113 bp) and GG (165 and 113 bp) in LWY boar samples taken under this study. In case of COX2, similar to the present findings *i.e.* the COX_2 locus was polymorphic (AA, AG, GG genotype) in different pig breeds *viz.* PI and PIHA population was reported by Kaewmala *et al.* (2012) in Chinese boars (Diniz *et al.,* 2014). Polymorphisms within the COX2 gene are reported to have significant association with the effect of prostaglandin production in pigs (Sironen *et al.,* 2010).

Table 7: Effect of genotypes of ESR_2 on different semen characteristics of LWY boar.

Parameter	AA	AG	GG	F-value
Volume (Gel) (ml)	81.66±2.58	79.08±2.19	79±4.06	0.281^{NS}
Volume (Sperm rich) (ml)	196.33±3.01	197.66±3.57	194±5.20	0.113^{NS}
Initial motility (Fresh) (%)	78.5±1.29^{b}	78.58±1.10^{b}	70±4.47^{a}	4.226*
Initial motility (Pre.) (%)	62.8±1.11^{b}	60.41±1.19^{ab}	54±3.71^{a}	3.848*
Mass activity (Fresh)	3.83±0.06	3.7±0.05	3.6±0.16	1.375^{NS}
Concentration(Fresh) (10^6/ml)	272.33±6.84	282.16±11.55	243±23.52	1.165^{NS}
Live sperm (Fresh) (%)	68.56±0.97^{b}	64.75±1.02^{ab}	59.8±5.02^{a}	4.575*
Live sperm (Pre.) (%)	62.96±0.91^{a}	59.75±0.80^{ab}	53.9±4.36^{b}	6.556*
Normal sperm (Fresh) (%)	90.43±0.40^{b}	89.11±.51^{b}	86±1.16^{a}	5.943**
Abnormal sperm (Fresh) (%)	9.56±0.40^{a}	10.88±0.51^{a}	14±1.16^{b}	5.943**
Normal sperm (Pre.) (%)	89.2±0.38^{b}	88.63±0.42^{b}	83.8±1.04^{a}	11.250**
Abnormal sperm (Pre.) (%)	10.8±0.38^{a}	11.36±0.46^{a}	16.2±1.04^{b}	11.250**
Intact acrosome (Fresh) (%)	91.46±.34	95.23±5.12	74.2±1.20	1.979^{NS}
Intact acrosome (Pre.) (%)	90.36±0.35^{b}	87.76±0.90^{b}	71.9±0.75^{a}	4.803**
Intact plasma membrane (Fresh) (%)	73.8±0.62^{ab}	75.35±0.76^{b}	70.2±2.55^{a}	3.885*
Intact plasma membrane (Pre.) (%)	71.5±0.60^{b}	72.43±0.83^{b}	66.1±2.14^{a}	5.206**
Litter size	9.11±0.17	8.61±0.18	8.16±0.30	2.592^{NS}
Conception rate (%)	94.44±1.45^{a}	91.66±1.08^{a}	66.66±0.00^{b}	45.091*

**P<0.01; *P<0.05.

Means bearing different superscripts in a row differed significantly.

Table 8: Effect of genotypes of COX_2 on different semen characteristics of LWY boar.

Parameter	AA	AG	GG	F-value
Volume (Gel) (ml)	88±4.16	78.75±3.69	79±1.87	1.507^{NS}
Volume (Spermrich) (ml)	198±4.66	199.5±4.19	196±3.11	0.178^{NS}
Initial motility (Fresh) (%)	79±2.33	82±1.37	76.28±1.15	3.320^{NS}
Initial motility (Pre.) (%)	64±2.21	63.25±1.27	59.2±1.16	2.490^{NS}
Mass activity (Fresh)	4±0.00^{b}	3.9±0.06^{ab}	3.6±0.06^{a}	4.990**
Concentration (Fresh) (%)	235±8.72	269.5±10.39	282.71±10.25	1.811^{NS}
Live sperm (Fresh) (%)	72.1±1.40^{b}	67.4±1.18^{ab}	63.87±1.1^{a}	4.991**
Live sperm (Pre.)(%)	64±0.90^{a}	61.95±61.95^{ab}	59.05±0.97^{b}	2.841**
Normal sperm (Fresh) (%)	91.1±0.62	90.45±0.56	88.57±0.47	3.643^{NS}
Abnormal sperm(Fresh) (%)	8.9±0.62	9.55±0.56	11.42±0.47	3.643^{NS}
Normal sperm (Pre.) (%)	89.8±0.72	89.35±0.54	87.81±0.45	2.511^{NS}
Abnormal sperm (Pre.) (%)	10.2±0.72	10.650.54	12.18±0.45	2.511^{NS}
Intact acrosome (Fresh) (%)	90.6±0.42	91.45±0.56	92.35±4.47	0.017^{NS}
Intact acrosome (Pre.) (%)	89.9±0.17	90.4±0.41	85.55±1.03	4.237^{NS}
Intact plasmamembrane (Fresh) (%)	74.8±1.10	74.95±1.01	74.14±0.75	0.182^{NS}
Intact plasma membrane (Pre.) (%)	72.2±0.69	73.2±0.89	70.94±0.80	1.182^{NS}
Litter size	8.66±0.21	8.58±0.25	8.76±0.16	0.150^{NS}
Conception rate (%)	100±0.00^{b}	91.66±1.91^{a}	88.40±1.38^{a}	5.630**

**P<0.01.

Means bearing different superscripts in a row differed significantly.

In the present study, polymorphism in COX2 and semen characteristic traits has failed to reach the significant level of association. There was no significant effect on mean semen volume, mean percentage of initial motility, normal and abnormal sperm count, plasma membrane integrity, intact acrosome and litter size at birth. However, genotypes of COX2 locus showed significant effect on mean mass activity, live sperm and conception rate. It was observed that AA genotype of COX2 gene contributed significantly to have higher initial motility, mass activity, sperm concentration, live sperm count, conception rate and a smaller number of abnormal sperm. The present findings were coincided with the observation reported by Diniz *et al.* (2014) in different Chinese pig breeds as the lack of differences between the phenotypes was mentioned. The PLCZ locus was monomorphic for all the blood samples from LWY boar investigated. No polymorphism in PLCZ gene of LWY boar was detected using specific primer sequences. The digestion of 488 bp PCR amplification product with the restriction endonuclease enzyme *Tsp509fI* didn't show any polymorphism as expected. The present findings were coincided with the observation reported by Kaewmala *et al.* (2012) in different pig breeds *viz*. PI and PIHA population.

CONCLUSION

AA genotype of COX2 locus and AG genotype of ESR2 locus showed better semen quality and fertility in LWY boars. This information can be used for selection of breeding boars. Further investigation with a larger sample size is recommended to validate the associations between the genotypes and semen quality of LWY boar.

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Conflict of interest

All authors declare that they have no conflict of interest.

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