Conservation Genetics Resources
https://doi.org/10.1007/s12686-022-01266-3

METHODS AND RESOURCES ARTICLE

A male-specific sex marker for the endangered western sawshelled turtle (*Myuchelys bellii*) using in silico whole-genome subtraction

Duminda S. B. Dissanayake[1,2] · **Louise M. Streeting**[3] · **Arthur Georges**[1] · **Deborah S. Bower**[3]

Received: 31 October 2021 / Accepted: 20 March 2022
© The Author(s) 2022

Abstract
Artificial incubation of eggs for the mass release of hatchlings is a common conservation intervention for imperilled turtle species. Programs designed to reinforce wild populations need to ensure that they are releasing appropriate male to female ratios into the wild. In many turtle species, the sex of juveniles cannot be identified using external morphology until they approach sexual maturity. For the endangered western sawshelled turtle, *Myuchelys bellii*, sexual dimorphism does not occur until at least 6 years of age. We aimed to develop a molecular test to identify the sex of *M. bellii* during the life stages where they cannot be sexed morphologically—embryos, hatchlings and small juveniles. We used in silico whole-genome subtraction of a female *M. bellii* (XX) from a male (XY) to identify a Y chromosome-specific sequence which we characterized and developed into a PCR sex test. Our research is the first to use a whole-genome subtraction method in-silico to successfully establish sex chromosome markers in a freshwater turtle species. Developing this technology provides an opportunity for conservation programs to ensure that populations are supplemented with a proportionate number of male and female hatchlings. Further, it allows large scale measurement of naturally occurring sex ratios in hatchlings and small juveniles, which in turn enables estimates of sex ratios within wild populations free from age-at-maturity bias. The application of sex-specific marker technology also provides an opportunity to quantify the influence of sex on behaviour, movement and survival in the segment of populations that cannot be morphologically sexed.

Keywords Sex-specific markers · Endangered species · Y chromosome · Turtle conservation

Introduction

Sexual dimorphism is absent or delayed in the development of many reptiles, fish, birds and frogs. The ability to distinguish the sexes in monomorphic species is of immeasurable value to understanding their ecology and behaviour. The identification of sex-specific sequences has essential practical value in ecological studies (Ferguson et al. 1995; Taberlet et al. 1999), conservation of threatened or endangered species (Boulanger et al. 2008; Carmichael et al. 2013; Taberlet et al. 1999), captive breeding (Sulandart and Zein 2012), aquaculture (Gui and Zhu 2012; Xue et al. 2020; Zhu et al. 2021; Han et al. 2021), elimination of mortality as a possible justification for sex ratio bias (Quinn et al. 2009; Dissanayake et al. 2021a, b), identification of sex chromosomes (Cornejo-Páramo et al. 2020) and sex forensics (Andréasson and Allen 2003). Sex-specific sequences provide an effective tool to address the ambiguity of sex in many species and life stages by the development of robust PCR sex tests.

Duminda S. B. Dissanayake and Louise M. Streeting have contributed equally to this work.

✉ Duminda S. B. Dissanayake
duminda.rusl@gmail.com

Louise M. Streeting
louisemstreeting@gmail.com

Arthur Georges
georges@aerg.canberra.edu.au

Deborah S. Bower
deborah.bower@une.edu.au

[1] Institute for Applied Ecology, University of Canberra, Canberra, ACT 2601, Australia

[2] Australian National Wildlife Collection, CSIRO, Canberra, ACT 2911, Australia

[3] School of Environmental and Rural Science, University of New England, Armidale, NSW 2351, Australia

Published online: 06 April 2022

Many chelonians, including the endangered western sawshelled turtle, *Myuchelys bellii* (Gray 1844), lack pronounced sexual dimorphism until sexual maturity. This medium-sized freshwater chelid is endemic to northern New South Wales (NSW) and south-eastern Queensland within the temperate zone of eastern Australia (Fielder et al. 2015a). *Myuchelys bellii* is a threatened species and a priority in conservation efforts (Tortoise and Freshwater Turtle Specialist Group 2016) because populations are ageing with insufficient juvenile recruitment to sustain them (Chessman 2015). Artificial, ex-situ incubation of eggs harvested from gravid female turtles and the release of hatchlings into the wild is a method widely implemented in an attempt to augment juvenile numbers in threatened freshwater turtle populations (Carstairs et al. 2019). Mature *M. bellii* are sexually dimorphic in that males are smaller than females and have proportionally longer tails (Fielder et al. 2015b), with the anterior margin of the cloaca located beyond the posterior margin of the carapace. However, below the size at which sexual dimorphism develops, juvenile turtles cannot be sexed using external morphology (Chessman 2015).

Myuchelys bellii has a genotypic sex-determination system where the sex of an individual is determined by the combination of sex chromosomes inherited from the parents at conception. *Myuchelys bellii* has male heterogamety (XX/XY sex chromosomes) typical of turtles in the family Chelidae (Martinez et al. 2008). The development of a sex-specific marker for *M. bellii* will provide a valuable tool for the sex identification of hatchling turtles produced for conservation interventions. A sex-specific marker would provide the opportunity to ensure that the population is supplemented with a proportionate number of male and female hatchlings. A sex-specific marker would also have the potential to aid further ecological studies of juvenile turtles below the age at which they display sexual dimorphism.

Our study aimed to identify sex-markers on the Y chromosome of *M. bellii* and to develop a robust PCR test that can be used to identify sex for all life stages but in particular immature individuals. We used in silico whole-genome subtraction to isolate sex-specific sequences unique to the Y chromosome (Dissanayake et al. 2020) of the endangered western sawshelled turtle and to develop a PCR sex test for the species.

Materials and methods

Samples

We caught adult turtles in the Gwydir River (30.622°S, 151.340°E) and Macdonald River catchments (31.075°S, 151.466°E) in New South Wales, Australia (Fig. 1) using cathedral traps (Georges et al. 2006) baited with lamb liver or ox heart. Sex and maturity were determined by observing tail length as is conventional for short-necked Australian chelid turtles (Gibbons 1990; Georges et al. 2006). Straight carapace length (SCL) and width were measured using Vernier callipers (± 1 mm). Blood samples (1 ml) were obtained from the jugular vein of 21 male and 21 female *M. bellii* using sterile syringes with 26-gauge needles. Samples were stored in EDTA vacutainers at − 80 °C.

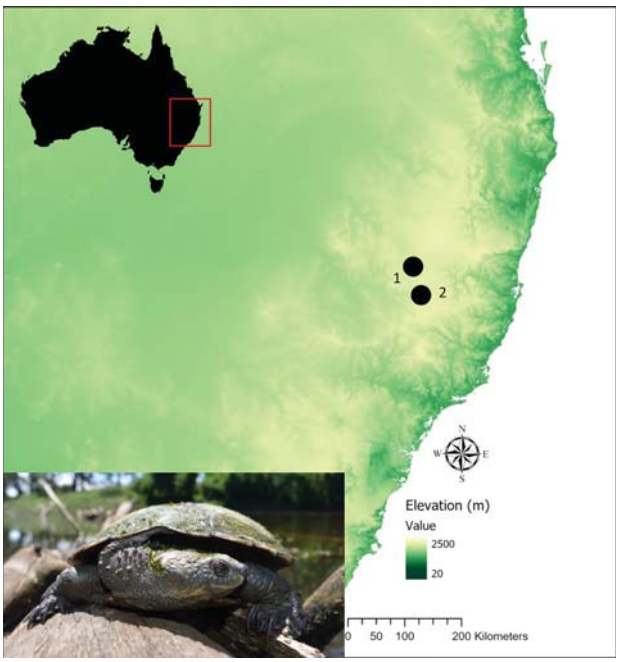

Fig. 1 *Myuchelys bellii* sampling localities—1. Roumalla Creek, Uralla NSW; 2. Macdonald River, Walcha NSW from which the focal and validation individuals in this study were sourced. Underlying map generated using ArcGIS 10.5.1 (http://www.esri.com) and data from the Digital Elevation Model (Geoscience Australia) made available under Creative Commons Attribution 3.0 Australia (https://creativecommons.org/licenses/by/3.0/au/legalcode, last accessed 19-Jul-20). Adult Bell's turtle photo by Paul McDonald

DNA extraction and sequencing

A focal male and a focal female of known phenotypic sex were chosen for whole-genome sequencing. According to the manufacturer's instructions, DNA was extracted using the Gentra Puregene Tissue Kit (QIAGEN, Australia). Final DNA suspensions were assessed for purity using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, 19810, USA) and quantified using Qubit 2.0 fluorometer (Invitrogen, Life technologies, Sydney, NSW, Australia). Library preparation and sequencing were completed at the Ramaciotti Centre for Genomics, University of New South Wales, Australia, using the Illumina HiSeq™ NovaSeq 6000 platform.

In silico whole-genome subtraction

Sequencing reads (both of the paired ends) for each of the focal male and the focal female were decomposed into k-mers of length 27 bp using Jellyfish 2.0 (Marçais and Kingsford 2011). Unique k-mers were counted again using Jellyfish 2.0; those with counts less than four were removed as sequencing errors and remaining k-mers in common between the male and female sets were removed from the male set using ksubtract.cgi (supplementary materials, Dissanayake et al. 2020). This subtracted k-mer set was enriched for the Y chromosome sequence, albeit mixed with polymorphic autosomal and X chromosome sequences. The Y-enriched k-mers were reassembled into contigs using a stringent inchworm assembler (kassemble.cgi available from supplementary material in Dissanayake et al. 2020).

PCR validation

Primers were designed for each of the 92 largest contigs using Primer 3 (Untergasser et al. 2012) implemented in Geneious version R8 (Kearse et al. 2012) for validation against a panel of known-sex animals (20 males and 20 females). Each PCR reaction contained 1 µl H20, 5 µl of MyTaq HS Red mix (2×, 5 ml 200 reactions), 1 µl of each of the forward and reverse primers at 4 µM, and 2 µl of the template DNA at approximately 25 ng/µl. The PCR cycling conditions involved a touchdown phase in increasing specificity of annealing, with conditions as follows: denaturing at 95 ˚C, annealing temperature stepping down from 64.6 °C by 0.5 °C for 10 cycles (20 s), extension at 72 °C at 60 s. This was followed by 30 cycles at 65 °C annealing and 72 °C extension (10 min).

PCR screening was conducted in three stages. First, the test was applied to the two focal individuals to confirm the successful identification of polymorphism or sex-linked markers in the focal male (XY) and their absence in the focal female (XX). Second, the test was applied to a panel comprising an additional four male and four female individuals, to yield a set of putative sex-linked markers accompanied by a reduced set of inter-individual polymorphisms that aligned with sex via sampling error. Finally, those presumptive markers were further screened against a panel of 16 additional males and 16 additional females to bring the overall validation set to 20 males and 20 females. The probability of an autosomal or X chromosome polymorphism being uniformly present in the focal male and 20 additional males, and uniformly absent in the focal female and 20 additional females, is vanishingly small, and enough to eliminate false positives. Thus, male-specific PCR markers that survive the validation process are Y-specific markers.

Results

In silico whole-genome subtraction

For each sex, we generated 33×10^9 150 bp PE genomics sequence libraries for the in-silico whole-genome subtraction pipeline which corresponded to 15.4 × read depth for the male and 17.3 × read depth for the female. We decomposed these reads into 1,829,456,700 and 1,814,035,674 k-mers (k = 27) for the focal male and female, respectively, the difference likely originating from differences in sequence error rates between sequencing runs. To eliminate k-mers arising from sequence errors, we observed the k-mer spectrum to determine suitable thresholds and eliminated k-mers with counts less than four for both males and females. A total of 1,781,903,147 k-mers were common to both sexes, and 47,553,553 k-mers were unique to males. The male-specific k-mers were reassembled to yield 78,243 contigs ranging from 80 to 3011 bp (Fig. 2).

PCR validation

We chose the longest 92 contigs from the subtraction for further investigation because they were sufficient to design robust primers and result in a PCR product easily visualized on an agarose gel. The 92 contigs ranged from 807 to 3011 bases in length (Fig. 3). As expected, all 92 contigs passed the subtraction first step of validation, where a product of the expected size was successfully amplified in the focal male and did not amplify in the focal female. Only 16 survived the second screening against the panel of four males and four females, and only one survived when screened against the panel of 20 male and 20 female individuals. This marker, sequence (5′–3′) forward TCCTGAGTCCTAGCATGG GA and reverse CCACGGTGAATCAAGGTCCA was thus validated as male-specific (Fig. 4) having proven concordant

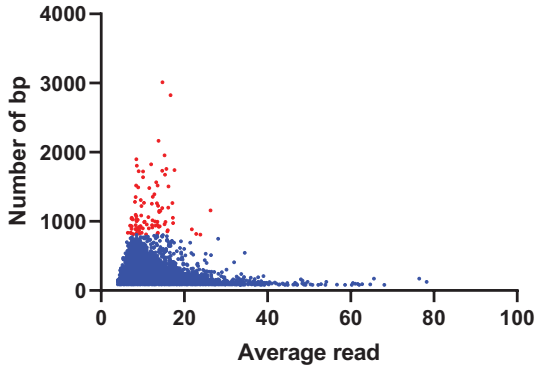

Fig. 2 Average read and number of bp in each contig resultant from the assembly. Red denotes primer designed contigs

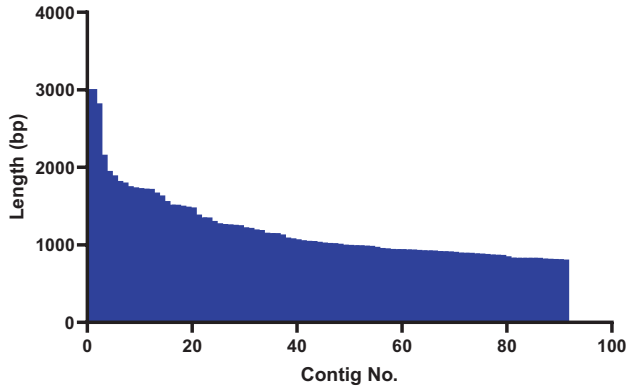

Fig. 3 Contig length (bp) for the 92 subtraction contigs selected for PCR-based screening

with phenotypic sex (present in male, absent in female) in a total of 42 animals.

Discussion

We successfully isolated a sex marker specific to the Y chromosome of *M. bellii* and developed a PCR sex test that can be used to identify the sex of *M. bellii* individuals at any life stage. The use of this sex-specific marker to identify the sex of immature *M. bellii* is of significant value to the ecological understanding and conservation efforts for this imperiled species. Our research is the first to use a whole-genome subtraction method in-silico to successfully establish sex chromosome markers in a freshwater turtle species.

It is essential to base conservation efforts on initiatives that are effective, beneficial and non-destructive. This male-specific sex marker was developed for application in a conservation program for *M. bellii* with the aim of ensuring that the proportion of male and female hatchlings being produced for release had an appropriate sex ratio, either 1:1 or biased toward females, not males.

Myuchelys bellii juveniles do not exhibit sexual dimorphism until approximately 6 years of age (Fielder et al. 2015b). Therefore, applying this sex-specific marker facilitates rapid sex identification for *M. bellii* at any life stage, including hatchlings and small juveniles that cannot be sexed morphologically. In contrast to laparoscopic examination or surgical dissection to identify gonads, the use of molecular markers to identify sex is a minimally invasive technique requiring the collection of a drop of blood on a Whatman Elute FTA card (QIAGEN Australia, Melbourne) whereby sampling can be performed in the field without the need to transport or hold animals.

Sex-specific marker technology has the potential to greatly assist in quantifying the influence of sex on behaviour, movement and survival in the portion of a population that cannot be morphologically sexed. For instance, *M. bellii* are a late-maturing species, with males taking almost 10 years to mature and females nearly 20 years (Fielder et al. 2015b). A sex-specific marker will aid ecological and demographic studies seeking to understand the conservation

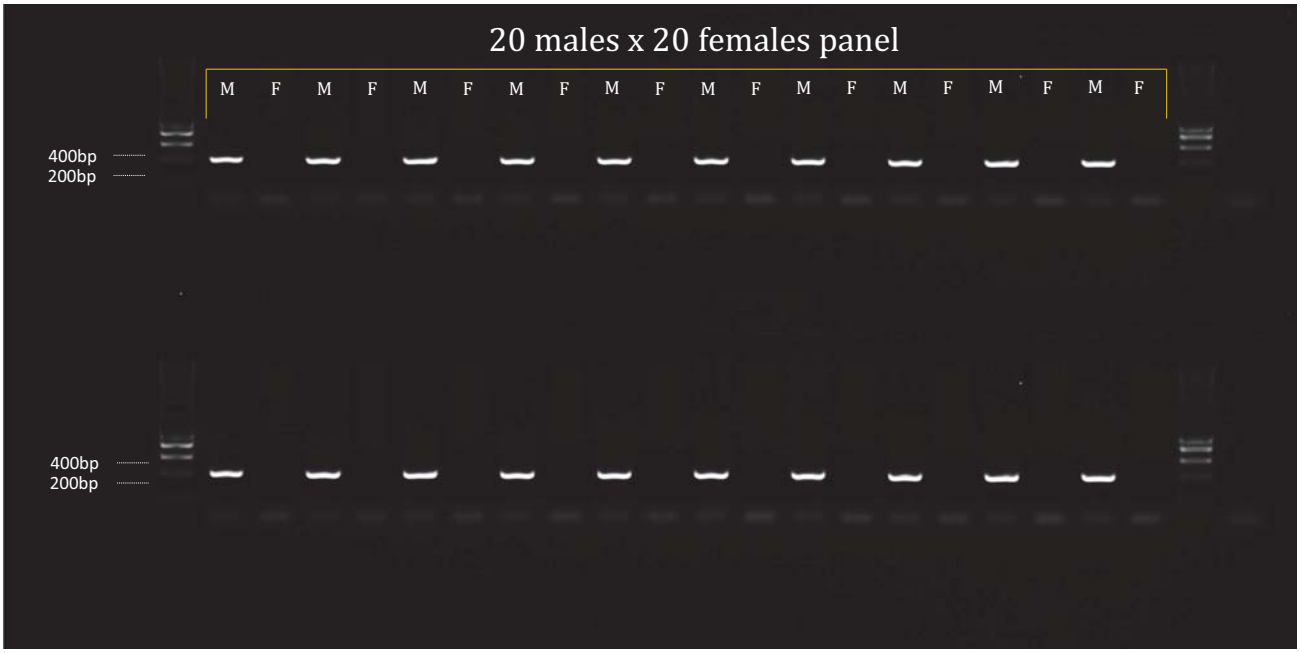

Fig. 4 Validation of the male-specific marker in *M. bellii* using a panel of 20 male and 20 female confirmed phenotypic sex individuals. Male specificity was defined as the presence of a distinct amplicon in males and the absence of amplification in females

ecology of this species throughout the immature life stages. The sex ratio for *M. bellii* in the Gwydir catchment has been reported to differ significantly from 1:1 but the analysis was constrained to turtles that were large enough to be morphologically sexed (Chessman 2015). The use of sex-specific molecular markers would allow large scale measurement and verification of naturally occurring *M. bellii* sex ratios in hatchlings and small juveniles and allow sex ratio estimation free from age-at-maturity bias (Georges et al. 2006). Therefore, the use of molecular markers will aid in the identification of any sex and age-specific bias in mortality rates.

Genetic sex identification will be particularly beneficial where immature turtles are collected to establish a captive breeding population for insurance purposes. In 2015 a novel nidovirus decimated the Bellinger River snapping turtle (*M. georgesi*) population (Zhang et al. 2018). *M. georgesi* is now critically endangered with an estimate of around 150 surviving individuals (Chessman et al. 2020). This mass mortality event disproportionately killed adult turtles and the remnant surviving population is dominated by immature individuals (Chessman et al. 2020). In 2015, 36 *M. georgesi* were taken from the wild to establish insurance populations, more than half of these were immature animals (Chessman et al. 2020). Had a sex-specific molecular marker been available for *M. georgesi* prior to this disease event, it would have greatly assisted in the selection of individuals to establish the captive breeding colonies.

The number of identified male-specific markers during the subtraction could be improved by accomodating autosomal and X bourne polymorphism by using multiple individuals from the homogametic sex in the subtraction (e.g. if three XX individuals and one focal XY individual are used for the whole genome subtraction pipeline, the capture of false positives arising from non-Y-specific sequence can be reduced). Isolated populations will typically display less variation among individuals because of genetic drift and inbreeding. Therefore, sequencing island populations may reduce the non-Y-specific variation detected in the subtraction. The success of the subtraction could also be improved through captive breeding programs to purposely reduce the level of autosomal variation between the two sequenced individuals in two ways: sequencing full siblings or inbreeding to reduce autosomal variation for several generations and then sequencing two highly related, inbred animals. Also, we can reduce the number of false-positive sex-specific contigs by increasing read depth, where some of the differences arise from random but differential calling of SNPs (see Dissanayake et al. 2020). Each of these approaches may increase the detection rate of sex-specific regions.

In this study, we introduced a novel approach for the isolation of a sex-specific region and the validation of sex-specific markers using in silico whole-genome subtraction from a single individual from both sexes of *M. bellii*. By isolating a male-specific scaffold, we obtained an accurate sex-specific molecular marker that can be directly used to identify genotypic sex for *M. bellii*. We demonstrate that where sex-specific molecular markers are successfully developed using the relatively low-cost in-silico whole-genome subtraction pipeline (Dissanayake et al. 2020) they can greatly assist conservation initiatives for imperilled turtles and may readily be applied to other vertebrate taxa.

Acknowledgements We gratefully acknowledge assistance from Martin Dillon of Northern Tablelands Local Land Services and Phil Spark and Zac Petersen of North West Ecological Services for their invaluable assistance in the trapping and collection of blood samples from adult western sawshelled turtles. We acknowledge the Anaiwan people, Traditional Custodians of the lands on which this study was conducted. We also thank the landowners who allowed us access to their properties. Turtle capture and handling procedures were authorized by the New South Wales Department of Primary Industry scientific collection permit P17_0062, New South Wales Department of Planning, Industry, and Environment scientific license SL101876, and University of New England Animal Ethics Committee approval AEC17-110.

Funding This study has been supported by the New South Wales Government through its Environmental Trust (Saving Our Species Partnership Grant 2015/SS/0017). Open Access funding enabled and organized by CAUL and its Member Institutions.

Data availability Data and materials are presented in the main paper and the perl scripts to undertake the subtraction and inchworm assembly available on the Dryad repository https://doi.org/10.5061/dryad.pvmcvdnj1 (Dissanayake et al. 2020). Illumina raw reads are available on the NCBI Short Read Archive (SRA) under bio project PRJNA776421 (Accession Numbers SAMN22789364, SAMN22789365).

Declarations

Conflict of interest Authors declare no conflict of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Andréasson H, Allen M (2003) Rapid quantification and sex determination of forensic evidence materials. J Forensic Sci 48(6):1280–1287. https://doi.org/10.1520/JFS2002416

Boulanger J, White GC, Proctor M, Stenhouse G, Machutchon G, Himmer S (2008) Use of occupancy models to estimate the influence of previous live captures on DNA-based detection probabilities

of grizzly bears. J Wildl Manag 72(3):589–595. https://doi.org/10.2193/2006-447

Carmichael SN, Bekaert M, Taggart JB, Christie HR, Bassett DI, Bron JE, Skuce PJ, Gharbi K, Skern-Mauritzen R, Sturm A (2013) Identification of a sex-linked SNP marker in the salmon louse (*Lepeophtheirus salmonis*) using RAD sequencing. PLoS ONE 8(10):77–832. https://doi.org/10.1371/journal.pone.0077832

Carstairs S, Paterson JE, Jager KL, Gasbarrini D, Mui AB, Davy CM (2019) Population reinforcement accelerates subadult recruitment rates in an endangered freshwater turtle. Anim Conserv 22(6):589–599. https://doi.org/10.1111/acv.12503

Chessman BC (2015) Distribution, abundance and population structure of the threatened western saw-shelled turtle, *Myuchelys bellii*, in New South Wales, Australia. Aust J Zoo 63(4):245–252. https://doi.org/10.1071/ZO15034

Chessman BC, McGilvray G, Ruming S, Jones HA, Petrov K, Fielder DP, Spencer RJ, Georges A (2020) On a razor's edge: status and prospects of the critically endangered Bellinger River snapping turtle, *Myuchelys georgesi*. Aquat Conserv: Mar Freshw Ecosyst 30(3):586–600. https://doi.org/10.1002/aqc.3258

Cornejo-Páramo P, Dissanayake DSB, Lira-Noriega A, Martínez-Pacheco ML, Acosta A, Ramírez-Suástegui C, Méndez-de-la-Cruz FR, Székely T, Urrutia AO, Georges A, Cortez D (2020) Viviparous reptile regarded to have temperature-dependent sex determination has old XY chromosomes. Genome Biol Evol 12(6):924–930. https://doi.org/10.1093/gbe/evaa104

Dissanayake DSB, Holleley CE, Hill LK, O'Meally D, Deakin JE, Georges A (2020) Identification of Y chromosome markers in the eastern three-lined skink (*Bassiana duperreyi*) using in silico whole genome subtraction. BMC Genomics 21(1):1–2. https://doi.org/10.1186/s12864-020-07071-2

Dissanayake DSB, Holleley CE, Deakin JE, Georges A (2021a) High elevation increases the risk of Y chromosome loss in Alpine skink populations with sex reversal. Heredity 126(5):805–816. https://doi.org/10.1038/s41437-021-00406-z

Dissanayake DSB, Holleley CE, Georges A (2021b) Effects of natural nest temperatures on sex reversal and sex ratios in an Australian alpine skink. Sci Rep 11:20093. https://doi.org/10.1038/s41598-021-99702-1

Ferguson AJ, Taggart JB, Prodöhl PA, McMeel O, Thompson C, Stone C, Mcginnity P, Hynes RA (1995) The application of molecular markers to the study and conservation of fish populations, with special reference to Salmo. J Fish Biol 47:103–126. https://doi.org/10.1111/j.1095-8649.1995.tb06048.x

Fielder D, Chessman B, Georges A (2015a) *Myuchelys bellii* (Gray 1844)—Western Saw-shelled Turtle, Bell's Turtle. Chelonian Res Monogr 1–7

Fielder DP, Limpus DJ, Limpus CJ (2015b) Reproduction and population ecology of the vulnerable western sawshelled turtle, *Myuchelys bellii*, in the Murray-Darling Basin, Australia. Aust J Zoo 62(6):463–476. https://doi.org/10.1071/ZO14070

Georges A, Guarino F, White M (2006) Sex ratio variation across populations of a turtle species with genotypic sex determination. Wildl Res 33:475–480

Gibbons JW (1990) Sex ratios and their significance among turtle populations. In: Gibbons JW (ed) Life history and ecology of the slider turtle. Smithsonian Institution Press, Washington, pp 171–182

Gray JE (1844) Catalogue of the tortoises, crocodiles and amphisbaenians in the collection of the British Museum. Edward Newman, London, p 80

Gui J, Zhu Z (2012) Molecular basis and genetic improvement of economically important traits in aquaculture animals. Chi Sci Bull 57(15):1751–1760

Han C, Zhou X, Lu H, Zhu Q, Han L, Li S, Lin H, Zhang Y (2021) A simple PCR-based genetic sex identification method in the blotched snakehead (*Channa maculata*) developed by high-throughput sequencing. Aquaculture 538:736579. https://doi.org/10.1016/j.aquaculture.2021.736579

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12):1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Marçais G, Kingsford C (2011) A fast, lock-free approach for efficient parallel counting of occurrences of k-mers. Bioinformatics 27(6):764–770. https://doi.org/10.1093/bioinformatics/btr011

Martinez PA, Ezaz T, Valenzuela N, Georges A, Graves JAM (2008) An XX/XY heteromorphic sex chromosome system in the Australian chelid turtle *Emydura macquarii*: a new piece in the puzzle of sex chromosome evolution in turtles. Chromosome Res 16(6):815–825

Quinn AE, Radder RS, Sarre SD, Georges A, Ezaz T, Shine R (2009) Isolation and development of a molecular sex marker for *Bassiana duperreyi*, a lizard with XX/XY sex chromosomes and temperature-induced sex reversal. Mol Genet Genom 281(6):665–672. https://doi.org/10.1007/s00438-009-0437-7

Sulandart SR, Zein MS (2012) Application of two molecular sexing methods for Indonesian bird species: implication for captive breeding programs in Indonesia. HAYATI J Biosci 19(4):183–190. https://doi.org/10.4308/hjb.19.4.183

Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap. Trends Ecol Evol 14(8):323–327. https://doi.org/10.1016/S0169-5347(99)01637-7

Tortoise & Freshwater Turtle Specialist Group (1996) *Elseya bellii* (errata version published in 2016) The IUCN Red List of Threatened Species eT40758A97265406. https://doi.org/10.2305/IUCN.UK.1996.RLTS.T40758A10362671

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3—new capabilities and interfaces. Nucleic Acids Res 40(15):e115. https://doi.org/10.1093/nar/gks596

Xue LZ, Guo XF, Zhou YL, Wang ZW, Fan HP, Li DP, Gui JF (2020) Screening and characterization of sex-specific markers by 2b-RAD sequencing in zig-zag eel (*Mastacembelus armatus*) with implication of XY sex determination system. Aquaculture 528:735550. https://doi.org/10.1016/j.aquaculture.2020.735550

Zhang J, Finlaison DS, Frost MJ, Gestier S, Gu X, Hall J, Jenkins C, Parrish K, Read AJ, Srivastava M, Rose K (2018) Identification of a novel nidovirus as a potential cause of large scale mortalities in the endangered Bellinger River snapping turtle (*Myuchelys georgesi*). PLoS ONE 13(10):e0205209. https://doi.org/10.1371/journal.pone.0205209

Zhu C, Liu H, Cheng L, Pan Z, Chang G, Wu N, Wang H, Ding H, Fang Y, Wang L (2021) Identification of sex-specific sequences through 2b-RAD sequencing in *Pseudobagrus ussuriensis*. Aquaculture 539:736639. https://doi.org/10.1016/j.aquaculture.2021.736639

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.