Review and Update: Genomic and Molecular Advances in Sex Determination and Differentiation in Small Animals

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Contents

Inherited disorders of sexual development are important to identify as a cause of inherited infertility or sterility in humans and animals. Investigation of these disorders in dogs and cats can identify new mutations, allowing us to eliminate inherited disorders from breeding populations, while contributing to the understanding of mammalian sexual development and differentiation. This review updates an overview of normal mammalian sexual development while discussing disorders of sexual development at three consecutive levels, as errors in sex chromosome constitution, gonadal sex determination or phenotypic sexual development. The molecular mechanisms controlling sexual development and current molecular methods to identify causative mutations are illustrated in three specific examples of abnormal sexual development reported in small animals: XX sex reversal, Persistent Mullerian Duct Syndrome and cryptorchidism. Identification of causative mutations and development of practical tests to identify carrier and affected animals will provide effective mechanisms to reduce the prevalence of these disorders in small animals.

Introduction

Both the canine and feline genome sequences are now available online (www.ncbi.nlm.nih.gov), which is facilitating efforts to identify genes controlling heritable traits and mutations causing inherited disorders in these species. Using these resources and new methods to study the genome, genes involved in sexual development are being identified. Three disorders are discussed below to illustrate this point, provide examples at different levels of sexual development and update the state of knowledge on these disorders.

In mammals, sexual development is controlled at three sequential steps; the establishment of chromosomal sex, the determination of gonadal sex, then development of phenotypic sex. Chromosomal sex is established at the time of fertilization, such that zygotes normally have either an XX or XY sex chromosome constitution. Normal XX and XY embryos develop similarly, being sexually indifferent when the bipotential gonad arises from the urogenital ridge. If the gonad is removed from either XX or XY embryos at this stage, the embryo develops as a phenotypic female. This indicates that the presence of a functional testis is critical to development of the male phenotype. The end of the sexually indifferent stage in XY embryos is marked by testis induction. The Y-linked SRY (sex determining region Y) gene encodes a testis determining factor that initiates testis formation in the bipotential gonad. Testis secretion is directly or indirectly required to masculinize the internal ducts and external genitalia. These include Mullerian inhibiting substance (MIS), testosterone and insulin-like peptide 3 (INSL3). In the absence of the Y chromosome and SRY, an ovary develops, although this process is incompletely understood, and female genitalia develop. To facilitate differential diagnosis, abnormalities of sexual development can be categorized according to the first step that differs from normal. In future, these disorders are likely to be categorized according to the causative genetic defect.

Abnormalities of chromosomal sex

These disorders are caused by abnormalities in chromosome number or structure. The best known examples are abnormalities in the number of sex chromosomes, such as XXY or X0 chromosome constitutions, in which underdeveloped rather than ambiguous sexual phenotypes are present. These can be identified by conventional karyotyping. However, chimeras, mosaics or chromosomal translocations affecting genes involved in sex determination also rarely occur. These have been reviewed in more detail elsewhere (Meyers-Wallen 1989). More specific methods may be needed to identify genes affected by structural abnormalities, such as polymerase chain reaction (PCR) to examine a specific gene sequence. More sophisticated techniques such as fluorescent in situ hybridization and comparative genome hybridization may be utilized in future, but have yet to be routinely applied for diagnostic purposes in small animals.

Abnormalities of gonadal sex

The process of mammalian gonadal sex determination is more complex than originally proposed. For example, it was hypothesized that testis determination was an active process while ovarian development occurred by default. Research now suggests that both testis- and ovarian-promoting pathways are active during gonadal sex determination, antagonizing one another in the bipotential gonad (DiNapoli and Capel 2008). Two genes involved in mammalian testis induction must exceed an expression threshold within a critical period to initiate normal testis development. These testis determining genes are the Y-linked SRY, and the autosomal gene SOX9 (sex determining region Y-box 9), which may be indirectly upregulated by SRY. A recently identified gene, R-spondin1 (RSPO1), probably acting in concert with the canonical pathway of the wingless-related protein family (WNT) through beta catenin, may have a role in suppressing SOX9-mediated testis pathways in the developing ovary (Kim and Capel 2006). Recent
evidence from other tissues indicates that \( SOX9 \) and beta catenin act antagonistically (reviewed in Kiefer 2007), raising the possibility that the \( RSPO1/\text{WT1} \) beta catenin pathway promotes ovarian development by blocking \( SOX9 \) before it can activate transcription.

Abnormalities identified at this level, termed sex reversal disorders, have increased our understanding of the genetic control of gonadal sex determination. In sex reversed individuals, the chromosomal and gonadal sex disagree. For example, in XX sex reversal (XXSR), the chromosomal sex is female (XX), yet the gonad contains testicular tissue. Affected individuals are categorized as XX males, having bilateral testes, or XX true hermaphrodites (XXTH), having both testis and ovarian tissue in one or both gonads. The most common aetiology of human XXSR is \( SRY \) translocation, which is not detected in a conventional karyotype, but can be identified by molecular assays such as PCR. However, approximately 10–20% of human XX males and most human XXTH do not have an \( SRY \) translocation, and thus have the \( SRY \)-negative form of XXSR (Koopman 1999), which is also the form of XXSR identified in domesticated animals.

**\( SRY \)-negative XX sex reversal**

In addition to humans, \( SRY \)-negative XXSR is a naturally occurring disorder in dogs (Meyers-Wallen et al. 1999), goats (Pailhoux et al. 2001) and several other domesticated species. A causative mutation is known only in the goat. This form of XXSR is not a naturally occurring disorder in the mouse, but has been modelled transgenically. Notably, both XX males and XXTH occur as siblings in some human families, suggesting a common genetic aetiology (Sarafooglou and Ostrer 2000).

At present, there are three known causes of \( SRY \)-negative XXSR in humans and animals. First, XX male mice are caused by transgenic \( SOX9 \) up-regulation during gonadal development (Bishop et al. 2000). Transgene transfer to a different mouse genetic background causes XXTH (Poirier et al. 2007). An \( SOX9 \) duplication has also been identified in a human XX male (Huang et al. 1999). Second, XX males in the polled intersex (PIS) goat are caused by a deletion in chromosome 1 (Pailhoux et al. 2001), which eliminates a regulatory region for \( FOXL2 \) and \( PISRT1 \) (Pannetier et al. 2005). Mutations in human \( FOXL2 \) are associated with premature ovarian failure, but not with sex reversal (DeBaere et al. 2003). Thirdly, a null mutation in \( R\)-spondin 1 (\( RSPO1 \)) was identified in human XX males from one family (Parma et al. 2006), while a different \( RSPO1 \) mutation was identified in an XXTH from a different family (Tomasselli et al. 2008). Interestingly, \( RSPO1 \) null mice develop as XXTH, but not XX males (Chassot et al. 2008). These three genes could be candidates for the canine disorder.

\( SRY \)-negative XXSR has been reported in at least 18 breeds, and an additional three were recently identified in our laboratory (Table 1). In a model studied in our laboratory, developed from the American cocker spaniel, XX males and XXTH occur as siblings, as in some human families. A complete uterus, hypospadias and cryptorchidism are present in XX males. While XXTH have ovotestes, they do not usually have other male internal genitalia, and externally may have normal female genitalia, or cranial displacement of the vulva and an enlarged clitoris with a bone. Rarely, affected XXTH have reproduced as females. The recommended treatment for affected dogs is gonadectomy and hysterectomy to prevent breeding of affected dogs and secondary testicular neoplasia or pyometra.

A number of methods have been used in the search for the causative genetic defect in the canine model, including candidate gene screening, linkage analysis using microsatellite markers, fine mapping using single nucleotide polymorphism (SNP) markers and direct genome sequencing. Screening of several candidates initially did not identify mutations that consistently segregate with the XXSR phenotype (Kothapalli et al. 2005, 2006; Pujar et al. 2005). Genome wide linkage disequilibrium analysis identified a region in CFA29 linked to \( SRY \)-negative XXSR in the model pedigree (Pujar et al. 2007). Fine mapping and direct sequencing in the linked region is underway. Significantly, no linkage was detected to chromosomal regions located near \( SOX9 \) (CFA9), \( RSPO1 \) (CFA15), or the region homologous to PIS (CFA23). Since it is not known whether the disorder is genetically homogeneous or heterogeneous among breeds, exon scanning was used to screen two conserved upstream regions and five exons of \( RSPO1 \) in affected dogs from the model and other breeds (DeLorenzi et al. 2008). Although a single base pair substitution in exon 4 was identified in affected soft coated wheaten terriers, the same mutation was also present in females in a control group of this breed, ascertained for inherited eye disorders but not sex reversal. More research is needed to determine conclusively whether this substitution is a rare SNP allele or a mutation contributing to the affected phenotype in this breed. This emphasizes the importance of canine pedigree, breed and population studies in evaluating candidate mutations. The possibility that \( RSPO1 \) polymorphisms play a modifying role in sex reversal, such as changing threshold or severity of phenotypic expression, cannot be excluded, but the primary cause of canine \( SRY \)-negative XXSR remains unknown. At present, it is recommended that affected dogs and their parents be removed from the breeding population. Although some siblings of affected dogs are likely to be carriers, there is currently no practical method to identify them.

<table>
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<tr>
<th>Breed</th>
<th><em>SRY</em>-negative XX sex reversal has been identified</th>
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<tr>
<td>American cocker spaniel</td>
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<td>English cocker spaniel</td>
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<td>French bull dog</td>
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* = Not reported previously
Abnormalities of phenotypic sex

Internal genital development is normally dependent upon gonadal sex. Although no ovarian secretions are necessary for female development at this stage, testis secretions are essential for genital masculinization. For example, MIS, secreted by Sertoli cells, is necessary for Mullerian duct regression. Testosterone, secreted by Leydig cells, directly stabilizes the Wolffian duct system and indirectly masculinizes the external genitalia after conversion to dihydrotestosterone by 5 alpha reductase in the urogenital sinus, genital tubercle and genital folds. Response to either androgen requires the androgen receptor (AR). Testis descent completes the male external phenotype. Factors involved in testis descent are: testosterone, AR, INSL3, secreted by Leydig cells, the INSL3 receptor (GREAT/LGR8) and calcitonin gene-related peptide (CGRP).

In disorders of phenotypic sex, the chromosomal and gonadal sex are in agreement, but the internal or external genitalia are ambiguous. Affected animals are female or male pseudohermaphrodites. The former have an XX sex chromosome constitution and ovaries, but the androgen-dependent genitalia are masculinized to some extent. The most frequently reported cause in dogs is iatrogenic androgen or progesterone administration to the dam during gestation, which masculinizes female foetuses (reviewed in Meyers-Wallen 2008). Male pseudohermaphrodites have failure of either androgen dependent masculinization or Mullerian duct regression. The former is rarely reported in dogs and cats. An example of the latter is Persistent Mullerian duct syndrome (PMDS).

Persistent Mullerian duct syndrome

Although Mullerian ducts are present in both male and female embryos at the sexually indifferent stage, they normally regress soon after testsis differentiation under the influence of MIS. Also called anti-Mullerian hormone, MIS is a secreted glycoprotein in the TGF-beta (Transforming Growth Factor-beta) family of growth factors. MIS signals through a complex of serine-threonine kinase receptors (type I and II) in the Mullerian duct. Upon ligand binding, the specific MIS type II receptor (MISRII) recruits a ligand-independent type I receptor and this complex activates downstream signalling mediators and/or other signalling pathways such as beta-catenin and nuclear factor kB (reviewed in Zhan et al. 2006). The ability of MIS to induce Mullerian duct regression is thought to be mediated via a paracrine mechanism involving mesothelial-epithelial interactions, since MISRII is expressed in the mesenchymal cells, whereas the overlying Mullerian duct epithelial cells undergo apoptosis. Although signalling pathway details are undefined, MIS and its receptors must be present concurrently during a critical period for Mullerian duct regression in male embryos. Failure of Mullerian duct regression can result from disruption at any of these steps.

Persistent Mullerian duct syndrome has been identified in humans (Brook et al. 1973), dogs (Brown et al. 1976) and several other species. In all of these, Mullerian derivatives (oviducts, uterus, cervix, cranial vagina) are identified in XY males having testes. The mode of inheritance is primarily autosomal recessive, and genetic defects in either MIS or MISRII have been identified in > 80% of PMDS patients (reviewed in Belville et al. 2004). All human MISRII mutations identified thus far affect ligand binding or disrupt receptor kinase activity.

Canine PMDS is inherited in two breeds, the miniature schnauzer (MS) in the United States (Meyers-Wallen et al. 1989) and the basset hound in Europe (Nickel et al. 1992). Remarks below refer to the canine PMDS model derived from the MS, which has a phenotype that is strikingly similar to that of humans. However, most human patients are identified due to cryptorchidism, whereas 50% of PMDS dogs have normally descended testes. Affected dogs have complete male internal genitalia, but develop bilateral oviducts, a complete bicornuate uterus and uterine body, a cervix, and the cranial portion of the vagina, which enters the dorsal prostate (Meyers-Wallen et al. 1989). Some have small connections between the cranial vagina and the prostatic urethra (Wu X et al., 2009) that are likely remnants of the Mullerian duct junction with the embryonic urogenital sinus, a connection that normally develops in female canine embryos but not in males (Gier and Marion 1969). Externally, PMDS dogs have a normal male phenotype, except that approximately 50% are either unilaterally or bilaterally cryptorchid (Meyers-Wallen et al. 1989). The cranial end of the uterine horn is firmly attached to the caudal pole of the testis, which may impair testis descent. Histological features of cryptorchid testes include absence of germ cells, whereas scrotal testes appear normal. PMDS males with bilateral scrotal testes were fertile, those with bilateral cryptorchidism were sterile and those with unilateral cryptorchidism had low sperm counts for body weight (Wu X et al., unpublished). As in other cryptorchid dogs, Sertoli cell tumour can occur secondarily (Brown et al. 1976; Marshall et al. 1982). Pyometra has also been reported (Marshall et al. 1982). Treatment includes gonadectomy and hysterectomy to prevent these secondary complications and remove affected dogs from the breeding pool.

To identify a candidate gene, initial studies investigated whether MIS was present in PMDS dogs. Both MIS mRNA and protein were expressed in testes of affected embryos of the PMDS model, as in normal embryos during the critical period for Mullerian duct regression (Meyers-Wallen et al. 1991, 1993). Bioassays for MIS activity (Meyers-Wallen et al. 1989) confirmed that the MIS produced by testes of PMDS embryos and neonates was biologically functional. These findings indicated that target organ insensitivity, caused by a mutation in either MISRII or a downstream gene in its signalling pathway, was likely to be causative (Meyers-Wallen et al. 1993). The causative genetic defect in this model was identified by exon scanning MISRII in genomic DNA from normal and affected dogs (Wu X et al., unpublished). Briefly, PCR primer pairs were designed using the canine genome sequence. PCR products, spanning each exon and its splice junctions, were sequenced and compared to the genome sequence. The mutation identified is predicted to cause a
truncated, non-functional MISRII protein. Genotypes at the mutation site are consistent with all phenotypes in the model pedigree and sex-limited autosomal recessive inheritance.

Identification of this mutation should enable the development of a screening strategy for the MS breed, allowing diagnosis of previously undetected PMDS males, heterozygous carrier males and homozygous and heterozygous carrier females. All dogs related to PMDS males should be tested, since female carriers appear normal and PMDS males must be differentiated from normal males having scrotal testes and males with isolated cryptorchidism (below). The frequency of the mutant allele within the MS breed, presently unknown, can be decreased by testing and selective breeding. Affected males can be prevented by avoiding matings of PMDS or carrier dogs to carriers. A strategy that should not jeopardize gene pool diversity is to first reduce the mutant allele frequency in the breed and then eliminate the mutant allele by removing all carriers from the breeding population.

Cryptorchidism
Cryptorchid males have an abnormality in development of phenotypic sex, but are otherwise normal males, and are not classified as male pseudohermaphrodites because they are not sexually ambiguous. Testis descent is a complex developmental process likely to involve several genes, including those directly controlling testosterone, AR, INSL3, GREAT and CGRP. For example, any genes encoding enzymes in the steroidogenic pathway, leading to testosterone production, or stimulating INSL3 secretion, such as steroidogenic factor 1, could theoretically be involved. However, failure of androgen-dependent masculinization elsewhere (male pseudohermaphroditism) should occur in addition to cryptorchidism if testosterone production is severely impaired. The type of cryptorchidism discussed below is isolated cryptorchidism, meaning the failure of testis descent unaccompanied by other phenotypic abnormalities. Mutations affecting factors listed above account for a small percentage of human cryptorchidism, so additional genes are likely to be involved (Virtanen et al. 2007), and none has been identified in canine cryptorchidism.

Past literature reviews (Gier and Marion 1969; Amann and Veeramachaneni 2007; Virtanen et al. 2007) vary in nomenclature for phases of testis descent. Further, a major comparative study, which includes the dog, reports differences in testis descent between domesticated mammals and rodents (Gier and Marion 1969). However, this review follows the three phases of testis descent proposed in Amann and Veeramachaneni (2007): abdominal testis translocation, transinguinal testis migration and inguinoscrotal testis migration.

Abdominal testis translocation
At the sexually indifferent stage, the bipotential gonad is held high in the abdomen by the cranial and caudal suspensory ligaments. The ovary remains in this position after gonadal sex determination, but after testis differentiation (d34 in the dog, Meyers-Wallen et al. 1993), the cranial suspensory ligament thins and elongates, freeing the testis for caudal translocation (Gier and Marion 1969). Contrary to previous opinions, testosterone is not required for these cranial suspensory ligament changes (reviewed in Amann and Veeramachaneni 2007; Virtanen et al. 2007). The testis is displaced by cranial migration of the metanephric kidney, leaving it caudal to the kidney. The gubernaculum incorporates the caudal suspensory ligament to form the gubernacular cord, which is attached to the caudal testis pole. These form a continuous cord from the testis to the retroperitoneal region where the gubernaculum is anchored (Gier and Marion 1969). The process vaginalis forms a pocket or hernia in the caudal peritoneum just ventral to the gubernaculum, and as the gubernaculum elongates, it fills the lumen of the process vaginalis as the hernia deepens (Gier and Marion 1969). Rodent studies indicate that INSL3 secretion is necessary and binds GREAT in the gubernaculum during this phase. Based on rodent studies, AR in the genitofemoral nerve and testosterone exposure at this phase are necessary for later CGRP release to guide testis descent (Amann and Veeramachaneni 2007). At the end of canine gestation, the testis lies on the peritoneal side of the internal inguinal ring, while the gubernaculum and tail of the epididymis lie within the inguinal canal (Gier and Marion 1969), and they remain in those positions for several days postpartum.

Transinguinal testis migration
In this phase, the gubernaculum bulb greatly enlarges, dilating the inguinal canal to allow testis passage. At the end of this phase, the testis is just external to the inguinal canal (Amann and Veeramachaneni 2007). Neither testosterone nor INSL3 secretion is required during this phase, according to rodent studies (Amann and Veeramachaneni 2007). In the dog, this phase is completed by 10 days after birth (Gier and Marion 1969).

Inguinoscrotal testis migration
During this phase, the gubernaculum bulb extends to the caudal scrotum, and at the end, the gubernaculum is compressed beneath the testis. Gier and Marion (1969) indicated that after the testis had passed through the inguinal canal 10 days after birth, ‘...the gubernaculum subsequently was compressed within the end of the processus vaginalis.’ However, in a figure of the 26-day neonate in that report, the gubernaculum was not compressed and the inguinal canal was not constricted. It is unclear when these processes are normally completed in the neonatal dog.

The role of CGRP is unclear in humans (Virtanen et al. 2007) and has not been studied in the dog. In rodents, the genitofemoral nerve guides testis descent, having a primary role in final positioning of the testis (Amann and Veeramachaneni 2007). Having been influenced earlier by testosterone, the genitofemoral nerve releases CGRP, which stimulates the gubernacular tip to grow.
towards the nerve, thus positioning the testis within the scrotum. This is followed by gubernacular regression and inguinal canal constriction. During this phase in rodents, testosterone is necessary for vaginal process expansion, cremaster muscle growth, gubernacular regression and inguinal canal constriction after final testis positioning.

It is important to note that the position of a cryptorchid testis depends on the phase at which failure of testis descent occurs. Thus, cryptorchidism is a term that encompasses several phenotypes, reflecting its complex genetic basis. To facilitate identification of genes involved at each phase of testis descent, our diagnoses should specify whether unilateral or bilateral cryptorchidism is present and identify the final position of the cryptorchid testis: adjacent to the caudal kidney pole, in the abdomen at the internal inguinal ring, within the inguinal canal, or external to the canal but cranial to the scrotum. Late descent (below) is another phenotype. Specific diagnosis can be achieved at the time of bilateral gonadectomy.

The type of cryptorchidism most frequently identified in dogs is isolated cryptorchidism. Thus major AR defects are unlikely to be causative, as these potentially affect all androgen-dependent masculinization. However, isolated cryptorchidism due to mutations in INSL3 or GREAT have been reported in mice and humans (reviewed in Adham and Agoulnik 2004). Homozygous null INSL3 mutations in mice cause bilateral cryptorchidism and abnormal gubernacular development, but have not been identified in humans. Interestingly, human males with heterozygous null INSL3 mutations are unilaterally cryptorchid at birth, but near the time of puberty the affected testis descends, which is termed “spontaneous resolution” (Tomboc et al. 2000). These mutations were transmitted by either parent to affected male offspring in a simple autosomal recessive manner. Mouse studies suggest that the INSL3 effect on testis descent is dosage dependent. Bilateral cryptorchidism was present at birth in homozygous null mice. Those with heterozygous null INSL3 mutations had unilateral cryptorchidism, and late descent occurred in 75% of these, which is analogous to the human spontaneous resolution phenotype observed with similar mutations. Thus, late descent in this example is yet another variation of the cryptorchid phenotype.

Using a candidate gene approach in pilot studies on canine cryptorchidism, we first exon-scanned canine INSL3 and GREAT in genomic DNA from normal and affected dogs of two breeds (Agoulnik A and Meyers-Wallen V, unpublished). No mutations were identified; however, as INSL3 mutations account for only 1.4% of cryptorchidism in humans, it is likely that other genes involved in testis descent remain to be identified (Tomboc et al. 2000).

Hospital surveys in the literature report a prevalence rate for cryptorchidism in dogs 6–12 month old as 13.6/1000 patients, and over a 3-year period as 9.1–1000 patients (Hayes et al. 1985). As observed in mice and humans (above), some canine cryptorchid testes undergo late descent. In one study, where cryptorchid dogs were monitored to 12 months of age, late testis descent occurred in 24.6% of cryptorchid testes, with 63.3% of those being unilaterally cryptorchid. In only 0.06% of cases did testis descent occur after 14 weeks of age, and none descended after 6 months of age (Dunn et al. 1968). However, inferring from human and mouse studies, dogs with late testis can be considered as having a variation of cryptorchidism, until genetic testing is available to pursue the matter further. In the United States, kennel clubs require every dog competing in a show to have two scrotal testes. Further, the American Veterinary Medical Association states that it is unethical for a veterinarian to surgically correct cryptorchidism without also rendering the animal incapable of reproduction. No medical treatment has been identified to reliably induce late testis descent (e.g. later than 10 days after birth), but medical treatment would not alter the affected dog’s genotype or its ability to transmit the trait to its offspring. For these reasons, the recommendation for cryptorchid dogs is early castration.

A different approach is needed to identify novel genes involved in canine cryptorchidism, since there are few candidate genes to pursue and the mode of inheritance is complex (polygenic). The approach being taken by our collaborative group is a genome-wide case-control association study. This method has been used successfully to map simple Mendelian canine traits (Carlsson et al. 2007). In the first stage of this approach, genome wide mapping is conducted in affected cases and normal controls from the same breed, utilizing SNP markers on a microarray. This identifies haplotypes (contiguous SNPs having the same allele) associated with the affected phenotype. In the second stage, chromosomal regions associated with the affected phenotype are narrowed to a few hundred kilobases by fine mapping cases and controls from a different breed(s). In this stage, SNPs are chosen at a high density over the entire chromosomal region(s) identified in the first phase. Simple autosomal dominant traits in dogs have been mapped by this method with as few as 10 cases and 10 controls (Carlsson et al. 2007); however, complexly inherited traits are likely to require many cases and controls to identify associated chromosomal regions. Again, these regions would be narrowed by screening several more affected and control dogs from different breeds. Mutations are verified by genotype-phenotype correlation within families.

Identification of causative mutations will provide a more effective way to reduce the frequency of cryptorchidism in a breed, since carriers could be identified by genetic testing. Presently, breeding recommendations to avoid production of cryptorchid dogs are derived from studies in farm animals, where the frequency of cryptorchidism has been decreased by removing affected and proven carrier animals from the breeding pool (Meyers-Wallen 1989). In the author’s opinion, dogs with late testis descent should also be removed from the breeding pool if possible, since they may carry mutations for cryptorchidism.

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Conflicts of interest

The authors have declared no conflicts of interest.

References


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