Identification of sex-linked SNP markers in wild populations of monomorphic birds

Aina Garcia-Raventós[1], Antonio Muñoz-Merida[2], Oriol Lapiedra[1], Mar Unzeta[1], Mariona Ferrandiz-Rovira[1], and Daniel Sol[3]

[1]Centre de Recerca Ecològica i Aplicacions Forestals
[2]Universidade do Porto Centro de Investigação em Biodiversidade e Recursos Genéticos
[3]Centre de Recerca Ecologica i Aplicacions Forestals

February 2, 2023

Abstract

Single-nucleotide polymorphism (SNP) analyses are a powerful tool for population genetics, pedigree reconstruction and phenotypic trait mapping. SNPs could also be useful for sexing individuals in species with reduced sexual dimorphism, yet this possibility remains poorly explored. Here, we develop a novel protocol for molecular sexing of birds based on the detection of unique Z- and W-linked SNP markers. Our method is based on the identification of two unique loci, one in each sexual chromosome. Individuals are considered males when they are heterozygotic for the Z-linked SNP and females when they are homozygote for the Z-linked SNP and have the W-linked SNP. We validated the method in the Jackdaw (Corvus monedula), a species whose reduced sexual dimorphism makes it difficult to sex individuals in the wild. We assessed the reliability of the method with 36 individuals of known sex, and found that their sex was correctly assigned in 100% of cases. The sex-linked markers also proved to be widely applicable to discriminate males and females from a sample of 927 genotyped individuals of different maturity stages with an accuracy of 99.5%. Given that SNP markers are increasingly used in quantitative genetic analyses of wild populations, the approach we propose has a great potential to be integrated into broader genetic research programmes without the need of additional sexing techniques.

Introduction

The growing development of advanced molecular and bioinformatic tools have revolutionized the study of ecology and evolution by allowing to sequence hundreds of samples in parallel at a whole-genome scale (Hudson 2008). Next-generation sequencing (NGS) techniques are sensible, accurate, low time-consuming, and can be applied to non-model organisms. Such availability of massive genomic data provides a promising potential in the field of genomics, transcriptomics, and proteomics, as well as a wide application for microsatellites and single-nucleotide polymorphisms (SNP) screening.

SNPs are increasingly used to study wild populations. They are suitable for population-level genotyping due to their abundance and widespread distribution along the genome, low genotyping error, high-throughput and low-cost per locus (Kaiser et al. 2017). The use of SNP markers is currently widely used in population genetic analysis, pedigree reconstruction, extra-pair paternity assignments and phenotypic trait mapping (Garvin et al. 2010), providing crucial information to address important aspects of evolutionary and conservation ecology.

Sex largely influences a wide array of key ecological and evolutionary processes, including habitat use, feeding specializations, parental care, dispersal and migration (Selander 1966; Durell 2000). Thus, it is not surprising that devising molecular tools for reliable sex identification has long been in the agenda of wildlife ecologists. Yet, tools for sexing using SNP markers have only been developed for commercial animals (Andrews et

al. 2016), including a range of aquaculture fish and crustacean species for which the factors involved in determining sexes are not identified yet (Palaiokostas et al. 2015; Shi et al. 2018; Wang et al. 2019; Fang et al. 2020). The use of SNPs for sexing remains, however, largely unexplored in wild animals, despite their great potential to reduce costs and improve performance of multiple genetic analysis from the same multi-locus panel including all SNPs of interest without the need of additional PCR amplifications and electrophoresis.

Here, we describe a novel protocol for molecular sexing of birds based on single nucleotide polymorphisms. Birds are well-known for their extraordinary diversity of sexually dimorphic characters. The elaborate breeding performances by colourful males with striking plumage-colours, bill sizes and shapes in birds-of-paradise (family Paradisaeidae) or the extreme adaptations in the tail of male peacocks (*Pavo cristatus*) are among the most emblematic displays (Beehler 1989; Owens and Hartley 1998). However, the sex of birds cannot always be easily identified by phenotypic traits. In fact, about half of all avian species are sexually monomorphic, with males and females showing very similar appearances (Price and Birch 1996). These include geese, cranes, rails, raptors, owls, parrots, doves, auks, shearwaters and many passerines (Volodin et al. 2015). Even in sexually dimorphic species, males and females rarely show sex-linked morphological differences shortly after hatching, making it difficult to obtain information on the sex ratio at birth exclusively from phenotypic measurements.

Our method is based on the identification of two unique loci, one in each sexual chromosome. Birds have the ZW sex-determination system, in which males are the homogametic sex (ZZ), while females are heterogametic (ZW) (Bloom 1974). As for the XY system in mammals, Z and W chromosomes share homologous sequences of nucleotides in the Pseudo-Autosomal Region (PAR) (Fridolfsson and Ellegren 1999). In this region, genes are inherited the same way as any autosomal gene rather than sex-linked, and both males and females have two copies of this region. Therefore, SNPs found in the PAR region would distinguish females and males only in the rare event that each allele variant is fixed and specific for each sex. For those cases, females would be heterozygotes whereas males would be homozygotes. Outside the PAR region, however, discriminating among sexes is expected to be easier because unique Z-linked SNPs would amplify two SNP alleles in heterozygotic males and only one in females, while unique W-linked SNPs would amplify only in females. Only hemizygotic (individuals in which only one member of a chromosome pair is present) or homozygotic males would express one Z allele call but not for W. ZZ males are then defined by either homozygote or heterozygote genotype calls for the Z-chromosome-linked SNP and the lack of the calling variants in the W-chromosome-linked SNP. Conversely, ZW females amplify for the W-chromosome-linked SNP but do not show heterozygosity for Z-chromosome-linked SNP.

To illustrate the method, we use the Western Jackdaw (*Corvus monedula*) as our study system. Jackdaws are small corvids from the Palearctic (Madge and De Juana 2019), characterized by a black plumage, grey nape and distinctive pale-blue irises. The identification of sexes in the wild is difficult because sexual dimorphism in plumage is absent and, although males tend to be larger than females, there is considerable overlap between sexes (Green and Theobald 1989; Henderson 1991; Fletcher and Foster 2010). The traditional molecular method for bird sexing based on the PCR amplification of the CHD sexual gene (Griffiths et al. 1998) works well in Jackdaws (e.g. de Kort et al. 2003; Arnold and Griffiths 2003; Salomons et al. 2006; Woods et al. 2018; Aastrup and Hegemann 2021; Hahn et al. 2021). However, the alternative use of SNPs holds great potential for its use in reliable high-throughput sexing within broader population genetic studies from the same multi-locus SNP panel. Despite the limitation of lacking a W-chromosome reference in the Jackdaw, our protocol made it possible to locate the Pseudo-Autosomal Region (PAR) in which both sexual chromosomes share homologous sequences as well as to detect unique W-linked markers that can be used to reliably sex individuals. Thus, our approach can easily be extended to other avian species.

Material and methods

Sample collection in the field

From 2015 to 2022, we captured both fledgling and adult Jackdaws as part of a long-term study of a

population breeding in nest-boxes in the Lleida plain, northeastern Iberian Peninsula (Unzeta 2020). We banded each individual with a metallic ring, measured the weight and the length of the tarsus and third primary wing, and took a sample of feathers from the abdomen for genetic analyses. To help developing the library of SNPs, we also used adults found dead during the visits to the nest-boxes. From each individual, we sampled a piece of tissue from the wing shoulder and placed it in Eppendorf tubes. All tissue samples were stored at -20^{o}C to prevent DNA degradation. Feathers were first stored in glassine bags at room temperature, and selected samples were later transferred to -20^{o}C freezers to be preserved until DNA extraction. Permits for animal manipulation and sample collections were provided for the Servei de Fauna i Flora from the Generalitat de Catalunya (SF/430-439/2016, SF/0473-0476/2018, SF/0039/2019, 4/2020/MP, SF/0093/21, SF/0018_2/22).

Sample dissection and DNA extraction in the lab

Feather dissections were performed by taking the cells from the basal tip of the calamus, following Morin et al. (1994). The lysis was performed by adding 400 µl lysis buffer and 40 µl protease K, and incubating samples at 56^{o}C overnight. DNA was extracted using an automated Chemagic 360 instrument (Perkin Elmer) from a total of 957 samples corresponding to 905 feathers from fledglings, and 49 feathers and 3 tissue samples from adult individuals. A duplicated sample collected from a recaptured individual was included as a genotyping control. The quality and concentration of the extracted DNA was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Laboratory procedures were performed at the SVGM laboratories (Molecular Genetics Veterinary Service) of the Faculty of Veterinary of the Autonomous University of Barcelona. Some feather samples, particularly those belonging to adults, had low DNA concentrations (below 50 ng/µL, which is the recommended DNA concentration to ensure proper sample genotyping according to the Thermo Fisher Scientific protocol for QuantStudio OpenArray® PCR Plates). For such low-quality samples (N=90), liquid was evaporated using a SpeedVac Vaccum Concentrator centrifuge to increase DNA concentrations. This process was carried out at the Centre for Research in Agricultural Genomics (CRAG) facilities. Further details on sample quality, applied thresholds and genotyping success related with DNA concentration are shown in Section S1 in Supplementary Information (see also Table S1, Fig. S2 and Fig. S3).

Identification of nine candidate sexual SNP loci

The exploration of candidate sexual SNP loci was carried out through the compilation of individual sequences using our own data (ten birds from Lleida) and data kindly provided by Weissensteiner et al. (2020) (four whole-genome sequenced birds from Sweden) (see Section S2 and Table S3 in Supplementary Information). We first located the sexual chromosomes and the PAR region within them based on the four whole-genome sequenced Jackdaws from Sweden. To do so, we assembled the sequences of the four individuals and assumed that the homologous regions between non-autosomal chromosomes should map within the PAR region. We annotated and identified the CHD gene (chromo-helicase-DNA-binding), traditionally used for sex identification in birds (Griffiths et al. 1998), to locate this region. Similar to Palmer et al. (2019), we used the amount of reads mapping to this gene to distinguish between males and females. Individuals J01, J02 and J08, with a high number of reads mapped, were classified as males while individual J03 was classified as a female based on the low number of reads mapped (see Section S2 in Supplementary Information). Since reads mapped heterogeneously in multiple regions along the chromosome Z, with variable coverage ranging from 4 to 30 and a median of 12, this approach did not allow us to identify the starting and ending positions of the PAR region. We located the chromosome W based on a sequence deposited in NCBI from a female of a close-related species, the New Caledonia Crow *Corvus moneduloides* (accession number CM018842.1). Reads not mapping to Z nor to any other part of the genome were assumed to be unique to W.

Once we identified the PAR region and the W chromosome, we used the sequences of the individuals from Lleida and Sweden to identify SNPs located in sexual chromosomes and outside the PAR region to assess their utility for sexing. From the W-chromosome, we selected four SNPs from different regions far from CHD and potentially unique from W. From the Z-chromosome, we selected one SNP previously tested in the studied population (F9A, see Section S2 in Supplementary Information) and four additional SNPs. These

four SNPs presumably presented fixed sex-specific allele variants at least within the analysed individuals from both populations, and were located in regions close to the CHD gene, assumed to be within the PAR region. Therefore, we used a total of nine SNPs located in sexual chromosomes to evaluate for their usefulness for sexing.

SNP genotyping with OpenArray Real-Time PCR

The OpenArray® technology is an advanced real-time PCR method that enables a broad range of applications, including SNP genotyping and gene expression analysis (Schleinitz et al. 2011; Broccanello et al. 2020). The thermocycler measures FAM and VIC fluorescence signals of the amplified product (SNPs sequences, in our case) that is generated during the reaction. Such fluorescence signals are expressed when the threshold cycle (C_T) value is reached, which is the number of PCR cycles until the genotype calling groups are specified. The fact that the detection of the DNA amplification occurs while the reaction is proceeding —instead of at the end-point— makes the technology faster and more precise and accurate than other PCR technologies (Valasek and Repa 2005). Excluding 3 blanks and 29 samples without amplified genotype variants of 960 samples, we analysed the genotyping results of 928 samples from 927 individuals.

Validation of the genetic sex assignments

Individuals with the combination of homozygote calls for Z-linked SNP and amplifying for W-linked SNP were assigned as females. Male individuals could show both homozygote or heterozygote calls for the Z-linked SNP. However, since males lack the W-chromosome, they should not show calling variants for the W-linked SNP. We validated these assignments using information on 36 adult breeding individuals (18 distinct females and 18 distinct males) that could be reliably sexed by morphological measures. Although males and females show certain overlap in body size, within a couple the male tends to have consistently larger tarsus and wings than the female (Henderson 1991; authors pers. obs.). During the breeding season and in early stages, we were able to identify breeding pairs because jackdaws are monogamic species forming long-term pair bonds and both sexes participate in building nests within cavities and in parental care (Hahn et al. 2021). To validate the genetic sex assignments, we selected ringed, sized and genotyped individuals from all pairs we identified in our population. We used these 36 individuals of known sex to assess how many were correctly sexed using our SNP approach.

Results

We found that six out of the nine candidate SNPs included in the OpenArray could not inform on sexual differences among individuals. Four of them (F1C, F2C, F4C and F6C) showed a fixed allele expression without variability between samples. Another SNP (F3C) did not amplify in any sample. The last SNP (F70A) did amplify for the "A" and "G" alleles but not for the heterozygote genotypes "A/G", indicating that the SNP was located within the PAR region from the Z chromosome. We therefore discarded these six SNPs and focused our attention to the remaining two W-markers SNPs (F5C and F7C) and the Z-marker F9A.

For F9A, 323 samples (34.8%) expressed the allele "A", 360 (38.79%) expressed the allele "G" whereas 236 samples (25.43%) expressed both "A" and "G". Nine samples (0.97%) could not be genotyped because they did not amplify in any of the 928 samples. Scatter plots for allelic discrimination confirmed the existence of three clusters for F9A (Fig. S1a; see also Table S1), corresponding to the homozygote genotype for allele 1 ("G/G" with high values of VIC and low values of FAM), the homozygote genotype for allele 2 ("A/A" with high values of FAM and low values of VIC) and finally the heterozygote genotype ("A/G" with high values of VIC and FAM). Since F9A was found in the Z-chromosome, heterozygote samples could only correspond to ZZ male individuals, while either male or female individuals could express a homozygote genotype.

The results for the F5C and F7C confirmed that they were W-linked and located outside the PAR region (Table 1). Fluorescence for F7C and F5C clustered into two groups separating samples that amplified ("A" with high values of FAM and VIC) from those which did not amplify ("NOAMP" with low values of FAM and VIC) (Fig. S1b and Fig. S1c respectively). In F7C, 466 samples (50.21%) amplified for the variant "A"

and in F5C, 443 samples (47.73%). No other allele variant was expressed in these two SNPs. The fact that only one copy was present indicated hemizygosity; thus, only female individuals carrying the W chromosome amplified for F5C and F7C. Being both W-linked SNPs complementary, one is enough to identify females instead of two, which would reduce the number of SNPs for genotyping. F7C showed less specificity to amplify other regions of the genome, suggesting the use of F7C primers was more reliable for sexing (see Section S3, Table S4, Table S5 and Fig. S4 in Supplementary Information for more details regarding this justification).

Therefore, we assigned an individual as female if amplified for the variant "A" or "G" in the Z-linked SNP F9A and "A" for the W-linked SNP F7C. We assigned an individual as male if amplified for the variant "A/A", "A/G" or "G/G" in the SNP F9A and did not amplify for the SNP F7C (Fig. 1 and Fig. 2). To validate the discriminating power of our method, we used 18 males and 18 females from 18 breeding pairs. Within couples, morphological measures can be reliably used to sex the bigger- and the smaller-sized individuals as males and females respectively (see Material and Methods). We found that our SNP approach correctly classified 100% of females and males (Table S2).

Using our method, we identified 456 individuals of the genotyped samples as females and 457 as males, including both adults and fledglings. This represents the 99.5% of all genotyped samples successfully sexed. The few cases in which individuals could not be sexed (N=5) showed a heterozygote genotype call for F9A but amplified for F5C (purple triangles in Fig. 1, observation in group b and c in Fig. 2). Accordingly, these individuals (with the sample codes CMF1598, CMF2121, CMF2223, CMF0858 and CMF1808) should be composed by two Z chromosomes and one W chromosome.

Discussion

We have proposed a general method for sexing wild birds using sex-linked SNP markers based on sequencing unique loci mapping outside the pseudoautosomal region where sexual chromosomes exchange genetic material. We hypothesized heterozygote genotypes for the Z-linked SNP would refer to males as the homogametic sex in birds. In contrast, the detection of W-linked SNP variants would occur only in females. We demonstrate the accuracy and reliability of the method in the monomorphic Western Jackdaw. Below, we provide further insights into the use of our molecular technique to sex wild birds, highlighting its benefits and limitations compared to other available tools for molecular sexing and the broader implications of the use of sex-linked SNPs on the study of ecology and evolutionary biology.

The proposed technique is expected to be particularly useful in studies aimed at genotyping large number of individuals within species and populations. On one hand, the method only requires sampling feathers. Feathers provide sufficient quality and quantity of DNA for molecular analyses like ours (Horváth et al. 2005). Indeed, we found that in feathers where the concentration of the DNA extracted was considered low (<50 ng/μL), genotyping was still successful in 86.55% of cases. On the other hand, our technique can be integrated into multiple genetic analysis based on SNP data without the need of additional PCR amplification and electrophoresis. Combined with the use of high-throughput sequencing method of OpenArray® genotyping based on real-time PCR technology (Broccanello et al. 2020), which results in a significant reduction on the sequencing time and costs required per sample and locus (Hudson 2008), the technique represents a cost-effective method for SNP genotyping in studies that require genotyping thousands of individuals (Jenkins and Gibson 2002).

Despite the capabilities of OpenArray® genotyping, its implementation is not exempt of difficulties. In our case, the genotyping variants of one locus included in the array was undefined for the totality of the samples. Because the estimated error rate of automated high-throughput methods for genotyping with SNPs is fewer than 1 in 2000 genotypes (Ranade et al. 2001), we can safely discard standard sequencing errors as the main source of the observed result. Instead, the allelic variants identified in individuals from the Sweden population for this locus in particular might not be present in Lleida's population. Furthermore, candidate loci meeting the screening criteria are more challenging to identify from sequencing artifacts in small sample sizes (N=3 males and N=1 females). In practice, multiple individuals of each sex would be required to avoid falsely identifying rare SNP variants as sex-linked contigs (Palmer et al. 2019).

The capabilities of OpenArray(r) genotyping are also limited by the quality of DNA samples. Although genotyping proved to be successful in poor-quality samples, a 3.03% of the samples (29 of the whole batch of 957) did not amplify for any loci due to low DNA concentrations. From the remaining 928 sequenced samples, the Z-allele could not be identified in 9 of them, leading to a total of 919 genotyped samples. The low mean DNA concentration (47.27 ng/μL) of these samples indicated, again, problems of sample quality rather than sequencing errors. Therefore, increasing the quality threshold should reduce the number of not genotyped samples.

More difficult to understand is the observation of five samples (5 of the 919 genotyped samples) that could not be sexed with certainty because showed two variants in the Z-chromosome and a third in the W-chromosome. A likely alternative is a differential specificity of the F5C and F7C primers for the W-unique loci. Indeed, in 2.4% of the cases where genotypes were defined, samples showed a mismatch between the genotypic variants amplified with the W-linked F5C and F7C SNPs. The existence of differential specificity of the F5C and F7C primers is further supported by a blast analysis, which shows that specificity was higher for F7C. Although in our sexing method we employed F7C, the fact that we still detected samples with two variants in the Z-chromosome may indicate that this may still be insufficient to fully resolve the specificity issue. Alternatively, the observation of ZZW may indicate possible cross-contamination events (mixing samples from male and female individuals) and/or sequencing errors (misassignment of genotype calls). We should also consider the possibility of trisomies which, although rare, have been documented in some bird species like *Gallus domesticus* (Lin et al. 1995), *Charadrius alexandrines* (Küpper et al. 2012), *Ara ararauna* (Tiersch et al. 1991) and*Acrocephalus arundinaceus* (Arlt et al. 2004).

Regardless of the limitations, the high accuracy (100% of individuals correctly sexed) and reliability (over 99% of samples correctly processed) of the proposed approach make it an efficient way to sex birds. Although there are other powerful approaches for sexing individuals (e.g. Griffiths et al. 1998; Lois-Milevicich et al. 2021), the use of sex-specific SNP markers located in unique fragments of Z- and W-chromosomes, as we propose here, will contribute to reliably sex individuals while genotyping samples for other purposes. While the approach requires reference sequences to identify and annotate sex chromosomes, this information is becoming increasingly available in non-model species through fast and affordable next generation (NGS) and whole-genome sequencing (WGS). Even in species for which only one reference sex chromosome is available, as in our case, sex-linked scaffolds can still be identified based on sex differences in genomic coverage (Palmer et al. 2019).

Currently, SNPs are widely used in a variety of research programs like human forensics, crop improvement, aquaculture, drug discovery and wildlife research (Garvin et al. 2010). In ecology and evolution, SNPs are increasingly used for population genetic analysis, pedigree reconstruction and phenotype mapping (Garvin et al. 2010). Given that males and females often differ in habitat preferences, feeding specializations, parental investment and dispersion, among many other ecological roles, these studies heavily rely on the accurate identification of the sex of individuals. Thus, we expect that the sex-linked SNPs protocol we present here will be useful to a broad range of fields because it will allow genotyping and sexing a high number of individuals in parallel and with independence of their life cycle stage.

Acknowledgements

We thank all people involved in field work, particularly Marcal Pou, Gerard Bota, David Giralt, Francesc Sarda, Joan Maspons, Simon Ducatez, Ferran Sayol, Joan Rodriguez, Ferran Broto and Lluis Cullere. We extend the gratitude to all people involved in lab work: Anna Maria Marcade and Maria dels Angels from SVGM, Lorena Serrano from Vetgenomics, Anna Castello from CRAG, and Miguel Fernandez and Teresa Porta from training programs. We are very grateful to Oscar Ramirez from CanID and Laura Balague who collaborated in the SNP panel design. Finally, an acknowledgement to Matthias Weissensteiner and Jochen Wolf for kindly providing genomic data which allowed the completion of the SNP library. This research was supported by funds from the Spanish Ministry of Science, Innovation and Universities to DS (CGL2017-90033-P and PID2020-119514GB-I00). AGR was supported by a FPI grant (PRE2018-084319). AMM was supported by the project NORTE-01-0246-FEDER-000063, supported by Norte Portugal Regional

Operational Programme (NORTE2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). OL was supported by a 'La Caixa' Junior Leader position under the Marie Skłodowska-Curie grant agreement no. 847648. MU was supported by a FI-DGR 2015 grant from the Generalitat de Catalunya.

References

Aastrup, C., & Hegemann, A. (2021). Jackdaw nestlings rapidly increase innate immune function during the nestling phase but no evidence for a trade-off with growth. *Developmental & Comparative Immunology* , 117, 103967. https://doi.org/10.1016/j.dci.2020.103967

Andrews, K. R., Good, J. M., Miller, M. R., Luikart, G., & Hohenlohe, P. A. (2016). Harnessing the power of RADseq for ecological and evolutionary genomics. *Nature Reviews Genetics* , 17(2), 81-92. https://doi.org/10.1038/nrg.2015.28

Arlt, D., Bensch, S., Hansson, B., Hasselquist, D., & Westerdahl, H. (2004). Observation of a ZZW female in a natural population: implications for avian sex determination. *Proceedings of the Royal Society of London. Series B: Biological Sciences* , 271, S249-S251. https://doi.org/10.1098/rsbl.2003.0155

Arnold, K. E., & Griffiths, R. (2003). Sex-specific hatching order, growth rates and fledging success in jackdaws *Corvus monedula* .*Journal of Avian Biology* , 34(3), 275-281. https://doi.org/10.1034/j.1600-048X.2003.03068.x

Beehler, B. M. (1989). The birds of paradise. *Scientific American,* 261(6), 116-123.

Bloom, S. E. (1974). Current knowledge about the avian W chromosome.*BioScience* , 24(6), 340-344. https://doi.org/10.2307/1296739

Broccanello, C., Gerace, L., Stevanato, P. (2020). QuantStudio 12K Flex OpenArray® System as a tool for high-throughput genotyping and gene expression analysis. In: Biassoni, R., Raso, A. (eds) Quantitative Real-Time PCR. Methods in Molecular Biology, vol 2065. Humana, New York, NY. https://doi.org/10.1007/978-1-4939-9833-3_15

de Kort, S. R., Emery, N. J., & Clayton, N. S. (2003). Food offering in jackdaws (*Corvus monedula*). *Naturwissenschaften* , 90, 238-240. https://doi.org/10.1007/s00114-003-0419-2

Durell, S. E. L. V. D. (2000). Individual feeding specialisation in shorebirds: population consequences and conservation implications.*Biological Reviews* , 75(4), 503-518. https://doi.org/10.1111/j.1469-185X.2000.tb00053.x

Fang, S., Zhang, Y., Shi, X., Zheng, H., Li, S., Zhang, Y., ... & Ma, H. (2020). Identification of male-specific SNP markers and development of PCR-based genetic sex identification technique in crucifix crab (*Charybdis feriatus*) with implication of an XX/XY sex determination system. *Genomics* , 112(1), 404-411. https://doi.org/10.1016/j.ygeno.2019.03.003

Fletcher, K., & Foster, R. (2010). Use of external biometrics to sex Carrion Crow *Corvus corone* , Rook *C. frugilegus* and Western Jackdaw *C. monedula* in Northern England. *Ringing & Migration* , 25(1), 47-51. https://doi.org/10.1080/03078698.2010.9674414

Fridolfsson, A. K., & Ellegren, H. (1999). A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology* , 116-121. https://doi.org/10.2307/3677252

Garvin, M. R., Saitoh, K., & Gharrett, A. J. (2010). Application of single nucleotide polymorphisms to non-model species: a technical review. *Molecular Ecology Resources* , 10(6), 915-934. https://doi.org/10.1111/j.1755-0998.2010.02891.x

Green, P. T., & Theobald, C. M. (1989). Sexing birds by discriminant analysis: further considerations. *Ibis* , 131(3), 442-447. https://doi.org/10.1111/j.1474-919X.1989.tb02795.x

Griffiths, R., Double, M. C., Orr, K., & Dawson, R. J. (1998). A DNA test to sex most birds. *Molecular Ecology* , 7(8), 1071-1075. https://doi.org/10.1046/j.1365-294x.1998.00389.x

Hahn, L. G., Hooper, R., McIvor, G. E., & Thornton, A. (2021). Cooperative nest building in wild jackdaw pairs. *Animal Behaviour* , 178, 149–163. https://doi.org/10.1016/j.anbehav.2021.06.004

Henderson, I. G. (1991). Sexing a population of Jackdaws on the basis of biometric characteristics. *Ringing & Migration* , 12(1), 23-27. https://doi.org/10.1080/03078698.1991.9673980

Horváth, M. B., Martínez-Cruz, B., Negro, J. J., Kalmar, L., & Godoy, J. A. (2005). An overlooked DNA source for non-invasive genetic analysis in birds. *Journal of Avian Biology* , 36(1), 84-88. https://doi.org/10.1111/j.0908-8857.2005.03370.x

Hudson, M. E. (2008). Sequencing breakthroughs for genomic ecology and evolutionary biology. *Molecular Ecology Resources* , 8(1), 3-17. https://doi.org/10.1111/j.1471-8286.2007.02019.x

Jenkins, S., & Gibson, N. (2002). High-throughput SNP genotyping. Comparative and Functional *Genomics* , 3(1), 57-66. https://doi.org/10.1002/cfg.130

Kaiser, S. A., Taylor, S. A., Chen, N., Sillett, T. S., Bondra, E. R., & Webster, M. S. (2017). A comparative assessment of SNP and microsatellite markers for assigning parentage in a socially monogamous bird. *Molecular Ecology Resources* , 17(2), 183–193. https://doi.org/10.1111/1755-0998.12589

Kupper, C., Augustin, J., Edwards, S., Szekely, T., Kosztolanyi, A., Burke, T., & Janes, D. E. (2012). Triploid plover female provides support for a role of the W chromosome in avian sex determination. *Biology Letters* , 8(5), 787-789. https://doi.org/10.1098/rsbl.2012.0329

Lin, M., Thorne, M. H., Martin, I. C. A., Sheldon, B. L., and Jones, R. C. (1995). Development of the gonads in the triploid (ZZW and ZZZ) fowl, *Gallus domesticus* , and comparison with normal diploid males (ZZ) and females (ZW). *Reproduction, Fertility and Development* 7, 1185–1197. https://doi.org/10.1071/RD9951185

Lois-Milevicich, J., Gomez, R. O., Ursino, C. A., Lois, N. A., & De la Colina, A. (2021). Rapid and low-cost molecular sexing of a corvid songbird using a single protocol with two universal primer sets. *Ardeola* , 68(2), 501-510. https://doi.org/10.13157/arla.68.2.2021.sc1

Madge, S., de Juana, E., del Hoyo, J., Elliott, A., Sargatal, J., Christie, D. A., & de Juana, E. (2019). Eurasian Jackdaw (*Corvus monedula*). *Handbook of the birds of the world alive.*

Morin, P. A., Messier, J., & Woodruff, D. S. (1994). DNA extraction, amplification, and direct sequencing from hornbill feathers. *Journal of The Science Society of Thailand* , 20(4), 994.

Owens, I. P., & Hartley, I. R. (1998). Sexual dimorphism in birds: why are there so many different forms of dimorphism?. *Proceedings of the Royal Society of London. Series B: Biological Sciences* , 265(1394), 397-407. https://doi.org/10.1098/rspb.1998.0308

Palaiokostas, C., Bekaert, M., Taggart, J. B., Gharbi, K., McAndrew, B. J., Chatain, B., ... & Vandeputte, M. (2015). A new SNP-based vision of the genetics of sex determination in European sea bass (*Dicentrarchus labrax*). *Genetics Selection Evolution* , 47(68), 1-10. https://doi.org/10.1186/s12711-015-0148-y

Palmer, D. H., Rogers, T. F., Dean, R., & Wright, A. E. (2019). How to identify sex chromosomes and their turnover. *Molecular Ecology,*28(21), 4709-4724. https://doi.org/10.1111/mec.15245

Price, T., & Birch, G. L. (1996). Repeated evolution of sexual color dimorphism in passerine birds. *The Auk* , 113(4), 842-848. https://doi.org/10.2307/4088862

Salomons, H. M., Muller, W., Dijkstra, C., Eising, C. M. & Verhulst, S. (2006). No sexual differences in embryonic period in jackdaws *Corvus monedula* and black-headed gulls *Larus ridibundus* .*Journal of Avian Biology,* 37, 19-22. https://doi.org/10.1111/j.0908-8857.2006.03614.x

Schleinitz, D., DiStefano, J.K., & Kovacs, P. (2011). Targeted SNP genotyping using the TaqMan(r) assay. In: DiStefano, J. (eds)*Disease Gene Identification* . Methods in Molecular Biology, vol 700. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-61737-954-3_6

Selander, R. K. (1966). Sexual Dimorphism and Differential Niche Utilization in Birds. *The Condor* , 68(2), 113–151. https://doi.org/10.2307/1365712

Shi, X., Waiho, K., Li, X., Ikhwanuddin, M., Miao, G., Lin, F., ... & Ma, H. (2018). Female-specific SNP markers provide insights into a WZ/ZZ sex determination system for mud crabs *Scylla paramamosain* ,*S. tranquebarica* and *S. serrata* with a rapid method for genetic sex identification. *BMC Genomics* , 19(981), 1-12. https://doi.org/10.1186/s12864-018-5380-8

Tiersch, T. R., Beck, M. L., & Douglass, M. (1991). ZZW autotriploidy in a Blue-and-Yellow Macaw. *Genetica* , 84(3), 209-212. https://doi.org/10.1007/BF00127249

Unzeta Lloret, M. (2020). Behavior and its role on reproductive performance under a life-history perspective (Doctoral dissertation, Universitat Autonoma de Barcelona).

Valasek, M. A., & Repa, J. J. (2005). The power of real-time PCR. *Advances in Physiology Education* , 29(3), 151-159. https://doi.org/10.1152/advan.00019.2005

Volodin, I. A., Volodina, E. V., Klenova, A. V., & Matrosova, V. A. (2015). Gender identification using acoustic analysis in birds without external sexual dimorphism. *Avian Research* , 6(20), 1-17. https://doi.org/10.1186/s40657-015-0033-y

Wang, L., Xie, N., Shen, Y., Ye, B., Yue, G. H., & Feng, X. (2019). Constructing high-density genetic maps and developing sexing markers in northern snakehead (*Channa argus*). *Marine Biotechnology* , 21(3), 348-358. https://doi.org/10.1007/s10126-019-09884-z

Weissensteiner, M. H., Bunikis, I., Catalan, A., Francoijs, K. J., Knief, U., Heim, W., Peona, V., Pophaly, S. D., Sedlazeck, F. J., Suh, A., Warmuth, V. M., & Wolf, J. B. W. (2020). Discovery and population genomics of structural variation in a songbird genus. *Nature Communications* , 11(1), 3403. https://doi.org/10.1038/s41467-020-17195-4

Woods, R. D., Kings, M., McIvor, G. E., & Thornton, A. (2018). Caller characteristics influence recruitment to collective anti-predator events in jackdaws. *Scientific Reports* , 8(1), 7343. https://doi.org/10.1038/s41598-018-25793-y

Data Accessibility and Benefit-Sharing

Data Accessibility Statement

Individual genotype data are available on Supporting Information (Table S2).

Benefit-Sharing Statement

Benefits Generated: Benefits from this research accrue from the sharing of our data and results on public databases as described above.

Author Contributions

D.S. conceived and financed the project. A.G.R., M.U. and D.S. collected samples in the field. A.G.R. and M.F.R. contributed to lab procedures. A.M.M. performed the data analysis. A.M.M. and A.G.R. interpreted the results. A.G.R. wrote the first draft, O.L. and D.S. revised successive versions, and all authors improved and approved the final manuscript.

Tables and Figures

TABLE 1. Sex-linked SNP markers information (Fw, forward; Rv, reverse; bp, base pairs).

Assay Name	F9A	F7C	F5C
Chromosome	Unique Z-linked, no PAR	Unique W-linked, no PAR	Unique W-linked, no PAR
Location (bp position)	28656895-28657166	1886358-18886578	10603258-10603378
Fw primer name	ANPRYWV_F	ANPRYWW_F	ANMGCR2_F
Rv primer name	ANPRYWV_R	ANPRYWW_R	ANMGCR2_R
Fw primer sequence	GGGTGTAGGTATAGA TTGGCTCTCA	GAAAATAATAACTAT CTGTGTATGGATGGG	ATTAAAAAATAAACC GTTTTCAAGCATTTGCA
Rv primer sequence	CTCAAAGCTCCATGG AACAAACTG	AACTCCATGCTTAAA CCGTCCTT	CACTTTGGAATCCTC TCCATTAGGA
Context sequence	ATTGGCTCTCAATTG ACCTCTAGCT[G/A] GTGGAGATTCTGCAG TTTGTTCCAT	GTAGTTAATGACAAT GCATGGATCT[A/C] TGTGTTGTGTATAAG GACGGTTTAA	AGCATTTGCATATTA TAAATTCGGG[A/G] ATTTTAGTTCTGAAT AAGTGGGTTT

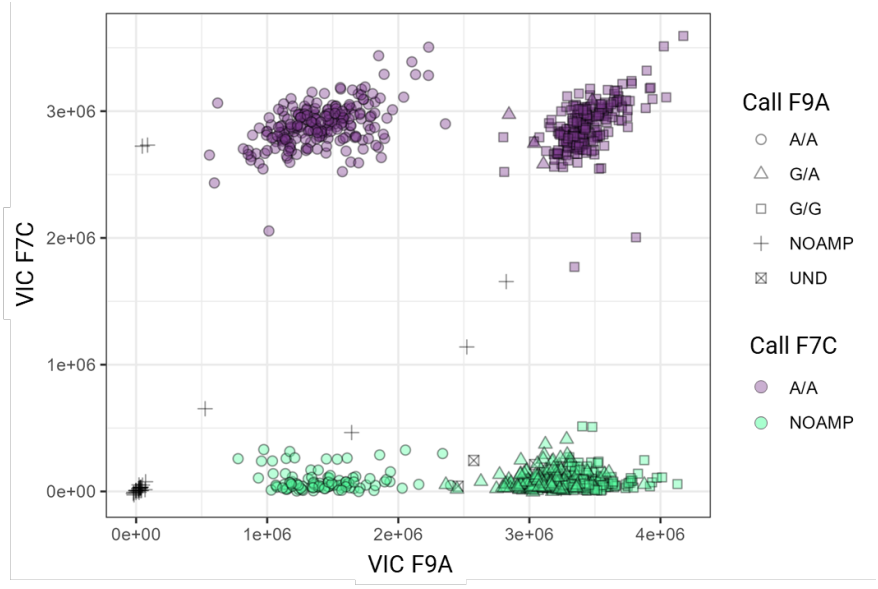

FIGURE 1. Allelic discrimination plot for F9A and F7C SNPs using fluorescence data from allele 1. Each dot represents the genotype of one sample at a specific SNP fluorescence value in VIC Rn units (normalized reporter signal). Genotype calls include F9A homozygotes (A/A and G/G in circles and squares respectively), F9A heterozygotes (G/A in triangles), not amplified (NOAMP in crosses for F9A and in green for F7C), or undefined (UND in crossed squares for F9A and in purple for F7C). Females are depicted in purple (N=456) and males in green (N=457).

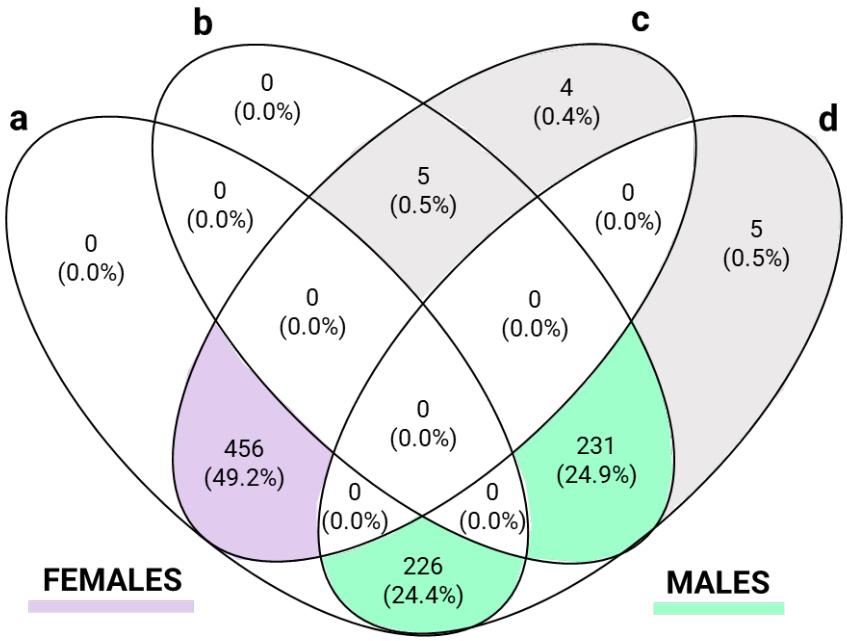

FIGURE 2. Summary of all possible genotype combinations using the Z- and the W-unique loci. The number and percentage of samples matching the conditions represented by the overlapping sets of the Venn diagram is shown in each area. The represented sets include homozygotes for F9A (a), heterozygotes for F9A (b), amplified for F7C (c), and not amplified for F7C (d). Females are depicted in purple (N=456) and males in green (N=457). Samples that could not be sexed are represented in the grey areas, while conditions not found in any sample are left empty.