




A novel swine sex-linked marker and its application across different mammalian species

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Received: 13 March 2020 / Accepted: 18 June 2020 / Published online: 30 June 2020
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Abstract Advances in genome editing tools have reduced barriers to the creation of animal models. Due to their anatomical and physiological similarities to humans, there has been a growing need for pig models to study human diseases, for xenotransplantation and translational research. The ability to determine the sex of genetically modified embryos, cells or fetuses is beneficial for every project involving the production of transgenic animals. This strategy can improve the time-efficiency and lower the production costs. Additionally, sex assessment is very useful for wildlife studies to understand population behavior and structure. Thus, we developed a simple and fast PCR-based protocol for sex determination in pigs by using a unique primer set to amplify either the *DDX3X* or *DDX3Y* gene. The sex was 100% correctly assigned when tail genomic DNA, Day-35 fetus and hair

samples from pigs were used. For both blastocysts and oocytes (84.6% and 96.5% of efficacy, respectively) the unidentified samples were potentially due to a limitation in sample size. Our assay also worked for domestic sheep (*Ovis aries*), American bison (*Bison bison*) and European cattle (*Bos taurus*) samples and by in silico analysis we confirmed X–Y amplicon length polymorphisms for the *DDX3* gene in 12 other mammalian species. This PCR protocol for determining sex in pig tissues and cells showed to be simple, specific, highly reproducible and less time consuming as well as an important tool for other livestock species and wildlife studies.

Keywords DEAD box protein (*DDX3X/Y*) gene · Porcine · Mammalian species · PCR · Sex determination

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Introduction

Pigs have become an important animal species for biomedical research and agriculture. Recent advances in genome editing technologies have facilitated the generation of pigs resistant to disease as well as models for studying human diseases and for xenotransplantation (Redel and Prather 2015; Ryu et al. 2018; Yang and Wu 2018). Sex selection of genetically modified embryos or cloneable cells is critical to

the efficiency of every project. For projects that involve sex-specific phenomena (lactation, spermatogenesis, etc.), the sex selection of founders reduces the number of animals required to determine the utility of individual genetic events or to determine the value of additional efforts. Similarly, since males can produce offspring at a much higher rate than females, it is often valuable to limit founder production to males only. Lastly, for projects that require founders of both sexes, there is value in being able to balance or bias the sex of founder animals.

The ability to distinguish males from females also has a substantial relevance for wildlife studies to understand population characteristics and to improve their management. To date, PCR-based methods for sex determination have been described for more than one hundred mammalian species (Hrovatin and Kunej 2018; Strah and Kunej 2019). Most of these assays were designed based on sex-specific insertions and deletions (SSIndels) to target the zinc finger protein (*ZFX* and *ZFY*) or the amelogenin (*AMELX* and *AMELY*) gene variants on X and Y chromosomes (Strah and Kunej 2019). In pigs, PCR-based methods for sex determination involve a duplex PCR-amplification of a Y chromosome specific sequence and simultaneous amplification of a non-sex discriminative sex region as endogenous control (Pomp et al. 1995; Sathasivam et al. 1995); or the amplification of X–Y homologous genes, more specifically the amelogenin gene variants *AMELX* and *AMELY* by using a single pair of primers (Fontanesi et al. 2008; Sembon et al. 2008). In humans and cattle, mutations at the amelogenin genes in the Y and X chromosomes have been reported (Grzybowski et al. 2006; Dash et al. 2020) showing inconsistencies in the expected amplicons size. This can result in incorrect sex identification, thus questioning the use of the amelogenin gene for sex determination. In pigs, the *AMEL*-PCR method was not sensitive enough to dependably amplify the expected products from low cell number samples—oocytes, and early embryos (Sembon et al. 2008). Therefore, the development of a new, rapid, sensitive, and inexpensive method for sex determination in pigs is desired.

Studies aiming to characterize the mammalian sex chromosomes have helped to identify the genes involved on sexual differentiation. Both Y and X chromosomes have been undergoing structure and content differentiation for more than 170–180 million

years (Skinner et al. 2016). The mammalian X chromosome has been shown to be very conserved between different mammalian species retaining much of its ancestrally. On the other hand, the gradual degeneration of the Y chromosome due to evolutionary events with the loss of 95% of the ancestral genes limits its characterization (Marshall Graves 1998; Liu et al. 2019). The presence of highly repetitive regions makes difficult to generate complete Y chromosomes sequence data, resulting in few species with a complete and well assembled Y chromosome sequence. However, after sequencing the Y chromosome from primates; mice, cats, dogs and cattle three genes (*USP9Y*, *DDX3Y* and *UTY*) were discovered to be conserved throughout suggesting a potential role on sexual development (Skinner et al. 2016; Liu et al. 2019).

DDX3X and *DDX3Y*, also known as *DBX* and *DBY* respectively, are members of the DEAD box protein family and are present on both sex chromosomes. In humans, *DDX3Y* gene is located in a Y chromosomal region required for spermatogenesis and its expression is restricted to the male germ line. *DDX3X* is present in a X chromosome region and it was found to be expressed ubiquitously in tissues (Ditton et al. 2004). Recently, an assay based on the *DDX3X/Y* gene for sex identification in bats was able to correctly confirm the sex in 100% of samples of known sex (22 males and 17 females) (Zarzoso-Lacoste et al. 2018). In bovine, it was shown that the DEAD box protein (*DDX3X/Y*) gene was able to determinate by PCR the sex origin of beef products. The assay showed to be reliable and resulted in 100% of success rate when meat samples (14 males and 14 females) of known sex were tested (Gokulakrishnan et al. 2012). Similarly, a sex determination assay using *DDX3Y* and *DDX3X* genes was developed for three Japanese Mustelid species using tissue samples which sex was previously recorded. The assay produced the expected results for both female and male samples (Sekiguchi et al. 2010).

The possibility of sex-specific selection by using the *DDX3X/Y* gene emerges as an important factor in the production efficiency of porcine models for biomedical and agriculture purpose. Based on the need to identify sex in embryos and early fetuses, we developed a novel method by using the *DDX3X* and *DDX3Y* genes to identify the genetic sex in pigs by using a unique primer set to amplify either *DDX3X* or *DDX3Y*. We also successfully applied the *DDX3X/Y*

method to sex confirmation and identification of porcine oocytes, embryo biopsies, Day-6 blastocysts and Day-35 fetuses, and also evaluate the application of this assay across different mammalian species.

Methods

Tissue samples and DNA extraction

Genomic DNA was extracted from tails of 87 pigs representing a variety of different lines (Table 1). Additionally, we collected pig hair samples and performed sex identification of 11 Day-35 fetuses. Female and male genomic DNA samples from *Bison bison*, liver samples from *Bos taurus* as well as endometrium and testicles samples from *Ovis aries* were kindly donated from Dr. Robert Schnabel, Msc. Emma Stephenson, Msc. Eleonore O’Neil and Dr. Jon Green, respectively, from the University of Missouri.

For DNA isolation (tail, liver, endometrium and testicle), tissue was resuspended in 500 μ L of lysis buffer I (LBI) (40 mM Tris, pH 8.9; 0.9% Triton X-100; 0.9% Nonidet P-40; 0.4 mg/mL proteinase K) and incubated at 65 °C for 15 min and then heated to 95 °C for 10 min. The DNA was purified by organic extractions of phenol and chloroform, and then precipitated with ethanol. Single hair follicles and a small sample of tissue from Day-35 fetuses were taken and suspended in 10 μ L of LBI; however, no organic extractions of phenol and chloroform were performed for these samples. Genomic DNA (0.5 ng) from tissue samples and cell lysate sample from fetuses and hair samples (1 μ L) were used to determine sex by PCR.

Table 1 Porcine lines utilized for tail DNA extraction

Lines	Females	Males
7/8 Minnesota mini 1/8 Domestic	2	4
1/2 Domestic 1/2 NIH c/c mini	16	17
1/2 Domestic 1/2 minnesota mini	10	3
1/4 Minnesota mini 3/4 domestic	11	18
Yucatan	4	0
Ossabaw	1	1

In vitro embryo production

Ovaries were collected from slaughtered prepubertal gilts according to the approved protocol and standard operating procedures by the Institutional Animal Care and Use Committee of the University of Missouri. Cumulus–oocyte complexes were aspirated from follicles 3–6 mm in diameter with a sterile 18-gauge needle attached to disposable syringe. Only cumulus–oocyte complexes with uniform cytoplasm and at least three layers of surrounding cumulus cells were subjected to in vitro maturation for 42–44 h at 38.5 °C in 5% CO₂/humidified air, as described previously (Zhang et al. 2010; Chen et al. 2018). After maturation, the cumulus cells were removed from oocytes by vortexing for 3 min in 0.1% (wt/vol) hyaluronidase and thirteen Metaphase II (MII)-stage oocytes, identified by the presence of the first polar body, were collected for DNA extraction. The remaining matured oocytes were washed and placed into 50 μ L droplets of IVF medium (modified Tris-buffered medium containing 2 mg/mL bovine serum albumin (BSA) and 2 mM caffeine) and incubated at 38.5 °C until sperm was added. For IVF, a 0.1-mL frozen sperm pellet was thawed in 3 mL of sperm washing medium (Dulbecco’s phosphate-buffered saline (Gibco, Gaithersburg, MD) supplemented with 0.1% FAF-BSA and 10 μ g/mL gentamicin). The sperm was washed by centrifugation in 45% Percoll solution and then in modified Tris-buffered medium. After, the semen pellet was resuspended in IVF medium and the concentration was adjusted to 0.5×10^6 cells/mL.

The fertilization drops containing the oocytes received 50 μ L of the sperm suspension (final concentration of 0.25×10^6 cells/mL) and gametes were co-incubated at 38.5 °C in 5% CO₂/humidified air for 4 h. Presumptive zygotes were washed and cultured in groups in 500 μ L of porcine zygote medium 3 plus 1.69 mM arginine and 5 μ M PS48 (MU2) in a four-well dish at 38.5 °C in 5% CO₂/humidified air. After 28–30 h of cultured the cleaved embryos were moved to a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 38.5 °C until day 6 post-fertilization. Day-6 blastocysts were washed in TL-Hepes-buffered saline and transferred to a physiological saline solution at pH 7.4 until the zona pellucida (ZP) and any sperm attached were removed. The embryos were washed in TL-Hepes buffered saline and collected in individual tubes for DNA isolation.

Parthenogenetically activation (PA) of porcine oocytes

Metaphase II (MII)-stage oocytes were selected for TPEN activation (200 μ M) for 30 min in TL-Hepes-buffered saline (Lee et al. 2015). Activated oocytes were cultured in MU2 medium supplemented with cytochalasin B (7 μ g/ μ L) for 4 h, washed three times and then cultured in MU2 in a four-well dish at 38.5 °C in 5% CO₂/humidified air. After 28–30 h of cultured the cleaved embryos were moved to a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 38.5 °C until day 7 post-activation.

Oocytes and embryos DNA extraction

Fifty-seven Day-6 in vitro-produced (IVP) porcine embryos and thirteen metaphase II (MII)-stage oocytes were collected for DNA isolation. Single porcine blastocyst and MII oocytes were suspended in 5 μ L of lysis buffer I (LBI) (40 mM Tris, pH 8.9; 0.9% Triton X-100; 0.9% Nonidet P-40; 0.4 mg/mL proteinase K) and incubated at 65 °C for 15 min and then heated to 95 °C for 10 min. Cell lysate from each blastocyst stage embryo (2 μ L) and each MII oocyte (2 μ L) were used to determine sex by PCR.

Parthenogenetically activated (PA) and in vitro fertilized (IVF) embryos biopsy

A total of twelve Day-7 in vitro-produced embryos and twelve parthenogenetically embryos (all female) were placed in drops of approximately 100 μ L of TL-Hepes-buffered saline for biopsy. Then, a portion of the trophectoderm (TE) was manually sectioned by using a needle blade (Fine Sciences Tools, Foster City, CA) (Bredbacka et al. 1995). The biopsies were collected in individual tubes and suspended in 3 μ L of lysis buffer I (LBI) (40 mM Tris, pH 8.9; 0.9% Triton X-100; 0.9% Nonidet P-40; 0.4 mg/mL proteinase K) and incubated at 65 °C for 15 min and then heated to 95 °C for 10 min. Cell lysate from each embryo biopsy (1 μ L) was used to determine sex by PCR.

Polymerase chain reaction

Primers *DDX3*-R(GCCACTAGAATTGGGCTTTT TCCT) and *DDX3*-F (TGCTTGCTCGTGATTTCTT GGA) were designed in regions of same identity

between the X and Y porcine chromosomes and were expected to yield a PCR-fragment of 485-bp for *DDX3X* target sequence and 545-bp for *DDX3Y*. PCR reactions were performed by using different sources of DNA polymerase as OneTaq Hot Start (NEB, USA) DNA Polymerase or LA Taq Polymerase (TaKaRa Bio). Two different melting temperatures (64 °C for *Sus scrofa*; 58 °C for *Bos Taurus*, *Bison Bison* and *Ovis aries*) and two different extension temperatures (68 °C for OneTaq protocol or 72 °C for LaTaq protocol) were defined according to the species and DNA polymerase applied. OneTaq PCR mix was prepared by adding the equivalent to 0.1 μ L of DNA polymerase, 2.5 μ L of 1 \times One Taq Standard Buffer, 0.5 μ L of 10 mM dNTP mix solution and 1 μ L of each forward and reverse primers (10 μ M) adjusting the final reaction volume with water to 12.5 μ L. For the LaTaq mix we added 0.2 μ L of DNA polymerase, 8.3 μ L of 2 \times GC Buffer I, 2.7 μ L of 2.5 mM dNTP mix solution, 1 μ L of each forward and reverse primers (10 μ M), and water to complete a final volume of 16.7 μ L. Amplification conditions were initial denaturation step at 94 °C for 30 s followed by 35 cycles of denaturation at 94 °C for 15 s, annealing for 30 s, extension for 1 min with an addition of 1 s per cycle, followed by a final extension for 2 min by using a Mastercycler EP Gradient machine (Eppendorf Hauppauge, NY). PCR products were separated by electrophoresis on a 2% agarose gel. Representative images from our sex determination assay are shown in Fig. 1. The sex determination assay developed by Hao et al. (2006) was applied to analyze samples for which the gender was not previously available. However, this assay was not successful for oocytes, biopsies and blastocysts samples because of their limited amounts of genomic DNA present.

Isolation and sequencing of *DDX3* fragments

DDX3 PCR products from one boar and one sow (1/2 Domestic 1/2 NIH c/c mini) were cloned into a vector for sequencing (pCR4- Topo, Invitrogen, Grand Island, NY) by the manufacturer's protocol. The TOPO cloning reactions were transformed into High Efficiency DH5 α chemically competent *Escherichia coli* cells (NEB) and plated onto Luria–Bertani/ChromoMax IPTG X-Gal (Thermo Fisher Scientific)/Kanamycin (30 mg/mL) agar plates overnight. For each pig, 5 clones were selected and submitted for

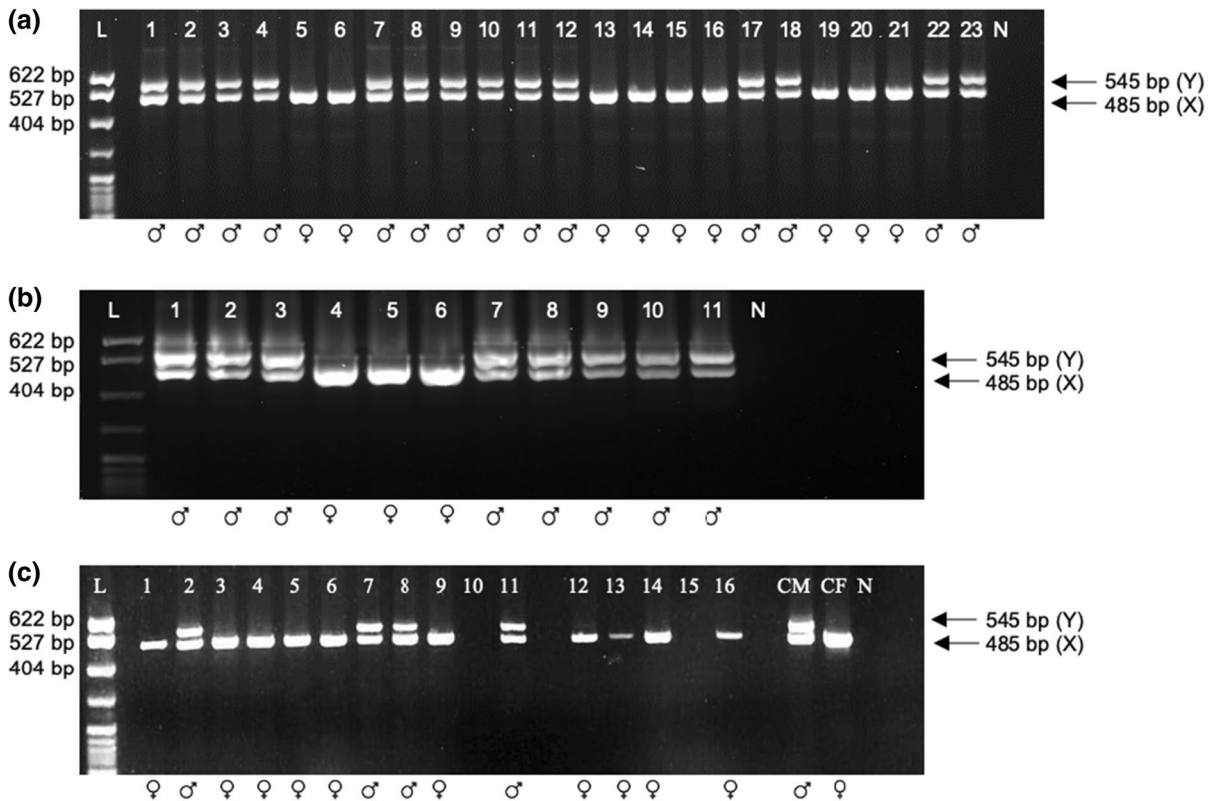


Fig. 1 Sex determination based on the amplification of porcine *DDX3X* and *DDX3Y* genes. **a** PCR-amplification of *DDX3X* and *DDX3Y* fragments from different pig breeds: 7/8 Minnesota Mini 1/8 Domestic (lanes 1 to 6), 1/2 Domestic 1/2 NIH c/c mini (lanes 7 to 16), 1/2 Domestic 1/2 Minnesota Mini (lanes 17 to 21), 1/4 Minnesota mini 3/4 Domestic (lanes 22 and 23) **b** Sex determination of Day-35 fetuses (lanes 1 to 11) **c** Sex determination of Day-6 in vitro-produced embryos (lanes 1 to

11) and GV-stage oocytes (lanes 12 to 16). In each lane the presence of two bands (545 and 485 bp) indicates male and one band (485 bp) female. L: pBR322/MspI ladder (New England Biolabs, Beverly, MA), CM: male positive control (male genomic DNA), CF: female positive control (female genomic DNA), N: Non-template control. Sex indicated in which line (♂: male; ♀: female)

DNA isolation by using a PureLink Quick Plasmid Miniprep kit (Invitrogen). The clones were subject to traditional bisulfite (Sanger) sequencing at the University of Missouri-Columbia DNA core facility and then, the sequences obtained were submitted to a global alignment based on Needleman-Wunsch algorithm by using the Pairwise Sequence Comparison (PASC) tool at the National Center of Biotechnology Information (NCBI) webpage.

In silico analysis of *DD3X* length polymorphism across different mammalian species

A sequence database was designed by first blasting (Blastn) our *DDX3 X/Y*-linked pig amplicons (including the primer binding sites) on all mammalian

(sub)species (taxid: 40674) reference genomic sequences (refseq_genomic) available. As only few species have a high-quality Y chromosome assembly, just the species that had a query coverage of 100% for the *DDX3Y* pig amplicon and not more than 3 mismatches at the primer binding sites were selected. Then, the corresponding chromosome, gene and sex information were obtained for each sequence analyzed and the size difference between the *DDX3Y* and *DDX3X* amplicons were predicted.

Statistical analysis

Statistical differences were analyzed by Fisher exact test (GraphPad Prism®). Significant differences were defined when $p < 0.05$.

Results

Porcine sex determination by *DDX3X/Y*

Amplification among different lines and samples

Representative images from *DDX3X/Y* PCR amplification using genomic DNA or cell lysate samples are shown in Fig. 1. Analysis of cell lysate samples from single hair follicles and genomic DNA extracted from 87 pigs among different breeds coincided with the

phenotypic sex (data partially shown, Fig. 1). Similarly, the results from Day-35 fetuses samples previously submitted to a different sex determination protocol (Hao et al. 2006) demonstrated 100% of concordance (Fig. 1 and Table 2). When genomic DNA from porcine blastocysts was used, the PCR analysis was able to identify the sex in 96.5% of the embryos with the ratio of male and female being 49.1 and 47.4%, respectively (Table 2). For GV-stage oocytes (all female) and embryo biopsies (Fig. 2 and

Table 2 Identification of the *DDX3X/Y* bands in different porcine samples by using a PCR-based method

Porcine tested samples	Total number of samples	PCR detection efficiency (%)	Number of samples (%) with only <i>DDX3X</i> bands	Number of samples (%) with only <i>DDX3X</i> and <i>DDX3Y</i> bands	Number of samples (%) with only <i>DDX3Y</i> bands	Number of samples (%) with no bands
MII-stage oocytes	13	11 (84.6) ^a	11(84.6)	0(0)	0	2(15.4) ^a
Day-6 blastocysts	57	55 (96.5) ^{ab}	28 (49.1)	27(47.4)	0	2(3.5) ^a
Embryo biopsies	24	20 (83.3) ^a	14 (58.3)	6 (25.0)	0	4 (16.7) ^a
Day-35 fetuses	11	11 (100) ^{ab}	3 (27.3)	8 (72.7)	0	0 (0)
Hair follicle	4	4 (100) ^{ab}	2 (50.0)	2 (50.0)	0	0 (0)
Tail genomic DNA	87	87 (100) ^b	44(50.6)	43 (49.4)	0	0 (0)

^{a-b}Values in the same column without a common superscript differ significantly ($P < 0.05$)

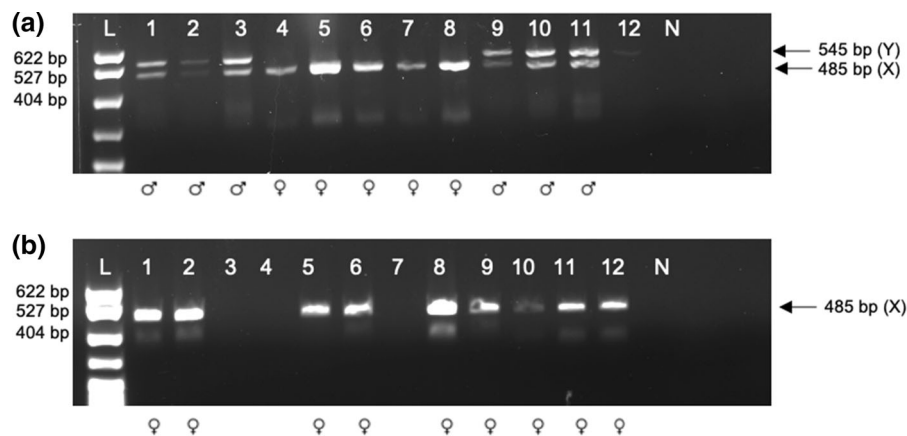


Fig. 2 Sex determination of porcine embryo biopsies based on the amplification of porcine *DDX3X* and *DDX3Y* genes. **a** Sex determination of biopsy samples from Day-7 in vitro-produced embryos **b** Sex determination of biopsy samples from Day-7

parthenogenetically activated (PA) embryos. L: pBR322/MspI ladder (New England Biolabs, Beverly, MA), N: Non-template control. Sex indicated in which line (♂: male; ♀: female)

DDX3-Y 1	GCCACTAGAAATTGGGCTTTTTCTGAGCGGAACTGGTGAAGGGCTTCCTCTCTATCTCTC	60
DDX3-X 1	GCCACTAGAAATTGGGCTTTTTCTGAGCGGAACTGGTGAAGGGCTTCCTCTCTATCTCTC	60
DDX3-Y 61	TGTGATCGGTCTCCATGAATACTGGTACAAGCATATCCTTCATGGTATAAGAAGTCTCTCC	120
DDX3-X 61	TGAGATCGGTCTCCATGGATACTGGTACAAGCATATCCTTCATGGTATAAGAAATCTCTCT	120
DDX3-Y 121	AAAGAATCGGCACCCTTTTTGGTCTCCACAAAACTAAAGTCAGTGAATCCTTCCCTGAA	180
DDX3-X 121	AGAGAATCTGCACCCTTTTTGGTCTCCACAAACACTAAGTTAGTGAATCTTTGCCT--A	178
DDX3-Y 181	AAATCACATTGAAAGTTCGTTTACTAAGAGCAACTTAGCATCATTGCCTGAGCTAAATA	240
DDX3-X 179	AAATTA-----AAGTA----TTAC-AAG-GTTTCT-----ATTAC-----TAA-TA	212
DDX3-Y 241	TTAACTAGTTTATTATCTCTCTTTGCACTCAGAACACATGCACAGGGAACATAAATAAAAA	300
DDX3-X 213	GTG----GTTTAGT---TCTCCTTACACTTAGTAAACTTGCAAATAGAGGCAATTAAAA-	264
DDX3-Y 301	TAAACTTGGTTTTAATGGCTAGAAAAG----TGGGCAGAGTTAGGTTATTGCTAAAGAGA	356
DDX3-X 265	TAGACCA--TTTTAAC---AGAATGGATTTGGATGGAGATTTCTTATTACCTAAG---	315
DDX3-Y 357	TGCAAACAGTCCCTTATACCTAATACTTTTTCTCAGAACTTTACCTGTTGCATTTAGGA	416
DDX3-X 316	--CCACCAGAG--TGATGC-TAA-----AATTATACCTGTTGCATTTAGAA	356
DDX3-Y 417	GGTCAAGCAGAAATGACCTTTTGTCTGACTCTTCTACCCAAACTACTTTCTGTGTGATGT	476
DDX3-X 357	GGTCAAGCAGAAATGACCGTTTGTCTGACTCTTCCACCCAAACTACTTTCTGTGTAATGT	416
DDX3-Y 477	TCTCAGAGGTAGAGCCAACCTACCTACAGCCAGAAAAGATATATTCATCCAAGAAATCAC	536
DDX3-X 417	TCTCAGAGGTTGAGCCAACCTTCCCTACAGCCAAAAGATATATTCATCCAAGAAATCAC	476
DDX3-Y 537	GAGCAAGCA 545	
DDX3-X 477	GAGCAAGCA 485	

Fig. 3 Alignment of the *DDX3Y* and *DDX3X* fragments of 1/2 Domestic 1/2 NIH c/c mini pigs. Underlined sequences indicate primers positions. -, alignment gaps; *, base substitution

Table 2) the identification rate was lower when compared to tail genomic DNA samples (84.6% and 83.3% vs. 100%), however there was no significant difference when compared to the other samples tested, $P > 0.05$ (Table 2).

Sequence analysis of the porcine *DDX3X/Y* gene amplified fragment

The PCR-fragments showed the expected size of 485 and 545-bp and matched the nucleotide sequence for the targeted *DDX3X* and *DDX3Y* regions, respectively (data not shown). Comparing the *DDX3Y* and *DDX3X* fragments showed the presence of regions of homology which resulted in an identity of 76% between the

Y and *X* chromosomes. In addition, several gaps resulting in size variation between the *DDX3Y* and *DDX3X* amplicons were found (Fig. 3).

Sex determination by *DDX3X/Y* amplification across different mammalian species

In silico analysis confirmed the reliability of the *DDX3X/Y* gene for sex determination across different mammalian species. A total of 13 species demonstrated a X–Y amplicon length polymorphism for the *DDX3* gene (Table 3). When we tested female and male DNA samples from *Bison bison*, we were able to confirm the sex previously recorded with PCR-fragments showing the expected in silico length

Table 3 In silico validation of the porcine DDX3X/Y primer set across different mammalian species

Sub(species)	Chromosome X				Chromosome Y					
	Females				Males and individuals without sex information					
	Sequence ID	Gene or product name	Sex	No. of bp	Chr : expected chr	Sequence ID	Gene or product name	Sex	N° of bp	Chr: expected chr
<i>Sus scrofa</i>	NC_010461.5	DDX3X	F	485	X: X	NW_006727254.1	ATP-dependent RNA helicase DDX3X	M	543	u : Y
<i>Balaenoptera acutorostrata scammoni</i>						NW_006728671.1	ATP-dependent RNA helicase DDX3X	M	509	u : X
<i>Bison bison bison</i>						NW_011495066.1	ATP-dependent RNA helicase DDX3X-like	M	547	u : Y
						NW_011494443.1	ATP-dependent RNA helicase DDX3X-like	M	500	u : X
<i>Camelus ferus</i>						NW_006212573.1	ATP-dependent RNA helicase DDX3X	M	553	u : Y
						NW_006212775.1	ATP-dependent RNA helicase DDX3X	M	509	u : X
<i>Capra hircus</i>						NW_017189685.1	ATP-dependent RNA helicase DDX3X-like	M	547	u : Y
						NW_017189629.1	DDX3X	M	501	u : X
<i>Desmodus rotundus</i>						NW_020092012.1	ATP-dependent RNA helicase DDX3X-like	M	559	u : Y
						NW_020093861.1	DDX3X	M	511	u : X
<i>Equus asinus</i>						NW_014638617.1	ATP-dependent RNA helicase DDX3X-like	M	542	u : Y
						NW_014637499.1	DDX3X	M	515	u : X
<i>Equus przewalskii</i>						NW_007678051.1	ATP-dependent RNA helicase DDX3X-like	M	542	u : Y
						NW_007679417.1	ATP-dependent RNA helicase DDX3X	M	516	u : X
<i>Globicephala melas</i>						NW_022135104.1	ATP-dependent RNA helicase DDX3X-like	M	549	Y : Y
						NW_022134945.1	DDX3X	M	509	u : X

Table 3 continued

Sub(species)	Chromosome X				Chromosome Y					
	Females				Males and individuals without sex information					
	Sequence ID	Gene or product name	Sex	No. of bp	Chr : expected chr	Sequence ID	Gene or product name	Sex	No. of bp	Chr: expected chr
<i>Homo sapiens</i>	NC_000023.11	DDX3X	u	999	X: X	NW_015504423.1	ATP-dependent RNA helicase DDX3X-like	M	549	u : Y
<i>Miniopterus natalensis</i>						NW_015504240.1	DDX3X	M	510	u : X
<i>Monodon monoceros</i>						NW_021703779.1	ATP-dependent RNA helicase DDX3X-like	M	549	u : Y
<i>Neophocaena asiakororientalis</i>						NW_021703779.1	DDX3X	M	510	u : X
<i>Odocoileus virginianus texanus</i>						NW_020171823.1	ATP-dependent RNA helicase DDX3X-like	M	549	u : Y
						NW_020174707.1	DDX3X	M	512	u : X
						NW_018343486.1	ATP-dependent RNA helicase DDX3X-like	M	560	u : Y
						NW_018335052.1	DDX3X	M	500	u : X
Sub(species)	Chromosome Y					Predicted base pairs difference between DDX3Y and DDX3X amplicons				
	Sequence ID	Gene or product name	Sex	No. of bp	Chr: expected chr	Sequence ID	Gene or product name	Sex	No. of bp	Chr: expected chr
<i>Sus scrofa</i>	NC_010462.3	DDX3Y	M	545	Y : Y					
<i>Balaenoptera acutorostrata scammoni</i>										60
<i>Bison bison bison</i>										34
<i>Camelus ferus</i>										47
<i>Capra hircus</i>										44
<i>Desmodus rotundus</i>										46
<i>Equus asinus</i>										48
<i>Equus przewalskii</i>										27
<i>Globicephala melas</i>										26
<i>Homo sapiens</i>										40
<i>Miniopterus natalensis</i>	NC_000024.10	DDX3Y or DBY	u	552	Y: Y					447
<i>Monodon monoceros</i>										39
										39

Table 3 continued

Sub(species)	Chromosome Y				Predicted base pairs difference between DDX3Y and DDX3X amplicons
	Sequence ID	Gene or product name	Sex	No. of bp	
<i>Neophocaena asiacaorientalis</i>					37
<i>asiacaorientalis</i>					
<i>Odocoileus virginianus texanus</i>					60

DDX3 X/Y pig amplicons (including the primers binding sites) were blasted across different mammalian (sub)species Sequence ID = NCBI reference accession format; No. of bp = number of base pairs; M, male; F, female; Chr., chromosome, u, unknown, Expected chromosome = expected chromosome assignment based on the level of sequence identity

differences of approximately 47 bp. Also, the assay was able to identify the sex in samples from *Bos taurus* and *Ovis aries* which were not included in the in silico analysis (Fig. 4). The number of species with query coverage greater than 95% for the X and Y chromosomes was 43 and 13, respectively. These discrepant values between X and Y assemblies restricted the number to 13 species analyzed.

Discussion

In this study, we developed a new simple and robust PCR-based assay for sex determination in pigs by using a unique primer set to amplify the *DDX3X/Y* gene. As expected, DNA from females produced one band and DNA from males generated two bands. This method proved to be applicable among different samples and across different mammalian species. This assay becomes a new alternative to improve the efficiency of projects involving genetically modified embryos or cloneable cells, and to wildlife studies. The creation of pig models to decipher the mechanism of human diseases, to enhance porcine disease resistance and to test the effects of gene therapy and stem cells treatments have proven a significant progress in biomedical and agriculture research (Madeja et al. 2019).

Our *DDX3X/Y* approach was able to identify the sex in pigs from six different lines and diagnosis the sex from samples containing small amounts of genomic DNA. This assay also could be applied to samples that were collected non-invasively such as (hair), which often contain highly degraded DNA. The sex was 100% assigned when tail genomic DNA, Day-35 fetuses and hair samples were used. For biopsies samples, blastocysts and oocytes the PCR detection efficiency was lower (83.3%; 96.5% and 84.6%, respectively), however no detection errors were observed. We believe that the unidentified samples were due to PCR failure and the different diagnostic rates between embryos, biopsies samples and oocytes is likely due to limited amounts of genomic DNA present at GV stage oocyte (1 cell, 4C) and the embryo biopsies in comparison to blastocysts (~ 40 cells). In a previous study performed by Sembon et al. (2008) where they used an *AMEL*-PCR method to sex identification in pigs, they could not detect any bands in several oocytes and EA and IVF embryos samples.

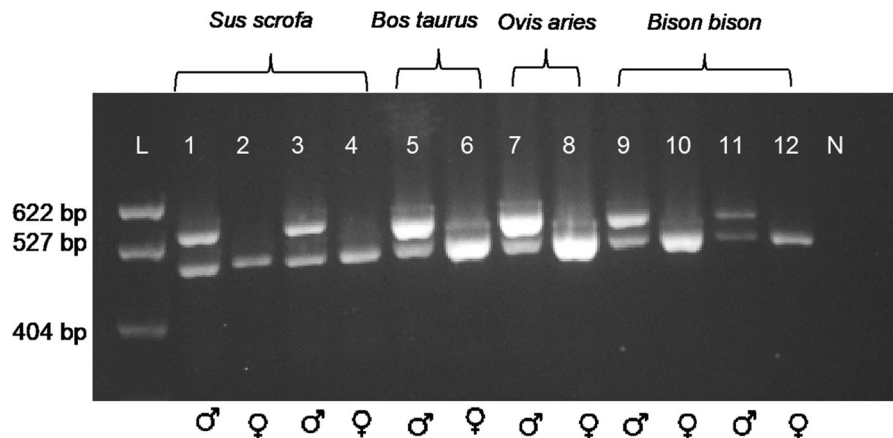


Fig. 4 Sex determination based on the amplification of *DDX3X* and *DDX3Y* genes across different mammalian species. *Sus Scrofa* hair (lanes 1–4), *Bos taurus* (lanes 5–6), *Ovis aries* (lanes 7–8) and *Bison bison* samples (lanes 9–12). Sex previously

recorded indicated in which line (♂: male; ♀: female). For all samples the expected results for both female and male samples were confirmed

They attributed it to human errors when extracting the DNA genomic or setting up the PCR reactions.

Alignment of the porcine amplicons for *DDX3Y* and *DDX3X* genes showed a difference of 60 bp between *DDX3Y* and *DDX3X* fragments. However, both *DDX3X* and *DDX3Y* demonstrated a 100% of identity with the *DDX3X/Y* nucleotide sequence for *Sus scrofa* deposited at NCBI. Although we used pigs from a specific line, we believe that the *DDX3* gene is very conserved when comparing different pig lines. Skinner et al. (2016) in an attempt to better understand the structure, the sequence, and evolution of the sex chromosomes observed that the *USP9Y-DDX3Y-UTY* is the only ancestral group of genes that have retained this pattern of rearrangement in pig, primates, mouse, cat, and dog. In cattle, a recent studied published by Liu et al. (2019) also demonstrated that the *DDX3Y*, *USP9Y* and *UTY* are still conserved in the X—degenerate region of the Y chromosome. *DDX3Y* gene has been shown an important role during human spermatogenesis suggesting its strong influence in sexual development. However, it may not be applicable for all primates and mice (Tyler-Smith 2008; Matsumura et al. 2019).

In vitro and in silico analysis confirmed the ability of the *DDX3X/Y* approach for sex diagnosis across different mammalian species. To our knowledge, this is the first study to confirm the application of the DEAD box protein gene for sex determination in pigs. Also, we were able to show that the same primers that

were used for pig *DDX3X/Y* amplification could be successfully used for domestic sheep (*Ovis aries*), American bison (*Bison bison*) and European cattle (*Bos taurus*) samples. Our in silico analysis confirmed X–Y amplicon length polymorphisms for the *DDX3* gene in other mammals as human, bat, dolphin, whale, deer, domestic goat, donkey and wild horse. Sex identification in some marine mammals are difficult to genotype based on their phenotype due to their poor sexual dimorphism and internal genitalia (Morin et al. 2005). We believe our *DDX3X/Y* PCR-based method can provide an easy and new alternative for sex determination in marine mammal specimens. Additionally, the ability to sex differentiate animals originated from *Bos taurus* and *Ovis aries* makes the *DDX3X/Y* PCR assay useful for reproductive and biomedical studies involving these important livestock animals. Lastly, another important application of this assay would be for the management of endangered species as *Camelus ferus*, *Equus przewalskii* and *Neophocaena asiaorientalis* by improving the propagation rate of those species. We believe that with the continued advances in the genome sequencing tools, it will allow us to expand the use of this sex assay on additional species.

In conclusion, the ability to rapidly determine the sex of porcine embryos and cloneable cells, without the need of restriction endonuclease digestion or simultaneous amplification of an internal control, makes the *DDX3X/Y* an invaluable tool for

transgenesis. Furthermore, the use of a unique primer for cross-amplification in different mammalian species can simplify sex-linked studies. Our *DDX3X/Y* assay showed to be simple, specific, and highly reproducible showing a wide range of applications for researchers and breeding programs.

Acknowledgements We would like to thank Dr. Robert Schnabel, Msc. Emma Stephenson, MSc. Paula Chen, Msc. Eleonore O'neil and Dr. Jon Green for generously provide genomic DNA and tissue samples from different species to this present study. The results presented here were funded by the National Institutes of Health via funding from OD, NIAID and NHLBI via U42OD011140.

Authors' contributions C. G. L., W. C. W., and K. D. W. designed the experiments. C.G.L., A.M.S., M. S. S. and L. D. S. conducted the experiments. C.G.L., K. D. W., and R. S. P. analyzed the data and wrote the manuscript.

Funding The results presented here were funded by the National Institutes of Health via funding from OD, NIAID and NHLBI via U42OD011140.

Compliance with ethical standards

Conflict of interest The authors have declared that no conflict of interest exists.

References

- Bredbacka P, Kankaanpää A, Peippo J (1995) PCR-sexing of bovine embryos: a simplified protocol. *Theriogenology* 44:167–176. [https://doi.org/10.1016/0093-691X\(95\)00166-6](https://doi.org/10.1016/0093-691X(95)00166-6)
- Chen PR, Redel BK, Spate LD, Ji T, Salazar SR, Prather RS (2018) Glutamine supplementation enhances development of in vitro-produced porcine embryos and increases leucine consumption from the medium. *Biol Reprod* 99:938–948. <https://doi.org/10.1093/biolre/iy129>
- Dash HR, Rawat N, Das S (2020) Alternatives to amelogenin markers for sex determination in humans and their forensic relevance. *Mol Biol Rep* 47:2347–2360. <https://doi.org/10.1007/s11033-020-05268-y>
- Ditton HJ, Zimmer J, Kamp C, Rapert-De Meyts E, Vogt PH (2004) The AZFa gene *DBY* (*DDX3Y*) is widely transcribed but the protein is limited to the male germ cells by translation control. *Hum Mol Genet* 13:2333–2341. <https://doi.org/10.1093/hmg/ddh240>
- Fontanesi L, Scotti E, Russo V (2008) Differences of the porcine amelogenin X and Y chromosome genes (*AMELX* and *AMELY*) and their application for sex determination in pigs. *Mol Reprod Dev* 75:1662–1668. <https://doi.org/10.1002/mrd.20903>
- Gokulakrishnan P, Kumar RR, Sharma BD, Mendiratta SK, Sharma D (2012) Sex determination of cattle meat by polymerase chain reaction amplification of the *DEAD* box protein (*DDX3X/DDX3Y*) gene. *Asian Australas J Anim Sci* 25:733–737. <https://doi.org/10.5455/vetworld.2012.526-529>
- Grzybowski G, Prusak B, Romaniuk B (2006) A novel variant of the amelogenin gene (*AMEL-X*) in cattle and its implications for sex determination. *Anim Sci Pap Rep* 24:111–118
- Hao YH, Yong HY, Murphy CN et al (2006) Production of endothelial nitric oxide synthase (eNOS) over-expressing piglets. *Transgenic Res* 15:739–750. <https://doi.org/10.1007/s11248-006-9020-8>
- Hrovatin K, Kunej T (2018) Genetic sex determination assays in 53 mammalian species: literature analysis and guidelines for reporting standardization. *Ecol Evol* 8:1009–1018. <https://doi.org/10.1002/ece3.3707>
- Lee K, Davis A, Zhang L et al (2015) Pig oocyte activation using a Zn²⁺ + chelator, TPEN. *Theriogenology* 84(6):1024–1032. <https://doi.org/10.1016/j.theriogenology.2015.05.036>
- Liu R, Low WY, Tearle R, Koren S, Ghurye J, Rhie A, Phillippy AM, Rosen BD, Bickart DM, Smith TPL, Hiendleder S, Williams JL (2019) New insights into mammalian sex chromosome structure and evolution using high-quality sequences from bovine X and Y chromosomes. *BMC Genom*. <https://doi.org/10.1186/s12864-019-6364-z>
- Madeja ZE, Pawlak P, Piliszek A (2019) Beyond the mouse: non-rodent animal models for study of early mammalian development and biomedical research. *Int J Dev Biol* 63:187–201. <https://doi.org/10.1387/ijdb.180414ap>
- Marshall Graves JA (1998) Evolution of the mammalian Y chromosome and sex-determining genes. *J Exp Zool* 281:472–481
- Matsumura T, Endo T, Isotani A, Ogawa M, Ikawa M (2019) An azoospermic factor gene, *Ddx3y* and its paralog, *Ddx3x* are dispensable in germ cells for male fertility. *J Reprod Dev* 65:121–128. <https://doi.org/10.1262/jrd.2018-145>
- Morin PA, Nestler A, Rubio-Cisneros NT, Robertson KM, Mesnick SL (2005) Interfamilial characterization of a region of the *ZFX* and *ZFY* genes facilitates sex determination in cetaceans and other mammals. *Mol Ecol* 14:3275–3286. <https://doi.org/10.1111/j.1365-294X.2005.02651.x>
- Pomp D, Good BA, Geisert RD, Corbin CJ, Conley AJ (1995) Sex identification in mammals with polymerase chain reaction and its use to examine sex effects on diameter of day-10 or-11 pig embryos. *J Anim Sci* 73:1408–1415. <https://doi.org/10.2527/1995.7351408x>
- Redel BK, Prather RS (2015) Meganucleases revolutionize the production of genetically engineered pigs for the study of human diseases. *Toxicologic Pathol* 44:428–433. <https://doi.org/10.1177/0192623315613160>
- Ryu J, Prather RS, Lee K (2018) Use of gene-editing technology to introduce targeted modifications in pigs. *J Animal Sci Biotechnol* 9:5. <https://doi.org/10.1186/s40104-017-0228-7>
- Sathasivam K, Kageyama S, Chikuni K, Notarianni E (1995) Sex determination in the domestic pig by DNA amplification using the *HMG*-box sequence. *Anim Reprod Sci* 38:321–326. [https://doi.org/10.1016/0378-4320\(94\)01371-R](https://doi.org/10.1016/0378-4320(94)01371-R)

- Sekiguchi T, Sasaki H, Kurihara Y, Watanabe S, Moriyama D, Kurose N, Matsuki R, Yamazaki K, Saeki M (2010) New methods for species and sex determination in three sympatric Mustelids, *Mustela itatsi*, *Mustela sibirica* and *Martes melampus*. *Mol Ecol Resour* 10:1089–1091. <https://doi.org/10.1111/j.1755-0998.2010.02842.x>
- Sembon S, Suzuki SI, Fuchimoto DI, Iwamoto M (2008) Sex identification of pigs using polymerase chain reaction amplification of the amelogenin gene. *Zygote* 16:327–332. <https://doi.org/10.1017/S0967199408004826>
- Skinner BM, Sargent CA, Churcher C et al (2016) The pig X and y chromosomes: structure, sequence, and evolution. *Genome Res* 26:130–139. <https://doi.org/10.1101/gr.188839.114>
- Strah R, Kunej T (2019) Molecular sexing assays in 114 mammalian species: in silico sequence reanalysis and a unified graphical visualization of diagnostic tests. *Ecol. Evol* 9:5018–5028. <https://doi.org/10.1002/ece3.5093>
- Tyler-Smith C (2008) An evolutionary perspective on Y-chromosomal variation and male infertility. *Int J Androl* 31:376–382. <https://doi.org/10.1111/j.1365-2605.2008.00889.x>
- Yang H, Wu Z (2018) Genome editing of pigs for agriculture and biomedicine. *Front. Genet.* 9:360. <https://doi.org/10.3389/fgene.2018.00360>
- Zarzoso-Lacoste D, Jan PL, Lehnen L et al (2018) Combining noninvasive genetics and a new mammalian sex-linked marker provides new tools to investigate population size, structure and individual behaviour: an application to bats. *Mol Ecol Resour* 18:217–228. <https://doi.org/10.1111/1755-0998.12727>
- Zhang X, Miao Y, Zhao JG et al (2010) Porcine oocytes denuded before maturation can develop to the blastocyst stage if provided a cumulus cell-derived coculture system. *J Anim Sci* 88:2604–2610. <https://doi.org/10.2527/jas.2009-2714>

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