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METHODS AND RESOURCES ARTICLE

Genetic sex test for the short-beaked echidna (*Tachyglossus aculeatus*)

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Abstract

Monotremes (echidnas and platypus) possess five X and four or five Y sex chromosomes, respectively, that evolved independently from the sex chromosomes found in therian mammals. While the platypus has obvious venomous spurs in the male, the short-beaked echidna (*Tachyglossus aculeatus*) lacks easily identifiable sexually dimorphic characteristics, making it difficult to sex adults out of the breeding season and almost impossible to sex juveniles or embryonic material. Here, we used restriction-site associated DNA sequencing (RADseq) to identify novel sex-specific markers in the short-beaked echidna. We identified and validated a subset of male-specific markers that can be used as a non-invasive genetic sex test for the short-beaked echidna. We also assessed how laboratory conditions, including DNA extraction protocol and number of PCR cycles, can influence the outcome of genetic sex tests. The combined use of these markers will provide a valuable toolkit for researchers, conservationists, and zoo-keepers to reliably and non-invasively determine sex in the short-beaked echidna.

Keywords echidna · monotreme · Sex chromosomes · RADseq

Introduction

The short-beaked echidna (*Tachyglossus aculeatus*) is one of five extant species of monotremes, a unique group of egg-laying mammals. Although the short-beaked echidna is very common and found throughout Australia and Papua New Guinea, the four remaining species of monotremes, the platypus (*Ornithorhynchus anatinus*, found only in eastern Australia) and the three species of long-beaked echidna (*Zaglossus bartoni*, *Zaglossus attenboroughi* and *Zaglossus bruijnii*, found only in Papua and New Guinea) are all either vulnerable or critically endangered. Very little is understood about monotreme reproductive physiology and only recently was the short-beaked echidna reliably bred in captivity (Wallage et al. 2015). The long-beaked echidna species have yet to be successfully bred in captivity. Echidnas have no readily apparent sexual dimorphisms (Rismiller and McKelvey 2000). They possess no external sexual organs, and the few sex-specific characters, such as the female pouch or male spurs, are transient or unreliable (Rismiller and McKelvey 2003; Johnston et al. 2006a). Other methods of sexing, such as ultrasound, require anesthesia, are time-consuming and are stressful for the animals. Furthermore, these features cannot be used to sex juvenile animals or embryonic material (Johnston et al. 2006a). Accurate and easy sexing methods are also necessary for ecological

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studies looking to assess wild populations and for conservation efforts (Robertson and Gemmell, 2006). Therefore, a simple, non-invasive method to identify sex in echidnas is needed.

Monotreme sex chromosomes are not homologous to the XX/XY sex chromosomes of therian (placental and marsupial) mammals (Wallis et al. 2007; Cortez et al. 2014). Instead, echidnas and platypuses independently evolved a sex chromosome system composed of multiple X and Y chromosomes: five X and Y chromosomes in platypuses and five X and four Y chromosomes in echidnas (Bick et al. 1973; Wrigley and Graves 1988; Rens et al. 2004, 2007; Grützner et al. 2004). The monotreme sex chromosomes share genes with the bird Z and mammal X chromosomes (Grützner et al. 2004; Rens et al. 2007; Veyrunes et al. 2008). The monotreme chromosomes form a multivalent meiotic chain in males connected by homologous regions in adjacent X and Y chromosomes (pseudoautosomal regions, Rens et al. 2004, 2007; Zhou et al. 2021). The sex chromosomes then segregate into haploid germ cells with all X or all Y chromosomes. In females, the X chromosomes simply organize into pairs. The identity of the monotreme sex-determining gene is unknown but the sex chromosomes include known sex-determining genes such as Doublesex and mab-3 related transcription factor 1 (*DMRT1*), the sex-determining gene for birds (Smith et al. 2009), African clawed frogs (*Xenopus laevis*, Yoshimoto et al. 2008), and medaka (*Oryzias latipes*, Matsuda et al. 2002; Nanda et al. 2002), as well as anti-Müllerian hormone gene (*AMH*), the sex-determining gene in a number of fish species (Hattori et al. 2012; Yamamoto et al. 2014; Li et al. 2015; Bej et al. 2017).

Advances in sequencing technology have allowed scientists to identify sex-specific genetic markers and sex chromosomes in a wide variety of taxa (Gamble and Zarkower 2014; Gamble 2016), including those with homomorphic sex chromosomes (Fowler and Buonaccorsi 2016; Gamble et al. 2015; Jeffries et al. 2018; Pan et al. 2016). PCR validation of these sex-specific markers can be used to sex individuals of unknown sex and only require enough tissue necessary for DNA extraction. These sex-specific PCR primers can be designed from Y-specific regions in XX/XY species, such that they amplify in males but not in females. The opposite is true for ZZ/ZW sex chromosomes, where W-specific markers will amplify in females but not in males. The first genetic sex test using hair with intact follicles of the short-beaked echidna was recently developed (Perry et al. 2019) with markers for *CRSPY*, a Y-specific gene on platypus Y5 (Tsend-Ayush et al. 2012), and *AMHX* and *AMHY*, gametologues of *AMH* which are found on platypus X5 and Y1, respectively (Cortez et al. 2014; Zhou et al. 2021). However genetic sex tests using just one marker are susceptible to uncertainty regarding their accuracy, particularly when well-known technical problems affect PCR amplification success and are subsequently misconstrued as evidence for one sex or the other (Robertson and Gemmell 2006). Here, we developed additional sex-specific markers using RADSeq that can now be used for reliable, non-invasive genetic sex testing of the short-beaked echidna, allowing for increased confidence in the sexing of this remarkable species.

Materials and methods

Animals

Adult male and female echidna tissues (muscle, liver, spleen and kidney, n = 10 individuals) were collected opportunistically from injured animals brought into the Currumbin Wildlife Hospital (SE Queensland) that required euthanasia for animal welfare reasons; no echidnas were killed for this research. Additional captive animals of known sex used for blood collection (n = 10) were housed and managed at Currumbin Wildlife Sanctuary (CWS; 28.1356° S, 153.4886° E) in Queensland, Australia. Animals were maintained on a beef mince–based diet (Wallage et al. 2015). Six female and four male sexually mature and healthy captive echidnas (average weight 5 kg) were used for blood collection. While the echidna was under isoflurane anesthesia, a blood sample (approximately 1 mL) was recovered from the rostral sinus (Johnston et al. 2006b) using a 25G butterfly needle and 3 mL syringe and stored in Queen's lysis buffer (Seutin et al. 1991).The University of Queensland Animal Experimentation Ethics Committees approved all sampling for echidnas, in accordance with the National Health and Medical Research Council of Australia Guidelines (2013).

Genomic DNA extraction and RADseq protocol

We extracted DNA for the RADseq from blood or tissue using the QIAGEN DNeasy Blood and Tissue Kit. We generated single-digest RADseq libraries using a modified protocol from Etter et al. (2012) as described in Gamble et al. (2015). Briefly, we digested genomic DNA using a high-fidelity Sbf1 restriction enzyme (New England Biolabs), and ligated individually barcoded P1 adapters to each sample. We pooled samples into multiple libraries, sonicated, and size selected for 200–500 bp fragments using magnetic beads in a PEG/NaCl buffer (Rohland and Reich 2012). We then blunt-end repaired, dA-tailed, and ligated pooled libraries with a P2 adapter containing unique Illumina barcodes. Pooled libraries were amplified using NEBNext Ultra II Q5 polymerase (New England Biolabs) for 16 cycles and size selected a second time for 250–650 bp fragments that

now contained both Illumina adapters and unique barcodes. Libraries were sequenced using paired-end 150 bp reads on an Illumina HiSeq X at Psomagen, Inc (Rockville, MD).

We analyzed the RADseq data from three males and seven females using a previously described method (Gamble et al. 2015). Raw Illumina reads were demultiplexed, trimmed, and filtered using the process_radtags function in STACKS (1.41, Catchen et al. 2011). We used RADtools (1.2.4, Baxter et al. 2011) to generate RADtags for each individual and identified candidate loci and alleles from the forward reads. We then used a custom python script (Gamble et al. 2015; Nielsen et al. 2019) to identify putative sex-specific markers from the RADtools output, i.e. markers found in one sex but not the other. The script also generated a list of "confirmed" sex-specific RAD markers that excluded any sex-specific markers found in the original read files of the opposite sex. Finally, we used Geneious (R11, Kearse et al. 2012) to assemble the forward and reverse reads of "confirmed" sex-specific RAD markers. These loci should correspond to the Y chromosomes, which are present in males but not in females.

Validating sex-specific markers

We PCR validated a subset of the male-specific RAD markers and visualized the results with gel electrophoresis. Male-specific PCR primers were designed using Primer3 (Untergasser et al. 2012), implemented in Geneious (R11, Kearse et al. 2012). Primers were tested twice in two laboratories - one in North America (US) and one in Australia (AUS) - with different samples to test their robustness to laboratory conditions and the consistency of the methods. Additionally, we also tested two markers from Perry et al. (2019), *AMH*, which amplifies both the X and Y gametologues and should produce two bands in males and one band in females, and the X-linked gametologue to *CRSPX*, which should produce bands in both males and females.

The first validation (US lab) used seven males and six females for validation, some of which were included in the RADseq dataset. The second validation (AUS lab) used three males and three females which were not included in the original RADseq dataset. Genomic DNA from the AUS animals was extracted using the Promega Wizard Genomic DNA Purification kit. PCR reactions in both labs were conducted with 6.5uL of Promega GoTaq Green Master Mix, 3.5uL of water, and 0.75uL of the forward and reverse primer using an Applied Biosystems Veriti 96-Well Thermal Cycler. All US lab PCR reactions were amplified with an initial denaturation at 94 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 45 s, and extension at 72 °C for 1 min, and finished with a final extension at 72 °C for five minutes. PCR conditions in the AUS lab were as described above except for optimization of annealing temperature to either 56 or 62 °C. The marker *AMH* was run a second time following the protocols of Perry et al. (2019) with an initial denaturation of 96 °C for 3 min., 40 cycles of denaturation at 96 °C for 30s, annealing at 58 °C for 1 min., and extension at 72 °C for 2 min., followed by a final extension at 72 °C for 7 min. We used the marker *CRSPX* as a positive control following the protocols of Perry et al. (2019). Following PCR, amplicons were visualized on a 1% agarose gel.

Table 1 Newly designed PCR primers used to validate sex-specific RADseq markers

Primer Name	Sequence (5' to 3')
Ta74_335-F	ACCAATTAGTCGGTGTTGGGT
Ta74_335-R	AGCATTGGGGTGGTTACAAGT
Ta129_196-F	AGACAAGGTCTCTTCCCCTCA
Ta129_196-R	TGCAGCATTCTAAGCAAAGGG
Ta65_317-F	TTCCAAGCCTGACTTCTAGCG
Ta65_317-R	TGACTCCTGCAGGAAACCAAG
Ta112_176-F	ACAAGTAAGCAAATACTGGGGC
Ta112_176-R	GGTACGTGGTTGTAGCTAGGG
Ta243-F	GCTGCATGGCTCCAAACTTAC
Ta243-R	TGACTGAACTGAGGCCCAATC
Ta116-F	CTCAGCCTCCCAATTCTCCTG
Ta116-R	CTGCCATCCCTCTCCTTTCTG
Ta61_332-F	GGGGGTTTCACTGTTATCACTCT
Ta61_332-R	AGTGCCTGACATATAGTAAGTGT

Identification of the chromosomal location of the RADseq markers

To determine the chromosomal location of the "confirmed" reads from the sex-specific RAD markers, we used BLAST (Altschul et al. 1990) to query the markers against the current versions of the echidna and platypus genomes (Zhou et al. 2021) using an e-value of 1e-50 and word_size 28 to filter the results to the best hits. For the few sequences which returned no hits, BLAST searches were repeated with no limitations to confirm their absence.

Results

We extracted DNA from the blood and tissue samples and generated RADSeq libraries. From these, we identified 112,773 RAD markers with two or fewer alleles. We then identified 401 male-specific markers and 3 female-specific markers. After checking the female-specific markers against the original male reads, we retained 385 **"**confirmed**"** male-specific markers and no female-specific markers.

We designed PCR primers and tested 18 of the male-specific RAD markers. Seven amplified in a sex-specific

manner in our first validation (US lab, Table 1), producing a single strong band in males and no bands in females (Fig. 1). Most of these markers also amplified in a male-specific pattern in our second independent validation (AUS lab, Fig. 1), except one marker (Ta61_332), that produced bands in females, although these were marginally fainter than those in males. Two additional markers (Ta243 and Ta65-317) also produced faint bands in females in the AUS lab validation, although they were substantially lighter than the male-specific bands and it was easy to distinguish male and female samples. *CRSPX*, the sex-specific marker previously identified from Perry et al. (2019) amplified in all samples (Supplemental Fig. 1).

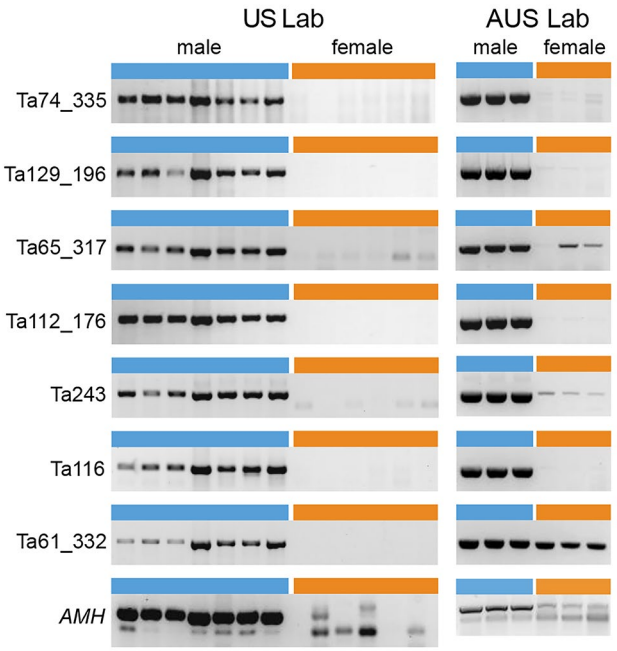

Fig. 1 PCR validation of male-specific amplification of sex-linked markers in *Tachyglossus aculeatus*. Markers were validated twice in two locations, North America (left, US Lab) and Australia (right, AUS lab) using seven males/females and three males/females, respectively. All PCR was run for 32 cycles with annealing temperature optimized at 54–62 °C except for *AMH* which was run with the protocol from Perry et al. (2019) amplifying for 40 cycles with an annealing temperature of 58°

Table 2 Results of BLAST query of male-specific RAD markers against the echidna and platypus genomes

	Number of RAD markers	
BLAST Hit Location	**echidna**	**platypus**
Chromosome X1	23	49
Chromosome X2	47	65
Chromosome X3	3	32
Chromosome X4	1	1
Chromosome X5	1	28
Chromosome Y1	7	0
Chromosome Y2	147	22
Chromosome Y3	39	0
Chromosome Y4	23	4
Chromosome Y5	N/A	8
autosomes	35	124
Unplaced scaffolds	2	7
No hit	6	35
Multiple hits to same location	14	1
Multiple hits to different locations	37	9
Total	**385**	**385**

We ran the marker *AMH* from Perry et al. (2019) with two PCR schemes, one from the original protocol with 40 cycles and one that matched the protocol used for the rest of the markers with 32 cycles. Perry et al. (2019) found that *AMH* produced two bands in males, representing the X and Y gametologues *AMHX* and *AMHY*, while females only produced a single *AMHX* band. In the US lab, we found that most samples matched this pattern when amplified for 40 cycles, but some males lacked the shorter *AMHX* band (Fig. 1). Additionally, some females had a very faint band that appeared to correspond to the Y-linked *AMHY* marker, while other females failed to display any bands. We performed PCR on the gene *16 S* as an additional positive control for all samples and ran the product on an agarose gel to confirm the samples amplified properly and the lack of X-linked bands in females was not due to low-quality DNA. All samples produced a strong band except for one female sample, which produced a much fainter band (Supplemental Fig. 1). This female was one of two female samples which did not produce an *AMHX* band. The second female without an *AMHX* band did have a strong 16 S positive control band, indicating that DNA quality alone does not account for lack of this X-linked amplicon. When we ran the *AMH* PCR for 32 cycles instead of 40 cycles, the males only produced *AMHY* bands and the females did not produce any bands in the US lab (Supplemental Fig. 1). In the second validation (AUS lab) when we ran *AMH* PCR for 40 cycles, males had a strong *AMHY* band and faint *AMHX* band whereas females had faint *AMHY* and *AMHX* bands (Fig. 1). When we ran *AMH* for 32 cycles in the AUS validation, males still had a strong *AMHY* and faint *AMHX* band, but females only had a faint *AMHX* band (Supplemental Fig. 1).

We used BLAST to identify the chromosomal location of the echidna sex-linked markers. Of the original 385 RAD-seq markers, 216 had clear hits to the Y chromosomes, 75 matched to the X chromosomes, 35 matched to autosomes, 2 aligned to unknown chromosome scaffolds, 51 aligned to 2 or more locations and there were no hits for the last 6 markers (Table 2). Most of the 7 markers tested in PCR had BLAST hits with the Y chromosomes with only 1 marker

that aligned to the X2 chromosome (Ta65_317). This marker produced fainter gel bands for the female samples in one PCR validation (AUS lab) but not in the other (US lab). We also used BLAST to identify the chromosomal location of the echidna sex-linked markers in the platypus genome. Of the original 385 RADseq markers, 34 had clear hits to the platypus Y chromosomes, 175 matched to the platypus X chromosomes, 124 aligned to platypus autosomes, 7 aligned to unknown chromosome scaffolds, 10 aligned to 2 or more locations and there were no hits for the last 35 markers (Table 2). When compared to the echidna, 26 of the 34 hits to the platypus Y chromosomes agreed with the markers identified in the echidna, including marker Ta243 tested with PCR on the echidna in this study.

Discussion

Conservation efforts are highly dependent on accurate sexing of the species of interest, both to examine the population dynamics of wild animals and to ensure success of captive breeding programs. When a species lacks easily identifiable sexual dimorphisms, genetic sex tests can be used instead (Taberlet et al. 1993; Ellergren 1996; Griffiths et al. 1998; Robertson et al. 2006). This requires identification of a sex-specific DNA marker, typically one from the Y or W sex chromosomes depending on the species. Genetic sex tests can also be non-invasive as the required DNA material can be extracted from scat, hair or quill samples (Perry et al. 2019, Summerell et al. 2019, Robertson and Gemmell 2006). Here, we identified several novel genetic markers for sexing short-beaked echidnas using RADSeq data. These, in conjunction with previously identified markers (Perry et al. 2019), provide biologists and conservationists an invaluable toolkit to quickly and easily identify sex in the short-beaked echidna, a task that previously required anesthesia and ultrasound examination by an experienced technician and which could only be used in sexually mature animals (Jackson 2007; Wallage et al. 2015). The genetic sex tests provided here are a viable option for both sexing juvenile animals, which cannot be sexed with ultrasound, and for sexing embryological material used for developmental studies. In addition, the markers identified here enhance the likelihood of being able to determine sex correctly from non-invasive samples that frequently yield only low-quality DNA.

This study identified seven novel sex-determining markers, of which four were confirmed to consistently amplify in a sex-specific manner regardless of DNA extraction method or thermocycler. Three of the novel markers produced ambiguous results based on the independent validations wherein females produced a fainter PCR band. The accuracy of one previously identified sex-linked marker, *AMH*, depended on the PCR conditions and lab under which it was run. While the conclusions do not change — males were easily identifiable in all reactions — the discrepancy does raise concerns regarding the difficulties that can occur with genetic sex tests.

A number of reasons, both biological and methodological, can explain the variation of gene amplification among samples. Cryptic genetic diversity within a species could render a pair of primers ineffective in one population while it works in another. The sex-limited chromosomes (Y and W chromosome) experience a higher evolutionary rate than the autosomes, the X chromosome, and the Z chromosome due to a lack of recombination and smaller effective population size that allows deleterious or nonsynonymous mutations to accumulate (Berlin and Ellegren 2006; Ellegren 2011); this may lead to sex-specific markers unique to a single population within a species (Nielsen et al. 2020). Such population structure could affect identification of sex-specific markers in two ways. If overly divergent samples are used in the RADseq pipeline, the number of identified sex-specific markers will be small as they must be shared across the population (Keating et al. 2020). However, these methods are typically robust to highly divergent samples (Hundt et al. 2019; Nielsen et al. 2020). Alternatively, if the samples used in the pipeline do not reflect the greater genetic diversity of a species, then markers may be sex-specific in one population and not in another due to population structure. For example, sex-specific primers designed for the developmental lizard model *Anolis sagrei* (Gamble et al. 2014) often fail to successfully identify sex in individuals from across its geographical range (Menke, *pers. comm*.), likely due to a high level of population structure (Reynolds et al. 2020). Similarly, certain populations of Chinook salmon (*Oncorhynchus tshawytscha*) display variation in the presence of Y-linked molecular markers, with some males lacking the markers while some females have the markers (Devlin et al. 2005). The majority of the echidnas used in this study originated from Queensland, Australia. However, short-beaked echidnas are widespread across all of Australia as well as some regions of Papua New Guinea. It has been suggested that there may be up to five subspecies of short-beaked echidna separated by geographical area, but this is yet to be confirmed (Griffiths 1978; Augee et al. 2006). Other evidence from mitochondrial phylogeographic analyses suggest there are three lineages instead, found in Papua New Guinea, Western Australia, and the rest of Australia, but this result was limited by the number of samples from Central Australia (Summerell et al. 2019). The short-beaked echidnas used in the Perry et al. (2019) study were all born in captivity in Perth Zoo, Western Australia, but their parental origins are unknown. Therefore, it is possible the variation we observed in the *AMH* amplification patterns could be due

to differences among individuals across their geographical range. Methodological problems can also lead to variation in amplification success, such as differences in DNA quality and quantity (due to reagents/protocol used or difficulty in extracting DNA from certain tissues such as hair), the PCR conditions including annealing temperatures and number of cycles, the type of thermocycler used for amplification, or even imperceivable differences such as ramp times between denaturation, annealing, and extension of PCR products (Frey et al. 2008; Ho Kim et al. 2008). In this study two different genomic DNA extraction protocols were used which were different again from the method used previously (Perry et al. 2019) which could explain the discrepancies observed. While the results are congruent in terms of sex-specificity, the differences do raise some concerns regarding best practices for developing genetic sex tests and the importance of providing detailed methods.

There are several ways to overcome the uncertainty regarding the accuracy of sex identification (Robertson and Gemmell 2006). First, a non-sex-specific gene can be amplified as a positive control to ensure DNA is of sufficient quality for PCR (Gamble et al. 2014). Second, primers can be designed to amplify both X and Y (or Z and W) alleles of different sizes, ensuring a signal of amplification in both sexes. Perry et al. (2019) designed the marker *AMH* in this way, although we found this was sensitive to DNA extraction method and PCR conditions. Finally, researchers can use multiple genetic markers to verify sex identification (Robertson and Gemmell 2006). Increasing the number of sex-specific markers used decreases, but does not entirely eliminate, the chance of a false negative or false positive leading to erroneous sex assignment. Furthermore, it also increases the chance of identifying non-polymorphic markers that can successfully identify sex across multiple populations within a species. By using multiple markers, scientists and conservationists can increase their confidence in echidna sexing, particularly when technical PCR difficulties lead to erroneous results (Robertson and Gemmell 2006).

This study identified a total of 216 sex-specific RADseq markers aligned to the echidna Y chromosomes. However, there was wide variation in their distribution across the four Y chromosomes with the majority present on Y2 (147 markers) versus the least represented chromosome Y1 (7 markers). The previously identified sex-specific markers (*AMHY* and *CRSPY*) both align to the echidna Y3 chromosome (Perry et al. 2019; Zhou et al., 2021). There was also wide variation in the distribution of the hits to the X chromosomes with the majority present on X1 and X2. Given the similarity between X and Y sequences, it was expected that at least some of the BLAST hits would be to the X chromosomes. This was highlighted by three of the markers which had BLAST hits to both the Y chromosome and the X chromosome. Additionally, despite the fact that the echidna lineage diverged from the platypus 55 million years ago (Zhou et al. 2021), this study identified 26 markers that had sex-specific hits to both the echidna and platypus Y chromosomes. If enough sequence similarity exists that the echidna male-specific markers can amplify Y-linked sequences in the platypus, these markers might be sufficient to identify sex in the platypus. Sex-specific markers designed in one species can sometimes work in a close relative if they share a homologous sex chromosome system (Keating et al. 2020). In addition, these 26 markers would be good candidates to test in the three vulnerable/critically endangered long-beaked echidna species.

Here, we identified and validated several new sex-specific genetic markers that can be used to identify sex in the short-beaked echidna (Table 1). These, alongside the markers of Perry et al. (2019), provide a toolkit for researchers, conservationists, and zoo-keepers interested in non-invasive sexing of the shorted-beaked echidna. These resources are also important for establishing the short-beaked echidna as a monotreme developmental model, as sexing embryological material is a vital step in understanding developmental processes. We recommend that researchers use a combination of the markers presented here and the markers of Perry et al. (2019) in order to confidently and accurately sex the short-beaked echidna. Future work will be needed to assess the efficiency of the sex-specific markers to work across the range of short-beaked echidna populations. In addition, application to the vulnerable and critically endangered New Guinea echidnas would significantly aid in conservation efforts and in establishing a breeding colony for these rare monotremes. Our results further highlight the utility of RADseq as a means to generate sex-specific markers and genetic sex tests in species that lack sexual dimorphism at some or all stages of their life history.

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Author Contributions M.B.R, J.C.F, and T.G. conceptualized the study. S.D.J, M.P, and J.C.F collected the material. S.E.K, B.J.P, I.A.G.M., and J.C.F performed the lab work. S.E.K, J.C.F., and T.G. performed the analyses. S.E.K., J.C.F., T.G., and M.B.R. co-drafted the manuscript. All authors contributed to reviewing and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Availability of data and material The RADseq data are available at the NCBI Short Read Archive under BioProject PRJNA816870 and BioSample accession numbers SAMN26725426-SAMN26725435.

Declarations

Conflict of Interest The authors declare no conflict of interests

Statement of Ethics The University of Queensland Animal Experimentation Ethics Committees approved all sampling for echidnas, in accordance with the Australian National Health and Medical Research Council of Australia Guidelines [2013].

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