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Single Nucleotide Polymorphisms of *Neurexophilin* Gene are Unrelated to Chicken Sperm Storage

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Abstract: Neurexophilin 1 (Nxph1) is considered as a potential candidate marker for sperm storage in hens. Neurexophilin 3 (Nxph3) functions as Nxph1 in Nxph1 knockout mice. The objective of this study was to characterize polymorphisms of *Nxph1* and *Nxph3* gene in 137 Hyline hens and 122 Yangzhou hens and evaluate their genetic effects on sperm storage. The hens were inseminated with spermatozoa on 2 consecutive days. Eggs of each hen were collected and set daily following the latter of two inseminations until two consecutive clear eggs. Duration of fertility, fertile egg number and early live embryos were used for association analysis. In this study, three Single Nucleotide Polymorphisms (SNPs), T-1378C, C-2953T and C797A were found at *Neurexophilin 1* gene and four SNPs, G236A, C507T, G564A and T612C were found at *Neurexophilin 3* gene. SNPs, C507T, G564A and T612C of Nxph3 resulted in synonymous substitution. SNP, C797A, led to the change of 266th amino acid of Nxph1 from proline to histine. SNP, G236A, led to the change of 79th amino acid of *Neurexophilin 3* from arginine to glutamine. SNPs, C-2953T, C797A, G236A did not influence characteristics of sperm storage in this study. The birds of Hyline hens with TT genotype at -1378 SNP had the lowest fertility duration, fertile egg number and early live embryos ($p<0.01$) while TT genotype birds of Yangzhou hens had the highest ones ($p<0.01$). There were only two TT genotype birds in Hyline hens and six in Yangzhou hens. The small number of TT genotype birds may be the cause of the contrary effect of SNP (T-1378C) on sperm storage in different chicken populations, suggesting that this SNP had no effect on chicken sperm storage.

Key words: *Neurexophilin 1* gene, *Neurexophilin 3* gene, polymorphisms, duration of fertility, chicken

INTRODUCTION

It is normal in chicken production that the fertility will decline if fresh semen are stored at room temperatures >0.5 h. Thus, diluents are used to extend semen and maintain the viability of spermatozoa *in vitro* as long as possible. However, only liquid storage of semen at refrigerated temperatures for up to 6 h in turkeys and 24 h in chickens can result in fertility levels comparable to freshly inseminated semen (Donoghue and Wishart, 2000). Because of the limited and variable, fertilizing ability obtained from frozen-thawed poultry spermatozoa, there is little or no commercial use of frozen-stored semen in the highly successful poultry industry. In contrast, female chicken can maintain viable and fertile sperm in the oviduct for 3-4 weeks following Artificial Insemination (AI) or natural mating (Pierson *et al*., 1988). The anatomical structures associated with prolonged sperm storage are the infundibulum and the Uterovaginal Junction (UVJ) and the latter is the primary Sperm Storage Tubules (SSTs) (Bakst, 1998).

Isolated neurones and small ganglia were identified in the uterovaginal junction of the turkey and chicken oviduct (Das, 2003; Freedman *et al*., 2001). Nerve fibres continued from the base of the tunica mucosa into the plicae and axons appeared to terminate on individual sperm storage tubules (Freedman *et al*., 2001). The evidence for the innervation of the SST in poultry oviduct suggest that neural factor may regulate sperm storage in and release of spermatozoa from the SST of hens. Liu *et al*. (2009) found that the expression level of one differentially expressed fragment was elevated in high fertility hens compared to low fertility hens. This fragment was similar to a hypothetical protein gene, the gene ID is linked to neurexophilin 1. Neurexophilin, belonging to a conserved family of neuropeptide-like glocoproteins is expressed in a subset of neurons that are scattered throughout the nervous system and represents a novel ligand for a-neurexins (Petrenko *et al*., 1996). There are at least four genes related to neurexophilins in mammals, referred to as neurexophilins 1, 2, 3 and 4. In rats and mice, only neurexophilin 1 (Nxph1) and 3 (Nxph3) bind tightly

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to the extracelluar domain of neurexin Iα (Missler *et al*., 1998) whose function involves calcium which are important factors for sperm motility (Froman *et al*., 2006; Thomson and Wishart, 1991). In the Nxph1 knockout mice, neurexin Iα is complexed with Nxph3 (Missler *et al*., 1998). Recently, neurexophilin 1 was found to suppress the proliferation of hematopoietic progenitor cells through neurexin Iα (Kinzfogl *et al*., 2011). These data indicate that nervous system may be involved in sperm storage and/or release through *Nxph1* and/or *Nxph3* gene in chicken.

To get information of gene function, the first step is to know its sequence variation because some Single-Nucleotide Polymorphisms (SNPs) may be relevant to characteristics. The objectives of the present study were to identify SNPs in *Nxph1* and *Nxph3* gene, detect those DNA polymorphisms in hens and evaluate their effects on characteristics of chicken sperm storage.

MATERIALS AND METHODS

Experimental populations: A total of 137 Hyline and 122 Yangzhou chicks were first raised collectively in floor pens and then individually caged at 16 weeks of age. The roosters were first raised with the hens and also individually caged at 16 weeks of age. They were fed standard diets *ad libitum* and kept under a 14 L:10 D photoperiod from 20 weeks to the end of the experiment.

Phenotypic measurements: Ejaculates from males were pooled and diluted to 2×10^9 viable spermatozoa per milliliter with 0.9% NaCl. Hens were artificially inseminated in the afternoon (from 15:00-16:00) on 2 consecutive days with 0.05 mL extended semen (1×10^8 spermatozoa). Artificial insemination was carried out within 30 min of semen collection. Eggs of each hen were collected and set daily from the day after the second insemination to the last fertile egg before two consecutive clear eggs. All eggs were candled on day 7 of incubation and those which did not contain an apparent live embryo were removed and opened for visual confirmation as infertile or early dead. Eggs classified as early embryonic death were counted as fertile. The fertile eggs, dead embryonic eggs and clear eggs (assumed infertile) were recorded. Duration of fertility, defined as the number of days from the day after AI to the last fertile egg before two consecutive infertile eggs (Goerzen *et al*., 1996; Liua *et al*., 2008), early live embryos and fertile egg number were used as characteristics indicative of *in vivo* sperm storage and their associations with the SNPs were analysed.

Genotyping: Following the fertility trial, blood samples of the birds were collected from wing vein. Genomic DNA was extracted from the blood samples according to standard procedures. Primers (Table 1) were designed to amplify *Nxph1* (GenBank accession No. XM 418683) and *Nxph3* (GenBank accession No. XM 427080) gene and 5'-upstream sequence of chicken *Nxph1* gene (GenBank accession No. NC_006089, Region: 25198092-25372913) using the Primer Premier 5.0 Software. Primer synthesis was completed by Shanghai Sangon Corporation, China. The 25 μL reaction included 100 ng of template, 0.5 μL of each primer (100 nmol μL^{-1}), 10.5 μL sterile distilled water and 12.5 μL PCR 2× mix reaction solution which contains Taq DNA Polymerase (0.05 units μL^{-1}), $MgCl_2$ (4 mmol μL^{-1}) and each of dNTPs (0.4 mmol μL^{-1}). PCR 2× mix reaction solution was from Beijing Zoman Biotechnology Co., Ltd. An Eppendorf thermal cycler was programmed for an initial incubation at 94°C for 5 min; 30 cycles each with denaturing at 94°C for 30 sec, annealing at 54-57°C (Table 1) for 30 sec and extension at 72°C for 30 sec and a final cycle at 72°C for 10 min.

To investigate sequence polymorphisms, PCR was carried out with mixed DNA of twenty hens and the PCR products were sequenced by Shanghai Sangon Corporation. There were two SNPs on the 5'-upstream sequence of the *Nxph1* gene. Restriction site of TaaI (ACN↓GT) and HindIII (A↓AGCTT) at the two SNPs were detected.

The PCR product of fragment1 was digested with 15U HindIII (Takara) at 37°C for 2 h. The reaction mixture was 16 μL nuclease-free water, 2 μL 10×M Buffer, 1 μL PCR reaction mixture and 1 μL HindIII. The PCR product of

Table 1: Sequences of primers used for amplifying *Neurexophilin 1* and *3* gene

Fragments	Position	Primer sequences	Temp.
Fragment1 (5'-upstream of neurexophilin 1)	-3004_-2632 bp	1F: TCTTCGCAATAACAGGG 1R: GTTCCAAAGGATTCCAG	54
Fragment2 (5'-upstream of neurexophilin 1)	-1630_-1109 bp	2F: TGTCTGAGGGGCAGGATGA 2R: GCCCGTGTCTTACCAATGTG	54
Fragment3 (exon of neurexophilin 1)	664-830 bp	3F: CAGACCCAGAGCCACGTGTCATG 3R: AGACACGTTCATCCTCACCCTGAGG	55
Fragment4 (exon of neurexophilin 3)	35-261 bp	4F: AAGGTGCCGAGCAGCGTGAG 4R: ACCCGAGGCCGATTCCAAGT	57
Fragment5 (exon of neurexophilin 3)	376-534 bp	5F: TTCTTCCGCCACAACTCC 5R: GGCACGGTCCACTTTCTC	55
Fragment6 (exon of neurexophilin 3)	515-715 bp	6F: ACGAGAAAGTGGACCGTGCCAAGAA 6R: CGTCGCTGTGGTAGTTGTAG	54

ragment 2 was digested with 7U TaaI (Fermentas) at 65°C for 2 h. The reaction mixture was 18.3 μL nuclease-free water, 2 μL 10 × Buffer Tango™, 10 μL PCR reaction mixture and 0.7 μL TaaI in 31 μL. The digested products were electrophoresed for 20 min at 100 V on a 1% agarose gel stained with ethidium bromide. Individual PCR-RFLP fragment sizes were determined by visualizing the banding pattern under ultraviolet light.

Three SNPs were found at *Nxph1* gene and four SNPs were found at *Nxph3* gene. Two of these seven SNPs change the amino acid sequence and they were detected using Polymerase Chain Reaction and Single-Stranded Conformational Polymorphism protocol (PCR-SSCP). Aliquots of 2 μL PCR products were mixed with 6 μL of loading dye (0.025% bromophenol blue, 0.025% xylene cyanol, 90% deionized formamide, 10% 10×TBE). The mixture was denatured at 98°C for 10 min and then placed on ice for at least 10 min. Electrophoresis was carried out on 10% polymerized gels (acrylamide: bisacrylamide, 29: 1, 90-130 V for 12 h) at room temperature in 1×TBE buffer. SSCP patterns on the gels were visualized by silver staining. The method of silver staining consisted of the following procedures: fixed with 10% ethanol for 10 min; stained in 0.2% silver nitrate for 15-20 min; developed in 1.5% sodium hydroxide (supplied with 0.05% formaldehyde after 5 min); terminating the development with distilled water. Individual genotypes were defined according to band patterns. The homozygous individuals of different genotype were sequenced by Shanghai Sangon Corporation, China.

Statistical analysis: The genotypic and allelic frequencies of the polymorphism site were calculated using Excel. Reliability (β) of the allelic frequency were calculated according to Liu *et al*. (2008). The SNPs were assessed for Hardy-Weinberg disequilibrium using the χ^2-test.

All data of the traits were checked for normality and the variables approximated a normal distribution. Association analysis of the genotype and the three characteristics were carried out using the GLM procedure of SAS 8.1. A $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Fertility characteristics: Duration of fertility from the 137 Hyline hens and 122 Yangzhou chickens ranged from 6-20 (average±SD = 14.59±2.83) and from 10-21 (average±SD = 14.87±2.66), respectively. Fertile egg number of Hyline and Yangzhou hens ranged from 6-19 (average±SD = 12.45±2.93) and from 5-17 (average±SD = 11.61±2.38), respectively. Early live embryos of Hyline and Yangzhou hens ranged from 5-18 (average±SD = 12.01±2.96) and from 5-16 (average±SD = 10.94±2.35), respectively. The three traits varied widely which suggested that the population might contain sufficient genetic variability and the data could be used to analyse correlations between genotype and these traits.

Sequence variation analysis: Sequencing of multiple individuals showed a C/T SNP at base -2953 and a T/C SNP at base -1378 of *Nxph1* gene. PCR-RFLP Method was developed successfully for genotyping the C-2953T and T-1378C SNPs of the *Nxph1* gene. Three genotypes of C-2953T SNP can be visulized using enzyme HindIII (Fig. 1) (The amplified PCR fragments with HindIII endonuclease digestion showed one fragment (373 bp) for C allele and two fragments (326 and 47 bp) for T allele. Accordingly, the genotype CC had one fragment (373 bp), genotype TC had three fragments (373, 326 and 47 bp) and genotype TT had two fragments (326 and 47 bp). Although, the 47 bp fragment was too small to detect in 1% agarose electrophoresis, the 373 and 326 bp fragments can accurately identify different genotypes) and three genotypes of T-1378C SNP can be visulized using enzyme TaaI (Fig. 2) (The amplified PCR fragments with TaaI endonuclease digestion showed one fragment (522 bp) for C allele and two fragments (271 and 251 bp) for T allele. Accordingly, the genotype CC had one fragment (522 bp), genotype TC had three fragments (522, 271 and

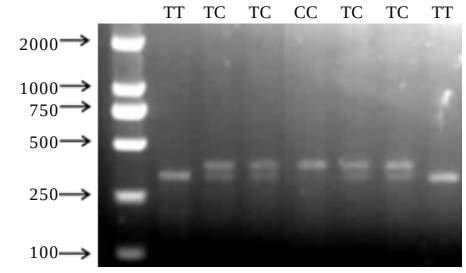

Fig. 1: The HindIII PCR-RFLP analysis of *Nxph1* gene at -2953 bp in chicken

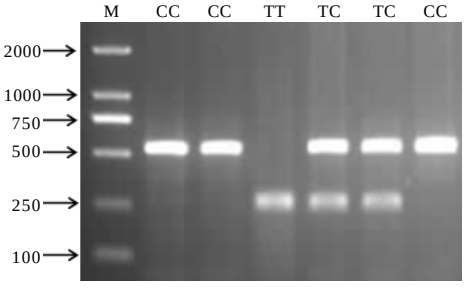

Fig. 2: The TaaI PCR-RFLP analysis of *Nxph1* gene at -1378 bp in chicken

251 bp) and genotype TT had two fragments. Although, it is difficult to distinguish the 251 and 271 bp fragments in 1% agarose electrophoresis, three genotypes can clearly be identified).

Sequencing of multiple individuals showed a C/A SNP at base 797 of *Nxph1* gene and this mutation led to the change of 266th amino acid of neurexophilin 1 from proline to histine but only two genotypes were detected using PCR-SSCP (Fig. 3).

Sequencing of multiple individuals showed four SNPs, G236A , C507T, G564A and T612C of *Nxph3* gene. Only SNP at base 236 of Nxph3 led to the change of 79th amino acid of neurexophilin 3 from arginine to glutamine and three genotypes can be visulized using PCR-SSCP (Fig. 4).

Gene frequencies and genotype distributions: Gene frequencies and genotype distributions were shown in Table 2. The reliability of the allelic frequency was above 0.99 which implied that the estimated allelic frequency was credible. The values of genotypic frequencies revealed that the SNPs at -1378 and -2953 base of chicken Nxph1 were deviated from Hardy-Weinberg equilibrium ($\chi^2>\chi^2_{0.01}$ = 6.64) in Hyline hens while it was in H-W equilibrium in Yangzhou chicken ($\chi^2<\chi^2_{0.05}$ = 3.84). SNP, C797A, of Nxph1 was deviated from H-W equilibrium in the two populations. SNP at 236 base of Nxph3 was in H-W equilibrium in the two populations.

Association of the SNPs with duration of fertility: None of the polymorphisms at -2953 base and 797 base of Nxph1 and 236 base of Nxph3 associated with live embryos, fertile egg number and duration of fertility. As shown in Table 3, the Hyline hens with genotype CC and TC at -1378 base of Nxph1 had more live embryos and fertile egg number and longer duration of fertility than those with genotype TT ($p<0.01$). Contrary to the situation in Hyline hens, the Yangzhou hens with genotype TT had the highest live embryos and fertile egg number ($p<0.01$). Very little is known regarding the mechanism of sperm storage and release in the hens however, utilizing molecular biological techniques and linking these findings to fertility characteristics might provide clues to how the SSTs function. In this study, seven SNPs were found at *Nxph1* and *Nxph3* gene. All the SNPs were single base pair substitutions which were

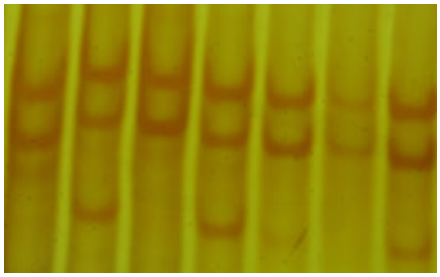

Fig. 3: SSCP analysis of PCR amplification using primer 3F and 3R in chicken. Genotype CC had C allele at 797 bp of Nxph1 and genotype CA had C and A alleles

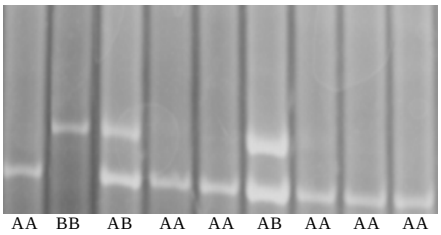

Fig. 4: SSCP analysis of PCR amplification using primer 4F and 4R in chicken. Genotype AA had G allele at 236 bp of Nxph3, genotype AB had G and A alleles and genotype BB had A allele

Table 2: Information of allelic frequencies

		Hyline chicken			Yangzhou chicken		
SNP	Allele	Frequency	β	χ^2	Frequency	β	χ^2
-1378 base of Nxph1	T	0.2518	0.99	8.12**	0.2418	0.99	0.13
	C	0.7482	1.00		0.7582	1.00	
-2953 base of Nxph1	T	0.4405	1.00	75.70**	0.4083	1.00	0.06
	C	0.5595	1.00		0.5917	1.00	
797 base of Nxph1	A	0.4182	1.00	54.62**	0.4675	1.00	56.75**
	C	0.5818	1.00		0.5325	1.00	
236 base of Nxph3	A	0.8540	1.00	0.38	0.8361	1.00	3.54
	B	0.1460	0.99		0.1639	0.99	

β is the reliability of the allelic frequency; **Loci showing significant deviation from Hardy-Weinberg equilibrium ($p<0.01$)

Table 3: Genotype distributions and multiple comparisons of sperm storage potential traits among the three genotypes at -1378 base of Nxph1

	Hyline chicken			Yangzhou chicken		
Parameters	TT	TC	CC	TT	TC	CC
Number of hens	2	65	70	6	47	69
Fertile egg number	6.50±0.71^a	12.49±3.06^b	12.57±2.66^b	13.17±2.23^a	10.89±2.45^b	11.96±2.21^a
Live embryos	6.50±0.71^a	11.91±3.17^b	12.27±2.65^b	12.50±2.35^a	10.19±2.40^b	11.32±2.19^a
Duration of fertility	7.50±2.12^a	14.58±2.76^b	14.80±2.67^b	15.33±3.14	14.32±2.72	15.20±2.55

In the same population, means in the same row with different superscript indicates significant difference ($p<0.01$)

by far the most common polumorphisms (Fullerton *et al*., 1994). SNPs, C507T, G564A and T612C of Nxph3 resulted in no amino acid substitution. No association was found between characteristics of sperm storage and SNPs C-2953T base and C797A of Nxph1 and G236A of Nxph3. The birds of Hyline hens with TT genotype at -1378 SNP had the lowest characteristics of sperm storage while the characteristics of TT genotype birds in Yangzhou chicken were the highest ones ($p<0.01$). There were only two TT genotype birds in Hyline hens and six TT genotype birds in Yangzhou chicken. The small number of TT genotype birds may be the cause of the contrary effect of SNP (T-1378C) on sperm storage in different chicken populations, suggesting that this SNP had no effect on chicken sperm storage.

The Hardy-Weinberg principle states that both allele and genotype frequencies in a population remain constant unless specific disturbing influences are introduced. Those disturbing influences contain non-random mating (including inbreeding, assortative mating, small population size), mutation, selection and so on. The results revealed a significant deviation from Hardy-Weinberg equilibrium at the SNPs T-1378C and C-2953T base of Nxph1 in Hyline hens. These hens used in this study are from a commercial lines. In general, commercial lines are systematically selected as closed populations and high selection intensity, small population size and nonrandom mating could be the reason for the disequilibrium (Li *et al*., 2006). Normally, the commercial lines were selected for the egg or meat production, not directly for sperm storage. What specific factors have effects on the *Neurexophilin 1* gene frequency needs further investigation.

Expression of *Nxph1* gene in adult chicken SSTs was found to correspond to fertility (Liu *et al*., 2009). Gene expression is often regulated by promoters or enhancers which locate at the 5'-upstream of the gene. Thus, the nervous system may be involved in sperm storage and neurexophilin 1 may play a role through its expression. If this is the case, the 5'-upstream sequences of neurexophilin 1 may be considered as a good candidate marker for sperm storage in SSTs.

Calcium is known to affect avian sperm motility and metabolism *in vitro*. The effect of calcium can be either stimulatory or inhibitory depending on the concentration (Thomson and Wishart, 1991). At 40°C, inhibition of chicken sperm motility can be reversed by the addition of calcium (Ashizawa and Wishart, 1987). The intracellular calcium concentration of cells forming the SSTs was similar to that in the mucosal epithelial cells of the utero-vaginal junction and that in the tubular fluid from chickens (Holm *et al*., 2000). The concentration of calcium in the tubular fluid is within the range known to inhibit the motility of spermatozoa, supporting this function for calcium during sperm storage (Holm *et al*., 2000). These studies suggest that calcium concentrations correlate to sperm motility during *in vivo* and *in vitro* storage.

In the nervous system, neurexophilin 1 is secreted and specifically binds to the extracellular domain of neurexin Iα. In the Nxph1 knockout mice, neurexin Iα is complexed with Nxph3 (Missler *et al*., 1998). Then, neurexin Iα binds to α-latrotoxin only in the presence of Ca^{2+} (Geppert *et al*., 1998; Missler *et al*., 1998; Petrenko *et al*., 1996). Binding of latrotoxin results in Ca^{2+} influx into nerve terminals (Dudanova *et al*., 2006; Missler *et al*., 1998). A possible mechanism may be that the elevated calcium concentration triggered by neurexophilin-neurexin-lactrotoxin complex within the hens SSTs could be to keep sperm immotile. This speculation led us to analyze the sequence of Nxph1 and Nxph3, test the gene frequencies distribution and evaluate the relationship between the SNPs and characteristics of sperm storage in hens. Unfortunately, no SNPs detected here significantly influenced the three characteristics measured in this study. Further research on this candidate gene is required to define how this gene involves in the process of sperm storage and/or release.

CONCLUSION

The results indicated that the seven SNPs found in this study did not influence characteristics of sperm storage and the detailed mechanism of neurexophilin on sperm storage in hens needs further studies.

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