Chapter B3: Molecular sex identification for applications in conservation, industry, and veterinary medicine

Authors and Affiliations

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Abstract

The ability to identify whether an organism is female or male is essential data feeding into a range of applications in biological research, animal-based industries and veterinary medicine. Many organisms show striking sexual dimorphism in secondary sexual characters and/or visible genitalia that make assigning a sex to an individual unambiguous. However, there are also many species where it is impossible to distinguish males from females on the basis of their outward appearance, and surprising complexity and lability exists in this seemingly binary trait (e.g., environmental sex reversal, natural sex change). Even in sexually dimorphic species, there are often life-stages where sex cannot be confidently assigned (e.g., embryonic development, juvenile life-stages and seasonal variation in reproductive plumage or colouration). Another challenge for sex identification exists when samples are collected without the researcher being able to inspect the organism, such as remote, non-invasive or environmental sampling. When phenotypes are challenging, researchers can instead use molecular data to establish the sex of individuals. Here we outline the application and importance of accurate molecular sex identification methods in a range of fields, including conservation, ecological research, food production, and veterinary medicine. We provide a framework to structure the sex-marker discovery process which will ensure that the strategy is appropriate for the biology of the organism and the budget of the researcher. Lastly, we detail how a core understanding of the evolution of sex chromosomes and sex determination mechanisms is crucial to successful sex marker development.

1. How to define the sexes?

Most vertebrates have a gonochoristic system of reproduction where two discrete phenotypic sexes exist – female or male (Bachtrog *et al.*, 2014; Stöck *et al.*, 2021). Differences in the size and investment in gametes distinguishes and defines the two sexes. Male animals produce many smaller gametes (spermatozoa, sperm) whereas females produce fewer larger ones (ova, egg cells). A greater investment is directed to offspring by the female than by the

male. Such investment can be material or behavioural, including striking an appropriate balance between risk to the parent and risk to the offspring. Sexual phenotypes might be strictly defined by the size and morphology of their gametes or the structure of the gonads that produce them, but internal character traits are generally not the most practical or observable trait to use for general research purposes. As most of us would know based on experience with our own species, female and male animals can be recognisable by the casual observer. Thus, more practically, sexual phenotype is often defined using the differences in primary and/or secondary sexual characteristics that vary between males and females. Some examples of definitive externally dimorphic traits are: body size, colouration, plumage or in extreme cases the often-spectacular outcomes of sexual selection (e.g., antlers in deer; outlandish plumage in peacocks and the dazzling structural colour displays seen in birds of paradise; Davies, Krebs and West, 2012). This chapter will focus on sex identification using vertebrate examples, but the analytical techniques and decision-making frameworks are broadly applicable across kingdoms.

2. Sex identification versus sex determination

Phenotypic sex is usually concordant with some underlying genetic foundation that governs developmental fate – male or female. In some species, the underlying propensity to be one sex or the other can be over-ridden by environmental factors, which requires us to make also a distinction between genetic sex identification and phenotypic sex identification. In this chapter, we focus exclusively on describing robust methodologies for developing genetic sex identification markers and discuss the application and utility of these molecular tools. You may notice that in some publications the term "sex determination" is used interchangeably with sex identification. However, we discourage this because it is imprecise and inaccurate to do so. Specifically, 'sex identification' should be the preferred term to refer to *methods* that a researcher uses to assign either the genotypic or phenotypic sex to an individual animal. The term 'sex determination' should be reserved exclusively for *biological processes* that initiate sexual differentiation in the embryo (e.g., gene-regulation, organogenesis) (Section 4). It is indeed possible for sex identification markers to inform the study of sex determination, in particular the characterisation of sex chromosome gene content. Likewise, studies of sex determination can provide valuable information that assists in the characterisation of sex identification markers. Thus, whilst they are interrelated, the terms should not be confused because they refer to fundamentally different areas of research with different goals, activities, and approaches.

3. Why identify genetic sex and what are the applications?

The sex of an individual is a critically important life history trait because it profoundly affects behaviour, phenotype, mode of reproduction, energetic investment and many other fitness components. The primary sex ratio of individuals in populations is a critical component in studies of sex allocation (the differential investment of the parents in female versus male offspring) and is the subject of a particular type of selection, that of Fisher's frequency-dependent selection (Fisher, 1930; Edwards, 2000). Fisher's frequency-dependent selection is a powerful form of natural selection that brings the primary sex ratio to equilibrium, typically a 1:1 sex ratio. The operational sex ratio, that is, the ratio of adult breeding males to adult breeding females in a population, which can depart dramatically from a 1:1 ratio because of differential mortality, has important demographic consequences, including a strong bearing on population viability. Thus, there are many reasons for wanting to unequivocally establish the sex of one or many individuals.

3.1 Population viability analysis

The use of molecular markers for sex identification has transformed the study of sexually monomorphic species in ecology, conservation, and wildlife management. In particular, operational sex ratios have a strong influence on effective population size. They can be used to determine if a population is experiencing problematic demographic shifts (Boyle *et al.*, 2014; Lambert, Ezaz and Skelly, 2021). These basic but essential biological data are required for population viability modelling, with sex ratio bias having both a direct and indirect influence on population viability (Heinsohn *et al.*, 2019) (Shaffer, 1981). For example, management of harvested furbearer species (e.g., beavers or bobcats) has been problematic owing to errors in sex and age data from harvested animals, which biased the population viability models. To rectify these errors, Hiller *et al.*, (2022) used a PCR-based test, developed by Pilgrim *et al.*, (2005), to identify the sex of harvested bobcats, *Lynx rufus*, where they found a 20% error between visual sex (identified by fur-takers) and genetic sex, mainly in juvenile individuals. In this case, the genetic sex identification increased precision of the population models and improved the local wildlife management strategies.

The demographic processes that influence local declines and extinction are the same processes that operate when species distributional boundaries shift (Andrewartha and Birch, 1954), such as under climate change. Extreme sex ratio skews can occur at the boundaries of climatic tolerance. Sex ratio monitoring to detect demographic disruption at the trailing edge of a species distribution could be an indicator of pending distributional shifts under climate change (Boyle *et al.*, 2014).

3.2 Animal behavioural ecology and sociobiology

Sex identification is critical for understanding the ecology and behaviour in species with little or no sexual dimorphism. An excellent example is the development of cross-species avian sex markers for non-ratites (Griffiths *et al.*, 1998; Fridolfsson and Ellegren, 1999) and ratites (Huynen, Millar and Lambert, 2002). The non-ratite sex markers are based upon a W-specific size polymorphism in the sex-linked and otherwise highly conserved CHD (chromo-helicase-DNA-binding) genes. The ratite markers are based on a size polymorphism at an anonymous locus, derived from random amplification of polymorphic DNA (RAPD) analysis. Although not without some technical challenges (Dawson *et al.*, 2001, 2015), these sex markers increased the power and accuracy of research in bird behaviour and also expanded the scope of questions that could be investigated (Hughes, 1998). Before these markers were available, the sex of monomorphic species was often inferred by painstaking but ultimately subjective behavioural observation or invasive techniques such as laparoscopy (Richner, 1989). Behavioural approaches rely on the assumption that there are suites of sexually dimorphic behavioural traits that are reliable indicators of sex. It is now appreciated the behavioural dynamics of avian populations are far more complex than previously assumed and that ecological, demographic, and behavioural methods to infer sex can be inaccurate, particularly in studies involving fewer than 200 birds (Dechaume-Moncharmont, Monceau and Cezilly, 2011). The new-found accuracy provided by sex-linked avian molecular markers has facilitated applications including: mating system reconstruction, accurate long-term monitoring, revealing group structure in co-operative breeders, study of sex-biased dispersal dynamics and a host of other applications (Hughes, 1998; Morinha, Cabral and Bastos, 2012).

3.3 Remote population monitoring

The capacity to identify the sex of animals from samples collected non-invasively without observing them, has generated a slew of new applications in wildlife management (Waits and Paetkau, 2005). Molecular sex identification markers have been applied to a wide range of

remote and/or non-invasive biological samples including: hair, scats and environmental DNA from water or soil samples. For example, Zarzoso-Lacoste *et al.*, (2018) used scat survey techniques for remote monitoring of endangered bat populations (*Rhinolophus hipposideros*) to assess variation in bat maternity colony demography. By combining a molecular capture–mark–recapture approach to estimate lesser horseshoe bat abundance (see chapter B4 for this technique) and a mammalian sex-linked PCR test, they discovered that colonies are heavily female biased (74.2% female) and that the sex ratios vary between colonies and through time within the same colonies. Using a sex-linked marker in combination with parentage assignment allowed this study to reliably delineate different categories of individuals (males, females, potential breeders, reproductive individuals) and contributed to a better understanding of bat reproduction. Remote sampling strategies can also combine sex identification with Y-chromosome haplotyping for unique biological inference. For example, Aarnes *et al.*, (2015) developed a Y-chromosome multiplex microsatellite PCR assay with the goal of using it to resolve the regional provenance of illegally traded hunting trophies. This approach can also be useful for detecting ancient or historical trade routes. For example, Barrett *et al.*, (2022) sex-typed ancient walrus remains to uncover a pattern of increased hunting pressure through time in the medieval European walrus ivory trade. Male walruses have larger tusks and are preferred for ivory harvesting. Female representation in ivory artefacts increased over time, suggesting that either males were depleted from the population or there was an increased harvest rate for both sexes. As with any study involving degraded or trace quantity DNA (Chapter C4), remote sex identification needs to plan an approach such that allelic drop-out, degraded DNA and a potentially high rate of false negatives do not bias the interpretation of the assay (Waits and Paetkau, 2005; Dawson *et al.*, 2015).

3.4 Ex-situ conservation and captive breeding programs

Genetic sex identification has a variety of uses in the management and breeding of captive animal populations such as endangered species breeding programs, zoos and wildlife reserves (Pereira *et al.*, 2021; Ryder *et al.*, 2021). Juvenile sex identification can assist the planning of animal exchanges between zoos, allowing animals to be transported before they are sexually mature. Accurate sex identification is crucial to all pedigree planning and stud book record-keeping. Molecular sex identification can also be used to inform assisted reproductive technology, for example, when selecting embryos to implant or sorting X and Y bearing sperm (see Section 3.5). Sex identification markers can also be useful to confirm unusual events, such as spontaneous parthenogenesis, that may occur in captivity for a range of vertebrate species (Watts *et al.*, 2006; Booth *et al.*, 2014; Miller *et al.*, 2019; Ryder *et al.*, 2021).

3.5 Food production efficiency

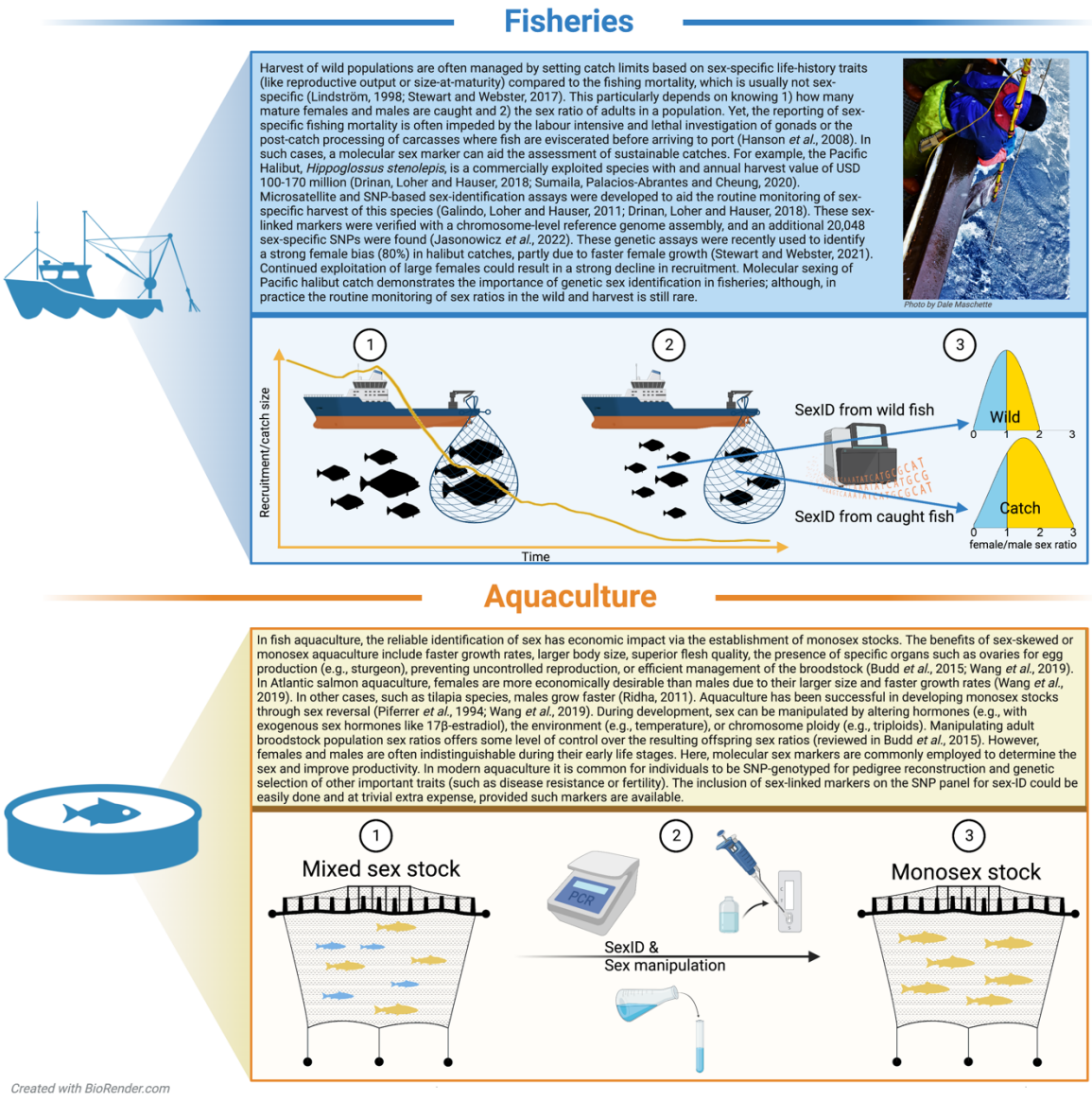
Sexually dimorphic traits are key factors to improve food production efficiency and sustainability (see Box 1). Sex identification and manipulation technologies have revolutionised food production from livestock since the 1980s (Hohenboken, 1981; King, 1984; Johnson and Clarke, 1988). For example, cattle farming for dairy production favours the production of female animals capable of lactation (Weigel, 2004), whereas in the beef livestock production, producing animals for eventual slaughter, there is a higher commercial value of male than female offspring for meat production (Hohenboken, 1999). Thus, even within the same livestock species, sex as a production trait can have opposing financial drivers. The manipulation of offspring sex ratios is routine now in the cattle industry via sex chromosome identification and the sorting of X and Y bearing sperm. Sexing of mammalian sperm is most efficiently done through flow cytometry, where the X-bearing sperm have a 3-4% higher DNA content (Johnson and Clarke, 1988; Garner, 2006). Another method includes

immunology sexing based on the H-Y antigen in the plasma membrane (Bradley, 1989); however, this reduces the motility of sperm and the conception rate due to the long processing time (Xie *et al.*, 2020).

Another example where there are clear cost, efficiency and ethical benefits to successful sex identification and manipulation is the poultry breeding and hatchery industry. Again, we observe a 50% efficiency cost of equal sex ratios in offspring. Egg production requires female poultry, while meat production generally favours male poultry. In both industries, the practice of culling day-old chicks of the undesired sex is standard (e.g., ~7 billion male chicks or ~40 million female ducklings per year globally). The sex of chicks is determined either by cloacal examination or, in some breeds, the development of sex-linked plumage traits. The culling of hatchlings in the poultry industry raises many ethical questions and public concerns (Krautwald-Junghanns *et al.*, 2018). Despite there being compelling incentives for the creation of monosex poultry lines, this remains an unsolved technical challenge, unlike in fish aquaculture (see Box 1; e.g., Curzon *et al.*, 2021). Sex-reversal of genotypically male chickens to produce reproductive egg-laying hens is rare to non-existent and is not viable at scale. Chickens possess cell-autonomous sex (Zhao *et al.*, 2010), which presents particular challenges for achieving sex reversal that endures beyond embryonic manipulations. Genetic sexing of chicken embryos may improve animal welfare and productivity. Novel *in ovo* sexing methods have been developed based on hormone measurement, DNA analysis and spectroscopy, thus eliminating the need to incubate the male eggs (Porat *et al.*, 2011; Weissmann *et al.*, 2013; Galli *et al.*, 2017). PCR-based tests, developed from sex-linked markers, have improved the efficiency of female-only egg-laying stocks (Porat *et al.*, 2011; Chen *et al.*, 2012; Clinton *et al.*, 2016). However, these sexing techniques can be time consuming and require a laboratory setup. Therefore, an *in vivo* method has been suggested which adds the green fluorescent protein (GFP) gene to the mother's Z-chromosome. This genetic modification facilitates the detection of male eggs (ZZ) through expression of this GFP in male, but not in female, offspring (Doran *et al.*, 2017). While this method is high throughput, it requires the production of genetically-modified organisms which causes other public concerns.

Box 1. Sex as a trait in seafood production

Seafood production (fisheries and aquaculture) often relies on accurate identification of sex. Unfortunately, many fish species do not exhibit any external sexual dimorphism (Devlin and Nagahama, 2002), which hinders productivity optimisation.



3.6 Studying monomorphic life stages

Similar to the challenge of sexual monomorphism in behavioural ecology (Section 3.2), sex identification remains difficult when studying the embryological development of most vertebrates. Indeed, during the bipotential phase of gonad differentiation, there are no observable structural differences between females and males. The study of embryogenesis underpins numerous research applications, from human disease and development to animal husbandry and assisted reproductive technologies (Section 3.4). Thus, the ability to balance experimental design and implement appropriate controls in developmental biology is desirable for methodological, ethical and financial reasons. A similar issue is that organisms that are sexually dimorphic as adults, are not always dimorphic as juveniles. Even though gonad differentiation occurs early in development, some species may exhibit external characteristics of the opposite sex until as late as sexual maturity (Neaves *et al.*, 2006; Martínez-Torres *et al.*, 2015; Whiteley *et al.*, 2018). In these cases, molecular sex markers are

the only way to determine whether an individual is female or male without lethal sampling and inspecting gonad morphology.

3.7 Veterinary medicine

There is often a medical need to identify the sex of domestically kept pets, to ensure their health (short or long term), make breeding recommendations and provide suitable behavioural enrichment conditions. The following veterinary treatments require sex identification to make clinical or breeding recommendations: 1) preventing and treating disorders of the reproductive system (Lumeij, 1997; Harr, 2002; Eatwell, Hedley and Barron, 2014), 2) interpreting biochemical blood analytes, 3) preventing accidental mating between relatives, 4) preventing combat in cohabitated individuals, 5) selecting successful male-female mating pairs, 6) advising on sex-associated disease risk (Tamukai *et al.*, 2011). The clinical need for sex identification methods is in addition to the natural curiosity that pet owners tend to have about the sex of their companion for naming purposes. For traditional companion animals (e.g., cats, dogs and other mammalian species) sex identification is a relatively simple matter of visual inspection. However, the recent increased uptake of exotic and unusual pet species means that sometimes the task is not so simple.

Avian pets are one of the most common species where sex identification is in high demand. Parrots are the most popular companion bird in Australia, many of which are not obviously sexually dimorphic. Typical methods employed for phenotypic sex identification in avian species include a visual assessment of sexual dimorphism, imaging such as coelomic rigid endoscopy, and radiographs, ultrasound, or computed tomography. Transcoelomic endoscopy requires a general anaesthetic and a surgical approach bringing increased risk to the patient and considerable cost. Endoscopy also requires specialised equipment and specially trained veterinarians. Ultrasound and CT are of limited use in young or small avian species. These procedures carry a risk to the animal (stress from handling, anaesthetic/radiation exposure), are expensive procedures and ultimately may not provide a definitive result. This is why commercial ventures now exist that provide avian molecular sex identification as a service either direct to the consumer or ordered through veterinary practices. Molecular sex identification carries very minimal risk to pet birds because the PCR amplification tests can be conducted using almost non-invasive sampling (e.g., a drop of blood, or a blood feather). It is also the safest method to conduct on pre-reproductive age juveniles.

Equivalent commercial molecular sex identification capacity exists for a small minority of the reptiles, amphibians and fish that increasingly present to general veterinary practice. Commercial testing is currently available for colubrid snakes, cobras, kraits, pitvipers and true vipers. In the absence of molecular tests, hobbyists and breeders will often resort to performing physical (and sometimes surgical) identification themselves, incentivised by the higher price commanded for guaranteed “breeding pairs”. Unfortunately, this means that sex identification injuries often present to the clinic such as: spinal damage, cloacal prolapse and hemipenal trauma resulting in abscessation, prolapse or necrosis of the organ and eventual morbidity to the animal. Accessible molecular sex identification for these (and other) pet species would reduce the risk of injury and infection, reduce procedure costs to owners, increase the accuracy of sex identification and vacate time for veterinarians to allocate to more critical cases, thus contributing to general improved animal health and well-being.

3.8 Detecting and monitoring sex reversal

As outlined briefly above, there is usually a very close functional relationship between genetic sex and phenotypic sex, however the relationship is not always perfect. There are

occasions when mismatches occur between genetic sex and phenotypic sex. This discordance can result from an environmental over-ride of genetic sex determination, which occurs naturally in response to temperature in several squamates (Van Dyke *et al.*, 2021; Whiteley *et al.*, 2021), or alternatively, in response to anthropogenic contamination or pollutants (Lange *et al.*, 2020; Nemesházi *et al.*, 2020), or because of mutational processes (e.g., SRY mutants; Délot and Vilain, 2018). There is an inherent assumption in any molecular test that genetic and phenotypic sex are equivalent, but practitioners need to be very careful to test this assumption and ensure that undetected processes are not disrupting the accuracy of the test. See Section 9 for detailed discussion.

4. Sex determination

Knowledge of the mode of sex determination is a critical aspect of developing sex identification markers. Female and male sexual development commences during embryogenesis. A developmental event occurs that pushes the embryo onto a trajectory that locks in the gene regulatory cascade that is required for it to develop either as a male or a female. This process is called sex determination. The initial factor that triggers sex determination can be genetic (genetic sex determination, GSD) or an external environmental factor such as temperature, pH, light exposure, or social hierarchies (environmental sex determination, ESD). Genetic sex determining factors or genes typically reside on the sex chromosomes; the alternative of polygenic sex determination, whereby differential combined expression of multiple independently segregating genes determine sex, is often considered transitory (Schartl, Georges and Graves, 2022). Examples of sex determining genes include: the male-specific gene SRY in most mammals, dosage based DMRT1 in most birds, variants of AMH in some fish, and many others. This chapter does not address the details of molecular sex determination. These regulatory processes are diverse, complex and labile across evolutionary time. See other reviews for current information on sex determination mechanisms (Capel, 2017; Nagahama *et al.*, 2021; Stöck *et al.*, 2021)

5. The formation of sex chromosomes and sex-linked sequence

Sex chromosomes are thought to evolve from an ancestral autosomal pair of chromosomes when a gene on those chromosomes captures the process of sex determination, that is, comes to direct the regulatory processes leading to female or male sexual fate (Marshall Graves and Shetty, 2001; Bachtrog *et al.*, 2014). Capture of the master sex determining role, is often achieved by a gene from the broader conserved vertebrate regulatory network that coordinates the process of sexual differentiation later in development. Many of these genes have potential to reverse sex under mutational influence. After the capture of a new master sex determining gene, a series of concurrent and consequential changes occur. Suppression of recombination in the chromosomal region with the novel sex determining gene occurs to cement the distinction between the male and female chromosomal complements (XX vs XY or ZZ vs ZW). This non-recombining region expands in stages along the sex chromosomes as new sexually antagonistic genes are recruited, those with alleles that are advantageous to the heterogametic sex and that incur a cost or are otherwise disadvantageous to the homogametic sex. Loss of recombination can result in accumulation of deleterious mutations that would otherwise be purged if there was recombination. Loss of gene function on the Y or W chromosome which, combined with the proliferation of repetitive sequence, leads to heterochromatisation and often degeneration of the Y or W. Loss of function of alleles or loss of the locus altogether on the Y or W results in dosage imbalance in the heterogametic sex, which needs to be accommodated by mechanisms of dosage compensation. We need to understand these processes of sex chromosome evolution if we are to devise strategies to identify sex-linked markers.

The progressive degeneration of the Y or W chromosomes, the proliferation of repetitive sequence, and the accumulation of mutations that would otherwise be purged in the presence of recombination, provide fodder for techniques to detect sex-linked markers (Charlesworth and Charlesworth, 2020). Such markers are quite diverse. They include the recruitment of a male specific gene on the Y which has no clear homologue on the X and so is an abundant source of sex-linked sequence. Loss of a gene from the Y, leads to its presence in double copy number in the XX females and single copy in the XY males. In this case, there are not necessarily any sex specific markers to be found, the difference lying instead in copy number signature. The sex-linked markers may establish on sequence that subsequently is involved in proliferation on the Y chromosome, such that there are multiple copies each embedded in a different context, and at different distances from the non-recombining region. Such markers have a common origin but can vary in sequence depending upon recombination, mutation and drift. Sex-linked and sex-specific markers can be in the form of SNPs, indels, microsatellites within exons or introns of coding genes or within promoter or enhancer regions, or in regions with no identifiable function. All can be a useful for sex identification marker development. These differences between species in the mode and degree of differentiation between the sexes can make genetic sex identification challenging and the most appropriate approach to detecting and characterising sex-specific sequence is typically species-specific.

6. Genomic approaches to characterise sex chromosomes and identify sex-linked sequence

To develop molecular sex-identification markers, researchers first must characterise genetic differences between the sexes. There is a huge body of literature discussing ways to achieve this (Palmer *et al.*, 2019). We have broadly categorised methodologies into nine analytical themes that share similarities in the basis of the approach (Table 1). The most effective strategies combine multiple analytical approaches to characterise sex chromosome sequence, but here we discuss each analytical theme separately to delineate their relative strengths and weaknesses. This will allow researchers to decide upon the best combination of analyses to suit species-specific sex chromosome evolutionary histories and genomic properties (Table 2). Successful hybrid approaches allow researchers to either validate the results independently (reducing the false discovery rate) or facilitate information from one technique to seed investigations using another (Cortez *et al.*, 2014; Koyama *et al.*, 2019; Cornejo-Paramo *et al.*, 2020). Bioinformatic approaches to characterise sex chromosome sequence is a rapidly evolving research area with new tools and software available all the time. For example, a recent hybrid approach (SexFindR) has broad applicability, with the capacity to identify both large sections of highly differentiated sex chromosome sequence and very small differences up to single-base resolution (Grayson *et al.*, 2022).

6.1 Cytogenetic differencing and mapping

Cytogenetic approaches were among the first techniques applied to identify sex chromosomes (Stevens, 1905). Since Nettie Stevens' seminal discovery that inheritance of the Y chromosome initiated male development, modern staining techniques for nucleic acids, increased resolution of microscopy and the integration of molecular resources, such as recombinant genomic libraries (Section 6.4) have increased the power of cytogenetic approaches. Cytogenetic approaches were particularly popular prior to the availability of high throughput and affordable next generation sequencing. Cytogenetic approaches remain a powerful method to associate anonymous sequence to specific chromosomes (including sex chromosomes). Cytogenetic techniques also remain the only way to directly visualise

chromosomes and inspect chromosome morphology. However, the time, cost, and considerable technical expertise required to successfully deploy these methods means that they are not in as regular use currently. An additional barrier to the use of cytogenetic methods is the need for cell lines or live tissue cultures to capture and fix metaphase cells. For some organisms, owing to rarity or remoteness of collection sites, or because of fundamental challenges in establishing cell lines, it can be infeasible or impossible to bring viable tissue back to the lab and to establish cultures. For example, cell cultures from sharks are notoriously difficult to establish because of a high urea & NaCl content in the blood (Uno *et al.*, 2020).

6.2 Direct sex chromosome sequencing

Physically isolating and direct sequencing of the heteromorphic sex chromosome generates high-confidence sex-chromosome derived sequences. However, this approach can be very technically challenging and dependent on how differentiated the sex chromosomes are. In mammals the highly degenerate small Y chromosome has been successfully isolated by flow sorting, which takes advantage of the disparate physical size of the Y compared to the X and autosomal chromosomes. For example, the first human Y chromosome of African descent was sequenced using long-read Oxford Nanopore sequencing of flow-sorted Y chromosome-enriched unamplified DNA template (Kuderna *et al.*, 2019). The advent of long read sequencing has improved both the feasibility and accuracy of this approach, which prior to long read technologies required the use of large-insert recombinant libraries (see 6.4) and painstaking manual curation, as was performed for the original Human Y chromosome assembly (Skaletsky *et al.*, 2003). Challenges faced by chromosome flow sorting include the difficulty of separating small Y chromosomes from the fraction of cellular debris during flow sorting. The method also fails when the heterogametic sex chromosome is a similar size to one or more other autosomes. An alternative approach is to physically isolate single Y or W chromosomes via microdissection, apply whole genome amplification techniques and directly sequence this material (Ezaz *et al.*, 2013; Zhu *et al.*, 2021). Micro-dissecting the X or Z chromosome is also important for the identification of sequence differences useful in establishing genetic sex tests, but this can be particularly challenging because the X and Z are not subject to degeneration and heterochromatism and so can be difficult to distinguish from autosomes. This is particularly the case where the sex chromosome pair is one among many microchromosomes, a characteristic feature of avian and reptile karyotypes.

6.3 Comparative genomic approaches and recombinant genomic library screening

Comparative genomic approaches use existing gene content information for one species and apply it to another species to discover novel sex chromosome sequence. This approach boasted early success in defining the sequence variation, gene content, copy number and structure of mammalian Y chromosomes (Raudsepp *et al.*, 2004; Murphy *et al.*, 2006; Perelman *et al.*, 2011; Cortez *et al.*, 2014; Bidon *et al.*, 2015). For example, Murtagh *et al.*, (2012) doubled the number of marsupial Y chromosome genes identified at the time, by developing a suite of Y-specific PCR loci for the five genes known to exist on both the Tammar wallaby and human Y chromosome. They then used conserved, invariant regions as an anchor point to screen a male bacterial artificial chromosome (BAC) library and identify and sequence new Y-specific BACs. This approach is sometimes referred to as “genome walking”. Genome walking can also be applied in the absence of recombinant large insert libraries, using short sex-linked markers (Section 6.6) extended by mapping to draft genome assembly contigs (Liu *et al.*, 2018).

The accuracy of comparative approaches improves if you combine other sources of information and genomic resources. For example, Murtagh also used flow-sorted Y-enriched DNA (Section 6.3) as a BAC probe and applied the biological knowledge that mammalian Y chromosomes tend to be enriched for genes with testis-specific expression to narrow the number of candidate BACs for full sequencing efforts. Whilst comparative approaches can be very successful in taxa with relatively stable sex chromosomes, the rapid turnover of sex chromosomes observed in other taxa (like many fish, amphibian, and squamate clades) means it can be a risky approach. Additionally, the dynamic process of novel gene acquisition and gene loss via degeneration in non-recombining regions means that the assumption of cross-species homology is by no means guaranteed, even in closely related species.

6.4 Pedigree-reliant approaches

Linkage group mapping uses a statistical framework to identify regions of the genome that show very low rates of recombination, which often corresponds to the non-recombining region of the Y or W chromosome (Palmer *et al.*, 2019). The challenge with linkage mapping approaches is that very large sample sizes with an accurately known pedigree are required. It is typical for experiments to require hundreds to thousands of progeny from multiple independent families to accurately estimate genome-wide recombination rates. It is these requirements that mean linkage mapping tends to be restricted to plants, insects and other easily bred, and reproductively prolific species (Goldberg, Spigler and Ashman, 2010; Charlesworth, 2018).

Segregation analyses take advantage of the different patterns of inheritance that occur in X and Y (or Z and W) alleles. Specifically, Y-linked alleles are exclusively paternally inherited (father-to-son) and X-linked alleles are passed from mother to son and/or father to daughter. Using the known relationships between the input parents and offspring, the program SEX-DETector assigns a likelihood to each SNP in the data set of being in one of three states: autosomal, X-linked with a Y-linked ortholog (X/Y pair) and those without (X-hemizygous) (Muyle *et al.*, 2016). Once assigned a mode of inheritance, SNPs can be mapped to a reference genome to assign sequence to the sex chromosomes.

6.5 Reduced representation methods

A popular approach to discover sex markers uses restriction enzymes (such as *EcoRI*, *MspI*, *PstI*, and *SphI*) to reduce the representation and complexity of whole genomes (e.g., (Gamble, 2016; Drinan, Loher and Hauser, 2018; Devloo-Delva *et al.*, 2022). In the past, randomly amplified polymorphic DNA (RAPD) or amplified fragment length polymorphisms (AFLP) were screened for an association to sex, but these methods had a low success rate and are labour-intensive (Quinn *et al.*, 2009; Lee *et al.*, 2011). Currently, approaches such as RAD-seq (restriction site-associated DNA sequencing), ddRAD-Seq (double digests RAD-seq), genotyping-by-sequencing (GBS), or DArT-seq are widely used because they are high throughput and cost effective (Jaccoud *et al.*, 2001; Baird *et al.*, 2008; Elshire *et al.*, 2011; Kilian *et al.*, 2012; Peterson *et al.*, 2012). See Campbell *et al.*, (2018) for an overview of available techniques. Large sample sizes and high read depths allow for robust detection of sex-linked markers. However, these different techniques target only a small percentage of the whole genome (typically 5-10%). Thus, failure to identify sex-linked sequences does not necessarily suggest that sex chromosomes are absent. This bias is especially relevant for species with homomorphic sex chromosomes that are not substantially differentiated. Despite this limitation, reduced representation methods can still be successful with complex sex chromosome systems, such as those observed in monotremes (Keating *et al.*, 2022).

Many analytical frameworks exist to identify sex-linked sequences from reduced-representation data by exploring distinct patterns of coverage and heterozygosity (e.g., Fowler and Buonaccorsi, 2016; Jeffries *et al.*, 2018; Hu *et al.*, 2019; Lange *et al.*, 2020). Here, the appropriate method to identify sex-linked sequences or SNPs depends on the sex-determination system (Palmer *et al.*, 2019). In species with an XX/XY system, unique Y-linked sequences will be present only in males whereas the opposite pattern is expected in ZZ /ZW systems (Trenkel *et al.*, 2020; Feron *et al.*, 2021; Devloo-Delva *et al.*, 2022). Of course, SNP differences between the XX and XY (or ZZ and ZW) individuals can arise from autosomal and X or Z polymorphisms, so particular care needs to be taken to ensure sufficient individuals are screened to constrain the likelihood of false positives. Statistical analyses need to admit the possibility of linkage owing either to the large number of markers screened or the presence of haploblocks. It is advisable to establish panels of validated sex markers to control for the possibility of low frequency recombination in any one marker misleading sex identification. Both types of sex-linked sequences were identified in the Pacific halibut (Drinan et al., 2018). Similarly, when read depth information per marker is available, the read depth for X-linked markers should be double in females (Devloo-Delva *et al.*, 2022). Generally, these methods require large sample sizes (n = 20-100 individuals) to acquire statistical significance, but where sample sizes are lacking, Bayesian inference methods with additional prior information can improve the marker classification (Gautier, 2014).

6.6 Genome subtraction

Genome subtraction can be conducted using either an *in vitro* approach using the annealing properties of double stranded DNA or computationally *in silico* using shotgun whole sequencing read data. The laboratory-based *in vitro* approach suppression subtractive analysis (Diatchenko *et al.*, 1996) is usually referred to as Representational Difference Analysis (RDA). Applied to naked genomic DNA, the method relies on PCR to preferentially amplify non-homologous DNA regions between digested fragments from XX and XY samples. 'Tester' DNA contains a sequence of interest, that is, unique Y sequence, that is non-homologous to the “driver” DNA of the XX individual. When the two are mixed, the driver sequence is added in excess to tester to anneal to homologous DNA fragments from the tester sample. This blocks PCR amplification and there is no increase in homologous fragments. Fragments that are different between the two samples will not anneal to a complementary counterpart and will be amplified by PCR. As more cycles of RDA are performed, the pool of unique sequence fragment copies will grow exponentially whereas fragments found in both samples, the XX and XY samples, will be proportionally eliminated. Various refinements to this technique have been developed (Luo *et al.*, 1999), but it remains technically challenging when applied to genomic DNA and is capable of driving the most meticulous researcher to leave science.

An alternative is to undertake genome subtraction *in silico* drawing upon recent techniques for accurately and comprehensively sequencing genomes (Dissanayake *et al.*, 2020). By this approach, two sets of genome sequences or “reads”, one for an XX individual and one for an XY individual are generated using one of the next-generation sequencing platforms (e.g., Illumina short-reads). These reads are then decomposed into k-mers of odd-integer length (to eliminate palindromic sequence). The k-mer sets are a highly redundant but unique representation of each genome. A subset of k-mers present in the XY set but not in the XX set are chosen to enrich for Y-specific sequence. The Y-enriched set of k-mers is then reassembled into contigs using a stringent inchworm assembler (Dissanayake *et al.*, 2020) to

provide a basis for identifying primers for a PCR sex test which can then be validated (Section 7.4).

A challenge with this approach is that it will identify both Y-specific sequence and interindividual polymorphisms that occur between the XX individual and the XY individual. Such autosomal polymorphisms can result in a large number of false positives, that is, contigs that are distinct between the two focal individuals, but that fail when applied to the panel of 20 males and 20 females. To avoid this high false positive rate, one can subtract multiple XX individuals from the focal XY individual. Alternatively, the false positive rate can be reduced by selecting samples from highly inbred populations (e.g., invasive populations with very few founders) or captive bred lines.

6.7 Sequencing read depth comparisons

When sex chromosomes are sufficiently diverged, the copy number of regions on the X and Y (or Z and W) chromosomes can differ. In non-recombining regions, females are expected to have double the read depth for X-chromosome regions and males are expected to have half the read depth of females, they having only one X, or vice versa for a ZW/ZZ system (Vicoso *et al.*, 2013; Vicoso and Bachtrog, 2015; Müller *et al.*, 2020; Sigeman, Sinclair and Hansson, 2022). This method is widely used because it can be applied with a few individuals (with associated risks of high false positive rate) and can be performed on samples that are pooled by sex (Nursyifa *et al.*, 2021). Additionally, examination of SNP density in the sex specific regions can provide information on the age of sex-chromosomes and synteny between species (Sigeman, Sinclair and Hansson, 2022). However, the analysis is generally performed on whole-genome sequencing data with high average read depth (>20x; Vicoso and Bachtrog, 2015; Palmer *et al.*, 2019). Obtaining such data is still expensive and requires high DNA quality and quantity from accurately sexed animals, which can be problematic for rare species or where sampling occurs in remote locations. Moreover, for comparing read depth between sexes, a good-quality reference genome (for a closely related species) is needed to accurately map sequencing reads. Mapping errors, owing to X-Y (or Z-W) orthology or repeated regions on sex chromosomes, can bias the observed coverage patterns (Palmer *et al.*, 2019; Nursyifa *et al.*, 2021). Any markers developed using this technique need to be validated against a panel of known sex individuals from across the range for which the markers are to be applied (Section 7.4).

6.8 Genome-transcriptome assisted methods

The advent of affordable shotgun genome and transcriptome sequencing has facilitated an array of approaches that use combinations of these two data types to characterise sex chromosomes (Cortez *et al.*, 2014). Such a combination of genomic sequence data and transcriptomic data is essential because many genes will become sex specific in expression well after sex determination and as the consequential sex differentiation process plays out. A common approach is to use the property of sex-specific and/or tissue-specific expression of Y or W genes to map sequence to the sex chromosomes. For example, Ayers *et al.*, (2013), used the genome assembly of a male chicken (ZZ) to map sex-specific *de novo* assembled transcripts to the draft Z chromosome reference. This allowed them to define and differentiate W gene sequences from their Z gametologue sequences. These Z/W variable sites are the necessary information to generate sex identification tests. Other approaches are similar to subtraction analyses (Section 6.6). Here, RNA-seq reads from the heterogametic sex are mapped to a homogametic refence genome, and unmapped reads are investigated as potential sex-limited regions (Cortez *et al.*, 2014). Expression-based approaches tend to be most successful in species with sufficiently differentiated sex chromosomes that sex-specificity

among RNA-seq reads is expected and/or there has been novel acquisition of genes to the Y or W chromosome (Palmer *et al.*, 2019).

6.9 *de novo* whole genome sequencing and haplotype phasing

The revolution in high throughput next generation sequencing is still progressing rapidly, and likely to transform our approaches to identifying sex-specific sequence. In particular, the advent of long-read sequencing technology (e.g., PacBio HiFi, Oxford Nanopore Technology, BGI srLTR) and chromatin conformation capture sequencing technology (e.g., Hi-C, DoveTail) has overcome many challenges of *de-novo* assembling sex chromosomes. Short-read assembly algorithms are not able to disambiguate regions of high sex chromosome homology, extensive tracts of repetitive elements and mega-base long high sequence-identity palindromes (Rozen *et al.*, 2003; Skaletsky *et al.*, 2003; Katsura, Iwase and Satta, 2012). It is now possible to generate high quality assemblies using PacBio HiFi sequencing as the backbone, Oxford Nanopore ultralong reads to gap fill difficult regions and scaffold the HiFi reads, and Hi-C sequencing to provide additional scaffolding including across difficult to assemble repetitive regions such as the centromeres. These techniques can be applied to single DNA strands and so deliver telomere to telomere fully-phased haplotypes (Xue *et al.*, 2021). Near complete X and Y or Z and W sequence can be obtained, with obvious benefits for those interested in the sequence differences between sex chromosomes.

Table 1: Analytical approaches and molecular methodologies to identify sex chromosome sequence which you can use as a template to develop sex identification markers. This table groups approaches thematically and provides examples for each but is not an exhaustive list.

Approaches and methodologies	Basis of the approach	Pros/Cons	Cost	Technical difficulty	Example references and software
1. Cytogenetic differencing and mapping					
Comparative Genomic Hybridisation	The application of labelled male of female DNA to chromosome spreads of the opposite sex, to highlight regions of the genome that differ between males and females.	Very labour intensive, requires specialised skills and laboratory equipment, low throughput	Moderate	High	(Traut, Eickhof and Schorch, 2001; Wang *et al.*, 2015)
Random repeat/microsatellite motif mapping	Identifies Y or W sex chromosomes if they are highly enriched repetitive elements and retroviral insertions.	Random selection of motifs is hit or miss, unless you complement it with a sequencing-based method to characterise sex-specific repeats.	Moderate	High	(Ezaz *et al.*, 2013; Matsubara *et al.*, 2016)
C- Banding	Identifies heterochromatin on chromosomes	Sex chromosomes not necessarily identifiable using this technique.	Moderate	High	(Ezaz, Quinn, *et al.*, 2009)
2. Direct sex chromosome sequencing					
Sex chromosome microdissection	The heterogametic sex chromosome is physically isolated either with a very fine probe or laser-microdissection. The single chromosome is whole genome amplified and sequenced.	Very challenging technically. Very low-input material results in low complexity libraries and potential biases arise during amplification of a single molecule.	High	High	(Ezaz *et al.*, 2013; Matsubara *et al.*, 2016; Kuderna *et al.*, 2019; Zhu *et al.*, 2021)
Sex chromosome flow sorting	Relies on the size of the sex chromosomes to physically separate them from the rest of the genome.	Difficult to isolate the W or Y in species with either small sex chromosomes or poorly differentiated sex chromosomes.	High	High	(Sankovic *et al.*, 2006)
3. Comparative genomic approaches and recombinant genomic library screening					

Cross-species probes/PCR amplification	Requires characterised sex chromosomes in at least one target species. Assumes homology of sex chromosomes across distantly related taxa.	Less successful in rapidly evolving or divergent sex chromosome systems. Risky because it is not targeted to detect sex differences.	Low	Moderate	(Bidon *et al.*, 2015)
BAC mapping and sequencing	Uses bacterial artificial constructs to map regions to sex chromosomes	Labour intensive, requires screening of large BAC libraries to identify sex chromosome specific sequences, low throughput	High	High	(Sankovic *et al.*, 2006; Ezaz, Moritz, *et al.*, 2009; Quinn *et al.*, 2010; Ayers *et al.*, 2013)
4. Pedigree-reliant approaches					
Linkage group mapping	Identifies areas of low recombination in the genome that might be the non-recombining region.	Requires known pedigree and very large sample size. May not work well in species with young sex chromosome at the early stages of recombination inhibition.	High	High	(Tao *et al.*, 2020) (Ayllon *et al.*, 2020)
Segregation analysis	Infers sex-linked genes using patterns of allelic segregation.	Requires data from parents and offspring, sensitive to pipeline parameters.	Moderate	Moderate	SEX-DETector (Muyle *et al.*, 2016)
5. Reduced representation methods					
RAPDs (randomly amplified polymorphic DNA)	Genome complexity reduction using short synthetic primers. Then identification of sex-specific amplified fragments.	No longer a recommended approach due to low reproducibility. Generates anonymous loci.	Low	Low	(Viñas *et al.*, 2012; Sun *et al.*, 2014)
AFLPs (amplified fragment length polymorphisms)	Genome complexity reduction via selective PCR amplification of restriction fragments from a total digest of genomic DNA. Then identification of sex-specific amplified fragments	Less informative than sequencing-based approaches. Generates anonymous loci.	Low	Low	(Quinn *et al.*, 2009; Lee *et al.*, 2011)
RAD-seq / ddRAD-seq	Genome complexity reduction via RE digest and then the identification of alleles that are sex-specific.	Fast turnaround. Can screen large numbers of individuals.	Low per sample; but many	Low	(Peterson *et al.*, 2012; Fowler and Buonaccorsi, 2016;

		But only surveying a small portion of the genome (typically 5-10%). Problematic for small sex chromosomes, especially repetitive ones.	samples required		Brelsford *et al.*, 2017; Gamble *et al.*, 2017)
DArT-seq	An equivalent method to RAD approaches.	Fast turnaround. Can screen large numbers of individuals. But only surveying a small portion of the genome (typically 5-10%). Problematic for small sex chromosomes, especially repetitive ones.	Low per sample; but many samples required	Low	(Kilian *et al.*, 2012; Lambert, Skelly and Ezaz, 2016; Hill *et al.*, 2018)
Sex-linked pattern analysis from fastq files or SNP genotype calls	Investigates contrasting heterozygosity and coverage patterns between sexes.	Existing SNP datasets can be combined and re-analysed for comparative studies. Yet, partial genome coverage and homomorphic sex chromosomes can yield false negative results.	Low if utilising existing data.	Low	(Gamble, 2016; Feron *et al.*, 2021; Devloo-Delva *et al.*, 2022)
F_{ST}-based approaches	Identifies allelic differences between sexes (e.g., via F_{ST} and outlier detection) to identify an association with sexual phenotype.	Able to identify sex-linked markers where sex-determining genes located on multiple chromosomes. However, F_{ST} -based methods can yield false positive/negative results due to technical error (e.g., genotyping error) or bias in sample schemes (e.g., population structure).	Low per sample; but many samples required	Low	(Benestan *et al.*, 2017; Drinan, Loher and Hauser, 2018; Dixon, Kitano and Kirkpatrick, 2019; Trenkel *et al.*, 2020)
Bayesian classification	Model-based approach that accounts for differences in allele frequencies	Provides higher confidence in the identified markers, even at relatively low sample	Low per sample; but many	Low	(Gautier, 2014)

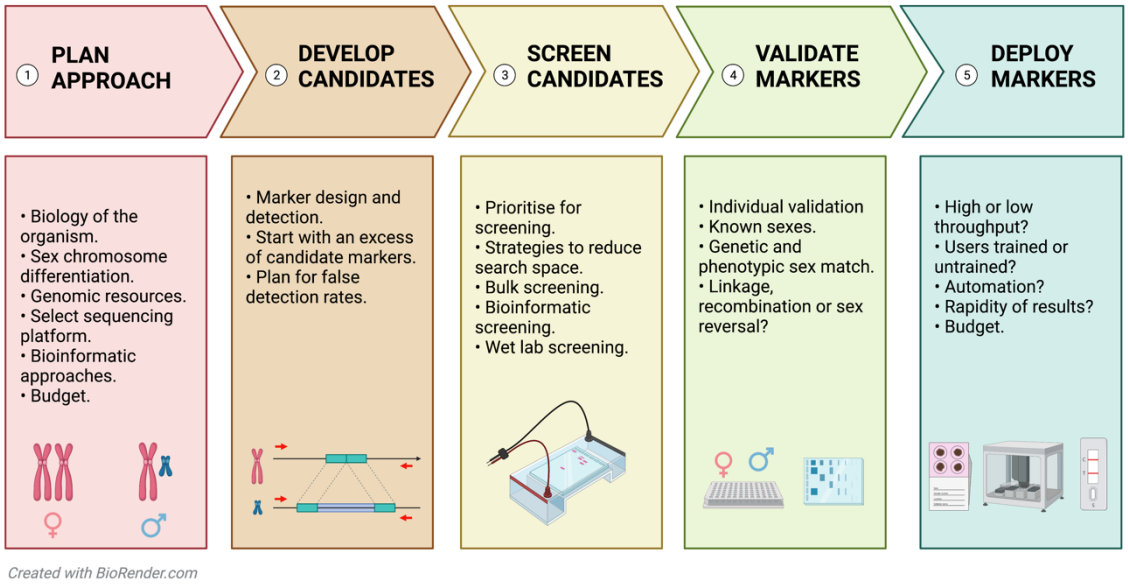
	due to distinct demographic histories and genotyping errors.	sizes (n>20). By accounting for genotyping error the model can combine data from different genotyping platforms.	samples required		
6. Genome subtraction					
Sequencing-based *in silico* subtraction and re-assembly	Computational approach to identify sex specific k-mers that are then re-assembled to form larger sex-specific contigs.	Reference genome free. Only requires low coverage sequencing (<10x). Can be conducted with a low number of individuals.	Moderate	Low	(Cornejo-Paramo *et al.*, 2020; Dissanayake *et al.*, 2020)
Subtraction to identify sex-specific repetitive motifs	After a genome subtraction, the sex-specific Kmer frequency distribution is plotted and inspected to identify very high frequency sequence motifs.	A bioinformatic approach to assist the selection of motifs for cytogenetic repeat mapping.	Moderate	Low	Arthur Georges - unpublished
in vitro genome subtraction and sequencing	A lab-based approach that uses the annealing properties of DNA from different sexes followed by rounds of PCR amplification to amplify sex-specific sequence.	PCR-bias towards sex-linked repetitive elements can swamp the signal and leave very few reads containing gene-rich single-copy regions.	Low	High	(Diatchenko *et al.*, 1996; Luo *et al.*, 1999)
7. Sequencing read depth comparisons					
Read depth ratios	Looks for hemizygous regions by comparing read-depths of reciprocally mapped reads.	Sequencing at relatively high read depth (>20*x*) is needed. Requires a good-quality reference genome and is sensitive to read-mapping algorithm parameters.	High	Moderate	(Chen *et al.*, 2014) (Wu *et al.*, 2021) (Nursyifa *et al.*, 2021)
Chromosome quotient	A variation of the read coverage ratio method that identifies the equidistant point between the peaks of the read distributions from female and male low coverage sequencing.	Can use lower coverage sequencing (>5X) at Y-chromosome regions. Reduces the noise from mapping algorithms, but still	Moderate	Moderate	Reviewed in (Palmer *et al.*, 2019) Original reference (Hall *et al.*, 2013)

		requires a good-quality reference genome.			
8. Genome-transcriptome assisted methods					
Opposite sex RNA-seq mapping	Mapping transcriptome reads to a reference genome of the same sex to identify either unmapped regions or hemizygous regions.	Requires reference genome of known sex. Only assesses coding regions of the sex chromosomes. Samples need to be from gonad tissues.	Moderate	Moderate	(Ayers *et al.*, 2013)
Pool-seq	Many individuals are grouped by sex and sequenced as two DNA or RNA samples. Male and female pools are then mapped to a reference to identify either unmapped regions or hemizygous regions.	Requires reference genome. Susceptible to biases from sex reversed individuals. Best used for species without environmentally sensitive sex determination.	Low - Moderate	Moderate	(Adolfi *et al.*, 2021; Kuhl *et al.*, 2021)
9. *de novo* whole genome sequencing and haplotype phasing					
Haplotype reconstruction	*De novo* assembly of a whole genome of the heterogametic sex, with long read sequencing at sufficient coverage to assemble and phase both sex chromosome haplotypes.	Very costly and computationally expensive. Over-kill if only short sex markers are required for the application.	Very high	High	(Xue *et al.*, 2021)

7. A framework to characterise molecular sex-identification markers

On the face of it, developing a molecular marker to identify the genotypic sex of individuals sounds like a simple task: discover sex-specific polymorphisms and screen individuals. However, it is important to recognize that there is a great diversity of sex determination modes in the animal kingdom, and this will greatly influence which analytical approaches are feasible. It is important for researchers inexperienced in the field of sex determination and sex chromosome evolution not to underestimate the complexity or the time it can take to develop robust sex markers. Here we outline a discovery framework that articulates the full process of sex marker discovery. This framework will guide decision making at all stages from articulating the necessary biological information, deciding on the sequence discovery strategy, to developing, validating, and deploying a test. The framework is a five-stage process summarised in Figure 1 and described in detail below.

Figure 1. Framework for the discovery of molecular sex-identification markers



7.1 PLAN a strategy to discover novel sex-identification markers

It is essential when planning a strategy to develop sex-identification markers, to first assemble and use all the available *biological information* and *genomic resources* you have at your disposal (Table 2). Once this is collected, an informed decision can be made about which of the multitude of approaches you should employ to discover sex chromosomes and/or sex-linked sequence (Section 6). However, a difficulty that regularly emerges, is that the information required to plan your approach is incomplete and the researcher will be forced to make decisions in the absence of reliable information.

Firstly, you must consider the biology of the study organism. A factor that will greatly influence how readily a sex test can be developed, and which analytical approach is suitable, is whether the sex determination mode of the species is known (GSD/ TSD/ gene-environment interaction). It is critically important to establish that the species does indeed have sex chromosomes, prior to any work attempting to develop genotypic sex-identification markers. If the mode of sex determination is unknown, there are a variety of experimental approaches to ascertain this (Whiteley *et al.*, 2021). If breeding experiments are not feasible, comparative phylogenetic methods exist to infer the likelihood of the species displaying a

thermosensitive aspect to their sex determination, that could disrupt marker development. In many species, particularly amphibians, fishes and non-avian reptiles, sex can be influenced by a variety of factors and sex reversal may occur under environmental influence. See Ayllon *et al.*, (2020) for a recent example of how a commonly used sex marker displays discordance with phenotypic sex in a salmonid.

After the presence of sex chromosomes is established, there are several characteristics of these sex chromosomes that you need to consider before selecting an appropriate analytical approach (Table 2). For example, can the sex chromosomes be distinguished using standard (G-banding) or advanced cytogenetic techniques (CGH, comparative genomic hybridization), are you expecting the sex chromosomes to be very similar in sequence content or very different, and/or is there an environmental override that can cause sex reversal in your species?

After weighing the biological and molecular considerations, it is also important take practical considerations into account, such as: the constraints of time and money, sample volumes (high throughput or low throughput), does the test need to be deployed by non-experts. Additionally, you should also decide what the ultimate goal of the project is. For example, if your project aims to develop a simple test to identify the species' chromosomal sex, it is not necessary to sequence and assemble the whole sex chromosome. In contrast, if the project aims to characterise the gene content of the sex chromosomes and discover sex determining genes then a much higher investment of resources is required.

Table 2. The ideal but often incomplete information needed to plan a strategy to characterise sex chromosomes and sex-linked sequence

	Planning implications	Options if information unknown or resource unavailable?
Biological and evolutionary knowledge		
Sex chromosomes present?	Informs appropriate sequencing techniques to identify sex chromosome complement and content.	1. Incubation experiments to detect the presence/absence of thermosensitive sex determination. 2. Studies of sex ratio variability in the wild. 3. Cytogenetic or karyotypic investigations.
Female or male heterogamety?	Can target discovery to the heterogametic sex.	1. Conducting analysis of sex linkage for both scenarios. This will increase the cost because it requires more individuals and computational investment.
Young/old sex chromosomes?	Young sex chromosomes require high depth sequencing approaches as they are typically not well differentiated from each other. They may not be identifiable using cytogenetic approaches (homomorphic). Old sex chromosomes with highly	1. Cytogenetic or karyotypic investigations. 2. Drawing inference from related species or lineages that have this data.

	divergent content are easier to detect cytogenetically or with lower coverage sequencing.	
The actual and relative size of sex chromosomes?	Can facilitate physical isolation methodology if the heteromorphic sex chromosome is larger than cellular debris and a unique size compared to the rest of the karyotype.	1. Perform necessary karyotypic analysis. 2. Draw inference from related species or lineages that have this data.
Existence of microchromosomes?	Increases the difficulty of cytogenetic approaches. Will require specialist expertise.	1. Cytogenetic or karyotypic investigations. 2. Draw inference from related species or lineages that have this data.
Environmentally sensitive sex determination and the possibility of sex reversal?	Need to combine genotyping and phenotyping to ensure that both the homogametic and heterogametic sex are compared. Be very rigorous in the selection of individuals analysed.	1. Incubation experiments to detect the presence/absence of thermosensitive sex determination. 2. Studies of sex ratio variability in the wild. 3. Study the offspring sex ratios of individuals chose for sequencing and avoid individuals that do not produce 50:50. 4. Use statistical methods that are robust to a low level of sex reversal (e.g., reduced representation methods).
Rapid sex chromosome turnover?	Do not assume homology of sex determination modes and/or sex chromosomes to sister taxa.	1. Drawing inference from based on lineages that have this data.
Molecular and genomic resources		
Assembled genome/s (M/F/Unknown)	Increases the choice of methods and will increase	Choose reference-free methods, such as reduced representation (6.5) or subtraction approaches (6.6).
BAC library	Enables screening and/or cytogenetic FISH approaches	It is now possible to synthesise large custom oligonuclotide probes if one is needed for library screening. Alternatively, you could use methods that do not rely on this resource.
Cell lines	Enables cytogenetic approaches.	Cytogenetic approaches are possible without established cell lines e.g., short-term blood cultures.
Karyotype	Can distinguish species with highly degenerate heteromorphic sex chromosomes from ones with	Rely on bioinformatic approaches where you can test for female/male heterogamety.

	weakly differentiated homomorphic sex chromosomes. Can assist in establishing female/male heterogamety.	

7.2 DEVELOP a suite of candidate markers

Developing candidate sex markers requires a characterised set of sequences associated with sex. This can be achieved by employing one or more of the sex chromosome sequence discovery approaches discussed extensively in Section 6 (Table 1). The next task is to use these sequences as a template to develop a suite of candidate sex-identification markers.

A common and cost-effective approach is to design PCR primers in regions that flank putative sex specific variation (e.g., a sex-specific insertion/deletion). By priming the reaction with sequence common to both sexes, the amplification of X or Z sequence functions as an internal PCR control band, that ensures that failed PCRs are not interpreted as a positive result for the homogametic sex. A similar style of assay can also be achieved by co-amplifying an autosomal control with Y or W specific primers in a multiplex PCR. After amplification, the result of the assay can be easily established using a range of common DNA fragment size analysis lab techniques (e.g., agarose/polyacrylamide gel electrophoresis). If you are limited to designing presence/absence style markers that only amplify in one sex (Y or W chromosome markers), the risk of PCR failure being misinterpreted as identification of the homogametic sex must be mitigated by performing additional positive control PCRs that amplify autosomal regions and/or amplifying several presence/absence loci. Another PCR-based marker development strategy involves using real-time quantitative PCR to detect differences in gene-dosage on the sex chromosomes (Rovatsos *et al.*, 2014; Wiggins *et al.*, 2020). However, quantitative PCR can be challenging due to the need for high accuracy to detect the difference in single versus double copy number. Technical replicates are necessary, which increase the time and cost investment. It is also best practise to use more than a single sex-linked locus to assign the sex of individuals based on this approach.

Designing assays to survey for sex-specific repeats is difficult if the flanking sequence is unknown. However, even if only the sex-specific repeat-motif is identified, this information can be used to develop chromosome-specific cytogenetic markers such as Y or W probes for use with fluorescent *in situ* hybridisation (FISH; Matsubara *et al.*, 2016).

For methods that identify sex-linked SNPs, implementing a sequencing capture-array that targets and enriches for many loci simultaneously is usually the most time and cost-effective strategy that has superseded methods for typing individual SNP loci. Additionally, a SNP panel or microarray can be designed to identify sex, species, and origin of tissue samples, which is a cost-effective method for the monitoring of wildlife and international trade of species or derived products (Arenas *et al.*, 2017). Alternatively, if there is a sufficiently high budget and a reference genome, it is possible to whole genome re-sequence all individuals of unknown sex at low coverage and use pipelines such as sex assignment through coverage (SATC; Nursyifa *et al.*, 2021).

7.3 SCREEN many sex markers on a few individuals to assess the success of your discovery approach

After developing a suite of sex identification markers, the next stage is to screen them to ensure sex-linkage. All the methods described to characterise sex chromosome sequence

(Section 6; Table 1) are imperfect, which will result in a false discovery rate for sex identification markers that can vary considerably. Therefore, you should always initially screen many more candidate markers than you require. The biology of the organism and differentiation of the sex chromosomes will also affect the false discovery rate for marker design. For example, the same *in silico* subtraction approach was recently applied to two Australian lizards with a large difference in marker discovery success. A skink with dimorphic XY sex chromosomes had a success rate of 8% (7 markers from 92 loci screened); whereas a dragon lizard with homomorphic micro sex chromosomes failed to discover any sex-linked loci, thus had an undefined success rate of <1% (0 markers from 90 loci screened; Zhang *et al.*, 2022) and instead revealed a high incidence of large polymorphic indels within wild populations. Sex is determined in the fish Fugu by a single base pair difference on the sex chromosomes (Kamiya *et al.*, 2012). This could easily be missed by representational approaches, buried in typically millions of autosomal SNP polymorphic variants assembled as contigs using genome subtraction, and mistaken for an infrequent read error using even the latest genome sequencing technologies. The biology of the organism matters. These studies demonstrate how the evolutionary history of sex chromosomes affects the power to detect sex differences, even when the same methods are applied. Marker screening can be a big job with a low success rate, so it is important to develop a strategy that will rapidly reduce the search-space, for example, this can be achieved through combinatorial sample pooling strategies (Dissanayake *et al.*, 2020). It is also important to consider the goals of your study and prioritise your candidates for full screening. For example, you may prioritise based on the size of the putative sex chromosome contig, the presence of open reading frames or gene ortholog BLAST hits if you are searching for sex chromosome genes. During the screening process it is essential to conduct appropriate positive, negative and no-template controls for every screen. Omitting these essential quality control measures risks false detections, biased results, wasted time, effort and money.

7.4 VALIDATE sex-linkage of the few putative markers to many individuals

After you have narrowed the search space and arrived at a small number of high-confidence sex-marker loci, it is time to invest in detailed individual-based validation of the test. Initially this involves employing the test across a panel of *known* genotypic males and females (minimum 20 individuals per sex) to show that the test is consistently sex specific. From these data, researchers can establish at least a preliminary estimate of the false positive and false negative rates for the test and use this as criteria to prioritise the use of the most accurate markers. In addition to reporting on the accuracy of the test, it is also important to quantify reproducibility (i.e. include technical replicates) and sensitivity (i.e. minimum required DNA quality and quantity). Once the test is routinely used, it is important to continually assess the accuracy of the test and to always use male, female and no-template PCR controls. These best practises will ensure that if mutation, recombination or transposition events occur that disrupt sex-linkage, they will be detected and will not generate spurious biological inference (Georges, Holleley and Graves, 2021).

7.5 DEPLOY the sex test in a research or industry setting

In many cases the molecular methodology used to screen and validate the putative sex markers will be appropriate, manageable and cost-effective enough for routine use. For example, PCR-based methods including presence/absence (Dissanayake *et al.*, 2020) and size polymorphism detection (Huynen, Millar and Lambert, 2002; Quinn *et al.*, 2009, 2010; Keating *et al.*, 2022), genomic dosage detection via quantitative real-time PCR (Rovatsos *et al.*, 2014; Wiggins *et al.*, 2020), or SNP-capture based arrays (Hill *et al.*, 2022). However, there are also situations where how you deploy a test requires additional consideration. For

example, if there is a need for a large volume of samples to be processed or if the results are needed rapidly for time-sensitive decision making or diagnostics, or perhaps both.

There are many ways to optimise and translate a successful low-throughput PCR based test, into a more time and cost-effective methodology. Each stage of the test needs to be investigated to make efficiency gains. For example, sample collection and other downstream processes can be streamlined and standardised using FTA cards. PCR set up can be miniaturised and/or automated using robotic liquid handlers. Thus, the significant gains in time and cost efficiency that depend on how you deploy the test are worth considering. Sometimes, it is the immediacy of the sex identification result, to enable rapid decision-making that is highest priority, above high throughput sample processing. The style of test where you can go from a fresh sample to a result in a matter of minutes in any location, is usually referred to as a "point of collection" test (POC). However, depending on the setting and application these can also be referred to by similar names, such as "point of care" tests in medical of veterinary science or "point of capture" tests for ecological research. Most people would be familiar with the style of test, due to the global deployment of at home rapid antigen tests (RATs) during the COVID-19 pandemic. In addition to the immediacy of the result, the potential benefit of this style of test is that it does not require specialised laboratory equipment and can be deployed by untrained personnel or citizens. However, budget can be a major constraint due to the high cost of consumables and reagents. To enable extraction, amplification and detection of nucleic acids in a point of collection sex test, requires thoughtful design and manufacture of the final test (Box 2).

For applications that require both high throughput and rapid decision making, *in vivo* test deployment is desirable. Industrial and commercial applications can have sufficient throughput of individuals to justify the extensive additional investment in research and development. For example, *in ovo* sex testing in the poultry industry is not yet deployed but is in active development, with a range of solutions in consideration, including hormone measurement, DNA analysis and spectroscopy, and even gene-editing CRISPR technology (Galli *et al.*, 2017; Khwatenge and Nahashon, 2021).

Box 2. Design, implementation and examples of point of collection sex identification tests.

Point of collection tests require significant additional optimisation. For example, the DNA extraction method will depend upon the intended sample type, which can vary in their composition and the presence/absence of inhibitors. Liquid samples are usually the easiest (e.g. blood, saliva) to transfer to a POC workflow and are readily implemented using either room temperature extractions (e.g. Alk-PEG) or high temperature incubations (e.g. HotSHOT).

Another major difference between standard PCR and POC tests is the method of DNA amplification, which requires newly designed primers and thermal cycling regimes. There are two main isothermal amplification approaches: Loop-mediated isothermal amplification (LAMP) and Recombinase Polymerase Amplification (RPA). LAMP utilises 4 - 6 primers to target a sex-specific locus and the primers amplify at a constant temperature continuously until the reaction is exhausted (between 55-65˚C). Continuous amplification can facilitate the use of less sensitive downstream detection methods. RPA utilises two primers and a probe to target a sex-specific locus. Similar to LAMP, the enzyme and primer mix bind and continuously amplify, but do so at a much lower constant temperature (~ 37˚C). The probe is designed to be 'blocked' until it binds to the target site, and upon binding is cut at the THF site by an enzyme (Endonuclease IV), enabling extension and generation of tagged, W-specific double-stranded DNA product. Significantly, RPA isothermal amplification occurs at approximately body temperature, and this has the benefit of eliminating the need to high temperature incubation equipment.

The final step of a POC test is to visualise the result by detecting the amplified DNA. A variety of detection methods exist depending on the specific application and amplification technology used. In the case of a binary presence/absence expected outcome for a number of methods are available, including simply observing turbidity in the reaction tube or adding standard DNA fluorescent dyes (e.g. SYBR Safe) and then shining a hand-held UV torch on the sample. If the diagnostic test produces a number of targets, then more complex methods are required. Tagging of RPA products is achieved through designing the primers to generate double-stranded DNA product which is specific to the target region, leading to naked-eye detection via commercially available lateral flow devices.

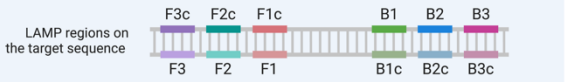
Raptor Sex Identification POC Test
Loop-Mediated Isothermal Amplification (LAMP)

LAMP Reaction 1: ACCI-W

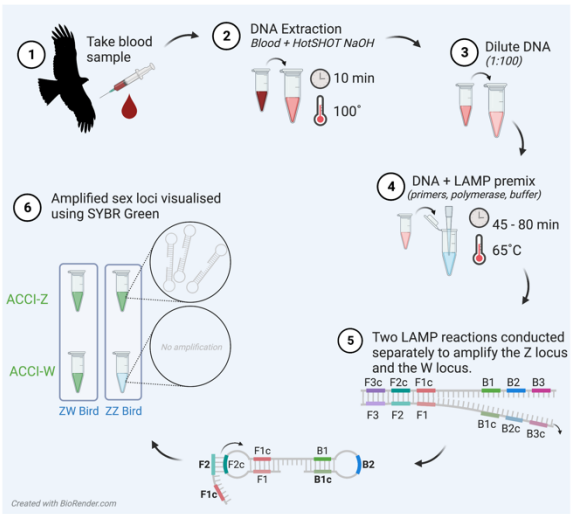
F3: 5'-TTTCACACATGGCACACC-3'
B3 5'-GTTTTCTTTCTGAGATGGAGTC-3'
FIP 5'-AGTTCAAAGCTACGTGACTAAAACATTTTTTCCCCCATTTTTGACAGG-3'
BIP 5'-ATTCCAGATCAGCTTTAATGGAAGTTTTTCAGATCCAGAATATCTTCTGCTC-3'

LAMP Reaction 2: ACCI- Z

F3 5-AMCAGCTGATATTGGAAGG-3'
B3 5'-TTTCTTTASTYTGAGGGTGA-3'
FIP 5'-GGCAACYTGCTTTMRCTGTYGTTTTACCTCTGGMTATSGTCTTG-3'
BIP 5'-CCAGGTGGCTTYTGAATGTCATTTTGCRCTGGAACAAGTTGTC-3'



Note: F indicates forward primer; B indicates reverse primer; c indicates complementary sequence.

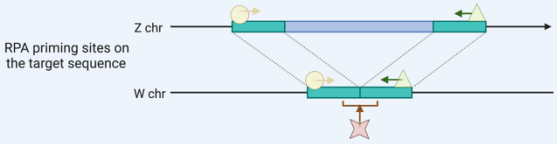


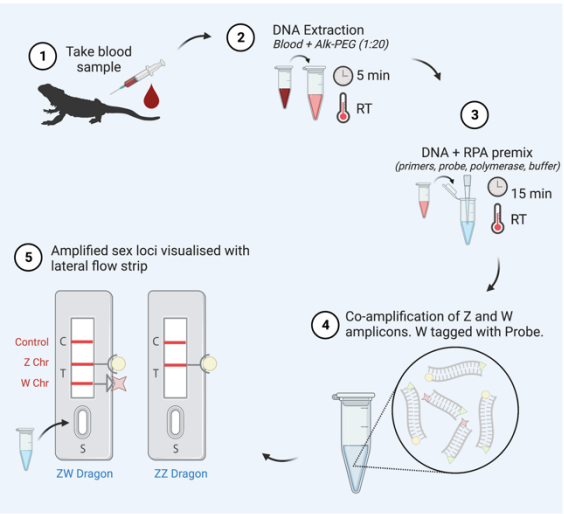
Bearded Dragon Sex Identification POC Test
Recombinase Polymerase Amplification (RPA)

Single RPA Reaction:

Z/W FWD: 5'-**DIGN**-TCACCTCGCCCTGAGTCCTCTCTCTAATTCTG-3'
Z/W REV: 5'-**BiosG**-TGGATCCAAGGCAAAGACCGTCTCCTGGAG-3'
W Probe: 5'-6FAM-AAAACCCCACCTGAGGCCACCAAGA-THF-CCTTCCTTGAGGAGACCTTCTTTCCC-3SpC3-3'

Note: THF and 3SPC3 oligo modifications eliminate extension from the probe binding site.





8. Sex reversal: when genetic and phenotypic sex don't match

In many species, environmental conditions can override the influence of sex chromosomes to cause sex reversal during embryogenesis, while in others, sex reversal can happen during adulthood (Radder *et al.*, 2008; Holleley *et al.*, 2015; Todd *et al.*, 2016). Sex reversal can also be induced via environmental contamination (for examples see Tubbs and McDonough, 2018; Chen *et al.*, 2019; Nemesházi *et al.*, 2020; Kar *et al.*, 2021; Mikó *et al.*, 2021). By definition, sex reversal is the discordance between the sex chromosome complement and phenotypic sex of an individual, so identification of sex reversal requires the reliable definition of both the genotypic and phenotypic sex of an animal. The first step is to develop a reliable molecular-based sex test for the species, following the principles discussed in sections above. The second step requires phenotypic sex identification. Phenotypic sex identification will be most accurate if the gonads are inspected, but for nonlethal methods phenotypic sex should be confirmed by several traits and/or reproductive status (e.g., pregnancy, gravidity, egg laying, maternity/paternity assignment).

Once sex reversal is established to occur in a species through reliable documentation of genotype-phenotype mismatch, they can be used as powerful indicators of environmental or anthropogenic change. In the central bearded dragon (*Pogona vitticeps*), sex reversal occurs in the wild (Holleley *et al.*, 2015), but at varying rates across the species range (Castelli *et al.*, 2020), showing the evolvability of sex determination systems in response to local climatic conditions. Similarly, wild populations of the eastern three-lined skink (*Bassiana duperreyi*) display sex reversal, but the rates of reversal are influenced by elevation in montane areas (Dissanayake *et al.*, 2021; Dissanayake, Holleley and Georges, 2021). For both species, modelling has shown the potential for the loss of the heteromorphic sex chromosome (W chromosome for *P. vitticeps* and Y chromosome for *B. duperreyi*) under certain climatic conditions. Sex chromosome loss would have profound implications for the affected populations of both species (Schwanz *et al.*, 2020; Dissanayake *et al.*, 2021).

Rapid evolutionary changes can occur in captive colonies maintained for research or aquaculture. Many studies using independently maintained captive zebrafish colonies yielded inconsistent results for sex linkage to one of three different chromosomes; sex ratios were far from 1:1. Studies of wild zebrafish show an unequivocal ZW system (Wilson *et al.*, 2014). Female-to-male sex reversal of fish with the ZW genotype during domestication appears to have led to the eventual loss of the Z chromosome (Wang *et al.*, 2022). The domesticated zebrafish strains are composed of only WW genotypes, some of which become females and other become fertile males. This is a case of polygenic sex determination, presumably transitory, and a wonderful opportunity to study the first steps in evolution of new sex determining genes (Schartl, Georges and Graves, 2022).

Lastly, some fish switch sexes as they grow, through a process of natural sex change. For example, Barramundi begin life as males and switch to females after reaching a threshold size (Davis, 1982). Others will switch sex on receipt of social cues, such as when the dominant male in a school dies (Gemmell *et al.*, 2019). In these and many other cases, genetic sex and phenotypic sex are decoupled, and the application of molecular sex markers is more complicated.

9. Molecular markers for phenotypic sex

Up until now, this chapter has focussed exclusively on developing molecular markers that are informative about *genetic* sex. However, there are species that do not have sex chromosomes

at all. In such species, sex is typically determined during embryonic development by some environmental factor, which is broadly known as environmental sex determination (ESD). The most well-known is temperature (temperature dependent sex determination, TSD), which is widespread in reptiles. Many fish species are sensitive to such cues in adulthood, which can trigger sex change during an individual's lifetime (e.g., in the wrasse, Todd *et al.*, 2016). In these instances, sex chromosome markers are either absent or insufficient in isolation to answer applied research questions.

Species with ESD systems are the most difficult to develop a sex test for. As these species do not have sex chromosomes, there is no difference in the genome between males and females for which a sex test can be developed. Instead "gene-expression-based" approaches will be required. It is important to note that these approaches are all based on assessing the expression of genes, or gene products (rather than sequence differences), involved in sex-specific functions, so ultimately will determine the phenotypic sex of the animal. Ideally, a gene-expression-based sex test will use a non-lethal sampling method, which currently presents considerable technical challenges.

The first epigenetic based sex test developed used a multiplex bisulfite sequencing approach to predict sex with ~90% accuracy using a panel of seven genes in the European sea bass (Anastasiadi *et al.*, 2018). However, this approach requires sampling of gonadal tissue (fatal to the animal) as methylation levels are tissue specific. As epigenetic techniques continue to advance there is hope that in the future methylation differences between sexes will be detectable using samples that can be obtained non-fatally (Piferrer *et al.*, 2019). Again, there are major advances in methylome sequencing on the horizon. Oxford Nanopore sequencers can distinguish methylated and non-methylated bases in its ultralong reads, and PacBio HiFi sequencing uses the dynamics of the florescence-based sequencing to achieve a similar goal. Generating such methylomes for tissues for which expression profiles differ between species will provide a basis for development of phenotypic sex markers that, once discovered, can be characterised and optimised for more targeted and cost-effective phenotypic sex tests.

Researchers studying sex in endangered sea turtles with TSD whose sex is notoriously difficult to identify at the embryonic, hatchling and subadult stages, have attempted to develop non-fatal or non-invasive techniques. These have generally been based on hormone levels, obtained either from blood or amniotic fluid in the egg, and have varying degrees of accuracy (Gross *et al.*, 1995; Xia *et al.*, 2011; Allen *et al.*, 2015). A novel approach used immunohistochemical staining of CIRBP, a known thermosensitive gene, on gonad tissue biopsies. This technique was used to identify sex with 93-100% accuracy for loggerhead and leatherback turtles (Tezak, Guthrie and Wyneken, 2017). More recently, Western Blots for AMH isolated from the blood of turtle neonates had between 90-100% accuracy (Tezak *et al.*, 2020). All of these approaches come with considerable caveats. Hormone levels, regardless of how they are detected, are influenced by age and reproductive state, affecting the accuracy of sex assignment. Antibody based approaches (like immunostaining and Western Blots) are often problematic for non-model species, as they may not cross-react if the evolutionary separation is too great.

10. Future directions and outstanding questions

The most likely future application of large-scale sex testing initiatives is in aquaculture and agriculture. We anticipate that the driving financial and ethical incentives will see a rollout of *in vivo* sex identification approaches. As genomic technologies advance and as knowledge of the genetic and epigenetic mechanisms of sex determination expands (Deveson *et al.*, 2017;

Whiteley *et al.*, 2022; Zhang *et al.*, 2022), sexual phenotype manipulation and management has the potential to become a commonplace food production technique. However for this to occur, regulatory restrictions, policy and the governance of genetically-modified organisms will also need to evolve (see recent review Xie *et al.*, 2020). Once the safety and efficacy of these techniques has been established by the deployment in industry, opportunities arise for technology transfer to more high-stakes applications, such as the conservation of endangered species. It is very unlikely that sex manipulation will be incorporated as a standard assisted reproductive technology until long-term data is available on the safety of this intervention.

An advance that is on the immediate horizon and is likely to change the shape of sex determination research is the generation of chromosome-length fully-phased reference genomes for the heterogametic sex, using long-read sequencing data. This technical and bioinformatic advance will accelerate sex chromosome research which has up until recently been stymied by almost exclusively homogametic reference genomes and plagued by insurmountable assembly challenges with short read sequencing and highly repetitive DNA templates. Fully characterising both sex chromosomes will rectify a genomic blind spot that the community has long experienced. Given the importance of sex chromosomes for healthy development, organogenesis and reproductive success later in life, we anticipate that the release of a new standard of completely characterised whole genomes, will facilitate medical research into sex-specific pathologies and spur evolutionary biologists to discover further complexities of sex determination and genomic novelty hidden in these unexplored regions. Further expanding on this area, the ability to simultaneously characterise genomic and epigenomic variation using single-molecule sequencing technology (e.g., PacBio HiFi and Oxford Nanopore Promethion) promises to transform our capacity to determine both genotypic and phenotypic sex.

12. Conclusion

Ultimately what we hope is for sex-identification markers to be a simple but powerful tool to advance ecological, evolutionary, and veterinary research. Sex markers have the potential to make huge practical contributions the implementation of conservation recommendations, the efficient production of more ethically farmed food, and the health and wellbeing of animals being treated by veterinarians. Now that you are armed with the evolutionary knowledge, the molecular strategies, and the analytical tools to develop robust sex-identification markers, their application is only limited by your imagination. Remember that sex is a complicated and surprising trait to study, it is not immutable and that evolutionary processes are actively at play. Do not be beguiled by the seeming simplicity of a trait with two phenotypes, instead use this chapter as a roadmap to navigate the complexity.

11. Discussion Topics

1. What do you see as the biggest challenge when developing a molecular sex identification test for your organism of choice and why?
    a) Use Table 2 to structure your argument.
    b) Use Table 1 to choose potential strategies.

2. What are the ethical and safety concerns regarding manipulation of sexual outcomes in populations?

Guiding questions:
    a) What is the potential for adverse unintended consequences with this intervention?

b) Does the risk-return equation change based on the intended application (e.g., conservation versus the food chain; captive versus wild populations)?
c) Are there disadvantages to having monosex lines?

3. How does the lability of sex determination and intermediate sexual phenotypes feed into our social and biological concept of sex?

Consider: species that undergo natural sex changes (sequential hermaphrodite life histories; Gemmell *et al.*, 2019), temporary pseudo hermaphroditism during reptile embryonic development (Whiteley *et al.*, 2018), intersex morphology (Real *et al.*, 2020) and environmental sex reversal (Whiteley *et al.*, 2021).

Abbreviations

GSD – Genetic sex determination
TSD – Temperature dependent sex determination
ESD – Environmental sex determination
BAC – Bacterial artificial chromosome
FISH – Fluorescent *in situ* hybridisation
POC – Point of collection
LAMP – Loop-mediated isothermal amplification
RPA – Recombinase polymerase amplification
SRY – Sex-determining region Y protein
DMRT1 – Doublesex and Mab-3 related transcription factor 1
AMH – Anti-Müllerian hormone
CHD – chromo-helicase-DNA-binding

Resources

1. Tree of Sex: A database of eukaryotic sex determination systems http://treeofsex.org/

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References

Aarnes, S.G. *et al.* (2015) Y-chromosomal testing of brown bears (*Ursus arctos*): Validation of a multiplex PCR-approach for nine STRs suitable for fecal and hair samples., *Forensic science international. Genetics*, 19, pp. 197–204. doi:10.1016/j.fsigen.2015.07.018.

Adolfi, M.C. *et al.* (2021) A duplicated copy of id2b is an unusual sex - determining candidate gene on the Y chromosome of arapaima (Arapaima gigas), *Scientific Reports*, 11, p. 21544. doi:10.1038/s41598-021-01066-z.

Allen, C.D. *et al.* (2015) First assessment of the sex ratio for an east pacific green sea turtle foraging aggregation: Validation and application of a testosterone ELISA, *PLoS ONE*, 10(10), pp. 1–25. doi:10.1371/journal.pone.0138861.

Anastasiadi, D. *et al.* (2018) Dynamic epimarks in sex-related genes predict gonad phenotype

in the European sea bass, a fish with mixed genetic and environmental sex determination, *Epigenetics*, 13(9), pp. 988–1011. doi:10.1080/15592294.2018.1529504.

Andrewartha, H. and Birch, L. (1954) *The distribution and abundance of animals*. Chicago: University of Chicago Press.

Arenas, M. *et al.* (2017) Forensic genetics and genomics: Much more than just a human affair, *PLoS Genetics*, 13(9), p. e1006960. doi:10.1371/journal.pgen.1006960.

Ayers, K.L. *et al.* (2013) RNA sequencing reveals sexually dimorphic gene expression before gonadal differentiation in chicken and allows comprehensive annotation of the W-chromosome, *Genome Biology*, 14(3), pp. 1–16. doi:10.1186/gb-2013-14-3-r26.

Ayllon, F. *et al.* (2020) Autosomal sdY Pseudogenes Explain Discordances Between Phenotypic Sex and DNA Marker for Sex Identification in Atlantic Salmon, *Frontiers in Genetics*, 11(October), pp. 1–10. doi:10.3389/fgene.2020.544207.

Bachtrog, D. *et al.* (2014) Sex Determination: Why So Many Ways of Doing It?, *PLOS Biology*, 12(7), p. e1001899. doi:10.1371/journal.pbio.1001899.

Baird, N.A. *et al.* (2008) Rapid SNP discovery and genetic mapping using sequenced RAD markers, *PloS one*, 3(10), p. e3376.

Barrett, J.H. *et al.* (2022) Walruses on the Dnieper: new evidence for the intercontinental trade of Greenlandic ivory in the Middle Ages, *Proceedings of the Royal Society B: Biological Sciences*, 289(1972), p. 20212773. doi:10.1098/rspb.2021.2773.

Benestan, L. *et al.* (2017) Sex matters in massive parallel sequencing: Evidence for biases in genetic parameter estimation and investigation of sex determination systems, *Molecular Ecology*, 26(24), pp. 6767–6783. doi:10.1111/mec.14217.

Bidon, T. *et al.* (2015) Genome-wide search identifies 1.9 Mb from the polar bear Y chromosome for evolutionary analyses, *Genome Biology and Evolution*, 7(7), pp. 2010–2022. doi:10.1093/gbe/evv103.

Booth, W. *et al.* (2014) New insights on facultative parthenogenesis in pythons, *Biological Journal of the Linnean Society*, 112(3), pp. 461–468. doi:https://doi.org/10.1111/bij.12286.

Boyle, M. *et al.* (2014) How do climate-linked sex ratios and dispersal limit range boundaries?, *BMC Ecology*, 14(1), p. 19. doi:10.1186/1472-6785-14-19.

Bradley, M.P. (1989) Immunological sexing of mammalian semen: current status and future options, *Journal of dairy science*, 72(12), pp. 3372–3380.

Brelsford, A. *et al.* (2017) Identifying homomorphic sex chromosomes from wild-caught adults with limited genomic resources, *Molecular Ecology Resources*, 17(4), pp. 752–759. doi:10.1111/1755-0998.12624.

Budd, A. *et al.* (2015) Sex control in fish: Approaches, challenges and opportunities for aquaculture, *Journal of Marine Science and Engineering*, 3(2), pp. 329–355. doi:10.3390/jmse3020329.

Campbell, E.O. *et al.* (2018) Would an RRS by any other name sound as RAD?, *Methods in Ecology and Evolution*, 9(9), pp. 1920–1927. doi:https://doi.org/10.1111/2041-210X.13038.

Capel, B. (2017) Vertebrate sex determination: evolutionary plasticity of a fundamental switch, *Nature Reviews Genetics*, 18(11), pp. 675–689. doi:10.1038/nrg.2017.60.

Castelli, M.A. *et al.* (2020) Evolving thermal thresholds explain the distribution of temperature sex reversal in an Australian dragon lizard, *Diversity and Distributions*, 27, pp. 427–438. doi:doi.org/10.1111/ddi.13203.

Charlesworth, B. and Charlesworth, D. (2020) Evolution: A New Idea about the Degeneration of Y and W Chromosomes, *Current Biology*, 30(15), pp. R871–R873. doi:10.1016/j.cub.2020.06.008.

Charlesworth, D. (2018) The Guppy Sex Chromosome System and the Sexually Antagonistic Polymorphism Hypothesis for Y Chromosome Recombination Suppression., *Genes*, 9(5). doi:10.3390/genes9050264.

Chen, L. *et al.* (2019) Perfluorobutanesulfonate exposure skews sex ratio in fish and transgenerationally impairs reproduction, *Environmental Science and Technology*, 53(14), pp. 8389–8397. doi:10.1021/acs.est.9b01711.

Chen, N. *et al.* (2012) Identification of avian W-linked contigs by short-read sequencing, *BMC Genomics*, 13(1), pp. 2–9. doi:10.1186/1471-2164-13-183.

Chen, S. *et al.* (2014) Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle, *Nature Genetics*, 46(3), pp. 253–260. doi:10.1038/ng.2890.

Chomczynski, P. and Rymaszewski, M. (2006) Alkaline polyethylene glycol-based method for direct PCR from bacteria, eukaryotic tissue samples, and whole blood, *BioTechniques*, 40(4), pp. 454–458. doi:10.2144/000112149.

Clinton, M. *et al.* (2016) Real-time sexing of chicken embryos and compatibility with in ovo protocols, *Sexual Development*, 10(4), pp. 210–216.

Cornejo-Paramo, P. *et al.* (2020) Viviparous reptile regarded to have temperature-dependent sex determination has old XY chromosomes, *Genome Biology and Evolution*, 12(6), pp. 924–930. doi:10.1093/gbe/evaa104/5841216.

Cortez, D. *et al.* (2014) Origins and functional evolution of Y chromosomes across mammals., *Nature*, 508(7497), pp. 488–493. doi:10.1038/nature13151.

Curzon, A.Y. *et al.* (2021) All-male production by marker-assisted selection for sex determining loci of admixed Oreochromis niloticus and Oreochromis aureus stocks, *Animal Genetics*, 52(3), pp. 361–364. doi:10.1111/age.13057.

Davies, N., Krebs, J. and West, S. (2012) *An Introduction to Behavioural Ecology*. 4th edn. Wiley-Blackwell.

Davis, T.L.O. (1982) Maturity and sexuality in Barramundi, *Lates calcarifer* (Bloch), in the Northern Territory and south-eastern Gulf of Carpentaria, *Marine and Freshwater Research*,

33(3), pp. 529–545. Available at: https://doi.org/10.1071/MF9820529.

Dawson, D.A. *et al.* (2001) A critique of avian CHD-based molecular sexing protocols illustrated by a Z-chromosome polymorphism detected in auklets, *Molecular Ecology Notes*, 1(3), pp. 201–204. doi:10.1046/j.1471-8278.2001.00060.x.

Dawson, D.A. *et al.* (2015) A marker suitable for sex-typing birds from degraded samples, *Conservation Genetics Resources*, 7(2), pp. 337–343. doi:10.1007/s12686-015-0429-3.

Dechaume-Moncharmont, F.-X., Monceau, K. and Cezilly, F. (2011) Sexing Birds Using Discriminant Function Analysis: A Critical Appraisal - Identification du sexe des oiseaux par analyse factorielle discriminante: une évaluation critique, *The Auk*, 128(1), pp. 78–86. doi:10.1525/auk.2011.10129.

Délot, E.C. and Vilain, E. (2018) Disorders of Sex Development, *Yen & Jaffe's Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management: Eighth Edition*, 16(2), pp. 365-393.e5. doi:10.1016/B978-0-323-47912-7.00016-0.

Deveson, I.W. *et al.* (2017) Differential intron retention in Jumonji chromatin modifier genes is implicated in reptile temperature-dependent sex determination, *Science Advances*, 3(6), p. e1700731. doi:10.1126/sciadv.1700731.

Devlin, R.H. and Nagahama, Y. (2002) Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences, *Aquaculture*, 208(3), pp. 191–364. doi:https://doi.org/10.1016/S0044-8486(02)00057-1.

Devloo-Delva, F. *et al.* (2022) An R-based tool for identifying sex-linked markers from Restriction Site-Associated DNA sequencing with applications to elasmobranch conservation. doi:10.21203/rs.3.rs-1797792/v1.

Diatchenko, L. *et al.* (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries., *Proceedings of the National Academy of Sciences*, 93(12), pp. 6025–6030. doi:10.1073/pnas.93.12.6025.

Dissanayake, D.S.. *et al.* (2021) High elevation increases the risk of Y chromosome loss in Alpine skink populations with sex reversal, *Heredity*, 126, pp. 805–816. doi:10.1038/s41437-021-00406-z.

Dissanayake, D.S.., Holleley, C.. and Georges, A. (2021) Effects of natural nest temperatures on sex reversal and sex ratios in an Australian alpine skink, *Scientific Reports*, 11(1), pp. 1–12. doi:10.1038/s41598-021-99702-1.

Dissanayake, D.S.B. *et al.* (2020) Identification of y chromosome markers in the eastern three-lined skink (*Bassiana duperreyi*) using in silico whole genome subtraction, *BMC Genomics*, 21(1), pp. 1–12. doi:10.1186/s12864-020-07071-2.

Dixon, G., Kitano, J. and Kirkpatrick, M. (2019) The origin of a new sex chromosome by introgression between two stickleback fishes, *Molecular biology and evolution*, 36(1), pp. 28–38.

Doran, T.J. *et al.* (2017) Sex selection in layer chickens, *Animal Production Science*, 58(3), pp. 476–480.

Drinan, D.P., Loher, T. and Hauser, L. (2018) Identification of Genomic Regions Associated With Sex in Pacific Halibut, *Journal of Heredity*, 109(3), pp. 326–332. doi:10.1093/jhered/esx102.

Van Dyke, J.U. *et al.* (2021) Australian lizards are outstanding models for reproductive biology research, *Australian Journal of Zoology*, 68(4), pp. 168–199. doi:10.1071/ZO21017.

Eatwell, K., Hedley, J. and Barron, R. (2014) Reptile haematology and biochemistry, *In Practice*, 36(1), pp. 34–42. doi:https://doi.org/10.1136/inp.f7488.

Edwards, A.W.F. (2000) Carl Düsing (1884) on The Regulation of the Sex-Ratio, *Theoretical Population Biology*, 58(3), pp. 255–257. doi:https://doi.org/10.1006/tpbi.2000.1482.

Elshire, R.J. *et al.* (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species, *PloS one*, 6(5), p. e19379.

Ezaz, T., Quinn, A., *et al.* (2009) Molecular marker suggests rapid changes of sex-determining mechanisms in Australian dragon lizards, *Chromosome Research*, 17(1), pp. 91–98. doi:10.1007/s10577-008-9019-5.

Ezaz, T., Moritz, B., *et al.* (2009) The ZW sex microchromosomes of an Australian dragon lizard share no homology with those of other reptiles or birds, *Chromosome Research*, 17(8), pp. 965–973. doi:10.1007/s10577-009-9102-6.

Ezaz, T. *et al.* (2013) Sequence and gene content of a large fragment of a lizard sex chromosome and evaluation of candidate sex differentiating gene R-spondin 1, *Bmc Genomics*, 14, p. 899. doi:10.1186/1471-2164-14-899.

Feron, R. *et al.* (2021) RADSex: a computational workflow to study sex determination using restriction site-associated DNA sequencing data, *Molecular Ecology Resources*, 21(5), pp. 1715–1731. doi:10.1101/2020.04.22.054866.

Fisher, R.. (1930) *The genetical theory of natural selection*. Edited by J.. Bennett. Oxford: Oxford University Press.

Fowler, B.L.S. and Buonaccorsi, V.P. (2016) Genomic characterization of sex-identification markers in Sebastes carnatus and Sebastes chrysomelas rockfishes, *Molecular Ecology*, 25(10), pp. 2165–2175. doi:10.1111/mec.13594.

Fridolfsson, A.-K. and Ellegren, H. (1999) A Simple and Universal Method for Molecular Sexing of Non-Ratite Birds, *Journal of Avian Biology*, 30(1), pp. 116–121. doi:10.2307/3677252.

Galindo, H.M., Loher, T. and Hauser, L. (2011) Genetic sex identification and the potential evolution of sex determination in Pacific halibut (Hippoglossus stenolepis), *Marine Biotechnology*, 13(5), pp. 1027–1037.

Galli, R. *et al.* (2017) In ovo sexing of chicken eggs by fluorescence spectroscopy, *Analytical and bioanalytical chemistry*, 409(5), pp. 1185–1194.

Gamble, T. (2016) Using RAD-seq to recognize sex-specific markers and sex chromosome systems, *Molecular Ecology*, 25(10), pp. 2114–2116. doi:10.1111/mec.13648.

Gamble, T. *et al.* (2017) The Discovery of XY Sex Chromosomes in a Boa and Python, *Current Biology*, 27(14), pp. 2148-2153.e4. doi:10.1016/j.cub.2017.06.010.

Garner, D.L. (2006) Flow cytometric sexing of mammalian sperm, *Theriogenology*, 65(5), pp. 943–957.

Gautier, M. (2014) Using genotyping data to assign markers to their chromosome type and to infer the sex of individuals: A Bayesian model-based classifier, *Molecular Ecology Resources*, 14(6), pp. 1141–1159. doi:10.1111/1755-0998.12264.

Gemmell, N.J. *et al.* (2019) Natural sex change in fish., *Current topics in developmental biology*, 134, pp. 71–117. doi:10.1016/bs.ctdb.2018.12.014.

Georges, A., Holleley, C.E. and Graves, J.A.M. (2021) Concerning an Article by Ehl et al.: False Premise Leads to False Conclusions, *Sexual Development*, 15(4), pp. 286–288. doi:10.1159/000518374.

Goldberg, M.T., Spigler, R.B. and Ashman, T.-L. (2010) Comparative genetic mapping points to different sex chromosomes in sibling species of wild strawberry (Fragaria)., *Genetics*, 186(4), pp. 1425–1433. doi:10.1534/genetics.110.122911.

Grayson, P. *et al.* (2022) SexFindR: A computational workflow to identify young and old sex chromosomes, *bioRxiv* [Preprint]. doi:10.1101/2022.02.21.481346.

Griffiths, R. *et al.* (1998) A DNA test to sex most birds., *Molecular ecology*, 7(8), pp. 1071–1075. doi:10.1046/j.1365-294x.1998.00389.x.

Gross, T.S. *et al.* (1995) Identification of Sex in Hatchling Loggerhead Turtles (Caretta caretta) by Analysis of Steroid Concentrations in Chorioallantoic/Amniotic Fluid, *General and Comparative Endocrinology*, pp. 204–210. doi:10.1006/gcen.1995.1103.

Hall, A.B. *et al.* (2013) Six novel Y chromosome genes in Anopheles mosquitoes discovered by independently sequencing males and females, *BMC Genomics*, 14(1), pp. 2–13. doi:10.1186/1471-2164-14-273.

Hanson, K.C. *et al.* (2008) Sexual variation in fisheries research and management: when does sex matter?, *Reviews in Fisheries Science*, 16(4), pp. 421–436.

Harr, K.E. (2002) Clinical Chemistry of Companion Avian Species: A Review, *Veterinary Clinical Pathology*, 31(3), pp. 140–151. doi:https://doi.org/10.1111/j.1939-165X.2002.tb00295.x.

Heinsohn, R. *et al.* (2019) Sex ratio bias and shared paternity reduce individual fitness and population viability in a critically endangered parrot, *Journal of Animal Ecology*, 88(4), pp. 502–510. doi:https://doi.org/10.1111/1365-2656.12922.

Hill, P. *et al.* (2022) Sex reversal explains some , but not all , climate mediated differences in sex ratio within a viviparous reptile., *In press*, pp. 2–9.

Hill, P.L. *et al.* (2018) Conservation of sex-linked markers among conspecific populations of a viviparous skink, *Niveoscincus ocellatus*, exhibiting genetic and temperature-dependent sex determination, *Genome Biology and Evolution*, 10(4), pp. 1079–1087.

doi:10.1093/gbe/evy042.

Hiller, T.L. *et al.* (2022) Sex identification and age estimation of bobcats and implications for management, *Wildlife Society Bulletin*, 46(3), p. e1328. doi:https://doi.org/10.1002/wsb.1328.

Hohenboken, W.D. (1981) Possibilities for genetic manipulation of sex ratio in livestock, *Journal of Animal Science*, 52(2), pp. 265–277.

Hohenboken, W.D. (1999) Applications of sexed semen in cattle production, *Theriogenology*, 52(8), pp. 1421–1433. doi:https://doi.org/10.1016/S0093-691X(99)00227-7.

Holleley, C.E. *et al.* (2015) Sex reversal triggers the rapid transition from genetic to temperature-dependent sex, *Nature*, 523(7558), pp. 79–82. doi:10.1038/nature14574.

Hu, Q. *et al.* (2019) Genome-wide RAD sequencing to identify a sex-specific marker in Chinese giant salamander Andrias davidianus, *BMC Genomics*, 20(1), pp. 1–8. doi:10.1186/s12864-019-5771-5.

Hughes, C. (1998) Integrating Molecular Techniques With Field Methods In Studies Of Social Behavior: A Revolution Results, *Ecology*, 79(2), pp. 383–399. doi:https://doi.org/10.1890/0012-9658(1998)079[0383:IMTWFM]2.0.CO;2.

Huynen, L., Millar, C.D. and Lambert, D.M. (2002) A DNA test to sex ratite birds, *Molecular Ecology*, 11(4), pp. 851–856. doi:10.1046/j.1365-294X.2002.01483.x.

Jaccoud, D., Peng, K., Feinstein, D., & Kilian, A. (2001). Diversity arrays: A solid state technology for sequence information independent genotyping. *Nucleic Acids Research, 29,* e25-e25. https://doi.org/10.1093/nar/29.4.e25

Jasonowicz, A.J. *et al.* (2022) Generation of a chromosome-level genome assembly for Pacific halibut (*Hippoglossus stenolepis*) and characterization of its sex-determining genomic region, *Molecular Ecology Resources* [Preprint].

Jeffries, D.L. *et al.* (2018) A rapid rate of sex-chromosome turnover and non-random transitions in true frogs, *Nature Communications*, 9(1). doi:10.1038/s41467-018-06517-2.

Johnson, L.A. and Clarke, R.N. (1988) Flow sorting of X and Y chromosome-bearing mammalian sperm: activation and pronuclear development of sorted bull, boar, and ram sperm microinjected into hamster oocytes, *Gamete Research*, 21(4), pp. 335–343.

Kamiya, T. *et al.* (2012) A Trans-Species Missense SNP in Amhr2 Is Associated with Sex Determination in the Tiger Pufferfish, *Takifugu rubripes* (Fugu), *PLOS Genetics*, 8(7), pp. 1–10. doi:10.1371/journal.pgen.1002798.

Kar, S. *et al.* (2021) Endocrine disruptors in teleosts: Evaluating environmental risks and biomarkers, *Aquaculture and Fisheries*, 6(1), pp. 1–26. doi:10.1016/j.aaf.2020.07.013.

Katsura, Y., Iwase, M. and Satta, Y. (2012) Evolution of genomic structures on Mammalian sex chromosomes., *Current genomics*, 13(2), pp. 115–123. doi:10.2174/138920212799860625.

Keating, S.E. *et al.* (2022) Research Article Genetic sex test for the short-beaked echidna (*Tachyglossus aculeatus*), *Conservation Genetics Resources* [Preprint]. doi:10.1007/s12686-022-01258-3.

Khwatenge, C.N. and Nahashon, S.N. (2021) Recent Advances in the Application of CRISPR/Cas9 Gene Editing System in Poultry Species, *Frontiers in Genetics*, 12(February). doi:10.3389/fgene.2021.627714.

Kilian, A. *et al.* (2012) Diversity Arrays Technology: A generic genome profiling technology on open platforms, in *Data Production and Analysis in Population Genomics*, pp. 67–89.

King, W.A. (1984) Sexing embryos by cytological methods, *Theriogenology*, 21(1), pp. 7–17.

Koyama, T. *et al.* (2019) A SNP in a Steroidogenic Enzyme Is Associated with Phenotypic Sex in Seriola Fishes, *Current Biology*, 29(11), pp. 1901-1909.e8. doi:10.1016/j.cub.2019.04.069.

Krautwald-Junghanns, M.E. *et al.* (2018) Current approaches to avoid the culling of day-old male chicks in the layer industry, with special reference to spectroscopic methods, *Poultry science*, 97(3), pp. 749–757.

Kuderna, L.F.K. *et al.* (2019) Selective single molecule sequencing and assembly of a human Y chromosome of African origin, *Nature Communications*, 10(1), p. 4. doi:10.1038/s41467-018-07885-5.

Kuhl, H. *et al.* (2021) A 180 Myr-old female-specific genome region in sturgeon reveals the oldest known vertebrate sex determining system with undifferentiated sex chromosomes, *Philosophical Transactions of the Royal Society B: Biological Sciences*, 376(1832). doi:10.1098/rstb.2020.0089.

Lambert, M.R., Ezaz, T. and Skelly, D.K. (2021) Sex-Biased Mortality and Sex Reversal Shape Wild Frog Sex Ratios, *Frontiers in Ecology and Evolution*, 9(October), pp. 1–14. doi:10.3389/fevo.2021.756476.

Lambert, M.R., Skelly, D.K. and Ezaz, T. (2016) Sex-linked markers in the North American green frog (*Rana clamitans*) developed using DArTseq provide early insight into sex chromosome evolution, *BMC Genomics*, 17. doi:10.1186/s12864-016-3209-x.

Lange, A. *et al.* (2020) A newly developed genetic sex marker and its application to understanding chemically induced feminisation in roach (Rutilus rutilus), *Molecular Ecology Resources*, 20(4), pp. 1007–1022. doi:10.1111/1755-0998.13166.

Lee, B. *et al.* (2011) Genetic and physical mapping for sex-link AFLP markers in Nile tilapia (*Oreochromis niloticus*), *Marine Biotechnology*, 13(3), pp. 557–562. doi:10.1007/s10126-010-9326-7.Genetic.

Lindström, J. (1998) Harvesting and sex differences in demography, *Wildlife Biology*, 4(4), pp. 213–221.

Liu, H. *et al.* (2018) Sex-specific markers developed by next-generation sequencing confirmed an XX/XY sex determination system in bighead carp (Hypophthalmichthys

nobilis) and silver carp (*Hypophthalmichthys molitrix*), *DNA Research*, 25(3), pp. 257–264. doi:10.1093/dnares/dsx054.

Lumeij, J. (1997) Avian Clinical Biochemistry, in Kaneko, J., Harvey, J., and Bruss, M. (eds) *Clinical Biochemistry of Domestic Animals*. 5th edn. Academic Press, pp. 857–883.

Luo, J.H. *et al.* (1999) Differential subtraction chain, a method for identifying differences in genomic DNA and mRNA., *Nucleic acids research*, 27(19), p. e24. doi:10.1093/nar/27.19.e24.

Marshall Graves, J.A. and Shetty, S. (2001) Sex from W to Z: evolution of vertebrate sex chromosomes and sex determining genes., *The Journal of Experimental Zoology*, 290(5), pp. 449–462. doi:10.1002/jez.1088.

Martínez-Torres, M. *et al.* (2015) Hemipenes in females of the mexican viviparous lizard *Barisia imbricata* (Squamata: Anguidae): An example of heterochrony in sexual development, *Evolution and Development*, 17(5), pp. 270–277. doi:10.1111/ede.12134.

Matsubara, K. *et al.* (2016) Amplification of microsatellite repeat motifs is associated with the evolutionary differentiation and heterochromatinization of sex chromosomes in Sauropsida, *Chromosoma*, 125(1), pp. 111–123. doi:10.1007/s00412-015-0531-z.

Mikó, Z. *et al.* (2021) Sex reversal and ontogeny under climate change and chemical pollution: are there interactions between the effects of elevated temperature and a xenoestrogen on early development in agile frogs?, *Environmental Pollution*, 285. doi:10.1016/j.envpol.2021.117464.

Miller, K.L. *et al.* (2019) Parthenogenesis in a captive Asian water dragon (Physignathus cocincinus) identified with novel microsatellites, *PLOS ONE*, 14(6), p. e0217489. Available at: https://doi.org/10.1371/journal.pone.0217489.

Morinha, F., Cabral, J.A. and Bastos, E. (2012) Molecular sexing of birds: A comparative review of polymerase chain reaction (PCR)-based methods., *Theriogenology*, 78(4), pp. 703–714. doi:10.1016/j.theriogenology.2012.04.015.

Müller, N.A. *et al.* (2020) A single gene underlies the dynamic evolution of poplar sex determination, *Nature plants*, 6(6), pp. 630–637.

Murphy, W.J. *et al.* (2006) Novel Gene Acquisition on Carnivore Y Chromosomes, *PLOS Genetics*, 2(3), p. e43. Available at: https://doi.org/10.1371/journal.pgen.0020043.

Murtagh, V.J. *et al.* (2012) Evolutionary history of novel genes on the tammar wallaby Y chromosome: Implications for sex chromosome evolution, *Genome Research*, 22(3), pp. 498–507. doi:10.1101/gr.120790.111.

Muyle, A. *et al.* (2016) Sex-detector: A probabilistic approach to study sex chromosomes in non-model organisms, *Genome Biology and Evolution*, 8(8), pp. 2530–2543. doi:10.1093/gbe/evw172.

Nagahama, Y. *et al.* (2021) Sex Determination, Gonadal Sex Differentiation and Plasticity in Vertebrate Species, *Physiological Reviews*, 101(3), pp. 1237–1308. doi:doi.org/10.1152/physrev.00044.2019.

Neaves, L. *et al.* (2006) Embryonic gonadal and sexual organ development in a small viviparous skink, *Niveoscincus ocellatus*, *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, 305(1), pp. 74–82. doi:10.1002/jez.a.249.

Nemesházi, E. *et al.* (2020) Novel genetic sex markers reveal high frequency of sex reversal in wild populations of the agile frog (*Rana dalmatina*) associated with anthropogenic land use, *Molecular Ecology*, (April), pp. 3607–3621. doi:10.1111/mec.15596.

Nursyifa, C. *et al.* (2021) Joint identification of sex and sex-linked scaffolds in non-model organisms using low depth sequencing data, *Molecular Ecology Resources*, (March), pp. 1–10. doi:10.1111/1755-0998.13491.

Palmer, D.H. *et al.* (2019) How to identify sex chromosomes and their turnover, *Molecular Ecology*, 28(21), pp. 4709–4724. doi:10.1111/mec.15245.

Pereira, D.C. *et al.* (2021) Molecular sexing in the formation of pairs of blue-and-yellow macaw (*Ara ararauna*) in reintroduction programs, *Research, Society and Development*, 10(10), p. 570101019330. doi:10.33448/rsd-v10i10.19330.

Perelman, P. *et al.* (2011) A Molecular Phylogeny of Living Primates, *PLOS Genetics*, 7(3), p. e1001342. Available at: https://doi.org/10.1371/journal.pgen.1001342.

Peterson, B.K. *et al.* (2012) Double digest RADseq: An inexpensive method for de novo SNP discovery and genotyping in model and non-model species, *PLoS ONE*, 7(5). doi:10.1371/journal.pone.0037135.

Piferrer, F. *et al.* (1994) Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males, *Journal of experimental zoology*, 270(3), pp. 255–262.

Piferrer, F. *et al.* (2019) The model of the conserved epigenetic regulation of sex, *Frontiers in Genetics*, 10(SEP). doi:10.3389/fgene.2019.00857.

Pilgrim, K.L. *et al.* (2005) Felid sex identification based on noninvasive genetic samples, *Molecular Ecology Notes*, 5(1), pp. 60–61. doi:https://doi.org/10.1111/j.1471-8286.2004.00831.x.

Porat, N. *et al.* (2011) Direct detection of chicken genomic DNA for gender determination by thymine-DNA glycosylase, *British poultry science*, 52(1), pp. 58–65.

Quinn, A.E. *et al.* (2009) Isolation and development of a molecular sex marker for Bassiana duperrey, a lizard with XX/XY sex chromosomes and temperature-induced sex reversal, *Molecular Genetics and Genomics*, 281(6), pp. 665–672. doi:10.1007/s00438-009-0437-7.

Quinn, A.E. *et al.* (2010) Extension, single-locus conversion and physical mapping of sex chromosome sequences identify the Z microchromosome and pseudo-autosomal region in a dragon lizard, Pogona vitticeps, *Heredity*, 104(4), p. 410. doi:10.1038/hdy.2009.133.

Radder, R.S. *et al.* (2008) Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard, *Biology Letters*, 4(2), pp. 176–178. doi:10.1098/rsbl.2007.0583.

Raudsepp, T. *et al.* (2004) A detailed physical map of the horse Y chromosome, *Proceedings of the National Academy of Sciences*, 101(25), pp. 9321–9326. doi:10.1073/pnas.0403011101.

Real, F. *et al.* (2020) The mole genome reveals regulatory rearrangements associated with adaptive intersexuality., *Science*, 370(6513), pp. 208–214. doi:10.1126/science.aaz2582.

Richner, H. (1989) Avian Laparoscopy as a Field Technique for Sexing Birds and an Assessment of Its Effects on Wild Birds (Laparoscopía en el Campo como Técnica para Determinar el Sexo de Aves), *Journal of Field Ornithology*, 60(2), pp. 137–142. Available at: http://www.jstor.org/stable/4513412.

Ridha, M.T. (2011) Evaluation of monosex culture of GIFT and non-improved strains of Nile tilapia Oreochromis niloticus in recirculating tanks, *International Aquatic Research*, 3(3), pp. 189–195.

Rovatsos, M. *et al.* (2014) Novel X-linked genes revealed by quantitative polymerase chain reaction in the green anole, *Anolis carolinensis*, *G3-Genes Genomes Genetics*, 4(11), pp. 2107–2113. doi:10.1534/g3.114.014084.

Rozen, S. *et al.* (2003) Abundant gene conversion between arms of palindromes in human and ape Y chromosomes., *Nature*, 423(6942), pp. 873–876. doi:10.1038/nature01723.

Ryder, O.A. *et al.* (2021) Facultative Parthenogenesis in California Condors., *The Journal of heredity*, 112(7), pp. 569–574. doi:10.1093/jhered/esab052.

Sankovic, N. *et al.* (2006) Construction of a highly enriched marsupial Y chromosome-specific BAC sub-library using isolated Y chromosomes, *Chromosome Research*, 14(6), pp. 657–664. doi:10.1007/s10577-006-1076-z.

Schartl, M., Georges, A. and Graves, J.A.M. (2022) Polygenic sex determination in vertebrates – is there any such thing?, *in review* [Preprint].

Schwanz, L.E. *et al.* (2020) Climate change, sex reversal and lability of sex-determining systems, *Journal of Evolutionary Biology*, 33(3), pp. 270–281. doi:10.1111/jeb.13587.

Shaffer, M.L. (1981) Minimum Population Sizes for Species Conservation, *BioScience*, 31(2), pp. 131–134. doi:10.2307/1308256.

Sigeman, H., Sinclair, B. and Hansson, B. (2022) FindZX: an automated pipeline for detecting and visualising sex chromosomes using whole-genome sequencing data, *BMC genomics*, 23(1), pp. 1–14.

Skaletsky, H. *et al.* (2003) The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes., *Nature*, 423(6942), pp. 825–837. doi:10.1038/nature01722.

Stevens, N. (1905) Studies in Spermatogenesis, *Carnegie Institution of Washington* [Preprint], (36).

Stewart, I.J. and Webster, R. (2021) *Overview of data sources for the Pacific halibut stock assessment, harvest policy, and related analyses*. Available at: https://protect-au.mimecast.com/s/vL3YC1WZg1fkG6pqIK0l_n?domain=iphc.int,.

Stöck, M. *et al.* (2021) A brief review of vertebrate sex evolution with a pledge for integrative research-towards 'sexomics', (July). doi:10.1098/rstb.2020.0426.

Sumaila, U.R., Palacios-Abrantes, J. and Cheung, W.W.L. (2020) Climate change, shifting threat points, and the management of transboundary fish stocks, *Ecology and Society*, 25(4). doi:10.5751/ES-11660-250440.

Sun, Y.-L. *et al.* (2014) Screening and characterization of sex-linked DNA markers and marker-assisted selection in the Nile tilapia (Oreochromis niloticus), *Aquaculture*, 433, pp. 19–27. doi:https://doi.org/10.1016/j.aquaculture.2014.05.035.

Tamukai, K. *et al.* (2011) Plasma biochemical reference values in clinically healthy captive bearded dragons (Pogona vitticeps) and the effects of sex and season., *Veterinary clinical pathology*, 40(3), pp. 368–373. doi:10.1111/j.1939-165X.2011.00329.x.

Tao, W. *et al.* (2020) High-quality chromosome-level genomes of two tilapia species reveal their evolution of repeat sequences and sex chromsomes, *bioRxiv*, pp. 1–37.

Tezak, B.M. *et al.* (2020) Identifying Sex of Neonate Turtles with Temperature-dependent Sex Determination via Small Blood Samples, *Scientific Reports*, 10(5012), pp. 1–8. doi:10.1038/s41598-020-61984-2.

Tezak, B.M., Guthrie, K. and Wyneken, J. (2017) An Immunohistochemical Approach to Identify the Sex of Young Marine Turtles, *Anatomical Record*, 300(8), pp. 1512–1518. doi:10.1002/ar.23589.

Todd, E. V *et al.* (2016) Bending genders: The biology of natural sex change in fish, *Sexual Development*, 10(5–6), pp. 223–241. doi:10.1159/000449297.

Traut, W., Eickhof, U. and Schorch, J.C. (2001) Identification and analysis of sex chromosomes by comparative genomic hybridization (CGH)., *Methods in cell science : an official journal of the Society for In Vitro Biology*, 23(1–3), pp. 155–161. doi:10.1007/978-94-010-0330-8_16.

Trenkel, V.M. *et al.* (2020) Methods for identifying and interpreting sex-linked SNP markers and carrying out sex assignment: application to thornback ray (*Raja clavata*), *Molecular Ecology Resources*, 00(June), pp. 1–10. doi:10.1111/1755-0998.13225.

Truett, G. *et al.* (2000) Preparation of PCR- Quality Mouse Genomic DNA with Hot Sodium Hydroxide and Tris (HotSHOT), *Biotechniques*, 29(1), pp. 29–30.

Tubbs, C.W. and McDonough, C.E. (2018) Reproductive Impacts of Endocrine-Disrupting Chemicals on Wildlife Species: Implications for Conservation of Endangered Species, *Annual Review of Animal Biosciences*, 6(November), pp. 287–304. doi:10.1146/annurev-animal-030117-014547.

Uno, Y. *et al.* (2020) Cell culture-based karyotyping of orectolobiform sharks for chromosome-scale genome analysis, *Communications Biology*, 3(1), p. 652. doi:10.1038/s42003-020-01373-7.

Vicoso, B. *et al.* (2013) Comparative sex chromosome genomics in snakes: differentiation, evolutionary strata, and lack of global dosage compensation, *PLoS biology*, 11(8), p.

e1001643.

Vicoso, B. and Bachtrog, D. (2015) Numerous Transitions of Sex Chromosomes in Diptera, *PLOS Biology*, 13(4), pp. 1–22. doi:10.1371/journal.pbio.1002078.

Viñas, A. *et al.* (2012) Mapping of DNA sex-specific markers and genes related to sex differentiation in turbot (Scophthalmus maximus)., *Marine biotechnology (New York, N.Y.)*, 14(5), pp. 655–663. doi:10.1007/s10126-012-9451-6.

Waits, L.P. and Paetkau, D. (2005) Noninvasive Genetic Sampling Tools for Wildlife Biologists: A Review of Applications and Recommendations for Accurate Data Collection, *The Journal of Wildlife Management*, 69(4), pp. 1419–1433. Available at: http://www.jstor.org/stable/3803503.

Wang, C. *et al.* (2015) Identification of sex chromosomes by means of comparative genomic hybridization in a lizard, *Eremias multiocellata*, *Zoological Science*, 32(2), pp. 151–156. doi:10.2108/zs130246.

Wang, H.-P. *et al.* (2018) *Sex Control in Aquaculture*, *Sex Control in Aquaculture*. John Wiley & Sons, Ltd. doi:https://doi.org/10.1002/9781119127291.fmatter1.

Wang, L. *et al.* (2022) Transposon-induced epigenetic silencing in the X chromosome as a novel form of dmrt1 expression regulation during sex determination in the fighting fish, *BMC Biology*, 20(1), p. 5. doi:10.1186/s12915-021-01205-y.

Watts, P.C. *et al.* (2006) Parthenogenesis in Komodo dragons, *Nature*, 444(7122), pp. 1021–1022. doi:10.1038/4441021a.

Weigel, K.A. (2004) Exploring the Role of Sexed Semen in Dairy Production Systems, *Journal of Dairy Science*, 87, pp. E120–E130. doi:https://doi.org/10.3168/jds.S0022-0302(04)70067-3.

Weissmann, A. *et al.* (2013) Sexing domestic chicken before hatch: A new method for in ovo gender identification, *Theriogenology*, 80(3), pp. 199–205. doi:https://doi.org/10.1016/j.theriogenology.2013.04.014.

Whiteley, S.L. *et al.* (2018) Developmental asynchrony and antagonism of sex determination pathways in a lizard with temperature-induced sex reversal, *Scientific Reports*, 8(1), pp. 1–9. doi:10.1038/s41598-018-33170-y.

Whiteley, S.L. *et al.* (2021) Temperature-induced sex reversal in reptiles: Prevalence, discovery, and evolutionary implications, *Sexual Development*, 15, pp. 148–156. doi:10.1159/000515687.

Whiteley, S.L. *et al.* (2022) Truncated jarid2 and kdm6b transcripts are associated with temperature-induced sex reversal during development in a dragon lizard, *Science Advances*, 8, p. eabk0275. doi:10.1126/sciadv.abk0275.

Wiggins, J.M. *et al.* (2020) Gene dose indicates presence of sex chromosomes in collared lizards (*Crotaphytus collaris*), a species with temperature-influenced sex determination, *Herpetologica*, 76(1), pp. 27–30. doi:10.1655/herpetologica-d-19-00036.

Wilson, C.A. *et al.* (2014) Wild sex in zebrafish: Loss of the natural sex determinant in domesticated strains, *Genetics*, 198(3), pp. 1291–1308. doi:10.1534/genetics.114.169284.

Wu, X. *et al.* (2021) Screening and characterization of sex-linked DNA markers and marker-assisted selection in blue tilapia (Oreochromis aureus), *Aquaculture*, 530(July 2020), p. 735934. doi:10.1016/j.aquaculture.2020.735934.

Xia, Z.R. *et al.* (2011) Evaluating noninvasive methods of sex identification in Green sea turtle (Chelonia mydas) hatchlings, *Chelonian Conservation and Biology*, 10(1), pp. 117–123. doi:10.2744/CCB-0852.1.

Xie, Y. *et al.* (2020) Sex Manipulation Technologies Progress in Livestock: A Review, *Frontiers in Veterinary Science*, 7(August), pp. 1–9. doi:10.3389/fvets.2020.00481.

Xue, L. *et al.* (2021) Telomere-to-telomere assembly of a fish Y chromosome reveals the origin of a young sex chromosome pair, *Genome Biology*, 22(1), p. 203. doi:10.1186/s13059-021-02430-y.

Zarzoso-Lacoste, D. *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, *Molecular Ecology Resources*, 18(2), pp. 217–228. doi:10.1111/1755-0998.12727.

Zhang, X. *et al.* (2022) Sex-specific splicing of Z- and W-borne nr5a1 alleles suggests sex determination is controlled by chromosome conformation., *Proceedings of the National Academy of Sciences of the United States of America*, 119(4). doi:10.1073/pnas.2116475119.

Zhao, D. *et al.* (2010) Somatic sex identity is cell autonomous in the chicken, 464, p. 237. doi:10.1038/nature08852 https://www.nature.com/articles/nature08852#supplementary-information.

Zhu, Z. *et al.* (2021) Diversity of reptile sex chromosome evolution revealed by cytogenetic and linked-read sequencing. bioRxiv. doi:10.1101/2021.10.13.462063.