SEX-DETERMINATION SYSTEMS AND THEIR EVOLUTION: MAMMALS

Sistemas de determinación de sexo y su evolución: caso mamíferos

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ABSTRACT

Sex-determination methods are very diverse as they have become an enduring research field. Understanding the causes of gonadal development and elucidating the main factors involved in sex-determination of offspring required relating information from far-ranging areas such as cytology, embryology, morphology, molecular biology and even ecology and evolution. This article presents an overview of sex-determination in placental mammals, encompassing several levels of biological organization. The importance of the underlying molecular tools in the context of sex-determination assays and their implications in conservation genetics is also discussed.

Key words: Sex chromosomes, *SRY*-HMG, sexual reversion, genetic conservation and sex-determination.

RESUMEN

Los sistemas de determinación de sexo son muy diversos y en consecuencia se ha aumentado la investigación en este campo. Entender el desarrollo gonadal y elucidar los factores involucrados en la determinación de sexo de la descendencia ha requerido tomar información de áreas como: citología, embriología, morfología, biología molecular e incluso de ecología y evolución. Este artículo presenta una visión de los mecanismos de determinación de sexo en mamíferos placentarios, abarcando algunos niveles de organización biológica. También se discute la importancia de las herramientas moleculares en las pruebas de determinación del sexo y sus implicaciones en conservación genética.

Palabras clave: cromosomas sexuales, *SRY-HMG*, reversión sexual, conservación genética y determinación de sexo.

Abbreviations:
SRY: Sex-determination Y chromosome region
ORF: Open Reading Frame

SOX: *SRY*-related gene
TDF: Testis Determining Factor

Abreviaciones:
SRY: Región determinante de sexo en el cromosoma Y
ORF: Marco abierto de lectura
SOX: Genes relacionados al *SRY*
TDF: Factor determinante de testículo

INTRODUCTION

A fundamental process in most species concerns the sexual phenotype, which is being determined in future individuals during the embryo period. Thus, studying sex-determination extends our knowledge about the genes and mechanisms involved in such a decision between two alternate embryonic routes: male or female phenotype.
The first attempts to understand sex-determination in mammals were made by removing the gonadal primordium in rabbit fetuses, resulting in all cases developing as females and therefore implying that the testicles were responsible for a male decision being made. Hence, it was established that differentiation into ovaries or testicles from the gonadal primordium determined the appearance of gender-specific structures during early development stages (Jost, 1953).
Subsequent research focused on finding a correlation between an individual's gender and their chromosomes, taking the case of *Drosophila melanogaster* as reference, where sex-determination depends on the relationship between the number of X chromosomes regarding autosomes, while the Y chromosome only carries ribosomal and fertility genes (Bernard, 1988; Hodgkin, 1990). However, studies in humans having chromosomal abnormalities (the so-called Turner and Klinefelter syndromes) have demonstrated the relevance of the Y chromosome in sex-determination in mammals (Ford *et al.*, 1959; Goodfellow and Lovell-Badge, 1993).

Sex-determination systems diversity

Sexual differentiation has proven to be a very complex process, as are all others in individual development. Although sex-determination is of vital importance in vertebrate reproduction, the great variety of sex-determination mechanisms across different taxa is surprising. They can be divided into two broad categories during the course of evolution: external environment-dependent or environmental mechanisms (also known as: Environmental-dependent Sex Determination or ESD) and genetic mechanisms (Genetic Sex Determination or GSD). The developing embryo's sex is determined by an initial signal provided by the environment in the former while the offspring's sex depends on primary genetic signals in the latter (Bull, 1983; Janzen and Paukstis, 1991).
Sexual differentiation occurs in ESD in response to external signals after fertilization, so that the sex into which zygotes finally differentiate is independent of their genetic/chromosomal composition. Many environmental factors are able to affect sex, although Temperature-dependent Sex Determination (TSD) is usually the most common form of environmental sex-determination (Korpelainen, 1990). This type of mechanism has been

described in invertebrates, fish (Strüssmann and Patiño, 1995), and mainly in reptiles and some species of turtles and lizards (Ferguson and Joanen, 1982; Smith and Joss, 1993; Johnston *et al.*, 1995; Pieau, 1996; Crews, 1996).

In organisms having genetic sex-determination, sex is "decided" at the moment of fertilization, depending on the gametes' chromosomal constitution. This does not necessarily mean that sex chromosomes should be heteromorphic, as evidenced by the fact that different degrees of differentiation can be shown, ranging from homomorphic sex chromosomes, to clear heteromorphism. The genes involved in sex-determination are thus present in a pair of chromosomes in some species that are indistinguishable under the microscope, while others are located on one of the heteromorphic chromosomes forming the sexual pair. In the latter case, heterogametic sex may be male (XX (♀) / XY (♂) systems), as in most mammals, or female (ZW (♀) / ZZ (♂) systems), as seen in birds and reptiles. The sex chromosomes may be highly conserved, as in the case of birds and mammals, or highly variable, even within species or genera, as in the case of amphibians (Wallis *et al.*, 2008).

The variability observed in the two categories of sex-determination mechanisms goes beyond what one would expect for so widespread a process, given that even for organisms in which sex is determined in a similar way, the genetic mechanisms by which sexual determination and differentiation routes are implemented vary widely. This is the case of sex-determination mechanisms in the three best-studied model organisms: the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans* and the mouse *Mus musculus* (McElreavey *et al.*, 1993; Ryner and Swain, 1995). Sex determination in *Caenorhabditis* and *Drosophila* in which the sex-determination signal depends on the relationship between the autosome and X chromosome dose is thus not comparable to what occurs in mice and most mammals where the presence of the Y chromosome leads to male development (Wallis *et al.*, 2008).

Likewise, sex-determination in mammals is made by the sex chromosome system XX (female)/XY (male), in which the presence of the Y chromosome is the dominant factor inducing male phenotype development. This pair of heteromorphic sex chromosomes originate from changes at evolutionary level, resulting from constant deletions and modifications which have led from originally homologous chromosomes to two distinct enough chromosomes X and Y differing in both morphology and gene content (Charlesworth, 1996). Such differentiation has been accompanied by the restriction or suppression of recombination between large regions of both chromosomes. Moreover, the Y chromosome has become drastically reduced in size, currently undergoing degeneration to become a generally small, gene-content poor, full of repeat sequences and mostly heterochromatic chromosome (Graves, 2006).

Thus, the presence of a Y chromosome in most mammals leads to morphological and hormonal changes which could be common to most species. One of the most significant changes is the development of testicles, although male gonads are indistinguishable from female organs during the early days of gestation. In fact, differentiation becomes morphologically visible in humans only six weeks after fertilization, while occurs 12.5 days post coitus (DPC) in mice (Koopman *et al.*, 1990; Lovell-Badge and Hacker, 1995). Masculinization in mammals can consequently be understood as an alteration of gonadal development which, by default, might have led to producing ovaries (Lovell-Badge and Hacker, 1995).

The presence of testicles gives rise to the production of two hormones, namely anti-Mullerian hormone (AMH) (or Mullerian inhibiting substance - MIS) and testosterone. The former is secreted by Sertoli cells and is responsible for degrading female structures which would eventually lead to the formation of the uterus and other female ducts. On the other hand, testosterone is produced by Leydig cells and is responsible for producing male ducts and external genitalia (Jost, 1953).

Exceptions to the importance of the Y chromosome in sex-determination in mammals have been observed in rodents, particularly in the wood lemming (*Myopus schisticolor*). Although the determination system is XX-XY (females and males, respectively), female XY can be found in this species in which, despite carrying the *SRY* gene (Y chromosome sex-determining region, a determinant of masculinization in most mammals), their X chromosome can mask its effect (Fredga, 1994). Species have been described which lack the Y chromosome in their genome. For example, species such as *Ellobius lutescens* and *Ellobius tancrei* lack this chromosome, so that both males and females have the same karyotype: XO in *E. lutescens* and XX in *E. tancrei* (Fredga and Lyapunova, 1991; Just *et al.*, 2002). Males from both species are fully fertile and have fully developed testicles. Despite the fact that the mammalian sex-determining gene, *SRY*, is also absent in these species, which indicates that could present a novel sex-determining mechanism that is independent of *SRY* and probably controlled by other master gene of the sex determination activation pathway. Recently, Kuroiwa *et al.*, 2011, have demonstrated that in males of *T. osimensis* and *T. tokunoshimensis* there are two or three more copies of *CBX2* per haploid genome than in females suggesting that these additional copies might be involved in the novel sex-determining mechanism in species that lack *SRY*. However, although important effort is made to identify the control candidate gene in these species for the moment sex determination mechanism in absence of *SRY* and Y chromosome is a great enigma.

Sex chromosomes and their evolution

Current thinking considers that reptiles, birds and mammals' sex chromosomes were independently originated from different autosomes in their common ancestor, given their lack of homology (Fridolfsson *et al.*, 1998). Part of such hypothesis has been confirmed by genetically mapping mammalian chromosomes XY and birds' ZW where there is no relationship between the members of the pairs (Nanda *et al.*, 1999). However, these studies have revealed that mammalian X chromosome exhibits some homology with birds' different autosome regions, suggesting that sex chromosomes evolved from a pair of autosomes which are likewise conserved in other vertebrates.

It has therefore been assumed that the X and Y chromosomes in mammals evolved from an ancestral autosomal pair about 300 million years ago (Ohno, 1967; Graves and Shetty, 2001). It is believed that this evolutionary process began when a locus playing a dominant role in determining sex (the *SRY* gene) appeared in one of the members of the ancestral chromosome pair (the proto-Y chromosome). Degeneration began from the proto-Y chromosome which has led to it becoming the small, heterochromatic and barely gene populated Y chromosome currently common in most mammals. Such degeneration has taken place as a result of deleterious mutation accumulation in the non-recombinant region of chromosome Y. Furthermore, suppressing recombination would have been favored by the evolutionary process in grouping together and

transmitting genes having male-specific functions (Graves and Shetty, 2001).
Contrasting with degeneration, sex chromosomes have also undergone increases in size during their evolution by autosomal regions becoming added. Thus, comparative studies between Eutheria mammals, marsupials and monotremes have shown that a wide region of Eutheria X and Y chromosomes exhibit autosomal location in marsupials and monotremes. This region, which was originally autosomal, has been transferred by translocation to the sex chromosomes in ancestral species of Eutheria (Graves, 1995a; Glas *et al.*, 1999). Another mechanism by which this chromosome might also have increased in size was repeat sequence amplification which would have accumulated on the Y chromosome during evolution (Graves, 2000).
Y chromosome degeneration, together with specific sequence amplification, resulted in this chromosome retaining only a small region of homology with the X chromosome, called the pseudoautosomal region (PAR; Graves, 1995b). Addition and degeneration are independent in each species or group of species and thus this region is neither the same nor has the same sequence in the sex chromosomes of all mammalian species, even when they are very close. However, this small homologous region is present in most mammalian sex chromosomes and is considered essential for allowing appropriate sex chromosome segregation during meiosis (Burgoyne, 1982; Ellis and Goodfellow, 1989). PARs are thus sex chromosome fragments which behave in an autosomal manner during meiotic crossover (Ellis and Goodfellow, 1989; Mohandas *et al.*, 1992; Freije *et al.*, 1992).
XY sex chromosomes are found in the three subclasses of mammals (monotremes, marsupials and placentals) but there are significant differences in their interactions during meiosis and their numbers. Male platypus (*Ornithorhynchus anatinus*) have five pairs of sex chromosomes (Rens *et al.*, 2004) and their recombination during meiosis (as in placental) is possible due to the PAR (Grützner *et al.*, 2004). This fact contrasts with these chromosomes' recombination inability in marsupials lacking homologous regions (Graves and Watson, 1991).
The X chromosome in placentaries is essentially euchromatic and usually represents 5% of each species' haploid genome (Ohno, 1967). This chromosome contains about 1,500 genes (many being constitutively expressed) contained in 165Mb (Ross *et al.*, 2005). The female produces random inactivation of X chromosomes in every cell to ensure equal doses of X-linked genes in males and females during embryonic development (Lyon, 1961). X chromosome size and gene content is highly conserved among different species, possibly ensuring correct functioning of the X chromosome inactivation mechanism (Ohno, 1967). It also has unique regions in monotremes and marsupials belonging to autosomes. In fact, it has been shown that autosomal translocation occurred after these groups' divergence (Graves, 1995a).
Placentaries' Y chromosome is small (60Mb), representing only 2% of the human genome. It contains a few genes (50 or so), most specializing in male functions, such as testicular determination and spermatogenesis (Graves, 2000; Graves, 2001; Skaletsky *et al.*, 2003). For the most part, the Y chromosome consists of heterochromatic repeat sequences (Graves, 2000; Graves, 2001); moreover, these repeat sequences are poorly conserved among Y chromosomes from different species, even if they are evolutionarily closely-related. Like the X chromosome, Y shares regions with other mammal species whilst others are unique (Waters *et al.*, 2001).

The *TDF* gene and its relationship with *SRY*-HMG: in search of the gene determining masculinity

Although the position of the testis determining factor (*TDF*) gene locus on human and mouse Y chromosome was already known in the 1980s, its cloning was not easy or as quickly as supposed at first. Throughout the history of the search for the testicle determining gene, several genes have once been considered equivalent to the *TDF* gene but have eventually been discarded due to evidence demonstrating their lack of involvement in testicular determination. Among these, the minor histocompatibility Y antigen (HY antigen) was one of the first candidates (Wachtel *et al.*, 1975), followed by satellite DNA sequences known as banded krait minor satellite DNA (*Bkm* sequences) isolated from the W chromosome of the snake *Bungarus fasciatus* (Family Elapidae; Singh *et al.*, 1976; Singh *et al.*,1979), and the zinc finger gene on the Y (*ZFY*) gene (Koopman *et al.*, 1989).

Final cloning of *TDF* was not possible until a detailed search was made of the minimum portion of the Y chromosome confering a male phenotype in humans became available. Thus, isolating specific regions of the Y chromosome (short arm distal), common in patients with testicular development and sex reversal (XX males), led to discovering an open reading frame (ORF) corresponding to the gene responsible for masculinity, known as the sex determining region of the Y chromosome (*SRY*). This gene has been confirmed as testicular determinant in subsequent research with mice (Sinclair *et al.*, 1990; Gubbay *et al.*, 1990; Page *et al.*, 1990).

It is currently assumed that activating the gonadal differentiation pathway is initially controlled by the *SRY* gene, although this does not exclude the possibility that other genes having loci on the Y chromosome, X or even autosomes are involved in regulating its expression (Goodfellow and Lovell-Badge, 1993; Lovell-Badge and Hacker, 1995). The *Sry* transcript location in the embryonic genital ridge and adults' testicles supports its role as a testicle determinant (Koopman *et al.*, 1990; Payen *et al.*, 1996; Daneau *et al.*, 1996).

The *SRY* gene is usually single copy in most placental species and some marsupials although, in some species, it has multiple copies, some of them found on the X chromosome (Bianchi *et al.*, 1993; Negamine, 1994; Bullejos *et al.*, 1997). However, due to the presence of multiple internal stop codons, it is considered that some of these copies are actually pseudogenes (Marchal *et al.*, 2008). Regarding the multiple copies found on the Y chromosome, they can be mono- or polymorphic, as in the case of some species from the family Microtidae, although it remains unknown whether these copies are tandemly repeated or dispersed in the chromosome (Bullejos *et al.*, 1999; Acosta *et al.*, 2010).

According to the rapid evolution of sex-determination systems hypothesis, research in some species of *Ellobius* (moles) and the spiny rat *Tokudaia* have confirmed that *SRY* is no longer responsible for determining masculinity, as it does not occur in the Y chromosome (the karyotypes for males and females being the same, XO in *E. lutescens* and *T. osimensis* and XX in *E. tancrei*; Just *et al.*, 1995; Soullier *et al.*, 1998). It is possible that even though there is a Y chromosome it may lack the *SRY* gene, as has been described in *Microtus mandarinus mandarinus* where the difference between males and females lies in X chromosome morphology (Chen *et al.*, 2008).

The *SRY* gene is highly variable among species regarding both chromosomal location and nucleotide structure. Regarding its location, this locus is most proximal to the centromere of the Y chromosome's short arm in the mouse, while it is found in the most distal short

arm in humans, precisely 5 kb from PAR (Sinclair *et al.*, 1990). This fact explains the high frequency of sex reversal cases, given that the *SRY* gene may be transferred to the X-chromosome due to errors in meiosis (Guellaen *et al.*, 1984; Page, 1986; Capel *et al.*, 1993).
Concerning the *SRY* gene's nucleotide sequence, the mouse's has a very unusual structure because it is composed of a single 2,739 bp region having a long ORF without introns which is flanked by two nearly identical inverted repeats of at least 17 kb (Gubbay *et al.*, 1992). This inverted repeat is absent in the human *SRY* gene (Sinclair *et al.*, 1990; Lovell-Badge and Hacker, 1995).
Despite the foregoing variations, all *SRY* genes identified to date are genes without introns and have a highly conserved region in most mammals, called the high mobility group (HMG) box which encodes a 79 aminoacid long protein domain, characteristic of proteins whose activity is DNA binding (i.e. the SOX protein). This domain confers the property of producing folds in DNA on proteins acting as transcription factors (Ner, 1992; Pontiggia *et al.*, 1994). Currently, there are no doubts about the importance of this HMG domain for proper *SRY* protein function, which has been confirmed in cases of sex reversal in humans resulting from mutations and small deletions in this domain (Goodfellow and Lovell-Badge, 1993).
However, the rest of the gene (regions flanking the HMG box) is highly variable in both nucleotide sequence and size, even among closely related species, thereby hampering their study. Thus, very little homology is shown when comparing nucleotide and aminoacid sequences outside humans, rabbits, mice and marsupials' *SRY*-HMG box. It has been proposed that these *SRY* sequences evolve faster because they present little functional significance, so that there are no functional restrictions preventing the accumulation of changes in sequence, so they are not subject to selective pressure (Whitfield *et al.*, 1993; Tucker and Ludrigan, 1993).
Most research related to *SRY* has usually been directed towards studying its role in sex-determination at molecular level. Interestingly, it has also been characterized and used for sex-determination in various species such as gorilla, gazelle, rat, sheep, pig, mouse (Margarit *et al.*, 1998), micro and mega bats (Bullejos *et al.*, 2000), humans (Drobnic, 2006), canines and felines (Meyers, 2006), cattle (Lu *et al.*, 2007) and goats (Shi *et al.*, 2008). Furthermore, due to the high rate of molecular change, the accumulation of nonsynonymous substitutions and the absence of recombination (Whitfield *et al.*, 1993; Tucker and Lundrigan, 1993), the *SRY* gene has been used as phylogenetic marker. Different authors have established phylogenetic relationships among species and genera within families of primates (Pamilo and O'Neill, 1997; Wang *et al.*, 2002; Moreira, 2002), bovines (Payen and Cotinot, 1994) or felines (King *et al.*, 2007).

The *SOX* gene family (*SRY*-related, HMG box-containing genes)

The *SRY* gene belongs to the *SOX* gene family consisting of genes whose proteins have greater than 60% identity in the HMG box regarding *SRY* (Goodfellow and Lovell-Badge, 1993). The DNA binding property afforded by that domain not only allows it to act as a transcription factor, but also as an architectural component of chromatin because it affects genetic material packaging (Canning and Lovell Badge, 2002). However, members of these proteins are not restricted to mammals and have been found in other groups such as insects, birds and reptiles (Griffiths, 1991; Foster *et al.*, 1992).

There are more than 20 known *SOX* genes in mammals and, although they have functions related to various aspects of embryogenesis, most of them are not involved in sex determination (Prior and Walter, 1996; Pevny and Lovell-Badge, 1997).
SOX3 is a member of this gene family; its locus is located in the X chromosome (Stevanovic *et al.*, 1993). There is evidence supporting the origin of the *SRY* gene from a primitive *SOX* gene present in the ancestral evolutionary line before the separation of Metatheria and Eutheria. The presence of the *SOX3* gene in the X chromosome of all mammals tested and the high degree of homology between the HMG box of the *SRY* genes and the chromosome suggests parallel evolution for these genes from an ancestral autosomal pair that gave rise to the sex chromosomes (Stevanovic *et al.*, 1993; Foster and Graves, 1994; Collignon *et al.*, 1996).
On the other hand, the *SOX9* gene is involved in sex determination (Foster *et al.*, 1992; Wagner *et al.*, 1994; Collingnon *et al.*, 1996). The *SOX9* gene is highly conserved among fish, birds and mammals and its expression is specific to the male gonads of birds and mammals, for example 11.5 DPC in mice, with no expression in females. This is why it is considered to be a gene which is involved in the formation of male gonads and may be regulated by the *SRY* gene (Morais *et al.*, 1996).
A finding related to the importance of *SOX9* in sex determination has been a case of sex reversal in humans (XX) resulting from this gene's duplication on chromosome 17 which led to the development of testes (Huang *et al.*, 1999). This fact corroborates *SOX9*'s potential (if expression threshold-dependent) to become a candidate gene for allowing the development of masculinity in the absence of *SRY*, mimicking the latter's function. This may have been due to the high degree of identity between HMG boxes from *SRY* and *SOX9* (76% at protein level), explaining why chimeric *SRY* genes (having *SOX9* boxes) have allowed masculinization of transgenic mice (Bergstrom *et al.*, 2000). Although little information is available about *SRY*-controlled genes, it has been suggested that *SOX9* is usually controlled by *SRY* (Canning and Lovell Badge, 2002).
When comparing the HMG box of distantly related species, the high identity between them becomes evident (Coriat *et al.*, 1993). However, the *SRY*-HMG box has specific and critical amino acids for its function to differentiate it from the HMG box of *SOX* genes (Gubbay *et al.*, 1990; Berta *et al.*, 1990; Coriat *et al.*, 1993).

Molecular sexing and genetic conservation

Sex identification is of great importance in evolutionary biology, ecology, prenatal diagnosis, forensic identification, population genetics, conservation and planning studies concerning the reproduction of threatened or clearly endangered species (Fridolfsson and Ellegren, 1999; Griffiths and Phil, 2000; Drobnic, 2006; Matta *et al.*, 2009). Sexing using molecular tools has been particularly important for testing and controlling poaching in which the capture and killing rate should be differential between males and females in bird breeding programs where sexual dimorphism is not obvious. It also represents an alternative to sexing methodologies such as laparotomy or laparoscopy (Fridolfsson and Ellegren, 1999; Griffiths and Phil, 2000; Matta *et al.*, 2009; North American Wildlife Enforcement Group-NAWEG-2000); research in its field is also relevant in conservation biology, ecology and wildlife management.
There is an increasing interest in the use of non-invasive sampling for the study of wild

animal populations to avoid disturbance of the animals and to get results with indirect and easy sampling, such as skin, hair, feathers, nails, feces, or simple drops of blood. Identification of the individual's sex is important for conservation studies of wild animals. However in some species, limitations regarding sex determination using molecular tools are associated with the problem of finding the specific sequences in sex chromosomes that differentiate between males and females and can be demonstrated by methodologies such as PCR amplification or differential cleavage with restriction enzymes. The gene does not necessarily have to involve pathways leading to sexual differentiation. Other genes or even non-encoding sequences may be responsible for other functions, although they must have differences between the sex chromosomes that are not in autosomes.

Several molecular methods for sexing can easily be adapted for a wide range of species. In mammals, RFLP analysis based on X- and Y- chromosome-specific polymorphism has been extensively used, as for example those observed in the AMELX/AMELY and *ZFX/ZFY* genes, located either on the X and Y chromosome respectively (Villesen and Fredsted, 2006; Lindsay and Belant, 2008; Xu *et al.*, 2008; Li *et al.*, 2010). However, in some species some sexing methods encounter difficulties in the amplification of long fragments from degraded DNA and in non-specific amplification from sample analysis. However, PCR products of considerable length are used, which is not suitable for degraded DNA such as faeces analysis. Hence the alternative approach consisted in the amplification of short Y-specific genes using conserved primers, as for example the *SRY* (Gupta *et al.*, 2006) and *Ube1Y* genes (Sloane *et al.*, 2000).

However, the use of Y-chromosome specific sequences alone is not informative because an absence of PCR product can indicate either a female or a failed reaction. Hence, these reactions are often performed in conjunction with another fragment to act as a positive control for amplification, such as a mitochondrial DNA or a nuclear locus, being preferable the nuclear one as mitochondrial DNA are present in more copy number than nuclear DNA and hence than the amplified Y chromosome specific markers.

Some limitations and considerations should be taken into account in molecular genetic analysis when the genome of the species of interest is scarcely studied and we are searching for target genes: 1) finding species-related genes (even if they are orthologous) does not necessarily involve a similar task, considering that the biological contexts of expression can be very different; 2) the genes of interest could not be located on the same chromosomes; 3) the gene sequences could not be conserved enough to permit to amplify with interspecific primers or cut with the same restriction endonucleases; 4) regarding the function itself, there is still a lot of uncertainty in terms of metabolic pathway flexibility when induced mutations might not be apparent in the phenotype as a result of possible substitutions by other proteins. These guidelines should be kept in mind when exploring little-studied species using databases and to avoid the pitfall of biological reductionism (Marin and Baker, 1998).

CONCLUSIONS

Studying sex determination is of wide interest due to the great variability of systems present even among species from the same gender, contrary to what has previously been

thought, i.e. that the relationship between XX/XY karyotypes and individuals' phenotype does not have to be so linear and, even less so, is always not only dependent on specific chromosomes but also on the genes and alleles responsible for masculinity.

DNA-based methodologies for identifying sex are valuable tools due to their speed, sensitivity and the possibility of easy sampling of meat, skin, feathers, nails, or simple drops of blood, thereby minimizing pain and stress during sampling. Moreover, they can be performed on very small tissue samples obtained from hunting-control programs. Correct sex determination is important when studying a population's behavior and social structure and for conservation plans where decisions must be taken by adopting an interdisciplinary approach.

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