Sex determination of non-model organisms in the absence of field records using Diversity Arrays Technology (DArT) data

Isabelle Onley[1], Jeremy Austin[1], and Kieren Mitchell[2]

[1]The University of Adelaide Faculty of Sciences
[2]University of Adelaide Faculty of Sciences

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Abstract

Conservation genomics research often relies on accurate sex information to make inferences about species demography, dispersal, and population structure. However, field determined sex data are not always available and can be subject to human error, while laboratory sex determination is costly and often challenging for non-model species. Conservation genomics programs increasingly use reduced-representation genome sequencing to assess neutral and functional genetic diversity, population structure, gene flow and pedigrees in threatened species. Here we demonstrate that sex can be determined from reduced-representation sequencing data produced by the increasingly popular Diversity Arrays Technology sequencing workflow (DArT-seq) using a program originally designed for application to shotgun data. This program – sexassign – compares the "dosage" of sequencing reads mapping to autosomes versus the X chromosome. In the present study, sexassign accurately determined the sex of 60 field-collected Greater Stick-Nest Rat (Leporillus conditor) samples, despite the absence of an annotated reference genome for the species. This "read-dosage" approach is not only more accurate and affordable than traditional sex determination methods, but can be applied to any diploid organism with a heterogametic sex determination system – including non-model and understudied species of conservation importance – by using FASTQs generated by DArT.

Keywords

Conservation genomics, sex determination, bioinformatics, DArT-seq

Introduction

Accurate sex determination is an integral aspect of conservation genomics research, particularly when studying parameters such as relatedness, dispersal, and philopatry. Sexing of individuals used in conservation genomics studies typically takes place in the field at the time of collection. However, sex assignments recorded in the field are not always reliable and there is a wide margin for human error, particularly for species that do not demonstrate sexual dimorphism or when researchers are working in difficult conditions. Further, field records can easily be lost or incorrectly transcribed during trapping and monitoring. Genetic sex determination is a favourable alternative or complement to field identification, as it is an objective, highly standardized, and accurate approach that eliminates the possibility of upstream sex misidentification confounding genomic studies (Hrovatin & Kunej, 2017).

While PCR-based sex identification methods have been used for several decades to identify and amplify sex chromosomes in individual samples (Akane et al., 1992; Clapcote & Roder, 2005; McFarlane et al., 2013), such processes can be time consuming and expensive. In addition, they require taxon-specific primers that are not always available or applicable to the target species. With the advent of high-throughput sequencing (HTS) technology it is now possible to produce high-resolution genomic data that may allow researchers to determine the sex of sequenced individuals bioinformatically. For example, single nucleotide polymorphisms (SNPs) in the genome can often be linked to the sex chromosomes in model organisms, allowing sex to be

determined on chromosomal presence-absence basis (Fowler & Buonaccorsi, 2016; Lambert et al., 2016). For non-model organisms where a well-assembled and well-annotated reference genome is unavailable, the overall "dosage" of sequencing reads mapping to the sex chromosomes can be assessed to determine whether the individual is heterogametic or homogametic (Bover et al., 2018; Gamble, 2016; Gower et al., 2019; Pečnerová et al., 2017).

Read-dosage-based approaches to sex determination have only been applied using shotgun sequencing data, where molecules are randomly sampled and sequenced (Flamingh et al., 2020; Motahari et al., 2013; Skoglund et al., 2013). However, many conservation programs employ reduced-representation sequencing approaches (e.g. RADseq), where sequenced molecules belong to a subset of genomic loci. One commercial provider of reduced-representation sequencing that is growing in popularity in the conservation genomics field is Diversity Arrays Technology (DArT) (Cummins et al., 2019; Ewart et al., 2019; Pazmiño et al., 2018; Sansaloni et al., 2011; Schultz et al., 2018; van Deventer et al., 2020). The DArT workflow uses restriction enzymes to reduce genomic complexity, allowing identification of informative markers that are subsequently sequenced for all submitted samples (Kilian et al., 2012). However, despite the growing popularity of DArT for conservation genomics projects, no simple and widely applicable sex-determination framework has emerged that can be applied to DArT data. In the present study we apply a read-dosage sex-determination approach to DArT data from an Australian rodent, the Greater Stick-Nest Rat (*Leporillus conditor*), and demonstrate that - despite being originally designed for application to shotgun data - this method remains robust when applied to FASTQ files generated as part of the DArT workflow.

Materials and Methods

DNA submitted to DArT was extracted from 60 *L. conditor* tissue samples collected by staff during routine trapping events at Arid Recovery Reserve, South Australia, between 1999 and 2003. DNA extraction was completed following the methods described by Barclay et al. (2006) and samples were subsequently stored at -20°C prior to sequencing by DArT. Following library preparation and sequencing by DArT using their proprietary workflow, we obtained the raw Illumina data in FASTQ format. We used the Paleomix v1.2.14 pipeline to process these data: AdapterRemoval2 v2.3.1 was used to trim residual adapter sequences (using default parameters) and filter reads shorter than 30 bp, after which all remaining reads were mapped against the repeat-masked house mouse genome assembly (GRCm38) using BWA v0.7.17 *mem* algorithm. We then used the *idxstats* command in SAMtools v1.10 to extract the number of reads mapping to each scaffold of the reference assembly.

We visualised read-dosage for each sample by using RStudio v1.3.1073 to plot the number of reads mapping to the autosomes and X chromosome (as a proportion of the total reads per sample) versus scaffold length. To determine the sex of the Greater Stick-Nest Rat samples we used Gower et al.'s (2019) python script *sexassign*(https://github.com/grahamgower/sexassign). This python script uses a likelihood ratio test to assign samples to either male or female on the basis of the observed ratio of reads mapping to the X chromosome versus the autosomes. However, because an underlying assumption of the calculations made by *sexassign* is that the X chromosome in homogametic females should receive the same read-dosage as an autosome of the same length (i.e. read dosage of ~1X versus ~0.5X in heterogametic males), we first multiplied the number of reads mapping to the X chromosome for all samples (regardless of putative sex) by a factor of 2.12 (the expected read-dosage for the X chromosome, 0.065, divided by the observed mean read-dosage for the X chromosome in putatively female samples, 0.0308, see Results).

Results

The proportion of reads mapping to each of the autosomes was highly consistent between samples (Fig. 1). Further, autosomal read-dosage appeared to be positively correlated with scaffold length, as expected if restriction motifs are randomly distributed. We tested this correlation by performing a linear regression in RStudio (proportion of reads ~ scaffold length), which resulted in a slope coefficient of $3.833e^{-10}$ (adjusted $R^2 = 0.7$, $p < 2e^{-16}$). Unlike the autosomes, values for the proportion of reads mapping to the X chromosome formed two clusters, putatively representing females (with higher read-dosage values) and males

(with lower read-dosage values). However, the mean proportion of reads mapping to the X chromosome (length = 171,031,299 bp) for the putatively female samples (0.0308) was substantially lower than the expectation (0.0656) based on the relationship between the proportion of reads mapped and scaffold length inferred from the autosomes. This difference may represent an inherent bias against sex-linked loci in the DArT pre-sequencing workflow or a depletion in the restriction motif on the X chromosome relative to the autosomes.

The read-dosage sex-assignment program (*sexassign*) allowed us to successfully assign all individuals in the dataset as either male (heterogametic, XY; X read-dosage = ˜0.5X) or female (homogametic, XX; X read-dosage = ˜1X, Fig. 2, Table 1). Of the 60 individuals sequenced, 33 were determined to be female and 27 to be male, consistent with the typical sex ratio in rodent populations under normal conditions (Labov et al., 1986; Rosenfeld et al., 2003). Genetic sex determination had a ˜94% concurrence rate with field determined sex, a typical human error margin considering the lack of obvious sexual dimorphism within the species and the difficulty of accurately sexing rodents in the field, particularly during non-reproductive periods (Hoffmann et al., 2010; Jacques et al., 2015).

Discussion

Our results demonstrate that the FASTQ-formatted data routinely generated by Diversity Arrays Technology (DArT) as an intermediate step in their workflow can reliably be used to determine the sex of samples from non-model organisms, confirming or replacing field-based sex identification and eliminating the need for additional costly laboratory sexing analyses. Importantly, a reference genome from the species of interest does not appear to be necessary, as we obtained robust results by mapping our data to the reference assembly for the house mouse (*Mus musculus*), which shared a common ancestor with*L. conditor* 10 million years ago (Steppan & Schenk, 2017). While the house mouse genome is assembled to the chromosome-level, making identification of reads mapping to the X chromosome straightforward, this approach should also work with scaffold-level reference assemblies. Indeed, Gower et al. (2019) identified X-linked scaffolds in the polar bear genome (UrsMar1.0) by first mapping all scaffolds against the chromosome-level dog reference assembly (CanFam3.1), then applied*sexassign* to shotgun sequencing data from a third species – brown bears (*Ursus arctos*) – that they mapped to the putative polar bear X-linked scaffolds. Given that scaffold-level assemblies are increasingly available for a wide range of taxa, our results suggest that most DArT end-users working on mammals (or indeed any diploid organism with a heterogametic sex-determination system) should be able use their FASTQ data to determine the sex of their samples.

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Conflict of Interest

The authors declare no conflict of interest.

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Data Accessibility Statement *(to be archived upon acceptance)*

The reads generated for this study have been deposited at the Sequence Read Archive (NCBI) with project number TBA

Animal Ethics Statement

Live animal trapping and sampling at Arid Recovery was conducted under South Australian Wildlife Ethics Committee permit number 58-2015.

Author Contributions

IRO and JJA coordinated submission of samples to DArT. IRO and KJM analysed the data. IRO drafted the abstract, introduction, results, and discussion. KJM drafted the materials and methods and figures. All authors contributed to the interpretation of results and provided feedback on the final manuscript.

Table 1. Results from *sexassign* , including sex assignment. The length of the X chromosome was 171,031,299 bp and the total length of the autosomes was 2,462,745,373 bp (Gower, 2019).

ID^{+}	**M$_X$**$^{++}$	**Sex**	**N$_X$**§	**N$_A$**¶
ET002	0.474	M	26392	802947

ID^{+}	**M$_X$**$^{++}$	**Sex**	**N$_X$**§	**N$_A$**¶
ET101	0.962	F	23290	349580
ET102	0.480	M	12663	380070
ET103	0.953	F	25446	385924
ET106	0.515	M	12856	359385
ET119	0.930	F	60581	941980
ET133	0.963	F	26462	396880
ET146	0.517	M	8416	234263
ET147	0.979	F	26407	388782
ET147B	0.976	F	12858	190022
ET148	0.507	M	14526	412334
ET149	1.020	F	29618	417327
ET151	0.975	F	24507	362393
ET152	1.002	F	28024	402617
ET153	0.970	F	26335	391810
ET154	0.946	F	25894	395471
ET155	0.4821	M	14054	420275
ET157	1.026	F	28484	399170
ET158	0.950	F	26525	403485
ET162	0.503	M	13867	397200
ET163	0.946	F	24215	370137
ET163B	0.473	M	14299	436150
ET17	0.942	F	58158	892183
ET173	0.956	F	27789	419868
ET176	0.938	F	22451	346121
ET177	0.952	F	26275	398926
ET18	0.905	F	39676	635045
ET183	0.495	M	13220	384279
ET184	0.487	M	32640	966813
ET185	1.010	F	26952	384146
ET186	0.473	M	28294	863292
ET187	0.996	F	25964	375434
ET188	0.503	M	12563	359345
ET189	0.972	F	22913	339929
ET192	0.500	M	14444	416297
ET193	0.489	M	13108	386485
ET195	0.960	F	25194	378761
ET196	0.977	F	27030	398915
ET198	0.512	M	28970	813496
ET198B	0.484	M	12733	378965
ET203	0.475	M	11469	348138
ET209	0.971	F	25533	379373
ET217	0.480	M	13460	404344
ET231	0.493	M	12745	372720
ET233	0.480	M	12353	370852
ET255	0.952	F	24282	368459
ET259	0.478	M	11357	342534
ET261	0.958	F	26557	400394
ET277	0.488	M	29029	857827
ET29	0.939	F	30250	465742
ET29B	0.959	F	23511	354200

ID^{+}	$M_X{}^{++}$	Sex	$N_X{}^{\S}$	$N_A{}^{\P}$
ET3	0.991	F	27316	397077
ET32	0.509	M	13059	369309
ET37	0.467	M	26816	828029
ET5	0.491	M	27564	809456
ET50	0.485	M	11802	350729
ET50.2	0.981	F	23708	348582
ET5967	0.958	F	26131	393719
ET61	0.491	M	7566	222226
ET62	0.987	F	36015	526076

+ ID = ear tag number for *L. conditor* individual,++ M_X = read dosage on X chromosome, §N_X = count of reads mapped to the X chromosome (after multiplying by 2.12), ¶ N_A = count of reads mapped to the autosome.

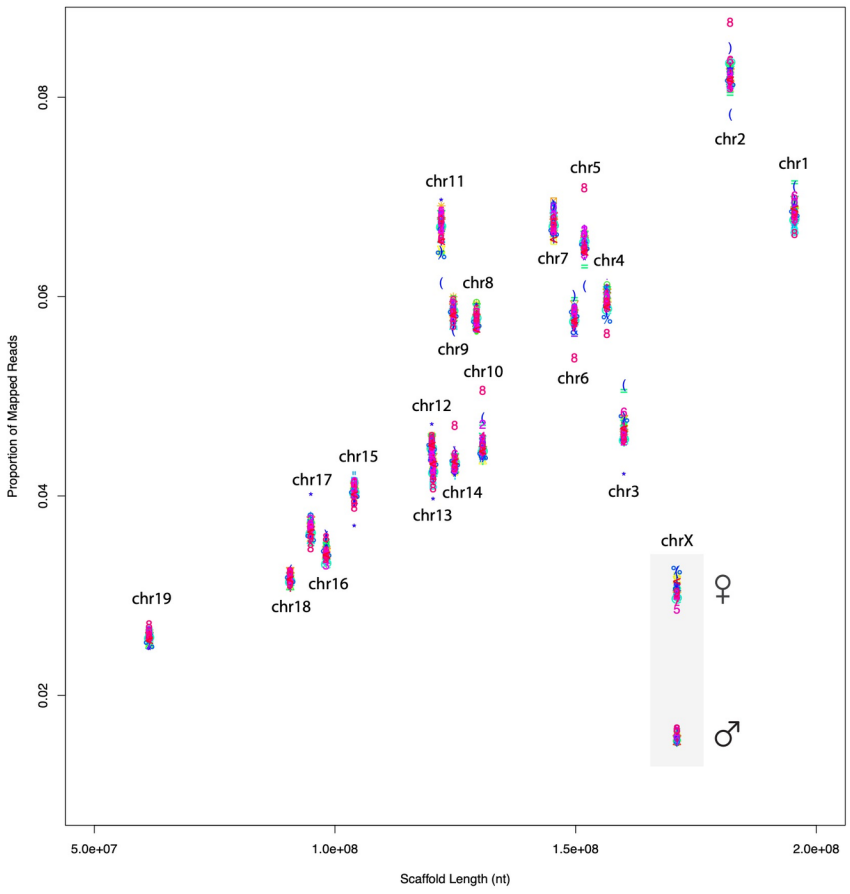

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Figure 2. Plot of X chromosome read dosages for all sequenced *L. conditor* individuals, with confidence intervals for male heterogametes (red) and female homogametes (blue).