UTS UNIVERSITY OF TECHNOLOGY SYDNEY

A Wildlife Forensic Genetic Toolbox to Combat the Illegal Trade of the Short Beaked Echidna

by Alexandra Summerell

Thesis submitted in fulfilment of the requirements for the degree of

Doctorate of Philosophy

under the supervision of Dr. Rebecca Johnson, Dr. Greta Frankham, Dr. Peter Gunn and Dr. Dennis McNevin

University of Technology Sydney
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February, 2021

Certificate of original authorship

I, Alexandra Summerell declare that this thesis is submitted in fulfilment of the requirements for the award of Doctorate of Philosophy in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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Acknowledgments

First and foremost I would like to thank my supervisors Rebecca Johnson and Greta Frankham, for the immense amount of help and encouragement over the many years. Rebecca, thank you for your constant support, knowledge, and guidance, even when being overseas, and for giving me amazing advice, both with my PhD and my career, every bit of which I value so much. Greta, an endless thank you the amount of time you put in, for teaching me so much, for your detailed edits and advice, and for your friendship. The opportunities you both have provided me are incredible and I am so grateful to have worked with you. I can't thank you enough for all the support you have given me, and for all the guidance, editing and encouragement.

Thank you to Peter Gunn for supervising the first few years of my PhD, and for your support and editing, and for allowing me to help so much with teaching. To Dennis McNevin, who graciously stepped in as my supervisor late in the game, and has been so helpful with editing and encouragement.

There have been so many people and organisations who have helped with this project. I am highly indebted to all the assistance, expertise, and time I have received from such amazing people. I hope I have not missed anyone, but if I have please know that I am forever grateful.

Firstly thank you to the sample donors for my project, without whom I would not have a thesis. Thanks to Australia Zoo, Currumbin Wildlife Sanctuary, Taronga Zoo, Perth Zoo, Los Angeles Zoo, San Diego Zoo and Featherdale Wildlife Sanctuary for donating live animal samples, and to the veterinarians and zookeepers who did the hard work of collecting blood or plucking quills. To the Australian National Wildlife Collection, Museum Victoria, Queensland Museum, Western Australia Museum, and of course the Australian Museum for the donation of tissue samples, and to all the people behind the scenes who did the hard work of getting them to me. To Stewart Nicol, Rachel Harris, and the University of Tasmania, for your generous donation of so many crucial samples, as well as your guidance, time and expertise you have provided. Particularly to Stewart for giving me a tour around Hobart to try and find a wild echidna, despite the lack of success it was an amazing day. Additionally, to Tahlia Perry and Frank Grützner from the University of Adelaide for lending me your primers and for your help surrounding the sex determination, as well as the amazing work you do each day in echidna research.

To the Australian Museum, and all of the amazing people who work there, I am so grateful for the enormous amount of help you have given me. To Kyle Ewart, I cannot thank you enough for the help I received from you (particularly with R), as well for your friendship and support over so many years. To Caitlin Morrison, for your help with R as well, thank you for always being such a beautiful person and friend. To the incredible AMRI team; Andrew King, Scott Ginn, Anja Divljan, Sandy Ingleby, Matt Lott, Mark Eldridge, David Alquezar, Linda Neaves, and Siobhan Dennison. You were all so welcoming, kind, and patient with me, and I have learnt so much from you. Particularly to Scott, Anja, and, Sandy for all your help with sample registration and retrieval, to Andrew for imparting so much wisdom and always being keen to help me troubleshoot whatever problem I had, and to David for helping with edits, as well as so much paperwork and lab logistics.

Thank you to the Centre for Forensic Science at UTS, while I spent most of my time at the museum, I am very grateful to be a part of such an incredible group of people. Thank you to Mark Barash for your help in the lab. To the forensic biology group, thank you for your support, particularly coming to watch my presentations, it is an honour to be amongst such an amazing group. Particularly thanks to Elisha Prasad for your friendship, understanding, and for aiding me so much with teaching. And to Dilan Seckiner, one of the most supportive

people, and has provided me with a huge amount of guidance and friendship, particularly nearing the end when I had so many submission questions.

Thank you to Carolyn Hogg and the Zoo and Aquarium Association for your early input and guidance, as well as the funding provided to kickstart this project. Additionally, thank you to my other funding sources; The Holsworth Wildlife Research Endowment, and the Australian Museum Research Institute Postgraduate Award. Thank you also to ANZFSS and NIFS for providing me funding to go to conferences, which have been the absolute highlights of my PhD time.

Thank you to the amazing group of people at the Royal Botanic Gardens, Sydney; particularly Ed Liew and Matt Laurence, for employing me, for your valuable knowledge, as well as for letting me use your space to write. The biggest thank you to Jason Bragg, your help was incredible, thank you for teaching me so much about R, I am forever indebted.

To my FASS/ERU family; thank you for your endless support and encouragement while I tried to do too much at once, and for not getting sick of asking me how much longer I had left. Thank you to the managers for allowing me study leave and flexible hours when I needed it. In particular, thank you to Tim Smith and Bec Douglas for the amazing training and constant reassurance and support you give me, and a huge thank you to Nicole Wyner for your friendship, proof-reading, and constant cheerleading, I am eternally grateful to you all.

To my amazing friends who I have seen much less of than I would have liked over this time, I love you all so much and am so thankful for your support. To my Commotion friends, and Kass and Bec for your constant cheerleading and support. To my online friends, Emily, Esther, Steph, Bean, Laura, Kim, Courts, Nadia, Maddy, Alex, Evan, Jill, you have been my rock and have provided me with so much support, guidance, and joy (and free accommodation!). Thank you to the large number of you who proof-read for me; I am so, so appreciative. Particularly big thank you to Kim who did a huge amount of proof-reading for me, you are an incredible person and friend. And to Nadia, for making my echidna gifs, which have been the absolute highlight of my presentations at many conferences.

Last but not least, to my family. My brother Patrick, thank you for always being there for me, despite being just as overwhelmed with university as I was, your support, guidance, (and proof-reading), means everything to me. To Taylah, I am so glad to have you as part of our family, you are always so positive and supportive. To Mum and Dad, you have provided unwavering support, in so many ways, and never falter your belief in me. Mum thank you for being the biggest provider of encouragement, advice, and positivity, and keeping me sane (and healthy!) over the years. Dad, you are a huge inspiration, and I would not have made it without your PhD knowledge and guidance, your proof-reading, and your level-headedness. Thank you all.

Thesis by Compilation Declaration

The following publication will be included in the thesis titled 'A Wildlife Forensic Genetic Toolbox to Combat the Illegal Trade of the Short Beaked Echidna' in the form of a thesis by compilation. Ph.D. candidate Alexandra Summerell researched, wrote, and edited the publication, while Dr Rebecca Johnson, Dr Greta Frankham, and Dr Peter Gunn contributed their guidance, edits and feedback to the article. The publication was accepted and published in Forensic Science International and will be included as Chapter 2 of this thesis.

DNA based method for determining source country of the short beaked echidna (*Tachyglossus aculeatus*) in the illegal wildlife trade (Accepted)

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The research and sample collection in this thesis was conducted under the University of Technology Animal Ethics (201500040) and Taronga Zoo Animal Ethics (3b/06/16). All samples from live animals in zoos were collected by trained veterinarians. Samples from University of Tasmania were collected by Stewart Nicol and Rachel Harris and were collected under several UTAS animal ethics spanning multiple years: 97006, A0005711, A0005452, A0007124, A0008659, A0010426, A12320, as well as the following Parks and Wildlife Permits: FA99096, FA011254,

FA02120, FA03080, FA04041, FA05057, FA06367, FA07020, FA08053, FA09048, FA10045, FA1103, FA12069.

The research in this thesis was supported by funding from:

- The Zoo and Aquarium Association Australasia
- The Holsworth Wildlife Research Endowment
- The Australian Museum Research Institute Postgraduate Award

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Peter Gunn

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Abstract

The international illegal wildlife trade is widespread and affects thousands of species. The illegal trade in 'captive bred' animals is one component of this trade, driven by the perceived value of unique species or those that are difficult to breed in captivity. 'Demand' for these species is met via poaching wild individuals to supplement 'captive breeding'. One of Australia's most iconic species; the short beaked echidna (*Tachyglossus aculeatus*) is one such species impacted by this trade. Echidnas are found throughout Australia, as well as New Guinea, and are notoriously difficult to breed in captivity, with less than 20 bred in Australian zoos in the last five years. However, in 2016 Indonesian breeding facilities listed a breeding quota of 50 echidnas raising suspicion around the origin of these animals. Exposing and combating illegal trade requires the development of robust forensic tools to aid enforcement. This thesis uses conservation genetics approaches to create a forensic genetic toolbox that can be implemented with short beaked echidnas of suspicious origin. C*hapter 2* outlines a validated mitochondrial DNA test that was able to determine source region (i.e. New Guinea or Australia) of short beaked echidnas, including with DNA extracted from non-invasive samples. Mitochondrial DNA provided limited resolution to determine the source finer than region, thus *Chapter 3* presents a single nucleotide polymorphism (SNP) marker set developed to investigate short beaked echidna subspecies, which to date had only been described based on morphology and geographic distribution. Genetic structure within the SNP data were congruent with current subspecies, but significantly wider sampling of echidnas, in particular, island populations and at subspecies overlap zones is needed to reach definitive conclusions. In *Chapter 4* I demonstrated these SNP markers also had the

power to elucidate relatedness between individuals, and using captive bred individuals, could be used to reconstruct pedigree, which I then applied to assess relationships within a wild population. *Chapter 4* includes a suite of SNPs that once validated could be used for forensic investigations of short beaked echidnas. Lastly, *Chapter 5*, outlines the attempted validation of a real-time PCR sex determination method using previously published methods. This test however failed multiple validation criteria so would require further optimisation before it could be used in a wildlife forensic context. This thesis presents the first set of genetic tools for the short beaked echidna in a forensic context, providing novel information on source region, subspecies and relatedness that can be implemented to combat the illegal trade of this iconic species.

Chapter One: General Introduction

1.1 The illegal wildlife trade

The quantum of global environment crime was estimated in 2016 by INTERPOL to be worth up to US $258 billion annually (Nellemann *et al.* 2016). The illegal trade in wildlife, both flora and fauna, is transnational, not only impacting biodiversity but also causing substantial economic losses around the world (Zimmerman 2003; Warchol 2004; Rosen and Smith 2010; Wilson-Wilde 2010; Nellemann *et al.* 2014; Van Uhm 2016). Countries such as South Africa, China, India, and many in South East Asia have some of the highest rates of illegal wildlife trade (Warchol 2004; Rosen and Smith 2010; Van Uhm 2016). While national laws controlling the hunting and trade of plants and animals have been in place for some time in many parts of the world, it is still one of the largest and most economically impactful crimes (Yi-Ming *et al.* 2000; Warchol 2004; Rosen and Smith 2010; Van Uhm 2016). The trade occurs both domestically and internationally, with illegal trade between countries gaining most attention (Yi-Ming *et al.* 2000; Nellemann *et al.* 2014, 2016; Van Uhm 2016).

Animal and plant products, and their derivatives in the illegal wildlife trade, are destined for uses such as food, traditional medicines, or traded as curios or status symbols because of their intrinsic value (e.g. ivory) (Warchol 2004; Rosen and Smith 2010; Wilson-Wilde 2010; Van Uhm 2016). They are also sold to private collectors, zoos, biomedical labs, pet shops, farms, and meat dealers (Warchol 2004; Nellemann *et al.* 2016; Van Uhm 2016). These crimes lead to the unsustainable harvest and subsequent decline of some rare species. These crimes can seriously impact broadscale biodiversity in many places, and impact ecosystems to the point where they become extremely fragile (Warchol 2004; Deeks 2006; Nellemann *et al.* 2016; Van Uhm 2016). In some cases the illegal wildlife trade poses a biosecurity and public health risk to humans, native species, and livestock by the introduction of

invasive species and products carrying potential disease, and can have serious social and health impacts (Deeks 2006; Rosen and Smith 2010; Henderson *et al.* 2011).

The illegal trafficking of animals is, in particular, a crime that is of concern worldwide (Warchol 2004; Wilson-Wilde 2010; Sollund 2011, 2013; UNODC 2016; Van Uhm 2016). Individual animals or animal products can fetch extremely high prices on the black market (Warchol 2004; Rosen and Smith 2010; Biggs *et al.* 2013; Van Uhm 2016). For example, in Vietnam and China, rhinoceros horns are highly valued and can fetch US $65,000-100,000/kg on the black market, which is a value higher than other well quantified commodities both legal and illegal, such as gold and cocaine (Warchol 2004; Rosen and Smith 2010; Biggs *et al.* 2013).

Other species, or their parts, that are commonly trafficked are elephants, tigers and other big cats, pangolins, great apes, many species of birds, fish, and reptiles; as well as many species of plants and timber (Brack 2003; Warchol 2004; Wasser *et al.* 2008; Van Uhm 2016). Even species such as pandas and antelopes have been documented as being illegally traded in parts of Asia (Yi-Ming *et al.* 2000). These species may be either killed or traded while still alive, with either scenario likely to be inhumane causing the individual animal great amounts of suffering (Sollund 2013; Van Uhm 2016). Other than animal parts destined for food, trophies, clothing, and traditional medicines, live animals may be illegally traded in many countries to be kept as pets, for entertainment, or to be displayed in zoos (Rosen and Smith 2010; Beastall and Shepherd 2013; UNODC 2016; Van Uhm 2016). In particular, species that are unusual, endangered, or perceived as ‘charismatic’ or ‘iconic’ by the general public are targeted. By their very nature this includes those species that are often difficult to breed in captivity (Warchol 2004; Rosen and Smith 2010; Beastall and Shepherd

2013). In most countries, the zoo industry is governed by specific and strict rules and regulations stipulating the circumstances animals can be kept in captivity (i.e. they must either have been bred in captivity or deemed by a veterinarian unsuitable for release to the wild). These guidelines have been determined by peak bodies such as The World Association of Zoos and Aquariums, or the Zoo and Aquarium Association Australasia, as well as by government legislation (e.g. in Australia the *Exhibited Animals Protection Act 1986*). The trade in illegally obtained species and the nature of their trade is of significant interest for the conservation of animals, the international zoo industry, and countries as a whole.

1.2 Conventions, laws, and legislation

1.2.1 CITES

Global efforts to prevent the illegal transnational trade of wildlife were brought about by the establishment of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). CITES came into force in 1975 when it was ratified by the governments of 80 countries. Since then a further 103 countries have signed up to the convention. CITES is an international convention that specifies the conditions and control of wildlife trade, and once ratified requires governments to introduce, define, and enforce their own legislation relating to wildlife trade (CITES 2015). There are currently approximately 35,000 species of plants and animals that receive protection under CITES by the countries that have ratified the convention. CITES identifies species where protection is needed, and trade (both legal and illegal) could pose a threat and places the trade of these species under certain controls. There are, however, many species that are not yet listed on CITES, and as a consequence, these species may only be protected nationally (in the countries to which they are

native). Once outside these boundaries, they are no longer protected by any form of legislation (CITES 2015).

1.2.2 National legislation

Once a country has ratified CITES and agreed to abide by the convention, the specific legislation with respect to the illegal trade in wildlife is quite variable between countries. Within Australia, the relevant legislation is the Commonwealth *Environmental Protection and Biodiversity Conservation Act 1999* (EPBC) that relates to illegal trade in native Australian species and non-native species traded within Australia. The EPBC Act enforces the illegal trade occurring with CITES listed non-native species, once they arrive in Australia, and provides conservation frameworks for many threatened native species. However, because Australia is a federation that shares governance between both federal and state/territory governments, each Australian state will also have legislation to permit if a species native to their states can be kept or sold, for example the *National Parks and Wildlife Act 1974* in New South Wales (NSW), and the *Wildlife Act 1975* in Victoria.

As previously mentioned, all native Australian wildlife is protected under Australian federal and state legislation, including those not listed on CITES. The Commonwealth *EPBC Act 1999* is a federal law governing such protection. Taking an animal from the wild without permission is prohibited under these laws, and hence punishable in court, and the sentences may often include large fines and detention as penalties such as a $500,000 fine for an individual or imprisonment up to seven years, or a $5 million fine for a corporate body (EPBC 1999). Additionally, there are also state compliance agencies, for example in New South Wales the Department of Planning,

Industry and Environment, that govern specific state laws regarding trade, and may be responsible for such tasks as issuing permits to zoos or licensed breeders.

Many other countries have similar legislation, including the *Wildlife and Countryside Act 1981* in the United Kingdom, and *Endangered Species Act 1973* and *The Lacey Act* which are Federal laws in the United States of America. In countries such as China, where there is a higher rate of wildlife crime (Wilson-Wilde 2010; Nowell 2012), increasingly strict laws have been implemented throughout the past 100 years (Yi-Ming *et al.* 2000). This includes the *Law of the People's Republic of China on the Protection of Wildlife 1988*. However, it has been suggested that enforcement is not as strict, and while China and many other countries ratified CITES in 1981, there have been challenges concerning the appropriate enforcement of these laws, and the severity of punishments for the crimes (Yi-Ming *et al.* 2000; Johnson 2010; Linacre and Tobe 2011; Wright 2011; Zhou *et al.* 2015a, 2015b; Van Uhm 2016). Other countries in Asia, that are often the source and demand countries of traded species, have poor (or non-existent) legislation, which has been described as difficult to interpret, or lacking implementation (Zhou *et al.* 2015a). It has been argued that stricter legislation and punishments, as well as thorough investigation and evidence, should be enacted. The UN has pointed out that governments often approach environmental crime from a conservation perspective, rather than criminalising it, with the consequence that it may not be dealt with as effectively as would be other organised crime. In addition, legislation and border control across many countries is not uniform, making it difficult to reach common positions on some laws, and consequently the conviction rate is relatively low (Yi-Ming *et al.* 2000; Linacre and Tobe 2011; Wright 2011). Furthermore, many of the convictions record relatively

minor penalties, far lower than the maximum allowable under the legislation, that likely do not deter the perpetrators (Yi-Ming *et al.* 2000; Johnson 2010; Linacre and Tobe 2011; Wright 2011). The rate of environmental crime has increased, possibly augmented by these low conviction rates and penalties, and hence is considered a low risk crime that still generates a high profit (Yi-Ming *et al.* 2000; Linacre and Tobe 2011; Wright 2011).

One of the limitations associated with enforcement of these laws in court is the requirement to provide sufficient evidence to support a prosecution (Alacs and Georges 2008; Johnson 2010). Robust forensic evidence to support claims made against the wildlife trade and prove species identification is necessary to categorically prove illegal crime has taken place, and to support cases that are taken to court and may result in prosecution (Alacs and Georges 2008; Johnson 2010; Linacre and Tobe 2011).

1.2.3 Non-CITES listed species

Numerous species found in the illegal wildlife trade are not listed by CITES, but this does not necessarily mean they are immune from risk of becoming endangered or extinct. Often there is not enough data regarding the impact of illegal trade on these species, or only anecdotal evidence suggesting they are targeted by such trade (Auliya *et al.* 2016; Jensen *et al.* 2018). The collection of trade data is often dependent on countries agreeing to share the information, and in many circumstances, declines in populations are detected too late for a CITES listing to make an impact. Additionally, there are multiple factors that influence such declines (e.g. habitat loss, climate change, feral species, disease), making it difficult to clearly demonstrate the impact of illegal trade. A study by Jensen *et al.* (2018) investigated the trade in non-CITES

listed African snakes and found that 23 species of snakes that were most frequently traded were not CITES listed. Furthermore, a review of the trade of reptiles by Auliya *et al.* (2016) found that at least 194 of the reptiles listed on the International Union for the Conservation of Nature (IUCN) red list as 'threatened by biological use' were non-CITES listed, and overall 90% of reptiles were not listed on CITES. While some of these species are nationally protected, those that are not regulated by CITES cannot be protected once they have been illegally traded outside of their range country (Auliya *et al.* 2016). Where possible, thorough research should be conducted as early as possible to assess key criteria such as endangered status and the species' presence in the trade (Auliya *et al.* 2016; Janssen and Shepherd 2018).

1.2.4 Known strategies of the illegal wildlife trade – laundering of wild-caught animals as captive bred

One worrying trend in the illegal wildlife trade is species being illegally trafficked within the zoo or animal breeding industry. Species in this trade that are particularly likely to be targeted may be unusual, endangered, or deemed 'charismatic' by the general public. Further, in many cases, species that are very difficult to breed in captivity are also targeted (Warchol 2004; Rosen and Smith 2010; Lyons and Natusch 2011; Janssen and Chng 2017). This specific trade is of significant concern to the international zoo industry as it undermines commitment to the conservation of wildlife, and the reputation of the zoo industry (Lyons and Natusch 2011). While CITES and national laws are important for species targeted within the illegal wildlife trade, there is often insufficient data to understand the extent and impact of this trade, and significant research is required before enforcement tools can be developed and put in place.

The zoo and aquarium industry in Australia and many other countries have strict protocols about the handling of animals and the legal trade between zoos (EPBC 1999). Animals may be legally taken in from the wild, particularly if they are injured or endangered and require captive breeding to boost numbers (Zoo and Aquarium Association, 2015). One of the main benefits of captive breeding species that have low population numbers is the potential to boost wild population numbers through reintroduction or ‘re-wilding’ of captive bred animals (Tribe and Booth 2003). However, there is evidence that some zoos, wildlife parks, or private collectors in parts of the world will not thoroughly check from where an animal has come, or if the paperwork supporting it shows obvious signs of forgery (Lyons and Natusch 2011; Beastall and Shepherd 2013; Janssen and Chng 2017). In instances where there are less regulations within zoos, parks, or selling to private collectors, it has been found that there are higher rates of animal trafficking and trade, particularly in South East Asia and surrounding areas (Beastall and Shepherd 2013; Nijman and Shepherd 2015; Janssen and Chng 2017; Willows-Munro and Kleinhans 2019).

Often animals sold to zoos by private breeders are claimed to have come from a licenced breeding facility or licenced breeder and thus are captive bred rather than wild-caught (Beastall and Shepherd 2013; Bush *et al.* 2014; Coetzer *et al.* 2017; Janssen and Chng 2017). For species which are difficult to breed in captivity, taking individuals (e.g. poaching) from the wild is often simpler, less resource intensive, and thus more cost effective (Nijman and Shepherd 2009, 2015; Beastall and Shepherd 2013; Janssen and Chng 2017). To sell these poached individuals, fraudulent paperwork and/or false pedigrees lacking genetic data are generated and offered up to recipient zoos to use should they wish to validate these claims (Beastall and Shepherd 2013). Traditionally zoos keep studbooks by recording data for each individual such

as parentage, birth date and location, and death date and location. Though genetic analyses have become cheaper it is still not routine to integrate genetic pedigree information into studbooks even in the many law-abiding organisations, leaving a loophole for those trading in illegally obtained species to exploit.

Evidence of this type of trade has been demonstrated in a range of species including bears, tigers, and some species of birds and reptiles (Warchol 2004; Nijman and Shepherd 2009, 2015; Lyons and Natusch 2011; Livingstone and Shepherd 2014; Janssen and Chng 2017). Such unregulated and illegal removal of animals can greatly impact demographics of wild populations as well as the wider ecosystem, particularly if there are certain biases such as age or sex. It can also be harmful for the animal involved, with many known animal welfare issues in the illegal wildlife trade. Animals are often poorly treated, provided with inadequate food and water, or are transported in inappropriate containers, resulting in injuries, disease, or death (Warchol 2004).

1.3 Tools utilised in the prevention of illegal wildlife trade

As with crimes involving human victims, environmental crime involving plants and animals has its own forensic discipline; *wildlife forensic science*, which can be implemented to assist in detecting this illegal trade (Linacre 2009; Johnson 2010; Linacre and Tobe 2011; Johnson *et al.* 2014). Wildlife forensic science is a specific sub-discipline of forensic science that relates to the analysis of non-human animals and plant material within and for the judicial system, and provides evidence in a range of cases including those involving illegal trafficking or handling of wildlife (Linacre 2009; Linacre and Tobe 2011; Johnson *et al.* 2014; Ogden and Linacre 2015). Unlike human forensics, which is focused on individualisation primarily after a crime has

occurred, wildlife forensics is usually required first to establish if a crime has taken place. The questions asked to establish that, also often vary from that in human forensics, and can include; what species is it, where has it come from, what level of individualisation is possible, and what sex is it (Johnson *et al.* 2014; Ogden and Linacre 2015; Ogden *et al.* 2016; Moore and Frazier 2019).

The analyses required in the illegal wildlife trade are typically concerned with the identification of species, geographic provenance and/or pedigree/paternity of wildlife that are found or seized (Linacre 2009; Linacre and Tobe 2011; Johnson *et al.* 2014; Ogden and Linacre 2015). Different tools within wildlife forensic science can be utilised, including morphology, which can involve, but is not limited to, identifying live and dead whole animals, skin, bones, feathers, and hair which may be done via microscopic analysis. Chemical tests and radioisotope analysis can also be implemented; for example, radiocarbon dating of elephant ivory can be used to determine an approximate age of the ivory (Uno *et al.* 2013). Radiocarbon dating can be applied in such examinations due to the significant increase in radiocarbon (^{14}C) due to nuclear testing during the mid 1900s and its subsequent decay, meaning a date can be determined along a 'bomb curve' of atmospheric ^{14}C (Linacre 2009; Uno *et al.* 2013). The possession of ivory is permitted if it can be demonstrated it was acquired or sampled prior to certain dates, which can vary between countries (Linacre 2009; Uno *et al.* 2013). Stable isotope analysis is elemental analysis that can also potentially be used for determining whether an animal originates from captivity or the wild (Bowen *et al.* 2005; Thomas *et al.* 2008; Brandis *et al.* 2018). A study by Brandis *et al.* (2018) used high-resolution x-ray fluorescence for elemental analysis of echidna quills. Based on the nutrients in the animals' diets, they could see that captive

animals, with a more varied diet, and wild animals, with a more specific diet of termites and ants could be differentiated based on this elemental analysis. DNA-based analyses are used widely for a range of purposes and are becoming increasingly useful for both casework and research questions (Linacre 2009; Ogden *et al.* 2009; Johnson 2010; Johnson *et al.* 2014; Moore and Frazier 2019), forming an ideal complement to the techniques outlined previously.

1.3.1 Validation in forensic science

Following the lead of human forensics, standardisation and validation are recommended within wildlife forensics to ensure high quality of testing and results (Linacre 2009; Johnson *et al.* 2014; SWFS Technical Working Group 2018). This is of particular importance when a result needs to be presented and defended in court. Human forensics has long been considered the gold standard, stemming from a long history of validation, and many guidelines, such as the process of validation described by the Scientific Working Group on DNA Analysis Methods (SWGDAM), have been implemented (SWGDAM 2016). Additionally, having laboratories certified by testing authorities and be compliant with international standards is commonplace. However, in comparison to human forensics which involves only one species, wildlife forensics, can require the analyses of potentially thousands of different species, thus developing and implementing validated tests can be significantly more challenging. Due to the increasing prevalence of wildlife crime, validation should remain a key goal to strive to, and it is recommended that where possible, new protocols and equipment should be validated and standardised (Linacre 2009; Johnson *et al.* 2014; SWFS Technical Working Group 2018; Moore and Frazier 2019).

1.4 The use of DNA in wildlife forensic science

Wildlife forensics has benefited significantly from ongoing advancements in molecular biology technology, in both new methodologies and in improvements in statistical analysis techniques (Linacre and Tobe 2013). Many studies have proven extremely beneficial in aiding the conservation of many species and providing tools that can give an appropriate deterrent to the crime and potentially facilitate a successful prosecution (Hsieh *et al.* 2003; Johnson 2010; Frankham *et al.* 2015).

DNA methods to assist in wildlife forensics are increasingly common and have become the preferred methodology for casework relating to wildlife crime for several decades (Thommasen *et al.* 1989; Shorrock 1998; Ogden *et al.* 2008, 2009; Dawnay *et al.* 2009; Johnson 2010; Ogden 2010, 2011; Linacre and Tobe 2011; Johnson *et al.* 2014; Ciavaglia *et al.* 2015; Ewart *et al.* 2018). DNA sequencing methods, both traditional and next-generation sequencing, have broadened the possibilities for producing evidence in criminal cases, in both human and wildlife forensics. This provides higher levels of accuracy and precision and as a result, a higher likelihood of securing a conviction (Linacre and Tobe 2013). Human forensics incorporates large amounts of technical developments to ensure accuracy and standardisation, something from which wildlife forensics can benefit. Often, many of the items illegally traded only consist of parts of animals, and consequently it may not be possible to use morphological features to determine the species identity. Additionally some species can be difficult to differentiate and look very similar morphologically (Linacre and Tobe 2011, 2013). For example, when rhinoceros horns are traded it is difficult to determine what species of rhinoceros it is from, or if it is even rhinoceros at all, as

sometimes water buffalo horns (which are not protected by CITES) have been substituted for rhinoceros (Ammann 2012; Ewart *et al.* 2018). DNA methods can combat such issues and be used to answer the key questions about species identity (when dealing with animal/plant parts, derivatives, or species complexes (Ewart *et al.* 2018)), geographic provenance (Ogden and Linacre 2015), genetic pedigree (Jan and Fumagalli 2016), or sex of an animal (Peppin *et al.* 2010). These are critical for many reasons, such as presenting evidence for court, and providing useful intelligence to law enforcement, as it is essential to know which animals are being targeted, and where, to put in place preventative measures (Johnson 2010; Ogden 2010; Linacre and Tobe 2011; Johnson *et al.* 2014). In addition, accurate identification of a species is critical, as the poaching or trafficking of many species have different legislation, enforcement, and penalties depending on the country of origin and the species itself (Johnson 2010; Linacre and Tobe 2011, 2013; Johnson *et al.* 2014; Ewart *et al.* 2018). If it is suspected that wild individuals are being substituted for captive bred animals, then it is imperative for enforcement agencies to be able to distinguish between wild and captive animals. However, this process can be difficult due to the need for larger reference datasets for each species.

1.4.1 DNA markers

The development of genetic markers is based on the identification of polymorphisms; a variation in the DNA sequence between individuals. There are several types of polymorphisms used in genetics, and the methodologies of identifying and selecting polymorphisms are continuously improving. Polymorphisms are the key to many aspects of paternity testing and forensic science in particular (Butler 2009). Forensic science has utilised polymorphisms that occur in the non-coding region of DNA as a means of identifying or excluding individuals associated with a crime (Butler 2009).

These can include both mitochondrial DNA or nuclear DNA depending on the questions that need to be answered.

1.4.1.1 *Mitochondrial DNA*

Mitochondrial DNA (mtDNA) is around 16,000 base pairs long in mammals and codes for consistent functions within cells across organisms. Mitochondrial DNA is often used for analysing phylogenetic relatedness and evolutionary processes between different organisms, and has the advantage of having a high mutation rate and lack of recombination, making it suitable for these tasks (Avise 1987; Ball Jr and Avise 1992). Within human forensics it is often used for specialist tasks such as disaster victim identification, bone and hair analysis, and missing persons cases. This is due to its high copy number and therefore persistence long after nuclear DNA has become unusable, as well as it being present only on the maternal line, and therefore useful for familial testing (Budowle *et al.* 2003; Butler 2009). Several genes within the mitochondria (e.g. Cytochrome B or CO1) are used widely for species recognition, particularly in wildlife forensic science, due to the ease of amplification and sequencing and the availability of reference data for those genes (Parson *et al.* 2000; Tobe *et al.* 2009; Johnson 2010). Additionally the control region is commonly used for phylogenetic studies in conservation, as it has more variability than CO1 and Cytochrome B, and therefore can be used for intraspecific questions such as geographic region (Budowle *et al.* 1999; Potter *et al.* 2012; Frankham *et al.* 2016).

1.4.1.2 *Nuclear markers*

Microsatellites, also known as short tandem repeats (STRs), are a relatively common feature of the nuclear genome, and are one of the most commonly used markers within human forensics due to their frequency throughout the genome and relatively

high mutation rate (Saferstein *et al.* 2011). Microsatellites are commonly used for individualisation in forensic science (Butler 2009) but are also well established in genetic disease studies, and have revolutionised the field of molecular biology as they allow us to detect variations between individuals relatively simply (Saferstein *et al.* 2011). Microsatellites are sections of repeated DNA, ranging from two to seven repeating base pairs, that get repeated in a sequence from five to up to fifty times (Saferstein *et al.* 2011). In contrast, single nucleotide polymorphisms (SNPs), are polymorphic sites where only a single base nucleotide varies between individuals at each site (Butler *et al.* 2007). SNPs are found abundantly throughout the genome and, like microsatellites, tend to occur in the non-coding regions, though they can occur in the coding regions and have effects on the protein that is produced (Sobrino *et al.* 2005). SNPs have become especially useful for analysing parentage, biogeographic ancestry, and phenotype in forensic science, and have also been used increasingly for wildlife genetics (Sobrino *et al.* 2005).

Both microsatellites and SNPs have advantages and disadvantages, and there has been much discussion over which methodology is preferred in forensic science (Amorim and Pereira 2005; Butler *et al.* 2007; Butler 2009; Ogden *et al.* 2013; Ross *et al.* 2014). In many cases, especially in wildlife genetics, SNPs are increasingly preferred over microsatellites, as they provide greater resolution at the individual relatedness level (Amorim and Pereira 2005; Ogden *et al.* 2013; Ross *et al.* 2014). SNPs are in theory easily transferable and reproducible between laboratories, and are more cost effective for the discriminating power they afford, though many more SNPs are required for similar discriminating power of microsatellites (Vignal *et al.* 2002; Morin *et al.* 2004; Ogden *et al.* 2013; Ross *et al.* 2014). Due to their larger size, the

amplification of degraded DNA can be difficult with microsatellites, compared to that of smaller sized SNPs (Butler *et al.* 2007). Additionally, as microsatellites need allelic ladders, this can hinder data sharing and standardisation between labs, which also presents a problem when there are many species in wildlife forensics (Moore and Frazier 2019).

SNP discovery can be carried out in a variety of ways. If a full genome sequence assembly is available, SNP discovery can be done in silico. However for species where no genome level data is available, de novo (no prior knowledge of genome) approaches such as reduced representation methods (or double digest restriction site associated DNA (ddRAD)) can be used (Ogden *et al.* 2013). This method uses two restriction enzymes to cut across the genome and allows a comparison of multiple copies of the same section of DNA so that SNP markers can be identified (Baird *et al.* 2008; Ogden *et al.* 2013).

1.4.2 Determination of geographic origin

Phylogeographic information based on genetic data can provide great insights into a species' evolutionary history and can potentially show a clear view of how and when specific divergences occurred. Within wildlife forensics, using such tests can provide biogeographic information to discern the origin of the individual animal in question, or where poaching hotspots and trade routes are located (Ogden and Linacre 2015; Zhao *et al.* 2019). This has been done within wildlife forensics using both mitochondrial (Rawlings and Donnellan 2003; Murray-Dickson *et al.* 2017; Zhao *et al.* 2019) and nuclear (Wasser *et al.* 2008; White *et al.* 2012; Nash *et al.* 2018) markers.

Murray-Dickson *et al.* (2017) completed a detailed phylogeographic study of the reticulated python (*Malayopython reticulatus*) to investigate the broad-scale population genetic structure of this species across its wide South-East Asian distribution. This species is also heavily traded both legally and illegally for its skins; with many individual animals claimed as captive bred though the origins are suspected of being wild. They used mitochondrial DNA to assess the genetic diversity across the species' range and found that there were 34 unique haplotypes within this species that varied between the geographic locations. This is crucial for species that are potentially being trafficked to pinpoint where such a species has been harvested to prove any trafficking claims, and aid with the repatriation of species to their geographic origin. Additionally, like most species whose distributions cross state or country borders distinguishing source populations can aid in management of this species by targeting smaller groups that may be at risk rather than the species as a whole.

1.4.3 Individualisation and pedigree reconstruction

Using genetic markers for individualisation is commonly used in human forensics and involves comparing a DNA profile with references to include or exclude an individual with a level of statistical confidence. Similar techniques can also be used for determining the relatedness between individuals and giving a statistical weight to that relationship. Within wildlife forensics individualisation and relatedness techniques are less routine compared to human forensics, but depending on the crime involved could be crucial to providing appropriate evidence. Both microsatellites and SNPs have been used to develop marker sets that are discriminatory down to an individual level for the species involved (Van Hoppe *et al.* 2016; Ciavaglia and Linacre 2018; Moore

and Frazier 2019; Dormontt *et al.* 2020). For example, the OzPythonPlex, a microsatellite assay for use with traded carpet pythons (*Morelia spilota*), which was developed in accordance with human forensic kits (Ciavaglia and Linacre 2018). This was also accompanied with a population database to provide statistical analysis on the profiles and allow for individualisation of carpet pythons. Additionally, Dormontt *et al.* (2020), developed a SNP panel which can individualise bigleaf maple (*Acer macrophyllum*), a species that is of high profile in the illegal logging trade. When determining relatedness between individuals it is imperative that specific markers are tested to ensure they can provide that level of detail and give an accurate representation of the pedigree of a species. This can then aid in determining whether an animal is captive bred or wild-caught, which is one of the key questions commonly asked within wildlife forensic science (Thommasen *et al.* 1989; Shorrock 1998; Nijman and Shepherd 2009; Bush *et al.* 2014).

Pedigree reconstruction provides family history information and is an invaluable tool for informing population studies, conservation and management of species, ecological and evolutionary history, and forensic science (Jones *et al.* 2010; Creel and Rosenblatt 2013; Huisman 2017). Genetic pedigrees are also useful to zoos and other sanctuaries that breed animals, in particular species that are rare or endangered (Tokarska *et al.* 2009; Creel and Rosenblatt 2013; Mucci *et al.* 2014; Farquharson *et al.* 2019). Validation of true definitive genetic pedigree in species is only possible through the development of highly variable species-specific DNA markers and the further calibration of these to allow for standardisation across laboratories (Jones *et al.* 2010).

The use of molecular genetic pedigrees are routinely used in other industries, in particular the domestic dog, cat, and equine industries for breeding purposes such as purebred breeding, as well as to avoid inbreeding (Mellersh *et al.* 1997; Coomber *et al.* 2007; Bower *et al.* 2013). Determining the genetic pedigree of a species reveals the extent of relatedness between individuals and has been a useful tool for cases involving the 'captive bred' trade (Ross *et al.* 2014; Frankham *et al.* 2015; Coetzer *et al.* 2017; Blåhed *et al.* 2018).

The broad-headed snake (*Hoplocephalus bungaroides*) is an example of the importance of ascertaining genetic pedigree in a wildlife forensics context (Webb *et al.* 2002; Frankham *et al.* 2015). This is an Australian reptile species, with a conservation status classified as endangered in NSW (NSW DPIE 2017) for which illegal trade was suspected as a key threatening process. This species can be kept legally with the appropriate licences, but it was suspected that dead animals were being substituted with wild animals under the same licence, or that wild clutches (from a gravid female taken from the wild) were being passed off as captive bred animals (Frankham *et al.* 2015; Hogg *et al.* 2018). To investigate these allegations and to assist law enforcement to develop a toolbox, Frankham *et al.* (2015) developed microsatellite markers to genotype all the broad-headed snakes in captivity (privately held and in zoos). These informative markers allowed differentiation among individuals and provided provenance and pedigree information. In a follow-up study, Hogg *et al.* (2018) assessed these markers and could determine that the privately held individuals appeared to have gene flow with the wild animals, whereas the zoo populations had not and were clearly differentiated. The authors recommended the use of bio-banking for this and other species, so that appropriate databases could be

generated and allow for the development of markers and tests for species in the trade, as well as acting as a deterrent (Frankham *et al.* 2015; Hogg *et al.* 2018).

1.4.4 Sex determination

Determination of sex has been a routine test in human forensic genetics for several years now, as it aids in the individualisation process, and can guide law enforcement in cases where the identity of a relevant party is unknown (Butler 2009). This is most commonly done by amplification of a section of the Amelogenin gene, which differs in size between males and females (Mannucci *et al.* 1994) and is including in STR kits routinely used for individualisation. Additionally, STRs solely on the Y-chromosome have also been developed for use in a forensic context, allowing the presence of specifically male DNA to be amplified (Ballantyne *et al.* 2012). These are often used in situations where standard STR kits aren't applicable or cannot determine sex (e.g. familial testing, mutations resulting in Amelogenin not amplifying, or mixtures where large amounts of female DNA are present) (Ballantyne *et al.* 2012).

Zoos, conservation programs, and breeders often use DNA sex determination tests as tools to aid with breeding programs or population management, particularly if a species does not show sexual dimorphism (Griffiths *et al.* 1998; Fridolfsson and Ellegren 1999; Griffiths 2000; Mucci *et al.* 2016).

Validated sex determination tests are minimal within wildlife forensic science (Gupta *et al.* 2006; Bidon *et al.* 2013; Joshi *et al.* 2019). This is likely due to the fact that often the sex is not needed for charges to be made. However, in some circumstances, penalties are increased when a female of certain species is involved (Piper 2015). Additionally hunting regulations in the USA include a range of rules and penalties

depending on the species, for hunting females, particularly with offspring (Burnham-Curtis *et al.* 2015; Piper 2015; Moore and Frazier 2019). Knowing the sex can also aid in resolving relationships for pedigree studies, or for individualisation purposes, both of which are important tools in providing wildlife forensic evidence.

1.5 The echidna (*Tachyglossus sp.* and *Zaglossus spp.*)

Echidnas, belonging to the family Tachyglossidae, are one of the two extant monotreme families along with the platypus. Monotremes are well known for their unique biology as the only mammals that lay eggs (Griffiths 1978; Rismiller and Seymour 1991; Nicol and Andersen 2007; Augee 2008). The platypus and echidnas diverged approximately 34 million years ago, sharing a now-extinct platypus-like monotreme common ancestor (Janke *et al.* 2002).

Echidnas are found throughout Australia and New Guinea (Griffiths 1978; Rismiller and Seymour 1991; Nicol and Andersen 2007; Augee 2008). There are four extant echidna species, with the short beaked echidna (*Tachyglossus aculeatus*: (Figure 1.1) being the most widespread Australian mammal species. The three long beaked echidnas (*Zaglossus spp*.) are endemic to New Guinea and include *Z. bruijnii*, the western long beaked echidna; *Z. attenboroughi*, Sir David Attenborough's echidna; and *Z. bartoni*, the eastern long beaked echidna (Flannery and Groves 1998).

1.6 Study species - the short beaked echidna - *Tachyglossus aculeatus*

The short beaked echidna (hereafter referred to as the 'echidna') is one of Australia's most recognisable and widespread mammal species. It features prominently in popular culture as it is portrayed on the five-cent coin, and given it is diurnal, it is often encountered in the wild by the general public (Rismiller and Seymour 1991).

The echidna is generally solitary and plays an important ecological role, being the only mammal feeding on mostly ants and termites, as well as contributing to soil turnover (Rismiller and Seymour 1991; Augee 2008; Clemente *et al.* 2016). It is easily recognised for the covering of spines on its dorsal surface (Figure 1.1), and for the long snout it uses to forage for food (Griffiths 1978; Rismiller and Seymour 1991; Augee 2008). Echidnas are toothless, except for one which, like birds and reptiles, is present in juveniles when they are in the egg, and used for hatching, then lost soon after (Davit-Beal *et al.* 2009). They have a long, quick tongue used to catch prey (Figure 1.1) (Davit-Beal *et al.* 2009). They often reside in dark more confined habitats, in hollowed out logs, under debris, or in thick bushes (Rismiller and Seymour 1991; Augee 2008). The echidna is a relatively small mammal, ranging from 30-45cm in length, and weighing around 2-7kg (Griffiths 1978; Rismiller and Seymour 1991; Augee 2008).

While echidnas are considered common throughout Australia, like many other Australian species they are impacted by the introduction of predators such as foxes, feral cats, and domestic dogs (Nicol and Andersen 2007). Native predators such as goannas have also been known to prey on echidnas (Rismiller and McKelvey 2000; Nicol and Andersen 2007). Habitat loss and urbanisation also impacts populations, with vehicles injuring and killing echidnas, and urban developments restricting suitable environments to live (Rismiller and McKelvey 2000; Nicol and Andersen 2007).

Figure 1.1 The short beaked echidna (*Tachyglossus aculeatus*) uses its long tongue to reach termites and ants; its main source of food. Their quills are used for protection and are a modified form of hair; they can be plucked or shed naturally (Source: Ben Beaden © Australia Zoo, used with permission.).

1.6.1 Reproduction in the short beaked echidna

Echidnas have an interesting and idiosyncratic reproductive process (Rismiller and McKelvey 2000; Ferguson and Turner 2012). Echidnas mate between June and September, the precise date depending on the location and the environmental conditions (Rismiller and McKelvey 2000; Ferguson and Turner 2012). Male echidnas pursue the females, with "trains" of several males, up to ten at a time, following female echidnas often for up to four weeks, with males sometimes changing which female they follow (Rismiller and McKelvey 2000; Morrow *et al.* 2009; Ferguson and Turner 2012). The trains are often in status order, with the youngest or smallest echidna at the end of the queue, and the female will choose one male to breed with (Rismiller and McKelvey 2000; Augee *et al.* 2006; Ferguson and Turner 2012). The actual mating process will take between half an hour and three hours, resulting in a single egg being produced (Rismiller and McKelvey 2000; Augee *et al.* 2006; Ferguson and Turner 2012). Females will usually only mate once every breeding season (Rismiller and McKelvey 2000).

The female echidna will construct a nesting burrow during the gestation period, which is on average between 21 to 28 days after initial mating (Rismiller and McKelvey 2000). The egg is laid into a 'pouch' on the echidna's abdomen; it is not a true pouch, rather a pouch-like fold of constricting muscles. The egg will remain there for approximately ten days, until it hatches, and the pouch young or 'puggle' stays within the pouch for approximately two months until it develops quills, after which the mother leaves the puggle in a nest until it is fully weaned (Rismiller and McKelvey 2000).

Due to the curious nature of echidnas there is interest in keeping these species in zoo collections. Wild echidnas needing rehabilitation after rescue from vehicle strikes or animal attacks are often introduced into zoos. Monotremes, however, are notoriously difficult to breed in captivity (Morrow *et al.* 2009; Ferguson and Turner 2012; Wallage *et al.* 2015). Their long and complicated reproductive process (as explained above) is extremely hard to replicate in a captive situation (Ferguson and Turner 2012; Wallage *et al.* 2015). To date there are only four zoos in Australia that have managed to successfully breed echidnas in captivity, Perth Zoo, Australia Zoo, Taronga Zoo, and Currumbin Wildlife Sanctuary. While improvements in husbandry have been made in the past five years, the birth rate is still relatively low and, of those young born in captivity, many do not survive past one year of age (Wallage *et al.* 2015).

1.6.2 Subspecies of the short beaked echidna

The short beaked echidna has been found in every state and territory as well as the island of New Guinea, and live in a wide variety of habitats, including deserts and snow-covered locations (Figure 1.2). Five subspecies of short beaked echidna have been named based on morphological differentiation (Griffiths 1978; Augee *et al.* 2006; Augee 2008). *Tachyglossus aculeatus acanthion* has a distribution in Western Australia, Northern Territory, and the arid zones of the other states on the mainland. *Tachyglossus aculeatus aculeatus* resides along the coasts of Queensland, New South Wales, Victoria and South Australia (Augee *et al.* 2006; Augee 2008). It is not clearly defined where the distribution of *T.a. acanthion* and *T.a. aculeatus* start and finish, likely due to the elusive nature of the echidna and no large-scale sampling or genetic testing. *Tachyglossus aculeatus setosus* is found only in Tasmania and some islands in

Bass Strait, and *Tachyglossus aculeatus multiaculeatus* is found solely on Kangaroo Island. A fifth subspecies, *Tachyglossus aculeatus lawesii,* dwells in the coastal regions and highlands of New Guinea. Limited information about population numbers and true distribution of T.a. lawesii, due to difficulties in data collection and monitoring. Depending on the location in which the echidna is found, there are small adaptations that have evolved in response to climatic conditions (Augee *et al.* 2006). For example, echidnas such as *T.a. setosus,* found in colder temperatures, will often have a greater mass of fur covering the body, whereas echidnas living in warmer climates will often have a majority of their covering consisting of quills. These subspecies show variation not only in their morphology but also between their physiological characteristics as well as their behaviour; however, little is known about any potential genetic differentiation between subspecies as no detailed phylogeography has been completed for the species (Augee *et al.* 2006).

Phylogeographic studies using a range of markers (mtDNA and nuclear DNA) have been employed in studies to understand taxonomy, evolutionary history, and conservation implications (Avise 1987; Neaves *et al.* 2009; Potter *et al.* 2012; Frankham *et al.* 2016; Eldridge *et al.* 2017). The echidna is widespread with some populations that are physically isolated (New Guinea; Kangaroo Island; Tasmania) so having a complete picture of the species would be helpful. When accompanied with biogeographic information (i.e. phylogeography), this information could give greater detail about the evolutionary history of the species and if there is genetic partitioning that mirrors the morphological variations.

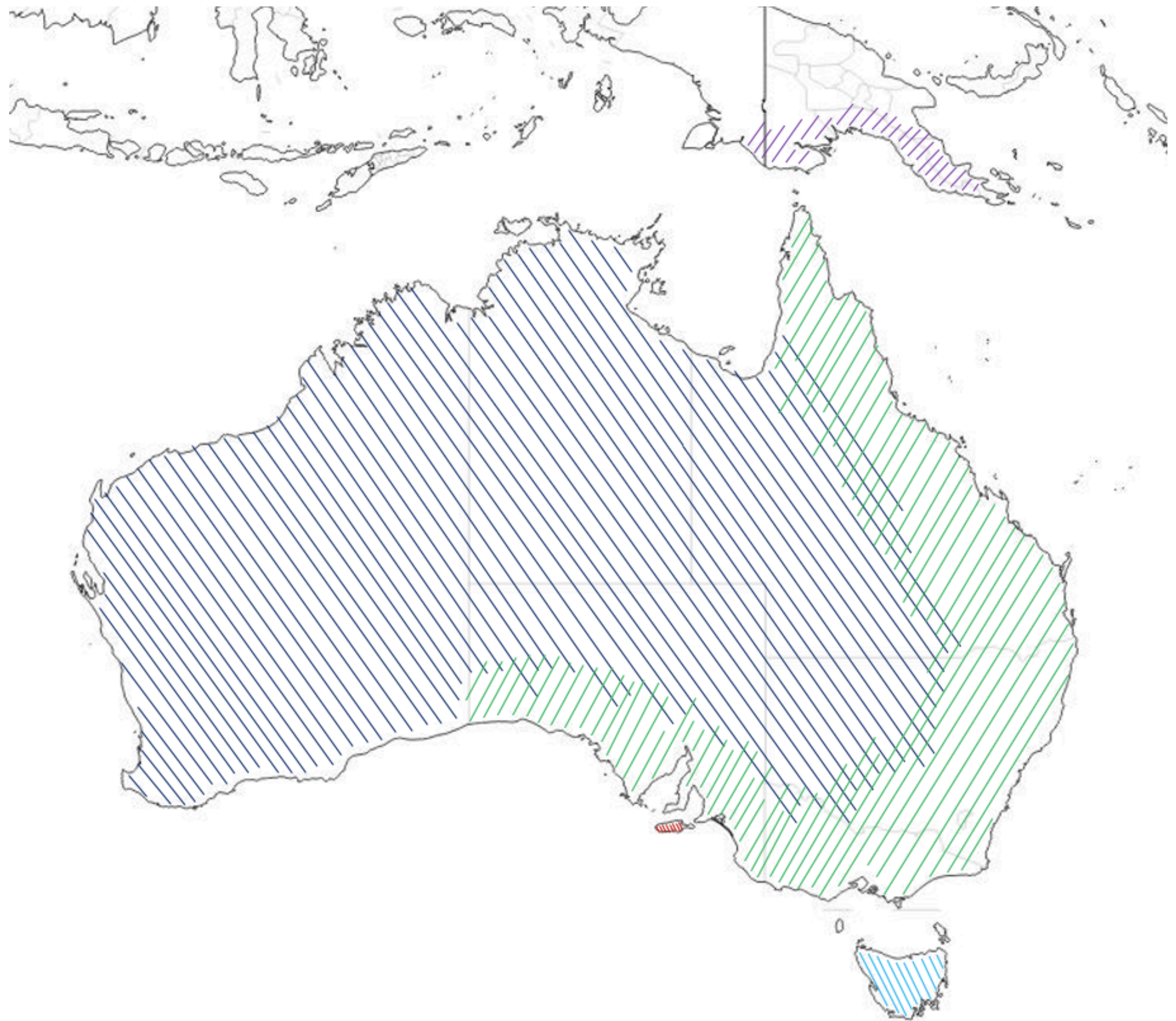

Figure 1.2 Distribution of the short beaked echidna (*Tachyglossus aculeatus*). The distribution and overlap of *T.a. aculeatus* and *T.a. acanthion* is ambiguous, hence the crossover presented in this map is an estimation by the author and warrants further investigation. Green = *T.a. aculeatus*; Dark blue = *T.a. acanthion*; Light blue = *T.a. setosus*; Red = *T.a. multiaculeatus*; Purple = *T.a. lawesii*.

1.7 Echidnas in the illegal wildlife trade

Due to the challenges in breeding these species in captivity, and the interest in having them in zoo collections, there is evidence that a 'black market' in echidnas exists (Beastall and Shepherd 2013; Janssen and Chng 2017). There is increasing evidence that echidnas are at risk of ending up in the zoo trade after being laundered as 'captive bred'. Considering the numbers that are being offered for sale, and based on breeding records from Australian zoos, it is likely they have been illegally taken from the wild rather than legitimately captive bred (Beastall and Shepherd 2013). The non-government organisation TRAFFIC, which monitors and works globally on wildlife trade, estimated that over 70 echidnas were claimed to be captive bred and traded in 2014. Janssen and Chng (2017) studied the biological parameters, such as number of females reproducing, annual number of breeding events, and survival rate of 129 species (including echidnas) in Indonesia's captive breeding production plan. These were compared with biological parameters from the literature and expert opinion to determine if the quotas allowed were realistic. For 99 out of 129 species it was concluded that the quotas were based on inaccurate parameters, and for 38 species, including the short beaked echidna, the quota exceeded the number of animals that could be realistically bred based on the biological parameters. In addition to the animal welfare issues associated with the illegal trade, this is a potential threat to the local echidna populations that are being targeted (Beastall and Shepherd 2013). While presently, short beaked echidnas are not listed as endangered, due to limited information recorded about the New Guinea subspecies, reduction of threat should still be a priority. Additionally, there is recent anecdotal evidence of trade in echidna parts reported within Australia, though this is not believed to be widespread (Lavelle, S, personal communication, June 2020.)

The short beaked echidna is not listed by CITES, creating challenges when it comes to the trade of these animals (Beastall and Shepherd 2013; CITES 2015). The echidna is also not expressly covered by the Australian legislation *(EPBC Act 1999)*, other than general protection as a native species, as the short beaked echidna is currently categorised as neither endangered or threatened species on the IUCN Red List. Therefore, despite being a well-known Australian species, significant research will be needed to implement any further protection.

As potential subspecies are geographically restricted, being able to genetically differentiate them will help to pinpoint potential localities where the short beaked echidnas are being poached from the wild. If the phylogeography of the echidna can be established and delineated, it would facilitate clear parameters for genetic testing on illegally traded echidnas. These could be targeted and give greater information about the area from which a particular animal may have been taken. Identifying poaching hotspots can better guide enforcement and protection of these populations. This can be investigated through the use of both mitochondrial DNA markers, as well as nuclear DNA markers.

Additionally, if relatedness between individual captive bred echidnas and their relatives are known, a pedigree can be used as a reference when an echidna is sold and claimed as ‘captive bred’. This could provide essential information to determine the legitimacy of these claims. However, to further complicate this, as is the case for many species, there are limited molecular data, at either the species or population level. Additional information on molecular variability in wild short beaked echidna

populations and in legitimate captive breeding programs is therefore required to build a solid foundation for this sort of research and to assist prevention of the illegal wildlife trade in a species such as the echidna.

Knowing the sex of an echidna can be beneficial information for zoos, due to adult echidnas being sexually dimorphic. To determine sex, it often requires an invasive exam, but recently a PCR based genetic test has been developed (Perry *et al.* 2019). The previously mentioned difficulties in captive breeding are a present concern, and therefore resolving the sex of an echidna could help inform captive breeding programs. Additionally, knowing the sex could be useful intelligence for when an echidna is traded, particularly when parentage and relatedness are important evidence for proving this trade is taking place. To use such a test in a wildlife forensic context, it would be ideal to have a test validated and make sure that it is a test of high quality and allows for minimal ambiguous results.

1.8 Study aims

My aim was to use molecular methods to develop a genetic toolbox that can be implemented in instances where a short beaked echidna is traded. The specific aims of each chapter are outlined below:

Chapter 2

This chapter aimed to develop a simple test that could distinguish between short beaked echidnas from New Guinea, and those from Australia. I sought to do this through the use of a mitochondrial DNA region that could be amplified from lower-template DNA. I also aimed to test non-invasive DNA sampling methods from echidna quills and apply the source region test on the extracted DNA. Lastly, it was

aimed to validate the method of source region determination to apply this in a forensic context.

Chapter 3

This chapter aimed to investigate further into the subspecies and phylogeography of the short beaked echidna and elaborate on what was unable to be resolved by Chapter 2. I sought to do this through the development of a SNP marker set that would potentially allow for further fine-scale detail and discrimination of the subspecies included in my study.

Chapter 4.

This chapter aimed to confirm the relatedness of individual echidnas with known pedigree. To do this my goal was to develop a robust and reliable marker set, which would be the first of its kind for the short beaked echidna. I aimed to test if this marker set could differentiate individuals by investigating the relatedness of those bred in captivity, as well as analyse and compare that with a wild population.

Chapter 5.

For this chapter I aimed to test two primer sets used to determine the sex of an echidna and develop them for use as a RT-PCR technique. To do this I aimed to use standard validation criteria to test the limits of the sex determination test to distinguish if it could be used in a wildlife forensic context.

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Chapter Two: DNA based method for determining source country of the short beaked echidna (*Tachyglossus aculeatus*) in the illegal wildlife trade

2.1 Abstract

The illegal trade in wild animals being sold as 'captive bred' is an emerging issue in the pet and zoo industry and has both animal welfare and conservation implications. DNA based methods can be a quick, inexpensive, and definitive way to determine the source of these animals, thereby assisting efforts to combat this trade. The short beaked echidna (*Tachyglossus aculeatus*) is currently one of the species suspected to be targeted in this trade. As this species is distributed throughout Australia and in New Guinea (currently comprising of five recognised sub-species), this project aimed to develop a DNA based method to definitively determine the source region of an echidna and explore the use of non-invasive sampling techniques. Here we use non-invasively sampled echidna quills and demonstrate the extraction of mitochondrial DNA and amplification of a region of the mitochondrial genome. Phylogenetically informative markers for analysis of a 322 bp segment of the D-loop region were developed, and subsequently validated, using animals with known source locations allowing us to reliably distinguish between echidnas from New Guinea, and Australia. This research presents the first validated forensic protocols for short beaked echidnas and will be an integral tool in understanding the movement of animals in this emerging trade.

2.2 Introduction

The illegal trade in protected wildlife is a serious and widespread crime, and was estimated to be worth USD $7-23 Billion in 2014 (Nellemann *et al.* 2014). Within this, the laundering of wild-caught animals as 'captive bred', sometimes with falsified or no supporting paperwork or genetic data, has been identified as an emerging issue (Beastall and Shepherd 2013; Bush *et al.* 2014; Frankham *et al.* 2015; Nijman and

Shepherd 2015; Hogg *et al.* 2018). There is increasing evidence that monotremes, including the short beaked echidna (*Tachyglossus aculeatus* but with five recognised subspecies across Australian and Papua New Guinea) and the long beaked echidna (*Zaglossus spp.),* are at risk of becoming part of this trade (Beastall and Shepherd 2013; Janssen and Chng 2017; Shepherd and Sy 2017). Monotremes have a complex mode of reproduction, which is notoriously difficult to replicate within captivity (Rismiller and Seymour 1991; Morrow *et al.* 2009; Wallage *et al.* 2015). Historic zoo records show that until 2009 there were less than ten short beaked echidnas born in captivity in Australia, with a low survival rate past 18 months of age (Ferguson and Turner 2012; Wallage *et al.* 2015). The quotas from the Indonesian Captive Breeding Protection Plan (Beastall and Shepherd 2013; Janssen and Chng 2017) in recent years have included approximately 70 echidnas in 2014, 150 in 2015, and 50 in 2016, however based on the low breeding success recorded by Australian zoos, it is possible the large number of Indonesian captive bred animals were supplemented by wild poached individuals, potentially from the New Guinea subspecies of short beaked echidnas (*T.a. lawesii)* (Beastall and Shepherd 2013; Wallage *et al.* 2015; Janssen and Chng 2017).

A vast number of animal and plant species are targeted in the illegal wildlife trade and, even in well-resourced countries, investment in enforcement and investigation of this trade is not at a commensurate level compared to 'human-victim' crimes (Broad *et al.* 2003). Furthermore, in developing countries, there are often additional challenges in conducting adequate compliance investigations and appropriate forensic testing for prosecution (Broad *et al.* 2003)**.** Often tests or vouchered reference data do not exist to confirm biogeographic provenance or alleged pedigree, or even confirm

species (Broad *et al.* 2003). Providing data for evidentiary or intelligence purposes in a meaningful timeframe can assist in maintaining momentum within an investigation and give direction on what should be done with seized animals requiring rehoming. Therefore, the establishment of non-invasive sampling methods, PCR protocols, and analyses that can be performed quickly and inexpensively within the laboratory can be of significant benefit to an investigation in both developed and developing countries.

Non-invasive sampling has many advantages, including reducing handling stress, and does not require anaesthetic or a vet to be present to take a sample. Previous studies have successfully extracted DNA using non-invasive methods from feathers, hair, scats and scales (Piggott and Taylor 2003; Piggott 2005; Suenaga and Nakamura 2005; Waits and Paetkau 2005; Hogan *et al.* 2008; du Toit *et al.* 2016), these methods can however, be limited by quality of the DNA that is extracted (Taberlet *et al.* 1999; Piggott 2005; Hogan *et al.* 2008). In wildlife forensic science, non-invasive sampling is particularly useful as seizures of live animals often occur without the presence of veterinarians and it may not be possible or time efficient to take a blood sample from an animal in an investigation (du Toit *et al.* 2016). Echidna quills are made of keratin, similar to hair, and can be plucked, or are naturally shed (James and Amemiya 1998). Quills also have a larger surface area than hair, and therefore retain more epithelial cells for DNA extraction.

Analysis of mitochondrial DNA (mtDNA) is well established as an effective tool for species identification, phylogenetic studies, and determining source populations; an essential component of not only wildlife forensics, but species conservation (Johnson

et al. 2014). Studies of mammalian evolutionary history, phylogenetics and phylogeography often utilise mtDNA analysis (Brown *et al.* 1979; Neaves *et al.* 2012; Ogden and Linacre 2015). For trafficked species that have a broad geographic distribution covering multiple countries, it can be important to establish the source, so that the correct jurisdiction and legislation involved can be identified, for example whether local wildlife legislation or legislation enforcing CITES should be enacted (Ogden and Linacre 2015). Short beaked echidnas are documented as having both a New Guinea and Australian distribution, with 4 Australian subspecies (*T.a. aculeatus* (East coast of mainland Australia), *T.a. acanthion* (Western Australia), *T.a. setosus* (Tasmania), *T.a. multiaculeatus* (Kangaroo Island)), and one New Guinea subspecies (*T.a. lawesii*) subspecies (Griffiths 1978; Augee *et al.* 2006). However, currently there is no published phylogeographic data to substantiate these delineations. Definitively identifying the region of origin of any traded echidna is the focus of this study, as this would benefit both investigative and enforcement efforts in order to identify the source of individuals poached from the wild. While the mtDNA regions CO1 and Cytochrome B are often used in wildlife forensics, because they provide useful interspecific resolution (Parson *et al.* 2000; Hsieh *et al.* 2003; Lee *et al.* 2008; Ferri *et al.* 2009; Wilson-Wilde *et al.* 2010), to determine source populations we require a genetic region that provides sufficient intraspecific resolution. Our candidate region, the mtDNA 'control region' or D-loop, is a hypervariable region of the mitochondrial genome which is often used in intraspecific or phylogeographic studies (Fumagalli *et al.* 1997; Budowle *et al.* 1999; Neaves *et al.* 2012).

This work aimed to evaluate DNA extraction methods using both plucked and shed quills of the short beaked echidna, utilising commonly available protocols and kits.

Further, to strive for recommended best practice we aimed to develop validated protocols for amplification of the phylogenetically informative region of the D-loop that effectively differentiates between echidnas of New Guinea or Australian origin, and determine the source region of any trafficked and seized short beaked echidnas.

2.3 Methods

2.3.1 Quills - sampling, storage and gDNA extraction methods

Shed and plucked short beaked echidna quills were collected from echidnas in captivity by zookeepers, as well as from one Australian Museum specimen (M.47764.004). Within each extraction trial, five quills were used - two from M.47764.004 which had been preserved in ethanol upon collection, and three from captive echidnas; two plucked and one shed (for detailed sample information see Appendix A1). The quills were transported to the laboratory in zip lock bags then frozen at -20°C on arrival.

Four different commonly used and readily available extraction kits were trialled (Table 2.1) to test the success of extracting DNA and the quantity of yield. Trials using purchased extraction kids were followed as to the manufacturer’s instructions, and the salting out technique as to instructions from Sunnucks and Hales (1996). Approximately one centimetre from the root of the quill (the weakest and thinnest part) was cut using tin snips to use for the extraction (Figure 2.1). DNA extractions were quantified using the Qubit 2.0 Fluorometer High Sensitivity Assay (Thermo Fisher Scientific, USA).

Table 2.1 Methodology for each extraction trial

Trial	*Method*	*Quill Roots Used Per Extraction*
1.	ISOLATE II Genomic DNA Kit (Bioline, Australia) animal tissue protocol. Automated extraction.	2x M.47764.004; M.48044.001; M.48042.001; M.48045.001
2.	ISOLATE II Genomic DNA Kit (Bioline, Australia), animal tissue protocol with the addition of 20µL of 1M DTT during the lysis stage.	2x M.47764.004; M.48044.001; M.48042.001; M.48045.001
3.	QIAamp DNA Investigator Kit (QIAGEN, Australia), following the Hair and Nails protocol.	2x M.47764.004; M.48041.001; M.48040.001; M.48043.001
4.	Salting out technique, following protocol published in Sunnucks and Hale (1996)	2x M.47764.004; M.48041.001; M.48040.001; M.48043.001

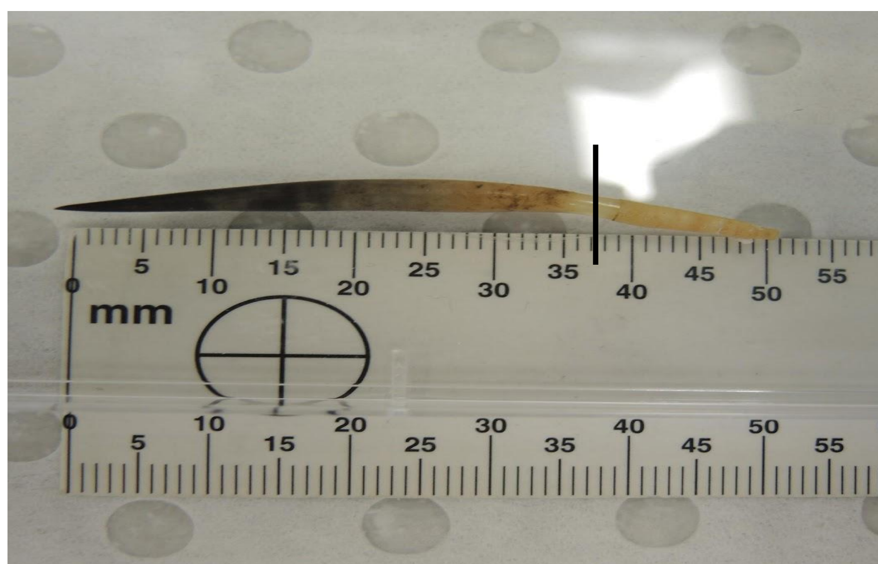

Figure 2.1 Example of quill used. Black line depicts where the quill was cut (~1cm from the root end of the quill)

2.3.2 Samples for phylogeographic analysis

Due to the difficulty of obtaining quill samples from New Guinea echidnas, only quill samples from Australian animals were tested. However, four echidna tissue samples from New Guinea, specifically Papua New Guinea, were included as part of the phylogeographic validation, along with one sample from Moa Island, Torres Strait, and one sample from a captive animal from San Diego Zoo, obtained legally from an Indonesia Zoo, with suspected New Guinea parents. Forty-one additional tissue and blood samples were included to give a wider variety of Australian locations. A total of 50 quill, tissue, blood, skin, or gDNA samples, with known collection localities were used. These animals were sourced from both museum/university collections (30 samples) and zoo collections (20 samples) (Table A1). For the reference samples, DNA extractions were carried out using either the ISOLATE II Genomic DNA Kit animal tissue protocol (Bioline, Australia), the ChargeSwitch Forensic DNA Purification Kit (Thermo Fisher Scientific, USA), or a salting out technique (Sunnucks and Hales 1996). DNA concentrations were determined using Qubit 2.0 Fluorometer High Sensitivity Assay (Thermo Fisher Scientific, USA).

2.3.3 Amplification conditions

Quill extractions were initially checked by amplifying a region of Cytochrome B (14841 – 15149), using the following universal primers; M5 – AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA and M6 AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA (Kocher *et al.* 1989). Primers to amplify a 430 bp region (15408 – 15859) of the short beaked echidna D-loop were then designed using Oligo 7 (Rychlik 2007) using the *Tachyglossus*

aculeatus whole mitochondrial genome (AJ303116.1) as a reference. The Primer sequences developed as part of this study are as follows: TacCRF – 5’-ACCATCAGCTCCCAAAGCTGA -3’ and TacCRR – 5’-CTTGAAGGGGAATTACCAGAGG-3’.

One sample from PNG (M5966) and the sample from Moa Island, Torres Strait (M4954) were extracted from historic museum study specimens (Australian Museum Mammalogy Collection). Due to the age and storage conditions of these samples, extracted DNA was found to be highly degraded. To amplify these samples, two additional primer sets were designed that would amplify the required region in two overlapping segments (15498 – 15698 and 15696 – 15864). These primer sequences are TacCR2F – 5’- TGCATTCATCTTTTATCCCCATAC 3’, TacCR2R – 5’-TAATCTGTCAGAACCTCAATTATG’, and TacCR3F – 5’-AACATAATTGAGGTTCTGACAG- 3’, TacCR3R – 5’-GTTCTTGAAGGGGAATTACC 3’.

Polymerase Chain Reactions (PCRs) were carried out in 25µL reactions, containing 10ng of DNA, 1x Bioline MyTaq Reagent Buffer, 10µM for each primer, and 5U of Bioline MyTaq DNA polymerase. PCRs were carried out on an Eppendorf MasterCycler pro S under the following conditions: 3 minutes at 94°C, 38 cycles of 94°C (20s) denature, 60°C (40s) annealing, and 72°C (40s) extension, with a final extension cycle of 72°C for 5 minutes. To amplify the two skin samples, touchdown conditions 55°C to 50°C (1°C step down per cycle) were employed for the annealing step. PCR success was checked using a 1% Agarose Gel and reactions were cleaned using Affymetrix ExoSAP-IT. Sanger sequencing was carried out by the Australian

Genome Research Facility (AGRF), Sydney on an AB3730 xl. Raw sequences were edited with reference to chromatograms using Sequencher version 5.3.

2.3.4 Analysis

D-loop sequences from all reference and quill samples were aligned using ClustalW in MEGA version 7.0 software (Kumar *et al.* 2016). To include all samples, sequences were edited to be the same length and analysis was conducted on a 322 bp fragment. The corresponding D-loop region from the western long beaked echidna (*Zaglossus bruijnii*; Accession number: AJ639865.1) was used as an outgroup. To perform phylogenetic analysis on all haplotypes generated, an appropriate model of evolution; T92+G, was determined in MEGA 7.0, using the Bayesian Information Criterion. Maximum likelihood trees were built using MEGA 7.0, Statistical confidence was calculated using 1000 bootstrap resamples. Bayesian inference of phylogeny analysis was conducted in MrBayes 3.2 (Ronquist and Huelsenbeck 2003). The analyses were run using default settings for priors. The chains were run for 10000000 generations and sampled every 1000 generations to obtain 2364 sampled trees. Tracer v1.6 (Rambaut and Xie 2014) was used to check for chain convergence and adequate Effective Sample Size (> 100). Posterior probabilities (decimals) were used to assess the level of branch support.

2.3.5 Validation

Validation was carried out based on the following characteristics: 1) *reproducibility/ repeatability*, 2) *limit of detection* 3) *specificity*, 4) *phylogenetic resolution* (as described above)

To test for *repeatability*, 50 samples were amplified, on four different thermocyclers. A temperature validation was conducted with eight samples undergoing amplification with the annealing temperature altered to both +/- 1.5°C of the regular annealing temperature (60°C). A blind study conducted by a different analyst, using regular PCR and sequencing conditions was also carried out using four unknown samples (two from PNG and two from Australia). *Limit of detection* was tested using 1 in 10 (1ng), 1 in 100 (0.1ng), and 1 in 1000 (0.01ng) serial dilutions of eight DNA samples, previously quantified using the Qubit 2.0 Fluorometer. Species *specificity* was analysed using the following species (Table A2) from the Australian Museum's Mammal, Bird and Fish collections; Western long beaked echidna *(Zaglossus bruijnii)* (M.47975.001), Platypus *(Ornithorhynchus anatinus)* (M.35614.001), Bilby *(Macrotis lagotis)* (WGM118-186), Little Penguin *(Eudyptula minor)* (B10002), Agile Antechinus *(Antechinus agilis)* (AM205-7), Eastern Crevice Skink *(Egernia mcpheei)* (R.150174.001), White-browed Woodswallow (*Artamus superciliosus)* (O.71701.001), Queensland Grouper (*Epinephelus lanceolatus)* (I.39681.003) and Rock Cod (*Lotella rhacina)* (I.31253.130). These samples were chosen either because they were closely related species, or to represent a range of genera and potential contaminants for our laboratory. They were run with the regular PCR conditions as well as both +/- 1.5°C of the optimal annealing temperature. All samples within the validation study were checked for PCR success using a 1% Agarose Gel and reactions were cleaned using Affymetrix ExoSAP-IT. Sanger sequencing was carried out by the Australian Genome Research Facility (AGRF), Sydney on an AB3730 xl. Raw sequences were edited with reference to chromatograms using Sequencher version 5.3 and species

2.4 Results

2.4.1 Quill extractions

DNA was successfully extracted from all quills used in each trial. The Qubit quantification indicated that the extraction Trials 1 and 2 both produced comparable DNA concentrations, on average <1ng/µL. Extraction Trials 3 and 4 both had quantification results that were higher than the previous two trials, with the average for Trial 3 = 24.2ng/µL and the average for Trial 4 = 3.26ng/µL. DNA was extracted successfully from both plucked and shed quills.

Comparison of sequences generated in this study to publicly available sequences to confirm species identification, returned 98 - 100% identity to the short beaked echidna (*Tachyglossus aculeatus* GenBank accession AJ303116.1) mitochondrial genome.

2.4.2 Phylogeographic analysis

A 322 bp region was sequenced from all quills and reference samples analysed. Three unique haplogroups were identified in the 50 samples; two Australian haplogroups and one PNG haplogroup. Four out of six samples from PNG and Moa Island shared the same haplotype, with the East PNG and Moa Island samples grouping closely with the West PNG samples. There were no control region haplotypes shared between samples of Australian and PNG origin. Both the maximum likelihood analysis and Bayesian Inference resolved trees with similar topologies showing low lineage resolution for most of the Australian reference samples, with the samples from PNG forming a distinct lineage. The samples from Western Australia also showed distinct lineage within in the sampling of this project, whereas the east coast, Tasmania, and

Kangaroo Island samples show little variation. The bootstrap value for the clustering of the PNG samples is 83% shown in the Maximum Likelihood tree (Figure 2.2), and 0.9901 in the Bayesian inference tree (Figure 2.3) indicating moderate to strong support for this lineage.

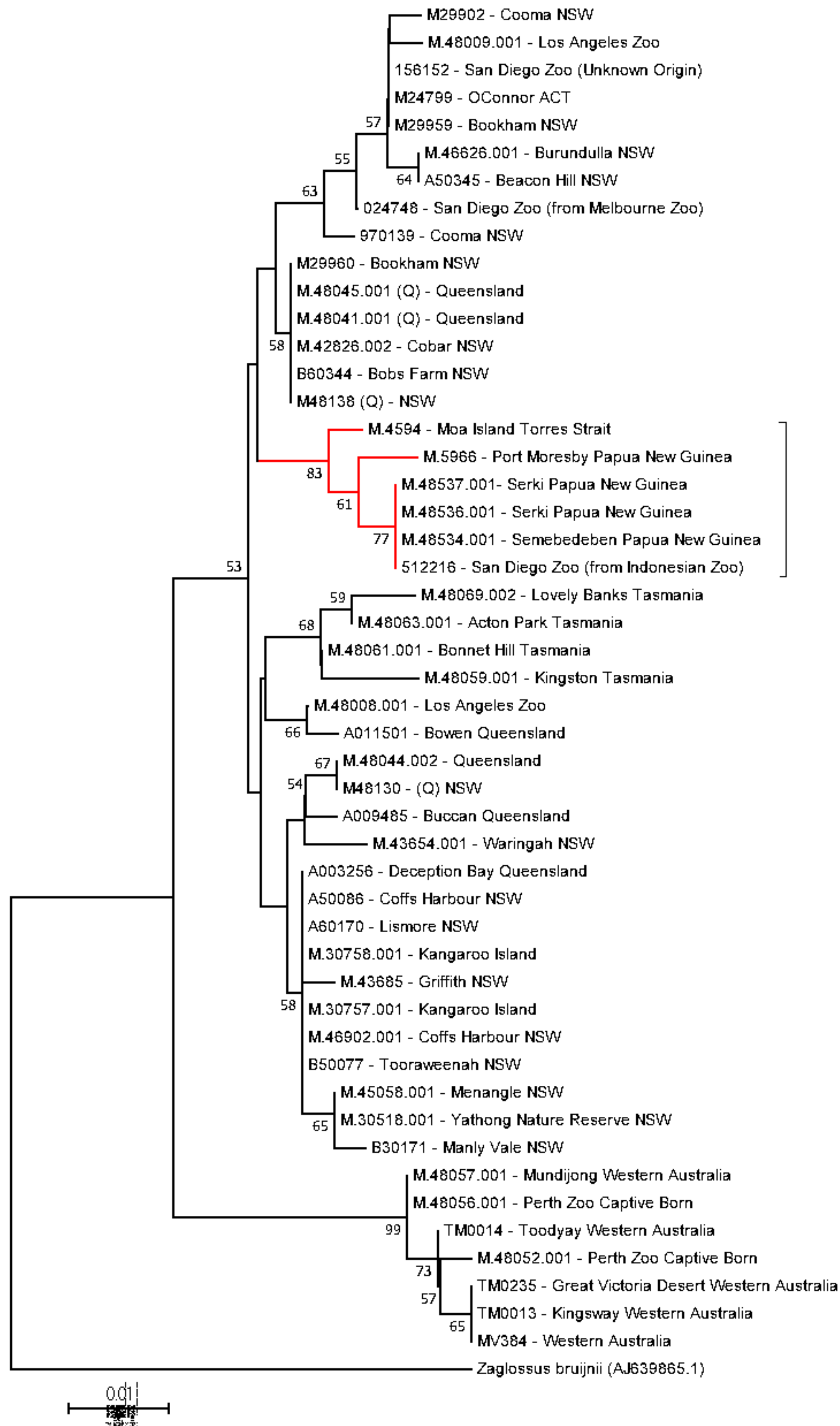

Figure 2.2 Maximum likelihood phylogenetic tree representing D-loop sequences from both Australian and Papua New Guinea short beaked echidnas. Bootstrap values (percentage) are listed above the nodes and no values less than 50 were shown. PNG samples shown in red. Respective museum or zoo numbers are used for sample identification.

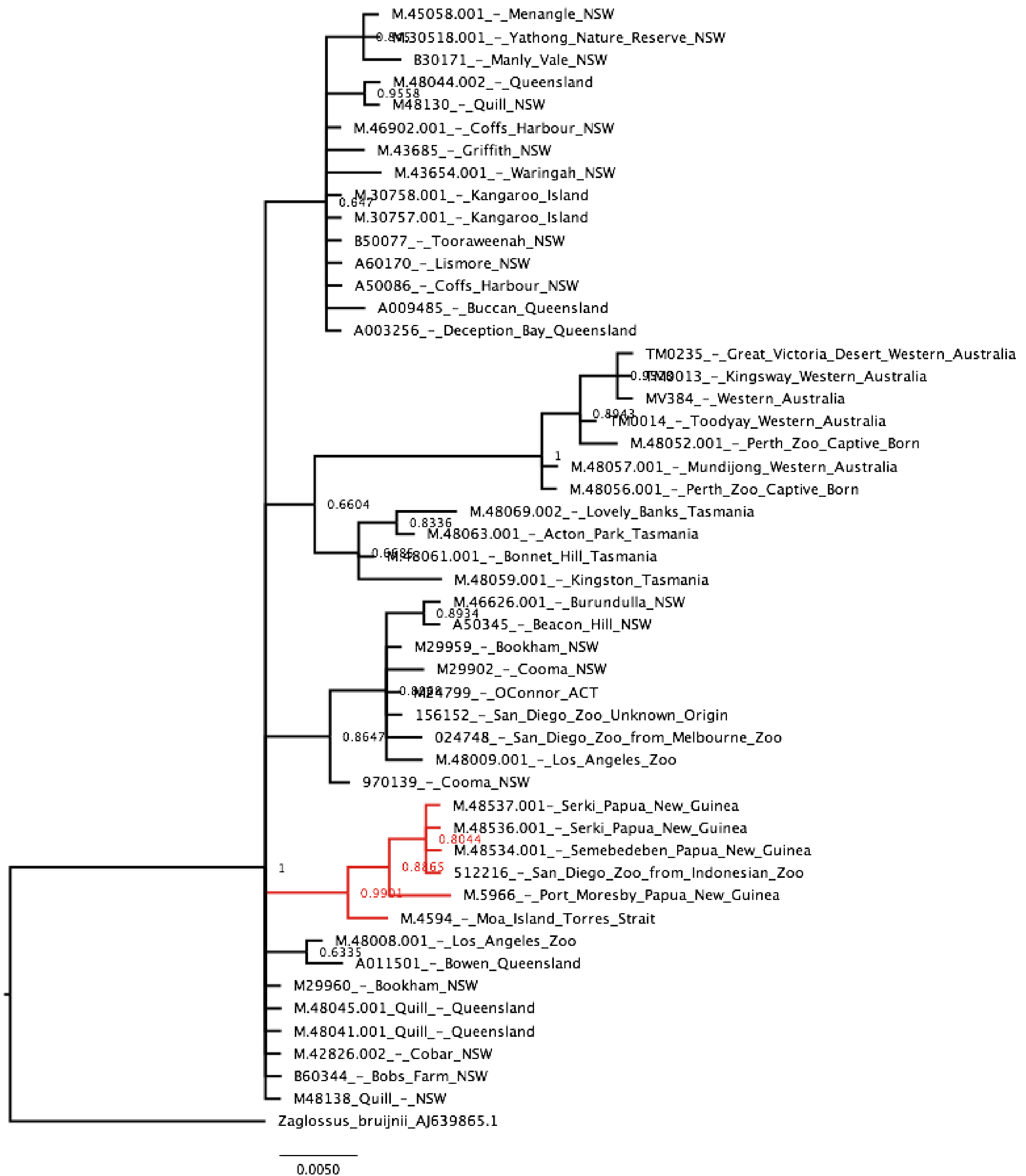

Figure 2.3 Bayesian Inference representing D-loop sequences from both Australian and Papua New Guinea short beaked echidnas. Posterior probabilities (decimals) are listed above the nodes and no values less than 50% are shown. PNG samples shown in red. Respective museum or zoo numbers are used for sample identification.

2.4.3 Validation

Reproducibility/repeatability: All samples were successfully amplified across the four different thermocyclers and both +1.5ºC and -1.5ºC annealing temperatures. The analyst conducting the blind test successfully determined the source region of all four unknown samples. *Limit of detection*: The 1ng and 0.1ng dilutions were also successfully amplified however the 0.01ng dilution samples were not able to be amplified. *Specificity*: The western long beaked echidna sample was the only species that was amplified and generated a sequence using these primers. There is distinct phylogenetic differentiation between the two genera of echidna at this region so there is no chance of incorrect generic identification.

2.5 Discussion

This study represents the first validated forensic protocol to successfully determine source region of the short beaked echidna using a short mtDNA D-loop region. Additionally, we have evaluated several commonly used extraction methods to obtain mtDNA from echidna quills. Our results demonstrate this non-invasive method is suitable for extracting and sequencing phylogenetically informative mtDNA down to a limit of detection of 0.1ng, which can be used for evidentiary or intelligence purposes to aid in combatting the illegal trade of the short beaked echidna.

The four extraction methods all produced suitable DNA quantity to successfully amplify the D-loop region. However, the QIAGEN QIAamp Investigator Kit 'Hair and Nails' protocol (following manufacturer's instructions) produced the most consistent results across both shed and plucked quills. While this is one of the more

costly kits, it is widely available, straightforward to use and produces consistent extracts with higher DNA concentrations on the whole. Importantly it would be the recommended kit for extraction from quills if blood or tissue were unavailable. With further optimisation, this kit has the potential to be used for nuclear DNA sequencing and genotyping, as has been shown with other non-invasive samples such as feathers, and preliminary results have indicated this (data not shown) (Sefc *et al.* 2003; Chan *et al.* 2007). In addition, there was also no remarkable quantitative difference between plucked and shed quills in the samples available for this study. Therefore, confirming that shed and non-invasively collected quills are appropriate to use for such a test, including those collected from enclosures containing seized animals. Further, sufficient template for mtDNA sequencing was recovered from both plucked and shed quills, however as these are from a zoo enclosure, further testing would be needed to investigate shed quills in a field context. This result provides insight into the benefit of this type of sampling, indicating there is the potential to collect shed quills from an echidna's natural habitat within a certain time frame. For many species in the illegal wildlife trade, including pangolins, tigers, and birds, non-invasive sampling has proved beneficial (Wan and Fang 2003; Speller *et al.* 2011; du Toit *et al.* 2016), and the methods presented here provide a sample preparation protocol that can also be implemented in better understanding the short beaked echidna trade.

Of the six samples analysed from PNG and Moa Island, four of the PNG samples shared the same haplotype. Three of these samples were from the same location in the Western Province of Papua New Guinea (in or around the village of Serki), but the fourth sample's specific location unknown because it was captive animal, with parents suspected to have come from New Guinea. The PNG sample from Port

Moresby (East PNG) while not an identical haplotype, still consistently grouped closely with the Western PNG samples with strong support, as did and the Moa Island sample (Figures 2.2 and 2.3). While we acknowledge that the sample size is small, the low haplotype diversity within these PNG samples suggests that, at least at the mtDNA level, there is limited structure in short beaked echidnas within the New Guinea population of short beaked echidnas, similar to what has been observed in eastern Australian echidnas (Figure 2.4).

Figure 2.4 Map of samples used in phylogeographic analysis. PNG/Torres Strait (Moa Island) samples in red, and Australian in black. Location of sample SD-2 (San Diego Zoo with Indonesian parents) unknown so excluded from map. Map made in QGIS software (QGIS, 2017).

For further insight into the extent the Torres Strait poses as a biogeographic barrier, it would be highly desirable to obtain further samples from echidnas across their New Guinea distribution and from the northern region of the Cape York Peninsula in Australia. All attempts were made to obtain DNA from the one Cape York study specimen available in the Australian Museum Collection, however we were unsuccessful in extracting DNA from this study specimen, which is likely related to its age and preservation method (Frankham *et al.* 2017).

New Guinea and Australia have been isolated for approximately 8000 years, a separation which has acted as a significant biogeographic barrier for many species that have distributions throughout the two countries (White *et al.* 1982; Osborne and Christidis 2001; Rawlings and Donnellan 2003; Malekian *et al.* 2010). Phylogenetic research on the green python (*Morelia viridis*), a species also prominent in the illegal wildlife trade, was also successfully resolved using the D-loop region (Rawlings and Donnellan 2003) and, as in our study, Australian and New Guinean pythons were able to be differentiated phylogeographically. The identification of geographic provenance of a species is often requested by enforcement agencies and can be of particular importance for CITES listed species with cross border distributions. It is of utmost importance to develop these tests that are validated if the end-point is to present this data in a legal context (Linacre 2009; Linacre and Tobe 2013; Ogden and Linacre 2015). The validated work presented here is reproducible/repeatable, specific to the echidna group (genera *Tachyglossus* and *Zaglossus*), with a limit of detection of 0.1ng (Linacre 2009). While this test amplifies both echidna genera, because this test also requires sequencing, a simple BLAST search under carefully specified criteria, or phylogenetic analysis using sequences from vouchered reference material (GenBank

accession AJ303116.1) can differentiate clearly between these two genera, and therefore does not interfere with determining species or source location (i.e. Australia or Torres Strait/PNG). Our phylogeographic analyses also indicate potential genetic variation between east and west Australian samples, however, this may be an artefact of sampling as we were unable to include many samples from central Australia. Identifying to state or population level within Australia may also be useful for state agencies, in cases where animals are found illegally in captivity within Australia. However, given that there was lack of variation along the east coast of Australia, this may need higher resolution data such as SNPs or microsatellites to obtain clarity, nor was it the focus of this study. We saw no phylogeographic structuring between the east coast, Tasmania, and Kangaroo Island populations to support their classification as separate subspecies (east coast: *T.a. aculeatus;* Tasmania: *T.a. setosus;* Kangaroo Island: *T.a. multiaculeatus*). Additional sample collection and analysis would be required to further investigate phylogeographic patterns of the Australian populations, and infer evolutionary history of the species.

This study demonstrated that non-invasive sampling, and subsequent DNA extraction from short beaked echidna quills, is possible and can be carried out using a range of commercially available DNA kits. Sample preparation from echidna quills can be useful for many cases, particularly in an illegal trafficking scenario where blood or tissue is unable, or unfeasible to be sampled. In addition, we were able to develop and validate phylogenetically informative markers to determine source region of any suspect individuals and direct any following enforcement actions. This also has potential to guide the establishment of presumptive indicators that could be used to flag the potential measures that could be put in place in areas where evidence shows higher levels of trafficking, as well as used by zoos to guarantee legitimate status of

any captive echidnas that may come into their collections. Implementation of the methods presented here will provide important intelligence about this trade, and aid in analyses involving the illegal trafficking of this iconic species.

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Chapter Three: Investigating subspecies of the short beaked echidna (*Tachyglossus aculeatus*) using single nucleotide polymorphisms for use in a wildlife forensic context

3.1 Abstract

Determining the geographic origin or source population of a species is often a key question asked within wildlife forensics, to investigate trade routes and poaching hotspots and gather information about the subspecies or populations of interest. The short beaked echidna (*Tachyglossus aculeatus*) is one species that is being sold as captive bred, but there is increasing evidence to suggest they have actually been taken illegally from the wild, likely from locations in New Guinea. There are five named subspecies of the short beaked echidna, all named based on minor morphological traits and/or geographical isolation; (*T.a. aculeatus, T.a. acanthion, T.a. setosus, T.a. multiaculeatus and T.a. lawesii)*. To date there has been no published phylogeographic study or genetic investigation into whether the subspecies are supported by molecular evidence. The mitochondrial test described in Chapter 2, was able to differentiate between New Guinea (*T.a. lawesii*) and Australian echidnas but lacked sufficient resolution for the other four subspecies. Here I aimed to develop a robust SNP marker set to investigate the level of phylogeographic structure in the four subspecies included in this study. I discovered some level of structure between Tasmanian (*T.a. setosus*) echidnas and the other three subspecies, as well as an east/west divide. There was a weak but significant correlation between genetic and geographic distance among the east coast populations (*T.a. aculeatus*). These SNPs also show clear differentiation between New Guinea and Australian echidnas, mirroring what was found in Chapter 2. I did not find any evidence to support taxonomic revision of subspecies but note that a large proportion of geographic locations were not included in this study. This chapter provides recommendations for

further sampling locations that should be included if a detailed phylogeographic study is to be carried out.

3.2 Introduction

Describing and recognising subspecies is often contested, and has led to debate about the necessity and implications of this taxonomic level (Wilson and Brown Jr 1953; Mayr 1982; O'Brien and Mayr 1991; Gippoliti and Amori 2007; Patten 2015; Weeks *et al.* 2016). Subspecies can often provide useful classification for different subpopulations within species that are morphologically or geographically different. Subspecies can be particularly useful for species conservation, especially if they represent distinct clades or lineages which are often observed across broad geographic ranges (O'Brien and Mayr 1991; Phillimore and Owens 2006; Luo *et al.* 2008). Recognition of subspecies descriptions can be contentious, especially depending upon what it is based, for example on morphology or geographic location, or molecular data (Phillimore and Owens 2006; Patten 2015). Advancements in genetic techniques, combined with reducing costs to make them more accessible, have increased the volume of population and phylogeographic data being generated for many species (Phillimore and Owens 2006). These have sometimes led to an increased use of alternate taxonomic levels, including subspecies (Phillimore and Owens 2006; Westerman *et al.* 2016) but with questionable benefit. Evolutionarily significant units (ESUs; independently evolving units of genetic variation) and management units (MUs; demographically independent but not necessarily evolutionarily independent) are often used in conjunction with subspecies or species recognition, and are often characterised in similar ways (Moritz 1994). Subspecies are commonly used in mammalian conservation, for example Eldridge *et al.* (2017), used molecular methods to separate the tammar wallaby (*Notamacropus eugenii* (*N.e. eugenii* in SA, and *N.e.*

derbianus in WA)). Similarly, Frankham *et al.* (2016) described subspecies of long-nosed potoroo (*Potorus tridactylus*) (based on geographically isolated mitochondrial DNA lineages which showed discordance from and the nuclear DNA. These authors argued this would be useful for conservation of genetic diversity within this species. Many other studies have employed molecular data to define subspecies for conservation or confirm subspecies described off morphology alone (Perez-Ponce de Leon *et al.* 1998; Firestone *et al.* 1999; Feulner *et al.* 2004; Degner *et al.* 2007; Janecka *et al.* 2017). Conversely, many other studies have not found molecular structure congruent with subspecies descriptions, this is more common with highly mobile species, for example the grey duck, sparrows, diamondback terrapins, and snowy plovers (Rhymer *et al.* 2004; Funk *et al.* 2007; Hart *et al.* 2014; Walsh *et al.* 2017).

Population and phylogeographic studies are common in biological conservation and molecular ecology research, and in a conservation genetics context are usually carried out to determine how to best manage the remaining genetic diversity within a species. They also have additional applications for those species threatened by illegal wildlife trade as they can be used for forensic investigative purposes (Ogden 2010; Linacre and Tobe 2013; Johnson *et al.* 2014). The ability to resolve an identification to the species or subspecies level, or its geographic origin (Zhao *et al.* 2019) can aid in determining if illegal activity has occurred as well as help build up intelligence around illegal trade for certain species (Johnson 2010; Johnson *et al.* 2014; Ogden and Linacre 2015). Furthermore, species level ID can be used in concert with tests that can assign parentage and relatedness between individuals or determine if an animal has been bred in captivity or is wild-caught (Coetzer *et al.* 2017).

Phylogeographic studies for wildlife forensic application have been developed across a range of taxa including birds, reptiles, and mammals (Ciavaglia *et al.* 2015; Coetzer *et al.* 2017; Nash *et al.* 2018; Zhao *et al.* 2019). These only represent a small number of species in the illegal trade; the rarity of such studies in a wildlife forensics context are due in part to the intensive resources required to sample robustly across a species range. Particularly those that are either widespread or cryptic, and therefore can be difficult to sample. In addition to sampling, having a robust and reliable set of genetic markers is crucial to understanding these relationships both at a broader geographic level as well as at finer population and individual levels.

Genetic analysis of wild populations has become more common with the advancement and reduced costs of molecular tools. This can be done using a range of genetic markers such as mitochondrial DNA, microsatellites, or single nucleotide polymorphisms (SNPs). Mitochondrial DNA is often useful for phylogeographic analysis due to its high mutation rate and lack of recombination, plus the ease of amplification and sequencing. If thorough analysis is needed, it is often via microsatellite analysis due to the powerful discrimination they can offer, combined with their relative ease of development (Butler *et al.* 2007; Ross *et al.* 2014). However, SNPs are becoming an increasingly popular choice within research due to their broad representation across the genome as well as decreases in cost and the wide range of tools available to develop and analyse these markers (Amorim and Pereira 2005; Butler *et al.* 2007; Städele and Vigilant 2016; Huisman 2017; Lemopoulos *et al.* 2019). Recent studies have shown SNPs have high levels of discrimination when analysing intraspecific differentiation, as well as individualisation (Kidd *et al.* 2006; Butler *et al.* 2007; Glover *et al.* 2010; Fernández *et al.* 2013; Deniskova *et al.* 2016),

demonstrating their value within a forensic context. SNP discovery for non-model species is commonly done using next-generation reduced representation methods to achieve a range of informative SNPs from across the genome (e.g. Ewart *et al.* 2019, Cruz *et al.* 2013, Robbana *et al.* 2019, Lah *et al.* 2016).

In recent years, there has been significant evidence emerging regarding the illegal trade of the unique and widespread monotreme, the short beaked echidna (*Tachyglossus aculeatus*) (Beastall and Shepherd 2013; Janssen and Chng 2017). There are five named subspecies of short beaked echidna across its distribution, which spans all of Australia and the southern regions of New Guinea. Ubiquitous across Australia, they are known to live in a wide range of climates, from deserts, to snow covered mountains, to tropical rainforests (Augee *et al.* 2006). These subspecies are primarily named for minor differences in morphology, as well as geographical location (Griffiths 1978; Augee *et al.* 2006). These are: *Tachyglossus aculeatus aculeatus*, (east coast Australia, Queensland through to South Australia), *T.a. setosus* (Tasmania), *T.a. acanthion* (Western and central Australia and arid zones of other states), *T.a. multiaculeatus* (Kangaroo Island), and *T.a. lawesii* (New Guinea). These subspecies have been designated based on differences in the length of their third and second toe in comparison to each other, and the fur to quill ratio (Rismiller and Seymour 1991; Augee *et al.* 2006). The Tasmania subspecies has a higher proportion of fur to quills compared to echidnas found in hotter climates. This is likely an adaptation to the colder climates of Tasmania (Griffiths 1978; Augee *et al.* 2006). It is not uncommon for species distributed across climatic and environmental clines to exhibit morphological differences, which may or may not be related to significant genetic differentiation. Koalas (*Phascolarctos cinereus*), exhibit a significant

morphological cline across their range with animals in southern parts of Australia (where the climate is colder) having larger body size, shorter limbs, and longer fur compared to the animals in the northern parts of their range (Briscoe *et al.* 2015) however genetic analysis did not support these populations being recognised as separate subspecies (Houlden *et al.* 1999; Neaves *et al.* 2016). To date there is limited information available in the literature that demonstrates that short beaked echidna subspecies are genetically distinguishable. It was suggested by Augee *et al.* (2006) that modern molecular techniques may result in a re-evaluation of subspecies taxonomy. As the short beaked echidna is not listed as endangered or vulnerable, the need for genetic data to guide conservation has not necessarily been a high priority, which may explain the absence of a systematic approach to obtain such a dataset. The emergence of the trade in 'captive bred' short beaked echidnas, however, is an increasing issue, and it has been suggested that it is the subspecies from New Guinea (*T.a. lawesii*) that is being targeted and laundered via this method (Beastall and Shepherd 2013; Janssen and Chng 2017). To aid with the investigation of this trade, it is first necessary to ascertain if it is possible to genetically differentiate these subspecies, followed by generation of markers that can also shed light on population and pedigree information.

To investigate if there is phylogeographic structuring in the short beaked echidna that is coincident with the morphologically described subspecies, a systematic analysis of samples from across the species range in Australia and New Guinea is necessary. The analysis of the control region of mitochondrial DNA (Chapter 2; Summerell *et al.* 2019) was able to differentiate between New Guinea and Australia, but other subspecies were not resolved. In this chapter a larger SNP dataset was generated to

determine if phylogeographic structuring within the species that could be used to assist investigations of the illegal wildlife trade in this species. This chapter presents an introduction and analysis of the marker set developed and its application to investigating if genetic data supports the five echidna subspecies.

3.3 Methods

3.3.1 Sample collection

A total of 192 samples from across as much of the echidna range as possible (Figure 3.1) were sourced from zoos, museums, and wild animals. Museum samples were all wild echidnas, and only zoo animals with known wild source localities were used in this study. Samples were sourced from New South Wales, Queensland, Victoria, Australian Capital Territory, Western Australia, Tasmania, and Papua New Guinea. For the complete list of sample information, see Table A3. 103 samples were sourced from the University of Tasmania (UTAS) collection, primarily consisting of wild echidnas from a population in and around Lovely Banks, Tasmania. Samples collected consisted of blood, tissue (muscle or liver), quills, and genomic DNA. Samples were stored at -20°C on arrival prior to DNA extraction. All samples were collected under UTS Animal Ethics 2015000040 and Taronga Zoo Animal Ethics 3b/06/16. Samples from UTAS were collected under several UTAS animal ethics spanning multiple years; 97006, A0005711, A0005452, A0007124, A0008659, A0010426, A12320.

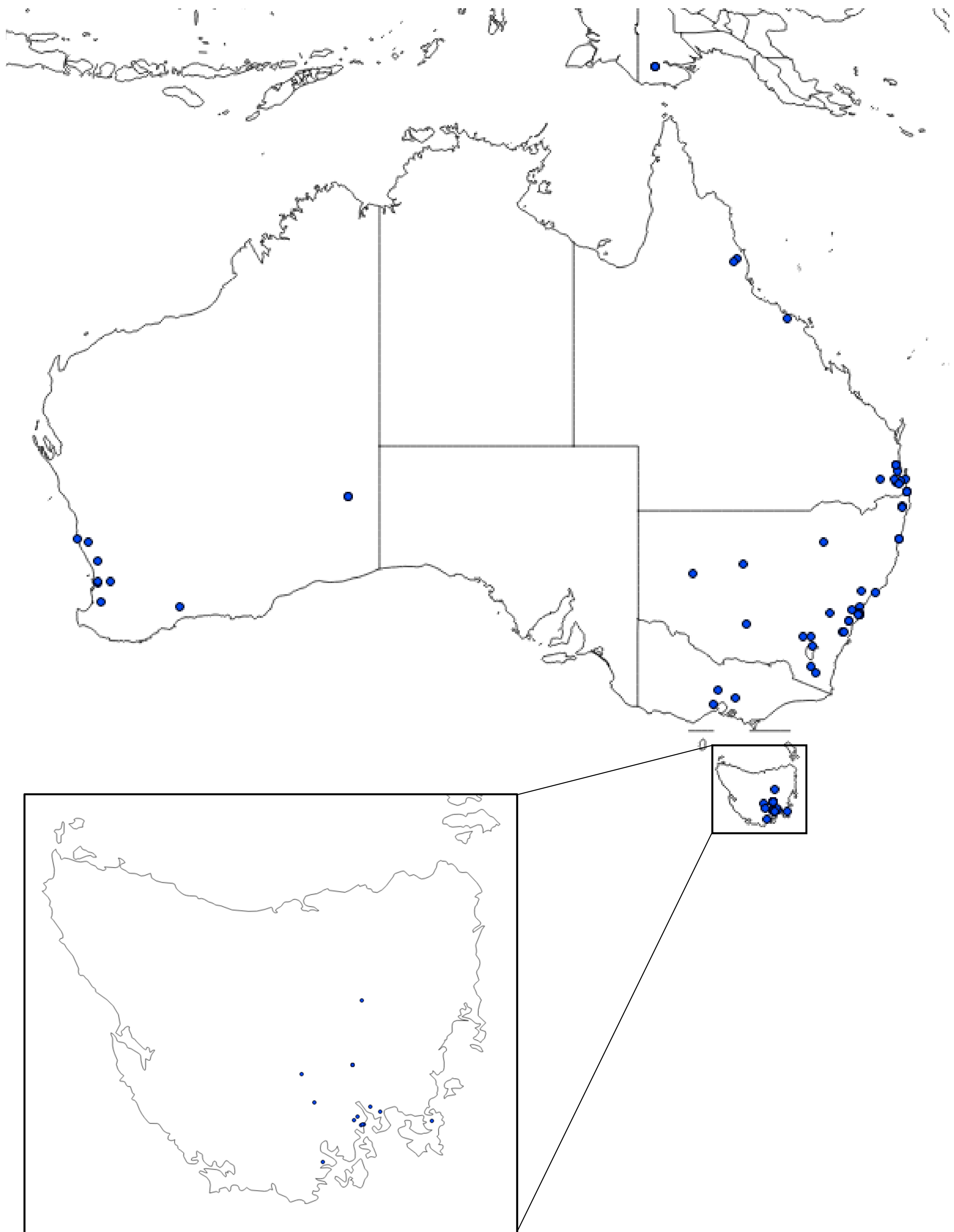


Figure 3.1 Map of short beaked echidna (*Tachyglossus aculeatus*) collection locations for samples used in Chapter 3. Enlarged section shows localities for Tasmania. Each blue dot represents a single sample.

3.3.2 DNA extraction

DNA extractions were carried out using either the ISOLATE II Genomic DNA Kit animal tissue protocol (Bioline, Australia), the QIAamp Investigator Kit Hair and Nails Protocol (Qiagen, Germany), or a salting out technique (Sunnucks and Hales 1996) (Table A4). DNA concentrations were determined using Qubit 2.0 Fluorometer High Sensitivity Assay (Thermo Fisher Scientific, USA) (Table A4). Dilutions were carried out to standardize the DNA at concentrations at ~20-50 ng/µL. 700-1000 ng of DNA were submitted to Diversity Arrays Technology (DArT) (Kilian *et al.* 2012; Cruz *et al.* 2013) for SNP discovery.

3.3.3 SNP sequencing

Sequencing for SNP markers was carried out at Diversity Arrays Technology (DArT) in Canberra, Australia, using their reduced representation sequencing method, DArTseq™ (DArT Pty Ltd). DArT uses a combination of complexity reducing restriction enzymes, implicit fragment size selection, and next-generation sequencing (NGS) (Sansaloni *et al.* 2011), and is described in detail by Kilian *et al.* (2012). For this study the restriction enzymes *Pst*I and *Sph*I were used. PCR conditions were: initial denaturation at 94°C for 1 min, then 30 cycles of 94°C for 20 sec, 58°C for 30 sec and 72°C for 45 sec, and a final extension step at 72°C for 7 min. After PCR, the resultant products underwent a c-Bot (Illumina) bridge PCR followed by single end sequencing for 77 cycles on an Illumina Hiseq2500. Sequences generated were processed using DArT analytical pipelines and were aligned using the Platypus (*Ornithorhynchus anatinus)* as a reference genome. Poor quality sequences were removed, and stringent selection criteria were applied to the barcode region to de-multiplex the sequence reads. Sequences were then trimmed to 69 bp and clustered

with a Hamming distance threshold of 3. Low quality bases from singleton tags were corrected where possible. SNP calling was then carried out using the proprietary DArT pipeline called DArTsoft14, which identified SNP markers using DArT PL's C++ algorithm. True allelic variants were discriminated from paralogous sequences by assessing a range of parameters within each sequence cluster including sequence depth, allele count and call rate.

3.3.4 SNP filtering

Filtering (Table A5) was carried out on the dataset using the R package '*dartR*' version 0.93 (Gruber *et al.* 2018), based on a call rate of 0.95. Loci that were not present in 95% of individuals were removed from the dataset. DArTSeq™ runs 30% of the samples in replicate in independent libraries and sequencing runs, and the consistency of each locus is measured across these replicates. Loci that were not 100% reproducible were removed from the dataset. Individuals with less than 80% call rate were filtered out and subsequently removed from the dataset (6 samples). Any monomorphic loci arising from the removal of individuals were also deleted. Any loci with multiple SNP loci in a fragment (secondaries) were also filtered out. Deviations from Hardy-Weinberg equilibrium were tested for after primary filtering, using the Bonferroni correction for sample size and after initial filtering no loci showed significant departure and therefore all were retained. A total of 2388 SNPs from 172 unique samples were in the final dataset (Table A3).

3.3.5 Phylogeographic analyses

Samples were assigned based on their nominate subspecies; *T.a. acanthion* (Western Australia (WA)), *T.a. setosus* (Tasmania (TAS)), *T.a. aculeatus* (eastern Australia) and *T.a. lawesii* (Papua New Guinea (PNG)). Two samples from the fifth subspecies

– *T.a. multiaculeatus* (Kangaroo Island) did not pass DArTSeq quality control and therefore could not be included in the final analyses. A hierarchical approach was taken, and analyses were initially conducted on all samples (Dataset A; *n=172; 2388 SNPs*); followed by analyses on only mainland (ML) samples after removing *T.a. setosus* samples (Dataset B; *n=69; 2289 SNPs*), and lastly conducted on samples only from *T.a. aculeatus* and *T.a. lawesii* after removing *T.a. acanthion* samples (Dataset C; *n=58; 2045 SNPs*). For each dataset locus metrics were recalculated and any monomorphic SNPs removed.

Three analyses were conducted on each of the datasets to explore population structure. Principle coordinates analysis (PCoA) was performed using the *dartR* R package (Gruber *et al.* 2018), and the *adegenet* R package (Jombart 2008) to obtain a broad summary and visualisation of any clustering between populations within the dataset. A discriminant analysis of principle components (DAPC) was then performed on all three datasets using the *adegenet* R package. DAPCs are known to effectively separate sub-populations by maximising the separation between groups while minimising the variation. The most likely number of clusters was determined using the Bayesian Information Criterion (BIC) and the appropriate number of principle components were retained for each dataset.

To compare with the PCoA and DAPC, inter-population structure was investigated using the Bayesian clustering methods in STRUCTURE version 2.3.4 (Pritchard *et al.* 2000) assuming no *a priori* population information. Analyses were conducted using the admixture model, alpha inferred from the data, independent allele frequencies, and lambda set at 1.0. A burn-in of 50,000 runs was used with 50,000 MCMC iterations

performed. The number of populations (K) was tested for all three datasets (K = 1 to K = 10). All simulations were carried out with 10 replicates of each K value. K was inferred from delta log likelihood (Evanno *et al.* 2005), which was visualised using STRUCTURE HARVESTER (Earl and vonHoldt 2012).

To test for isolation by distance, a Mantel test was carried out to determine if there was a correlation between genetic distance and geographic distance within the *T.a. aculeatus* samples, after removing the *T.a. lawesii* samples. This was calculated in GENALEX 6.5 (Peakall and Smouse 2006, 2012).

3.4 Results

3.4.1 SNP dataset

DArTseq successfully genotyped 178 samples and their pipeline called 27,529 SNPs. Fourteen samples were not able to pass the DArTseq quality control standards so were excluded from library preparation. Six samples had a call rate of <80% so were removed from the dataset during filtering. After filtering and quality control 2388 SNPs remained from 172 samples.

3.4.2 PCoA

The PCoAs (Figure 3.2 a, b, c) showed various distinct clusters. PCoA 3.2a, which included all samples identified three distinct clusters; one consisting of only *T.a. acanthion* samples, one of only *T.a. setosus* samples, and one consisting of *T.a. aculeatus* and *T.a. lawesii* samples. The latter was not a tight cluster and rather spread along axis 1. The first axis accounted for 52.9% of the variation, with the second accounting for 6.9%.

PCoA 3.2b, consisted of all samples from mainland Australia, after removing *T.a. setosus* samples. The first axis consisted of 26.1% of the variation, with the second accounting for 13.3%. This showed a similar separation of *T.a. acanthion* and *T.a. aculeatus*/*T.a. lawesii* samples. The *T.a. aculeatus* and *T.a. lawesii* samples had more distinct separation in this dataset, compared to that of 3.2a.

PCoA 3.2c, which consisted of solely the *T.a. aculeatus/T.a. lawesii* samples, showed further separation, consistent with the two subspecies. The first axis accounted for 26.8% of the variation, with the second accounting for 6%.

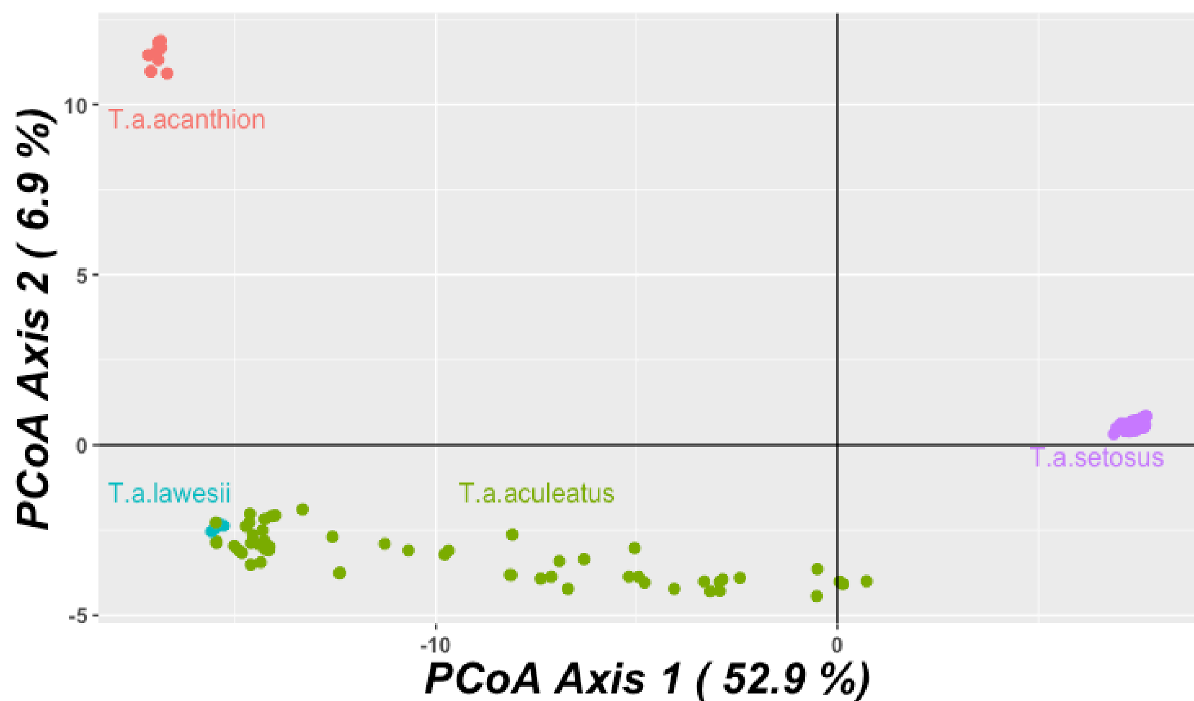


Figure 3.2a PCoA of Dataset A from 172 samples, representing four subspecies of the short beaked echidna. Each dot represents a single sample. Subspecies are separated by colour (Red = *T.a. acanthion*; Blue = *T.a. lawesii;* Green = *T.a. aculeatus*; Purple = *T.a. setosus).*

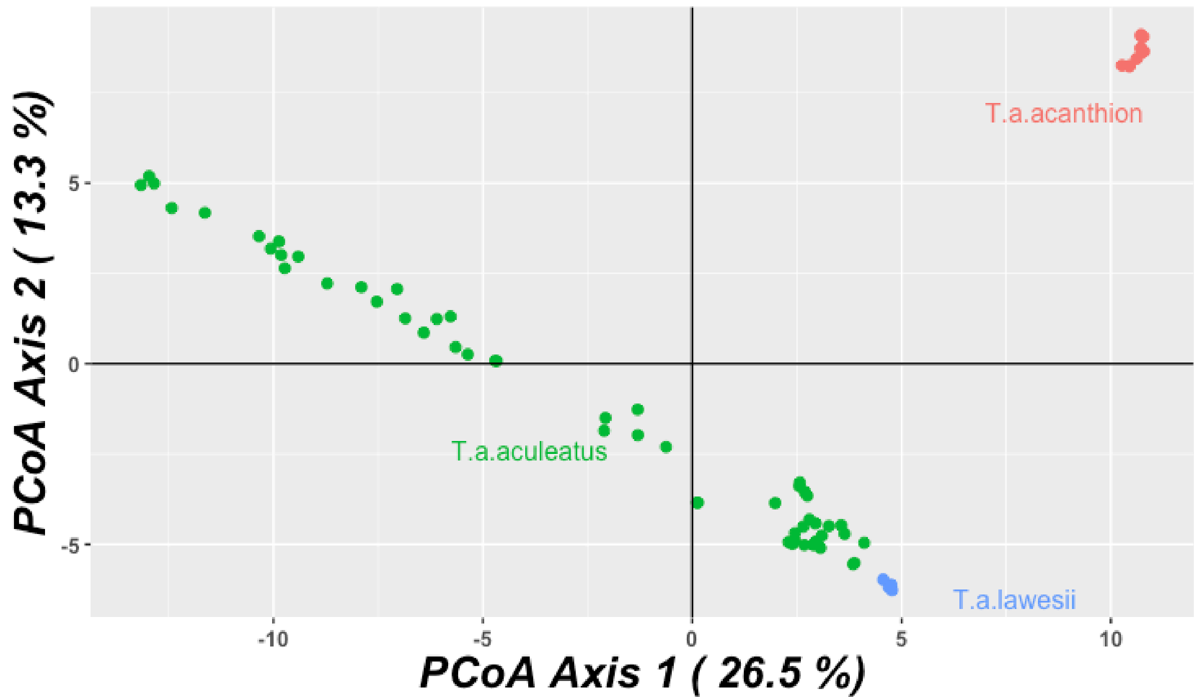


Figure 3.2b PCoA of Dataset B from 69 samples, representing three subspecies of the short beaked echidna. Each dot represents a single sample. Subspecies are separated by colour (Green = *T.a. aculeatus;* Blue = *T.a. lawesii;* Red = *T.a. acanthion).*

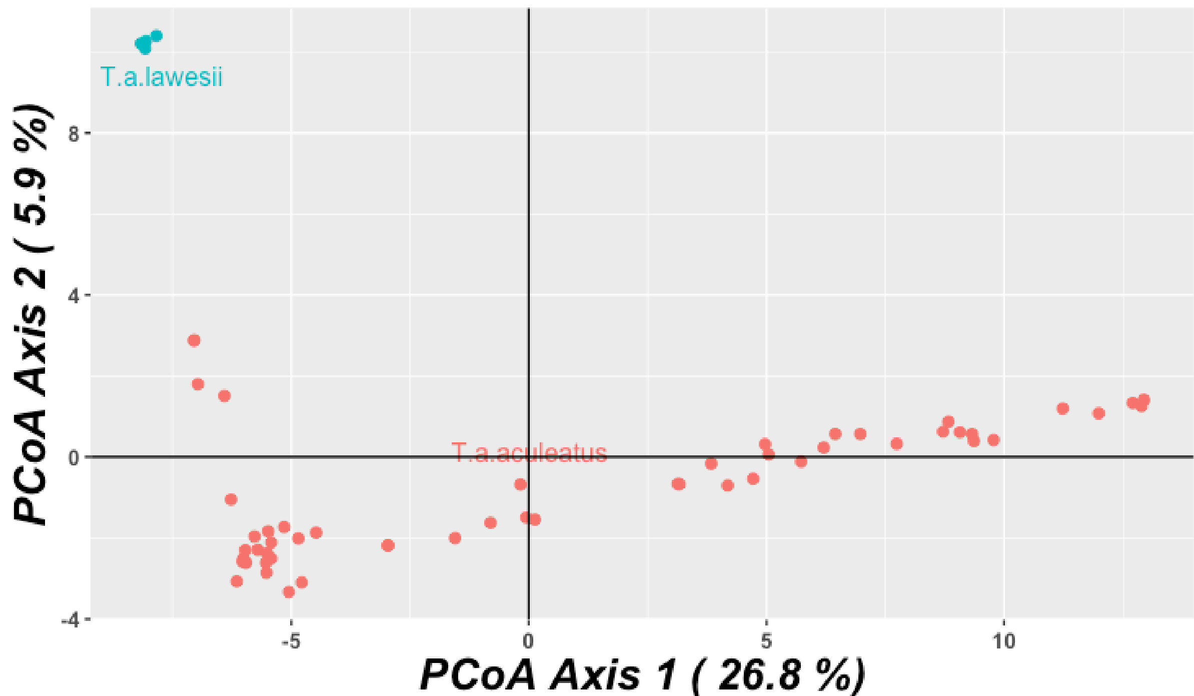
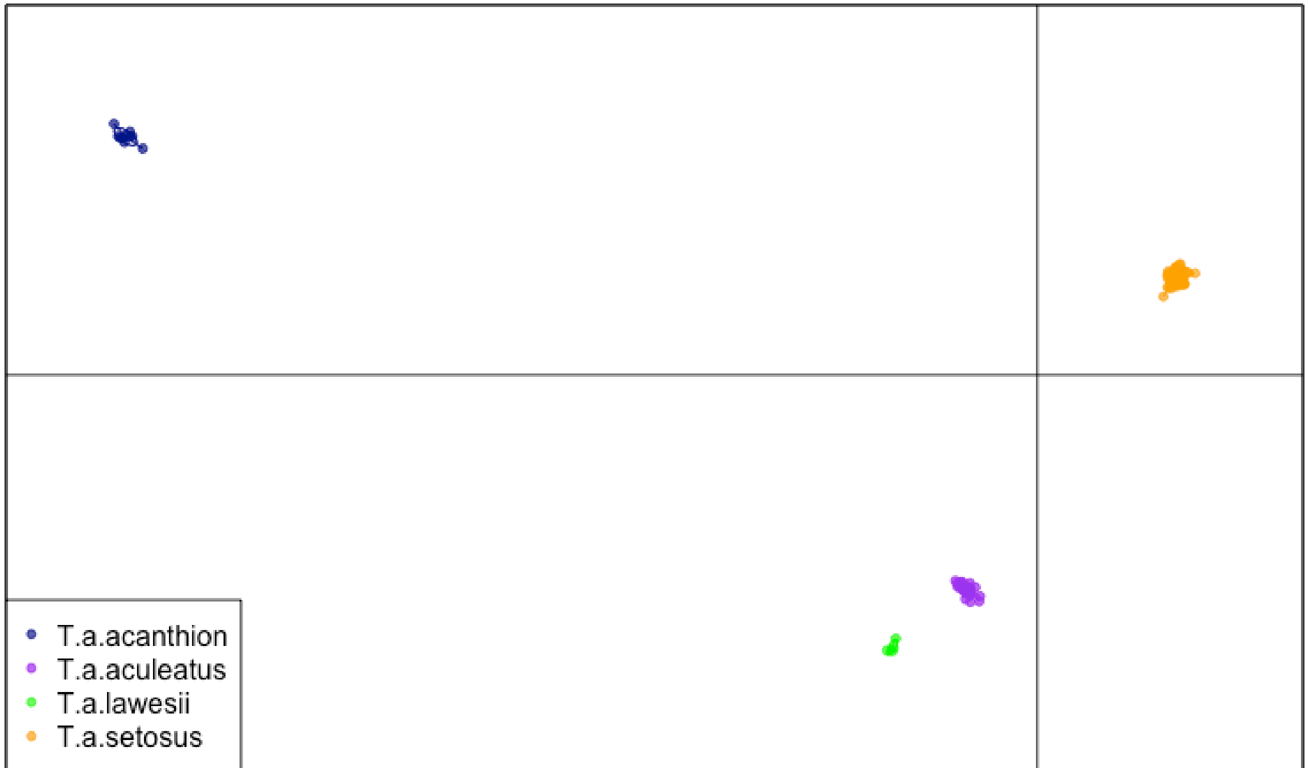


Figure 3.2c PCoA of Dataset C from 58 samples, representing two subspecies of the short beaked echidna. Each dot represents a single sample. Subspecies are separated by colour (Red = *T.a. aculeatus*, Blue = *T.a. lawesii).*

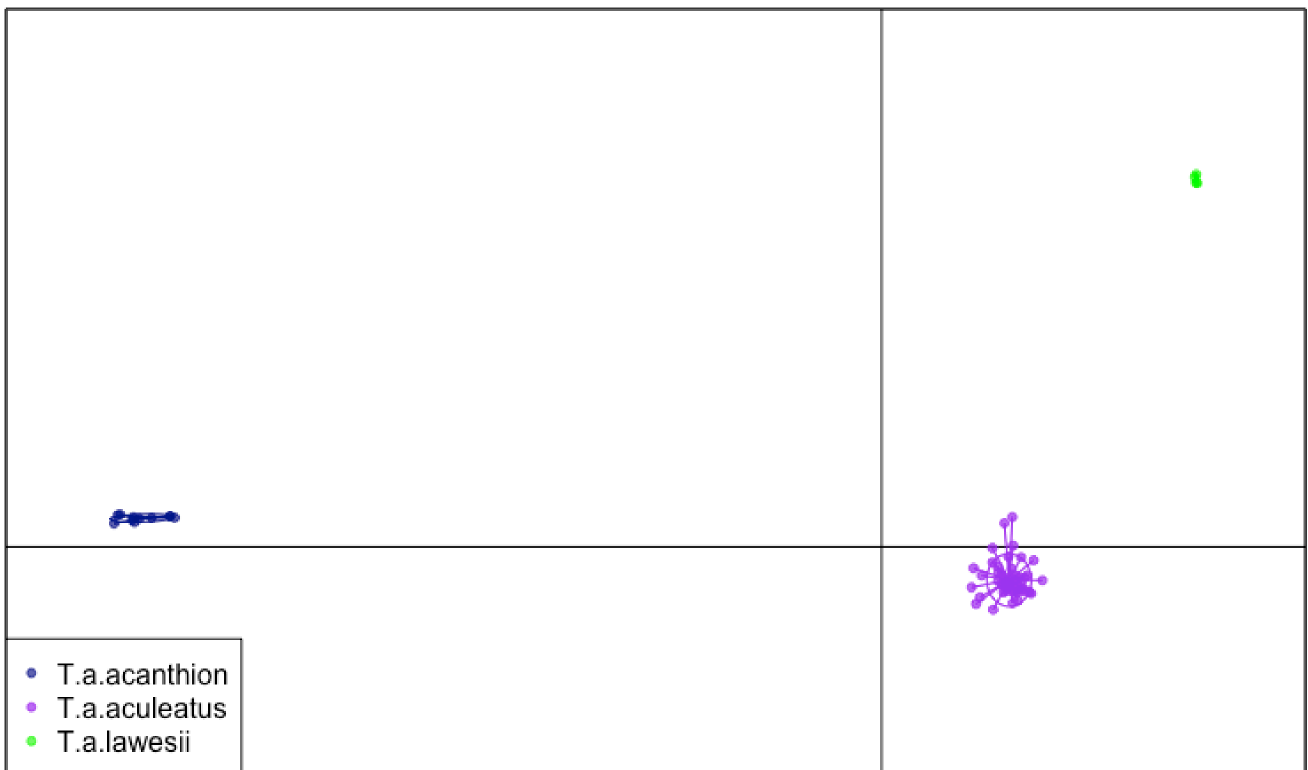
3.4.3 DAPC

The DAPCs (Figures 3.3a, b, c) show similar separation of the populations to that of the PCoA. Dataset A had 50 principle components retained. In Figure 3.3a the clusters of the four subspecies can be seen clearly. Unlike the PCoA, the *T.a. lawesii* samples were separated from the *T.a. aculeatus* samples. This was again more obvious when examining Dataset B (Figure 3.3b) with *T.a. setosus* samples removed; *T.a. aculeatus, T.a. acanthion* and *T.a. lawesii* form distinct clusters.

a)



b)



c)

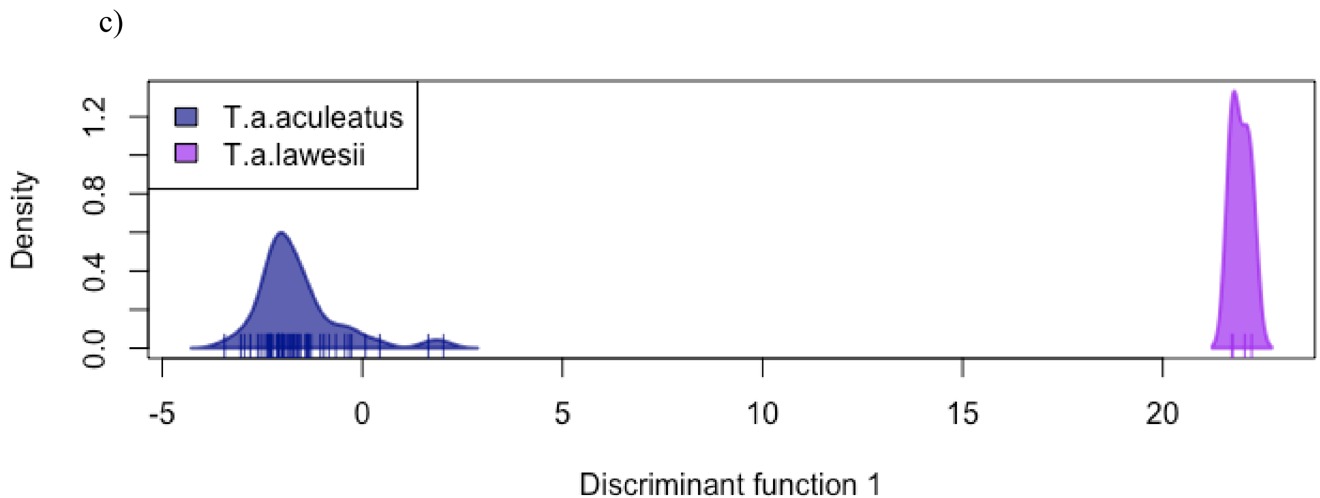


Figure 3.3 Discriminant analysis of principle coordinates (DAPC) for subspecies of the short beaked echidna based on over 2000 Single Nucleotide Polymorphisms (SNPs). a) Dataset A; all sampled individuals, 50 PCs retained; b) Dataset B, all mainland populations, 10 PCs retained; c) Dataset C, all Eastern Australian and PNG samples, 5 PCs retained.

3.4.4 STRUCTURE

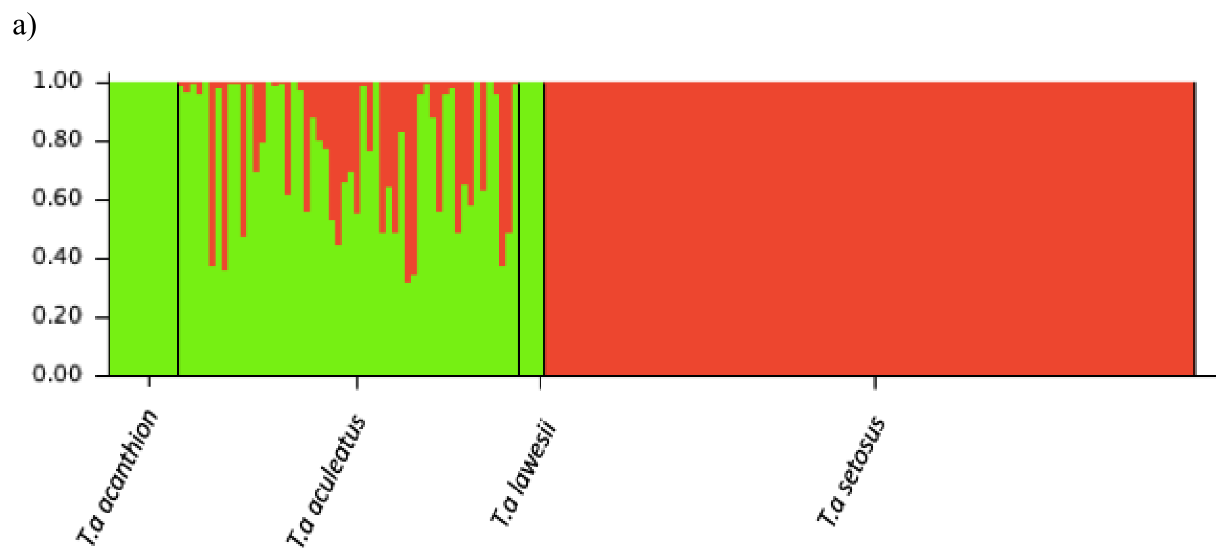
STRUCTURE analysis identified some population structure, with the analysis of all samples suggesting K = 2 (determined by Evanno *et al.* 2005) was the best fit for the Dataset A (Figure 3.4a). This clustered the mainland Australian samples (*T.a. aculeatus; T.a. acanthion*) with *T.a. lawesii* (PNG) samples together, with the *T.a. setosus* (Tasmania) samples as the other cluster. There was some admixture between *T.a. aculeatus* samples and *T.a. setosus* samples.

Within Dataset B, once the *T.a. setosus* samples were removed, more population differentiation could be seen (Figure 3.4b), with K = 4 (determined by Evanno *et al.* 2005) being the best fit for this data. The *T.a. acanthion* and *T.a. lawesii* samples are clearly distinct from the *T.a. aculeatus* samples. The *T.a. aculeatus* samples have more variation and admixture. This corresponded with geographic location;

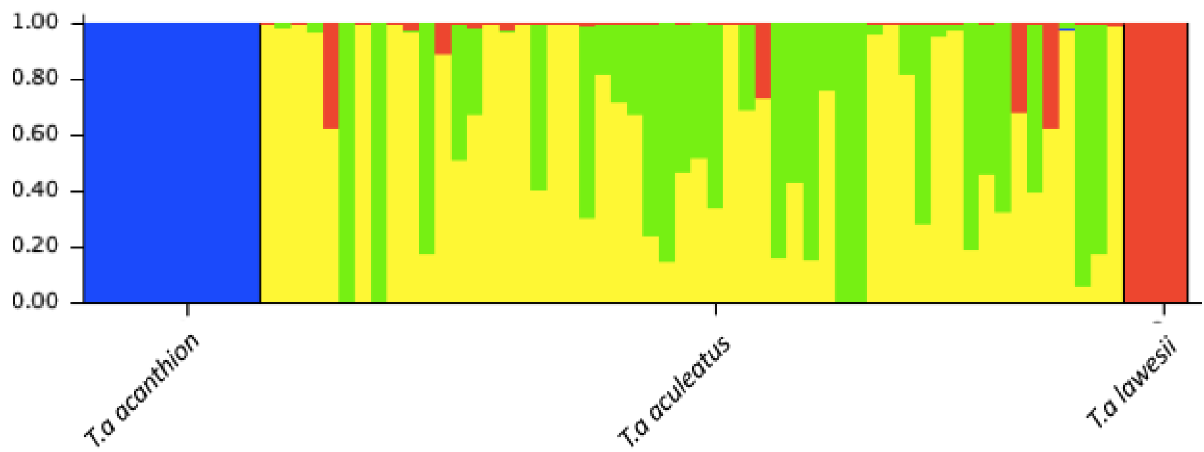
Queensland forming one group (Figure 3.4b yellow individuals) and southern NSW, Victoria and Australian Capital Territory forming another (Figure 3.4b green individuals). Samples found in the mid-region of NSW appeared to show admixture between these two, and some samples from northern Queensland displayed some admixture with the *T.a. lawesii* samples.

Dataset C follows a similar pattern once the *T.a. acanthion* samples were removed, with K = 3 (determined by Evanno *et al.* 2005) being the best fit. This separated out the *T.a. lawesii* samples from the *T.a. aculeatus*, with the *T.a. aculeatus* further separated into two groups, which corresponded to geographic location similar to Dataset B.

a)



b)



c)

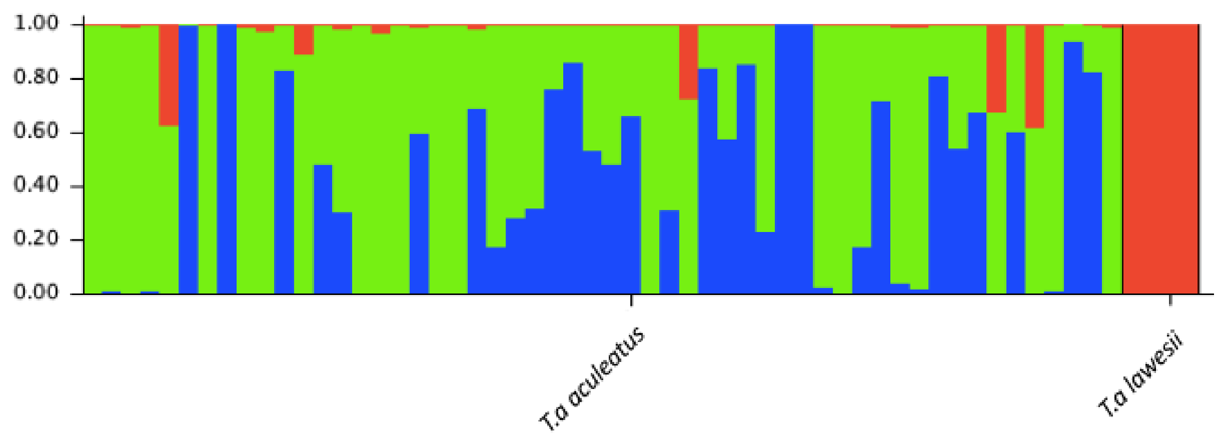


Figure 3.4 Bayesian population assignment (STRUCTURE) plots for short beaked echidna subspecies based on over 2000 Single Nucleotide Polymorphisms (SNPs). Each vertical column represents a single individual, the partitioning of different colours in each column is directly proportional to the membership coefficient to that cluster. a) Dataset A, all sampled individuals K =2; b) Dataset B, mainland populations only, K = 4; c) Dataset C, eastern Australia and PNG samples only, K = 3.

3.4.5 Isolation by distance

The isolation by distance analysis (Figure 3.5) showed a weak but significant (p = 0.0011; $r^2 = 0.0802$) correlation between geographic and genetic distance with the *T.a. aculeatus* samples.

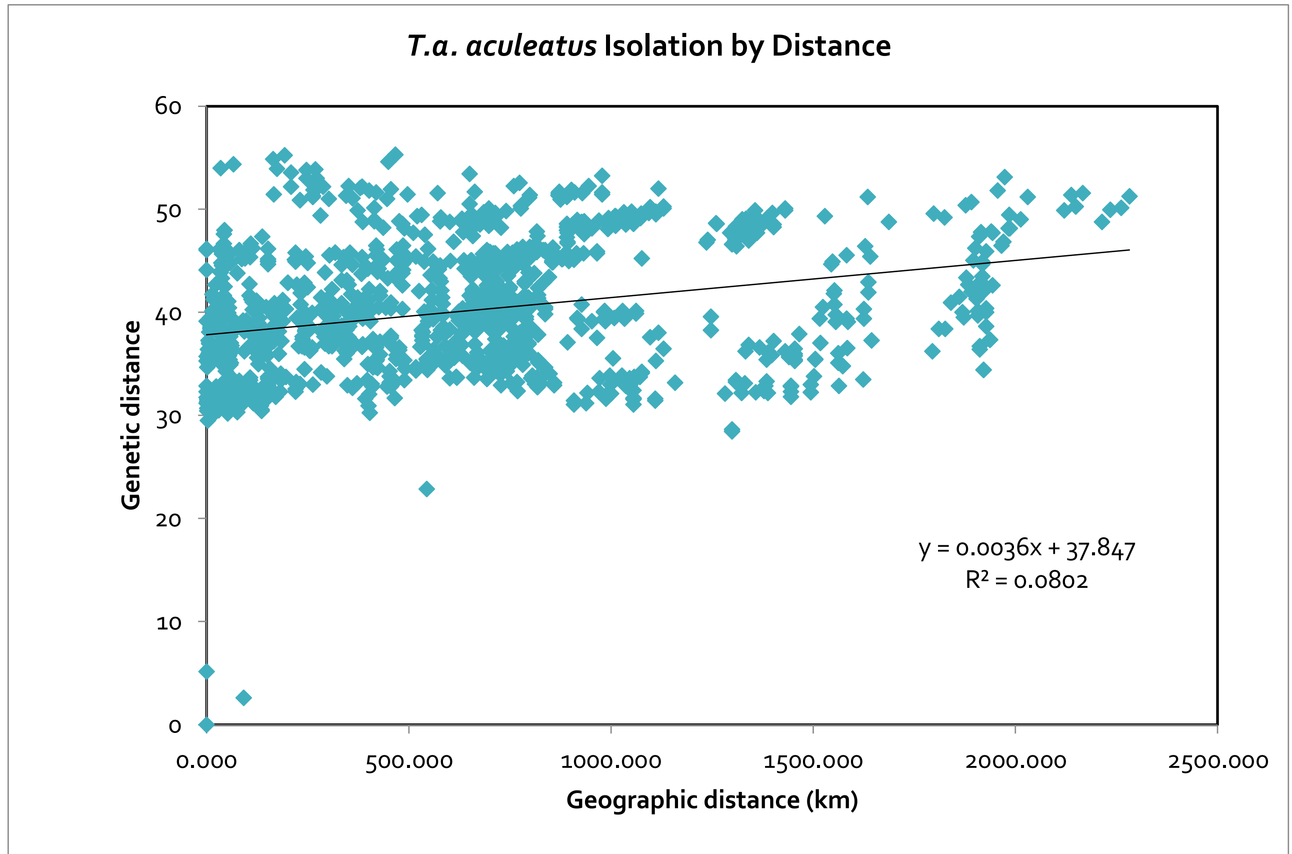


Figure 3.5 The relationship between geographic and genetic distances for the *T.a. aculeatus* samples using the Isolation By Distance Mantel test in GENALEX.

3.5 Discussion

3.5.1 SNP analysis

The five subspecies of short beaked echidna, described based on morphology and geographic location, to date have not been analysed using genetic data, therefore this is the first study to produce genetic data from across the short beaked echidna's range. I was able to generate 2388 informative SNPs that could be used for larger scale genetic analysis of the short beaked echidna (*Tachyglossus aculeatus*). Despite some limitations in the sample collection, I was able to infer some overall trends and insights into the named subspecies when using this SNP dataset.

The SNPs developed as part of this study are a more powerful tool for understanding phylogeographic structure and other population level questions than previous microsatellite markers developed for the short beaked echidna by Vanṕe *et al.* (2009). Only nine out of 43 microsatellite loci had sufficient diversity to be used for population level studies, giving them little resolving power and no published studies have used these markers since their initial publication. Several similar studies have demonstrated the power of SNP datasets over microsatellite markers including (Aitken *et al.* 2004; Tokarska *et al.* 2009; Morin *et al.* 2012; Lah *et al.* 2016). Such studies can often provide additional support for mitochondrial DNA based phylogeography (Morin *et al.* 2004; Esquerré *et al.* 2019; Kornilios *et al.* 2020).

3.5.2 Subspecies of the short beaked echidna

The analyses performed here identified support for the echidna subspecies (*T.a. aculeatus, T.a. acanthion, T.a. setosus, T.a. lawesii*) included in this study. These results should be treated with caution however, given echidnas are widespread, and

that there are some large sampling gaps within my sample set, in particular across central Australia where two subspecies (*T.a. aculeatus, T.a. acanthion*) overlap. The patterns of isolation by distance seen across *T.a. aculeatus* and the fact echidnas have been shown to move large distances (Rismiller and McKelvey 1994) suggests that this overlap zone should be further examined. At this stage, in the absence of a systematic sampling from all Australian states, assigning a sample to a particular Australian subspecies should be done with caution.

3.5.2.1 T.a. setosus

My analyses revealed distinct structure between the mainland samples and the Tasmanian *T.a. setosus* samples. This is not a surprising result, as Bass Strait has existed as a major biogeographic barrier for many species for ~14,000 years (Lambeck and Chappell 2001) resulting in subspecies designations across this break (Firestone *et al.* 1999; Norgate *et al.* 2009; Frankham *et al.* 2016; Cooper *et al.* 2019; Martin *et al.* 2019). While there is likely merit in the recognition of the Tasmanian subspecies *T.a. setosus,* this study does also include a large proportion of samples from a single population (Lovely Banks) gathered by researchers at the University of Tasmania, therefore even the Tasmanian sampling is not yet widespread enough to ascertain if this subspecies is supported or if a high proportion of sampling from one population is biasing the data. When observing the STRUCTURE plot for Dataset A, the main structure is found be between the Tasmanian samples and the mainland samples (K = 2), which is why the hierarchical analyses were employed to investigate further possible structure. Meirmans (2015) addresses issues of utilising only one value of K, as hierarchical genetic partitioning could mean that different K values are still biologically relevant, and therefore suggests considering more than one value.

Additionally, in this plot there are some mainland samples that appear to be admixed, between *T.a. aculeatus* and *T.a. setosus* populations, which warrants further investigation and wider sampling and could be indicative of ancestral genotypes. Samples from the Bass Strait islands in particular, would give an indication of how much divergence there has been between the populations. Frankham *et al.* (2016) noted that most species that don't show high levels of divergence across the Bass Strait are those that are highly mobile and habitat generalists (e.g. Zenger *et al.* 2003; Norgate *et al.* 2009; Chapple *et al.* 2011; Ng *et al.* 2014), like the short beaked echidna.

3.5.2.2 T.a. acanthion/ T.a. aculeatus

The samples from mainland Australia showed structure between the two subspecies; *T.a. aculeatus* and *T.a. acanthion*. This was also reflected in the mitochondrial DNA results (Summerell *et al.* 2019; Chapter 2), showing the *T.a. acanthion* samples clustered with strong support. Short beaked echidnas are found throughout the whole of mainland Australia and the descriptions of the extent of these subspecies, of their distributions and if there is any overlap, are inconsistent (Griffiths 1978; Griffiths and Greenslade 1990; Augee *et al.* 2006). Occurrence records indicate that short beaked echidnas are widespread throughout the country, including the centre of Australia (ALA 2020), and there is no hard demarcation or clear biogeographic barrier between the two subspecies. The *T.a. acanthion* samples used in this study have collection localities mostly from the greater Perth region, so this is a very limited representation for this subspecies. However, one sample collected from near the Western Australian and South Australian border still clustered tightly with the rest of the *T.a. acanthion* samples, providing some insight into clustering of the more western samples. This

was in contrast to the *T.a. aculeatus* samples which were more widespread along the east coast of Australia from Queensland through NSW and Victoria. As there were no samples from the north of Western Australia (WA), Northern Territory, or South Australia, I am reluctant to comment on whether the distinct separation of WA samples would continue to display the same results if more samples from a wider range were added, or if it would merge to a more continuous cline across the mainland. This is particularly important as other studies have noted significant divergence due to biogeographic barriers on mainland Australia for species such as the brush-tailed phascogale, grey kangaroos, and honeyeaters (Spencer *et al.* 2001; Toon *et al.* 2010; Potter *et al.* 2012). Barriers such as the East-West Kimberley divide (Eldridge *et al.* 2011), or the Carpentarian barrier in the north (Toon *et al.* 2010), the Nullarbor and Eyrean barriers in the south (Kearns *et al.* 2009; Neaves *et al.* 2009) have all had influences on the divergence of species. A number of genetic studies have shown east-west divergence within species distributed across the continent (Cooper *et al.* 2000; Spencer *et al.* 2001; Strasburg and Kearney 2005; Neaves *et al.* 2009). It would be informative to determine whether any such barriers have significantly impacted the short beaked echidna enough to continue supporting the two mainland subspecies, although this would be a surprising result given their adaptability to such a wide range of environmental conditions (Augee *et al.* 2006).

3.5.2.3 *T.a. aculeatus*

Relatively weak sub-structuring was identified in the *T.a. aculeatus* samples; with evidence of gene flow across the east coast. The STRUCTURE analysis suggested that three populations was the best fit for Dataset C which also included two clusters representing the *T.a. aculeatus* samples, loosely related to geographic location

(north/south), with admixture across central NSW. This supports the isolation by distance analysis, suggesting there is genetic cline across this region. The number of admixed samples does indicate that there has been gene flow between the populations. Echidnas have the potential to travel large distances; there is evidence of an echidna travelling five kilometres in a single day (Rismiller and McKelvey 1994), though it is also suggested they have small home ranges (~0.4-1.1km^2), particularly females who keep young in a burrow that they need to return to during the breeding season (Abensperg-Traun 1991; Rismiller and McKelvey 1994, 2000). Their ability to travel large distances, and their adaptability to habitats, could support the admixture and give an indication of the gene flow seen in the *T.a. aculeatus* samples. As there was a slight trend seen in the isolation by distance analysis of the east coast, there is the potential for this to be seen across east and west as well, given the ubiquitous nature of echidnas. More sampling would allow for further investigation into this, and whether there is a biogeographic barrier that has resulted in the slight structure seen here. A wide review on the use of subspecies and species in cetaceans by Rosel *et al.* (2017) noted that many studies often do not have sufficient sampling, and recommended that species and subspecies delimitation should rely on a sufficient sample set that captures the genetic variation and is unbiased to ensure accuracy. I would recommend sampling not just populations on the east coast, but also more western locations of the states to truly be representative.

3.5.2.4 T.a. multiaculeatus

Unfortunately during sample collection I was only able to obtain two samples from the fifth subspecies of echidna from Kangaroo Island (*T.a. multiaculeatus)* (both sourced from Australian Museum collections), and these both failed the quality

control process at DArTseq. Incorporating samples from Kangaroo Island would have been valuable, to have representative samples from all subspecies. This would again be pertinent to include in a wide scale analysis, due to many populations of other species on Kangaroo Island showing divergence from mainland populations in species such as Rosenberg's goanna (Smith *et al.* 2007), the superb fairy wren (Dudaniec *et al.* 2011) and the tammar wallaby (Eldridge *et al.* 2017). Given that Kangaroo Island has been separated from the mainland for ~10,000 years, it can be hypothesised that *T.a. multiaculeatus* subspecies may show similar separations to what is seen with the *T.a. setosus* samples. Island populations do typically have reduced diversity compared to mainland populations (Frankham 1997) and given the endangered status of the *T.a. multiaculeatus* (Australian Government 2009), genetic analysis of this population should be prioritised.

3.5.2.5 T.a. lawesii

Despite the small sample size of four, the *T.a. lawesii* samples clustered separately in all the analyses except the PCoA and STRUCTURE analyses using Dataset A. The results from Dataset B and C reflect the results described in Summerell *et al.* (2019; Chapter 2), supporting the classification of the New Guinea subspecies. Studies analysing species such as logrunners, green pythons, the king brown snake, and blue winged kookaburras, across the two regions have found similar structure between New Guinea and Australian populations (Joseph *et al.* 2001; Rawlings and Donnellan 2003; Kuch *et al.* 2005; Peñalba *et al.* 2019; Dorrington *et al.* 2020). The consistency between the mitochondrial DNA test and the SNP marker analyses is particularly encouraging due to the evidence of illegal trade of the short beaked echidna, which is likely of this New Guinea subspecies *T.a. lawesii* (Beastall and Shepherd 2013). To

further analyse this subspecies a larger sample size would be ideal, though samples from this region are more difficult to collect. It would also be useful to include samples from the areas bridging New Guinea and Australia such as Torres Strait and Cape York. As seen in STRUCTURE from Dataset C, there were a small number of samples that appeared to share more alleles with the PNG samples; these were originating from North Queensland, which potentially indicates retention of ancestral genotypes, or more recent geneflow during periods of lower sea level, but further sampling of those regions would be warranted to investigate these dynamics. A sample from Torres Strait was included in the mitochondrial study, however, was taken from museum skin and did not yield enough quality DNA to use for SNP sequencing.

3.5.3 Wildlife forensic application

This study contributes to my aim of providing a range of methods that can be implemented as a wildlife forensic technique to support or deny claims that an echidna is legitimately captive bred. In a casework scenario it would be recommended to first perform the validated mtDNA control region test (Chapter 2) as it was designed to work with low amount of template DNA, which is sometimes all that is available in a casework scenario. That test could distinguish the source region of any short beaked echidna with an unknown origin while also being time and cost efficient (Summerell *et al.* 2019). This mitochondrial test was limited to determining if an echidna came from Australia or New Guinea, which is particularly important to determine for species protection as well as establishing jurisdiction. SNPs should be used if further genetic resolution is required in an investigation; this may be key in supporting claims that an echidna is from New Guinea or roughly which Australian

subspecies, and will provide further confidence in any reports made about the origin of the animal. Additionally, this could then be combined with information about the pedigree of an individual (Chapter 4) and present stronger evidence to law enforcement regarding any suspicious origins of this echidna. Being able to determine subspecies or source populations of an echidna will help direct enforcement and compliance resources to the appropriate areas (Alacs and Georges 2008; Kitpipit *et al.* 2012; Gupta *et al.* 2013; Ogden and Linacre 2015). Additionally, it could help with repatriation of any seized animals to their population of origin.

Alternatively, SNPs could be integrated into studbooks kept by zoos to provide assurance that any newly acquired echidnas have come from a legitimate source, whether it be New Guinea or Australia. This could provide greater resolution for Australian populations and may be able to determine which subspecies an echidna came from, which could aid and promote the legal trade between both Australian and international zoos.

The data presented here shows promising results for development into a routine wildlife forensic technique. Development of a SNP panel could be implemented using informative SNPs that could determine the difference between New Guinea and Australia, in conjunction with SNPs that could distinguish the pedigree of an individual (Chapter 4). Similar genetic marker panels have been constructed in a range of species both for wildlife forensics and population genetics purposes (Tokarska *et al.* 2009; Clarke *et al.* 2014; Ciavaglia and Linacre 2018; Willows-Munro and Kleinhans 2019; Dormontt *et al.* 2020). With further sampling, this could be refined and validated so that being able to identify the subspecies, or geographic

origin, potentially beyond just determining the source region (i.e. New Guinea or Australia), could become a routine wildlife forensic technique for the short beaked echidna.

3.6 Conclusion

This study uses high resolution markers to contribute to the understanding of phylogeographic structure and subspecies of the short beaked echidna, and if there is any support using genetic data for these subspecies. While the sampling gaps make it difficult to make concrete conclusions or recommendations regarding synonymising any current subspecies, it is clear that there is some genetic structure between known subspecies, in particular those which are physically isolated from other subspecies, namely *T.a. setosus* (Tasmania), and *T.a. lawesii* (New Guinea). The distinction of *T.a. lawesii* samples could be crucial in combating the illegal wildlife trade, as many of the laundered animals are suspected to be members of this subspecies. The tight clustering of the *T.a. acanthion* (Western Australian) samples is of interest, suggesting there is not complete gene flow across Australia. However, more sampling across the boundaries of the *T.a. acanthion* and *T.a. aculeatus* subspecies is needed to further investigate this, given the evidence of gene flow on the east coast. This study adds to the toolbox that can be implemented when an echidna is traded, and forms the basis of the populations when understanding relatedness levels.

3.7 References

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Chapter 4: Development of forensically informative DNA markers for the short beaked echidna: their use in pedigree testing and application in the illegal wildlife trade

4.1 Abstract

The illegal trade in species poached from the wild, but claimed to have been bred in captivity, is increasingly a crime of concern. Often there is insufficient evidence to confirm the captive bred status of animals in the wildlife trade. Determining the relatedness between traded individuals has the potential to aid law enforcement, support or disprove captive breeding claims, and provide peace of mind for zoos or sanctuaries acquiring new animals for their collections. To determine relatedness between individuals, a robust genetic marker set is required and should ideally be piloted using individuals of known pedigree as a proof of concept. There is evidence to suggest the short beaked echidna (*Tachyglossus aculeatus*) is being traded illegally; echidnas are notoriously difficult to breed in captivity, which raises suspicion when echidnas are sold and claimed to be captive bred. Prior to this study no suitable genetic marker set existed to test relatedness in the short beaked echidna, therefore I aimed to develop a robust single nucleotide polymorphism (SNP) dataset for this species that could test pedigree claims by comparing them to individuals legitimately bred in Australian zoos and a population of wild animals that has been monitored for over 20 years (and included some known relationships). Using DArTseq marker discovery a set of 2406 SNPs was generated that was used to confirm the relatedness of 34 individuals of known pedigree from three Australian zoos using Identity by Descent (IBD) analysis. In the wild population this analysis identified more distantly related individuals than the captive populations. These data demonstrate these SNPs are suitable for determining relatedness between individual echidnas as well as set the baseline for relationships expected in captive bred and wild-caught individuals. Lastly, I determined the minimum number of SNPs required to individualise an echidna and recommend further validation with a smaller subset of SNPs that would allow for cost and time efficient testing within a forensic context.

4.2 Introduction

4.2.1 DNA methods for relatedness testing

The ability to use DNA methods to reconstruct relationships between individuals has been widespread and instrumental across a range of fields. This is best demonstrated by human biological science with examples ranging from genealogy and ancestry, to paternity testing, through to forensic science applications. Paternity testing is a large and growing market globally, which uses validated genetic markers to determine the likelihood of a man being the biological father of a child (Gjertson *et al.* 2007). Familial testing (comparing DNA profiles to determine relatedness) is also used in human forensic science, particularly in missing persons or unidentified remains cases, such as Disaster Victim Identification (Butler 2009; Maguire *et al.* 2014). The most comprehensive range of examples involve reconstruction of pedigrees with the use of molecular data obtained from the individual in question and one or both of their parents (Egeland *et al.* 2000; Weir *et al.* 2006; Gjertson *et al.* 2007).

Pedigree reconstruction to provide information on non-human family history is commonly used in economically important animal industries such as show dogs, racehorses and livestock (Tunnell *et al.* 1983; Hall and Bradley 1995; Leroy 2011). Traditional (non-molecular) pedigree reconstruction involved documentation of relationships ('studbooks'), often noting key traits, presence of diseases, and strengths and weaknesses of individual animals to ensure optimum breeding results (Jones and Wang 2010). Nowadays it is recognised that to achieve this, most pedigree analyses should also incorporate genetic testing, which provides higher precision and accuracy.

Pedigree reconstruction is also used in population studies (Blouin 2003; Jones and Ardren 2003; Creel and Rosenblatt 2013), ex-situ and in-situ conservation management of species (Allendorf *et al.* 2010; Lacy 2012; Fienieg and Galbusera 2013), ecological and evolutionary

history (Jones *et al.* 2010) and wildlife forensic science (Moore and Frazier 2019). Knowledge of pedigrees and relatedness levels provides information on inbreeding and unknown relationships which can be crucial when genetic diversity is needed for the long-term resilience of populations, particularly small or fragmented populations. Further, genetic pedigrees are particularly beneficial for industries that breed species, such as zoos and agriculture to provide data that can inform and manage breeding programs (Hall and Bradley 1995; Paiva *et al.* 2011; Creel and Rosenblatt 2013; McLennan *et al.* 2018; Farquharson *et al.* 2019). Zoos that manage breeding programs use pedigree information to maintain genetic diversity and reduce inbreeding in order to avoid deleterious alleles becoming prevalent in their closed populations, which can result in diseases and a reduction in fitness and life-span (Ballou *et al.* 2010).

4.2.2 Pedigree in the illegal wildlife trade

Pedigree reconstruction is a proven tool in wildlife forensics to aid in individualisation tests, and for determining whether an animal is captive bred or poached from the wild (Shorrock 1998; Johnson *et al.* 2014; Moore and Frazier 2019). With the emergence of large scale wildlife breeding facilities, farms, and increasing licenced breeders, having the capacity to determine the legitimacy of captive bred status is valuable (Nijman and Shepherd 2009; Janssen and Chng 2017; Moore and Frazier 2019). Industries such as the wildlife tourist trade (e.g. tiger tourism), entertainment (such as zoos/circuses), and the high interest the general public has in ‘exotic’ pets, are additional drivers for the trade. Breeders may turn to the ‘easier’ and more profitable route of animal trafficking/poaching over legitimate captive breeding (Rosen and Smith 2010; Janssen and Chng 2017; Moore and Frazier 2019). Appropriate tools are required to detect this activity.

To detect this trade it is imperative to have the ability to infer relatedness between individuals (White *et al.* 2012; Mucci *et al.* 2014; Frankham *et al.* 2015; Jan and Fumagalli 2016; Hogg *et al.* 2017). Species particularly vulnerable to this trade tend to be those not protected by national or international laws or conventions such as The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), as trade of these species may not be so stringently controlled. Relatedness information could be crucial intelligence; providing the data required to demonstrate that questionable practices are being carried out, and thus higher levels of protection may be warranted. One example is the Tokay gecko (*Gekko gecko*) a species native to Asia, including Indonesia. It was not listed on CITES, nor on Indonesia's list of protected species and appeared to be exploited by the 'captive bred' trade primarily for use in a range of traditional medicines. A report by Nijman and Shepherd (2015) from the non-government organisation TRAFFIC, found that six permitted breeding facilities were producing three million geckos per annum, however when all costs associated with breeding (i.e. food, utilities, staff wages) were factored in it was determined that this could not be a profitable business if these animals were indeed all captive bred. It was the compilation of data like this that was important to the Tokay gecko being listed on CITES in 2019 (CITES 2019), which should curb the impacts of this trade and allow for ongoing monitoring of this species.

While the Tokay gecko recommendations were based on a rationale that there was no defensible business case, genetic relatedness and individualisation testing would also provide important evidence if wild animals are being laundered through breeding facilities. Molecular analyses could determine if they have been legitimately bred (i.e. could show close relatedness), or if they are unrelated, suggesting wild origins (Frankham *et al.* 2015; Jan and Fumagalli 2016; Städele and Vigilant 2016; Hogg *et al.* 2017). Relatedness relies on

understanding the proportion of shared genetic information, and ideally in a wildlife forensic context it is drawn from individuals from captive and wild populations so that any information derived from known trafficking hotspots can be understood (Johnson *et al.* 2014; Städele and Vigilant 2016). A study by Jan and Fumagalli (2016) demonstrates a useful example of molecular analysis that was conducted on a range of parrots (e.g. red-tailed amazon (*Amazona brasiliensis*), yellow-headed amazon (*Amazona oratrix)*, and red-spectacled amazon (*Amazona pretrei*)) that are found in the 'captive bred' trade. They developed and tested 106 microsatellite markers, which were found to be robust and suitable for individualisation and parentage analysis, and the authors recommended the development of these markers for use as a wildlife forensics tool.

4.2.3 Relatedness

Relatedness refers to the measure of how many alleles are shared between individuals (identical by state: IBS) are, in fact, identical by descent (IBD) (Blouin 2003; Weir *et al.* 2006), and can be measured in the absence of parentage information, or by inferring parentage (Blouin 2003; Jones and Ardren 2003). Alleles that are identical by descent are ones that have 'recently' descended from a single ancestral allele (Blouin 2003). In terms of relatedness studies, 'recently' is generally understood to be a few generations prior to the individual/s currently in question. IBS is used to refer to alleles that currently have the same allelic state, which are either IBD or could be IBS by chance. A SNP variant could be IBS but not IBD if a (back) mutation reverses the difference that occurred as a result of genetic distance (Blouin 2003). It is only possible to confirm IBS; IBD must be inferred by probabilistic calculations. When using Single Nucleotide Polymorphism (SNP) data, if an individual, and a potential parent are both homozygous for different alleles at any locus, this can be classed as an 'exclusion' (Jones and Ardren 2003; Jones *et al.* 2010; Hayes 2011). While this is useful for close relationships (i.e. parent/offspring, full siblings), for more

distant or complex relationships (such as cousins, second cousins, great-grandparent/offspring) a comprehensive genetic data set can infer this level of relatedness (Jones and Ardren 2003). SNPs are increasingly being used to determine relatedness between individuals, with powerful statistical algorithms and compute allowing the analysis of thousands of SNPs (Weir *et al.* 2006; Huisman 2017). SNPs with high minor allele frequency (MAF: the frequency of the second most common allele in the population) are known to be useful for determining relatedness between individuals, and are often selected when developing SNP panels for parentage analysis (Murray *et al.* 2004; Matukumalli *et al.* 2009).

4.2.4 Categories of relationships

'Categories of relationships' refer to the different levels within a pedigree, such as 'full-siblings' or 'uncles and aunts'. Full-siblings and parent-offspring usually will share approximately 50% of their alleles based on IBD and are referred to as first-order relatives. Grandparent-grandoffspring, half-siblings, and aunts/uncles niece/nephew are second-order with approximately 25% of alleles shared. First cousins, great grandparent/offspring are third-order with approximately 12% shared, and so on (Weir *et al.* 2006). Not every set of genotypes can accurately classify every individual into one of these categories, especially in wild populations, where intergenerational mating may be more common. Often in captive breeding situations at least one parent is known, making the determination of pedigree a more straightforward task (Blouin 2003). However, when individual animals are traded, there may be very little or no data available to infer or reconstruct their pedigree. These situations rely on a robust genetic marker set that has been tested using a range of individuals of known relationships.

4.2.5 Genetic markers

Microsatellites have been used for several decades to infer genetic relatedness levels (Butler *et al.* 2007). In recent years single nucleotide polymorphisms (SNPs) have been increasingly used, particularly within wildlife genetics (Vignal *et al.* 2002; Glover *et al.* 2010). Microsatellites have the advantage of being more polymorphic and having high heterozygosity, i.e. the proportion of loci that have two different alleles. SNPs however, have been shown to be present in high abundance and representation across the genome, though more SNPs are needed due to their lower statistical power per locus (as they are most commonly biallelic and therefore heterozygosity can only have a maximum of 0.5). SNPs have also been shown to be a useful marker when working with degraded DNA (Sobrino *et al.* 2005) because of their small loci size compared to microsatellites which may be a few hundred base pairs long. While microsatellites still remain the preferred choice in human forensic casework, SNPs are increasingly used in human forensics for familial searching and biogeographical ancestry prediction (Maguire *et al.* 2014).

For non-human species, determining relatedness is limited when there are no suitable genetic markers available. The development of genetic datasets is important for use in combatting the illegal wildlife trade, to distinguish captive bred or wild-caught status. Kinship patterns in wild populations compared to captive populations can be varied, particularly in captive populations with multiple generations bred in captivity (Ellegren 1999; Farquharson *et al.* 2019). Inbreeding, dispersal, and kin-based behaviour are altered under captive conditions and thus will impact the relatedness structure. Wild populations pose an additional challenge due to the difficulty in sampling the entire population over multiple generations, inbreeding, and intergenerational breeding (Ellegren 1999; Blouin 2003). However with a comprehensive dataset that can be continuously updated, and a thorough analysis of captive populations,

methods can be applied to individual animals (Hayes 2011; Städele and Vigilant 2016; Blåhed *et al.* 2018; Farquharson *et al.* 2019). A forensic dataset should be comprehensive, robust, with well-characterised limitations (e.g. validated). Marker sets (most commonly microsatellites) used in human forensics have been vigorously tested, and standard commercial kits are used across the world for casework (Butler 2009). For wildlife forensic application, a new dataset set should be developed for each species if individualisation is needed. Ideally this should follow similar guidelines to human forensics (Linacre *et al.* 2011). Several datasets using both microsatellites and SNPs have been validated and developed into a routine test that can be used in wildlife forensics for species including but not limited to bigleaf maple (Dormontt *et al.* 2020), hen harrier (Van Hoppe *et al.* 2016) African grey parrot (Willows-Munro and Kleinhans 2019), carpet pythons (Ciavaglia and Linacre 2018), and rhinoceros (Dicks *et al.* 2017).

4.2.6 Echidna trade

There has been limited success of breeding echidnas within captivity, with only four Australian zoos having bred them in recent years and low juvenile survival rates of captive bred echidnas (Ferguson and Turner 2012; Wallage *et al.* 2015). This lack of success is due to the complex nature of echidna mating, with wild male echidnas forming ‘trains’ to follow the female echidna for several days (Rismiller and Seymour 1991). Based on this it is suspected that echidnas being sold as ‘captive bred’, through Indonesian captive breeding facilities, are likely to have been taken illegally from the wild (Beastall and Shepherd 2013; Janssen and Chng 2017). It is imperative that empirical data is collected to analyse the relatedness between individuals being sold. For sound evidence to be collected, first methods need to be developed that are specific for the short beaked echidna and have been tested against individuals of known pedigree.

4.2.7 *De novo* discovery of highly variable markers in the short beaked echidna

A previous study by Vanṕe *et al.* (2009) developed microsatellites for the short beaked echidna. The initial panel consisted of forty-three loci however only nine of these microsatellites amplified reliably making them limited for larger scale genetic analysis such as population studies, nor did they have the power required for pedigree reconstruction.

Reduced representation sequencing is a commonly used method for *de novo* discovery of highly variable SNP markers for individualisation in non-model organisms (e.g. Cruz *et al.* 2013; Kjeldsen *et al.* 2016; Blåhed *et al.* 2018; Ewart *et al.* 2019). Such data can assist in developing genetic pedigrees for the captive breeding of this species and for detecting the illegal trade of short beaked echidnas.

In this study DArTseq™, a method of reduced representation sequencing, was used to discover highly variable SNP markers in short beaked echidnas to be used for relatedness testing. I aimed to include individuals of known pedigree, and wild individuals with some known parentage to test the utility of SNPs for this, as well as for implementation in wildlife forensic investigations involving echidnas.

4.3 Methods

4.3.1 Sample collection

In total 236 samples were sourced from zoos and wild animals. Samples of known pedigree were obtained from four zoos in Australia that have successfully bred echidnas (Currumbin Wildlife Sanctuary, Australia Zoo, Perth Zoo, Taronga Zoo). For the complete list of sample information, see Table A6. Of the total 236 samples, 165 samples were from a single population (Lovely Banks; 42° 28' 00" S, 147° 13' 59" E) in Tasmania, collected by the

University of Tasmania (UTAS). This population was part of a long-term study with some known relationships (e.g. mother-offspring) while others were of unknown pedigree. Within the Tasmanian samples, 26 of these were replicate samples, and 12 replicates from zoo samples were also included. Samples were from a range of blood, tissue (muscle or liver), and genomic DNA. Samples were stored at -20°C prior to DNA extraction. All samples were collected under UTS Animal Ethics 2015000040 and Taronga Zoo Animal Ethics 3b/06/16. Samples from UTAS were collected under several UTAS animal ethics spanning multiple years; 97006, A0005711, A0005452, A0007124, A0008659, A0010426, A12320.

4.3.2 DNA extraction

DNA extractions were carried out using either the ISOLATE II Genomic DNA Kit animal tissue protocol (Bioline, Australia), or a salting out technique (Sunnucks and Hales 1996) (Table A6). DNA concentrations (Table A6) were determined using Qubit 2.0 Fluorometer High Sensitivity Assay (Thermo Fisher Scientific, USA). Dilutions were carried out to standardize the DNA at concentrations at ~20-50 ng/µL. 700-1000 ng of DNA were submitted from a total of 236 samples to Diversity Arrays Technology (DArT) (Kilian *et al.* 2012; Cruz *et al.* 2013) for SNP discovery, this included 39 replicates within and across plates.

4.3.3 SNP sequencing

Discovery and sequencing for SNP markers was carried out at Diversity Arrays Technology (DArT) in Canberra, Australia, using their proprietary reduced representation sequencing method, DArTseq™ (DArT Pty Ltd). DArT uses a combination of complexity reducing restriction enzymes, implicit fragment size selection, and next-generation sequencing (NGS) (Sansaloni *et al.* 2011), and is described in detail by (Kilian *et al.* 2012). For this study the restriction enzymes *Pst*I and *Sph*I were used. PCR conditions were: initial denaturation at

94°C for 1 min, then 30 cycles of 94°C for 20 sec, 58°C for 30 sec and 72°C for 45 sec, and a final extension step at 72°C for 7 min. After PCR, the resultant products underwent a c-Bot (Illumina) bridge PCR followed by single end sequencing for 77 cycles on an Illumina Hiseq2500. Sequences generated were processed using DArT analytical pipelines and were aligned using the Platypus (*Ornithorhynchus anatinus)* as a reference genome. Poor quality sequences were removed, and stringent selection criteria was applied to the barcode region to de-multiplex the sequence reads. Sequences were then trimmed to 69 bp and clustered with a Hamming distance threshold of 3. Low quality bases from singleton tags were corrected where possible. SNP calling was then carried out using the proprietary DArT pipeline called DArTsoft14. DArTsoft14 identified SNP markers using DArT PL's C++ algorithm. True allelic variants were discriminated from paralogous sequences by assessing a range of parameters within each sequence cluster including sequence depth, allele count and call rate.

4.3.4 SNP filtering

Filtering (Table A7) was carried out on the set of 182 samples that passed quality control using the R package '*dartR*' version 0.93 (Gruber *et al.* 2018), based on call rate (0.95). Loci that were not present in 95% of individuals were removed from the dataset. DArTseq runs 30% of the samples in replicate in independent libraries and sequencing runs, and the consistency of each locus is measured across these replicates. Loci that were not 100% reproducible were removed from the dataset. Individuals with less than 80% call rate were filtered for and subsequently removed from the dataset (4 samples). Any monomorphic loci arising because of the removal of individuals were also removed. Any loci with multiple SNP loci in a fragment (secondaries) were also filtered out. Error rates and missing data of replicates were analysed to decide which replicate of each sample would be kept for the final dataset. Deviations from Hardy-Weinberg equilibrium were tested, using the Bonferroni correction for sample size, and after initial filtering no loci showed significant departure and

therefore were all retained. 2406 SNPs from 139 unique samples were in the final dataset after removing replicates for further analysis.

4.3.5 Relatedness analyses

Five different datasets were constructed to analyse relatedness between individual echidnas (Table 4.1). Dataset A) included all samples (including replicates) to assess accuracy; Dataset B) included all samples after removing replicates; Datasets C) and D) involved three different captive populations: Currumbin Wildlife Sanctuary (CWS) and Australia Zoo (C), and Perth Zoo(D). Only four samples from Australia Zoo passed quality control so due to the small sample size were analysed with CWS samples. All of the key known pedigree samples from Taronga Zoo failed quality control so samples from this zoo were not analysed further. Finally, Dataset E) included all samples from Tasmania, once replicates had been removed.

Table 4.1 Source of short beaked echidna samples used in each dataset used to analyse IBD.

Dataset	***Description***	***Number of samples***	***Number of SNPS***
A	All samples including replicates, to confirm they were producing expected kinship values for identical samples	178	3667
Bi	All samples with replicates removed	139	2406
Bii	Dataset B filtered for MAF ≥ 0.4	139	167
C	Captive Currumbin Wildlife Sanctuary/Australia Zoo samples	28	1701
D	Captive Perth Zoo samples	8	430
E	Samples obtained from Tasmania wild population	88	979

The R package *SNPRelate* (Zheng *et al.* 2012) was used to obtain Identity by Descent (IBD) kinship (k) values and assess relatedness between individuals. Kinship coefficient values were generated for each dataset using the IBD script within *SNPRelate* which gives standard kinship results as 0.5 for identical samples, 0.25 for first-order relationships (parent/offspring or full-siblings), 0.125 for second-order relationships (half-siblings or grandparents), and so on. The Maximum Likelihood (ML) estimator was chosen as it is commonly preferred for estimating relatedness (Milligan 2003). Kinship values were compared to expected values for the known relationships within the captive populations. For Dataset E (the wild dataset), IBD kinship values were also generated and analysed in order to compare to the captive population results. I compared the proportion of unrelated (k = 0), first-order relationships (k = 0.25), half-siblings (k = 0.10-0.18), and distantly related (k < 0.10), to the captive population of CWS. Additionally, within Dataset E, a 'real world' example test was conducted by comparing the previously calculated kinship values of single offspring (M.50841) with three possible fathers (M.48072; M.48073; M.48075). From this, a pedigree was reconstructed demonstrating the confirmed relationships.

A subset of Dataset B (named Bii) was additionally filtered for loci with a high (≥ 0.4) minor allele frequency (MAF), as high MAF SNPs are informative for use in relatedness testing (Murray *et al.* 2004; Matukumalli *et al.* 2009). IBD analysis was then conducted on this subset to determine if the kinship values of each individual were able to be generated from this smaller dataset and then was compared to values from Datasets C, D and E. Probability of identity (PI) for individuals and first-order relatives (PIsibs) was calculated using GENALEX 6.502 using standard parameters. This allowed for determination of the minimum number of SNPs needed for individualisation and relatedness testing.

4.4 Results

182 samples that passed DArTseq quality control initially generated 27,258 SNPs before filtering. 54 samples failed quality control, primarily due to low DNA quantity. Once all filtering was conducted (as per methods section 4.5.1 and Table A7) and replicates were removed, the dataset consisted of 139 samples and 2406 SNPs with 0.99% missing data. No samples in the final dataset were found to have deviated from Hardy-Weinberg Equilibrium. 1049 SNPs were found to have a minor allele frequency of greater than or equal to 0.1.

4.4.1 Relatedness and kinship

Identity by Descent (IBD) testing was conducted on all datasets to produce kinship values that reflect the level of relatedness between individuals. These values were then compared to the expected values extrapolated from the studbook pedigree provided by the donor zoos, to demonstrate the utility of these SNPS for use in a wildlife forensic context.

Dataset A: Replicate analysis confirmed that all replicates of individual samples were producing expected results between and across the three plates. Using IBD analysis, the expected replicate kinship value is 0.5 (with first-order relationships giving 0.25, and so on). When all replicates were compared with corresponding sample to ensure that value was approximate to 0.5, no outliers were observed (Table A8). The average observed kinship coefficient for 38 samples was 0.491 ± 0.007SD, with a standard error of 0.002.

Dataset C: Currumbin Wildlife Sanctuary (CWS) and Australia Zoo captive animals

Kinship coefficients were calculated using IBD, ML from Dataset C and then compared to the known pedigree samples. Figure 4.1 compares the kinship values obtained for all samples, with the expected value (i.e. k = 0.5 for identical samples, k = 0.25 for

parent/offspring or full-siblings, k = 0.125 for half-siblings) extrapolated from the known pedigree. This figure shows there was a slight spread of values along the y-axis. Figures 4.2 and 4.3 show the reconstructed pedigrees for Currumbin Wildlife Sanctuary and Australia Zoo respectively. For raw kinship values see Tables A9 and A10.

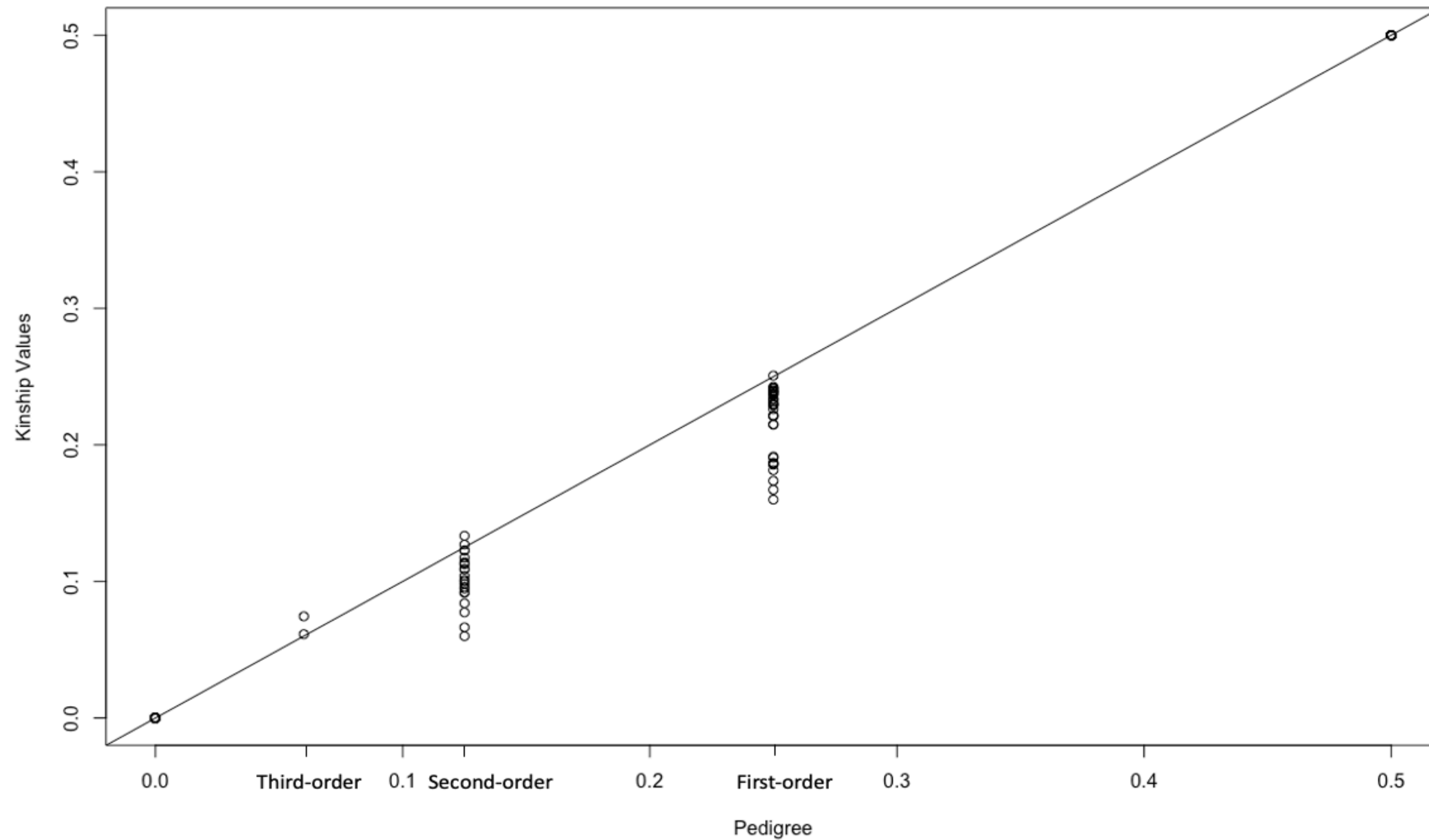

Figure 4.1 Comparison plot of short beaked echidna samples in Dataset C (Currumbin Wildlife Sanctuary and Australia Zoo) kinship values obtained from IBD Maximum Likelihood Estimator (*SNPRelate*), compared to the expected values from the known pedigree. First-order: k = 0.25, Second-order: k = 0.125, Third-order: k = 0.06.

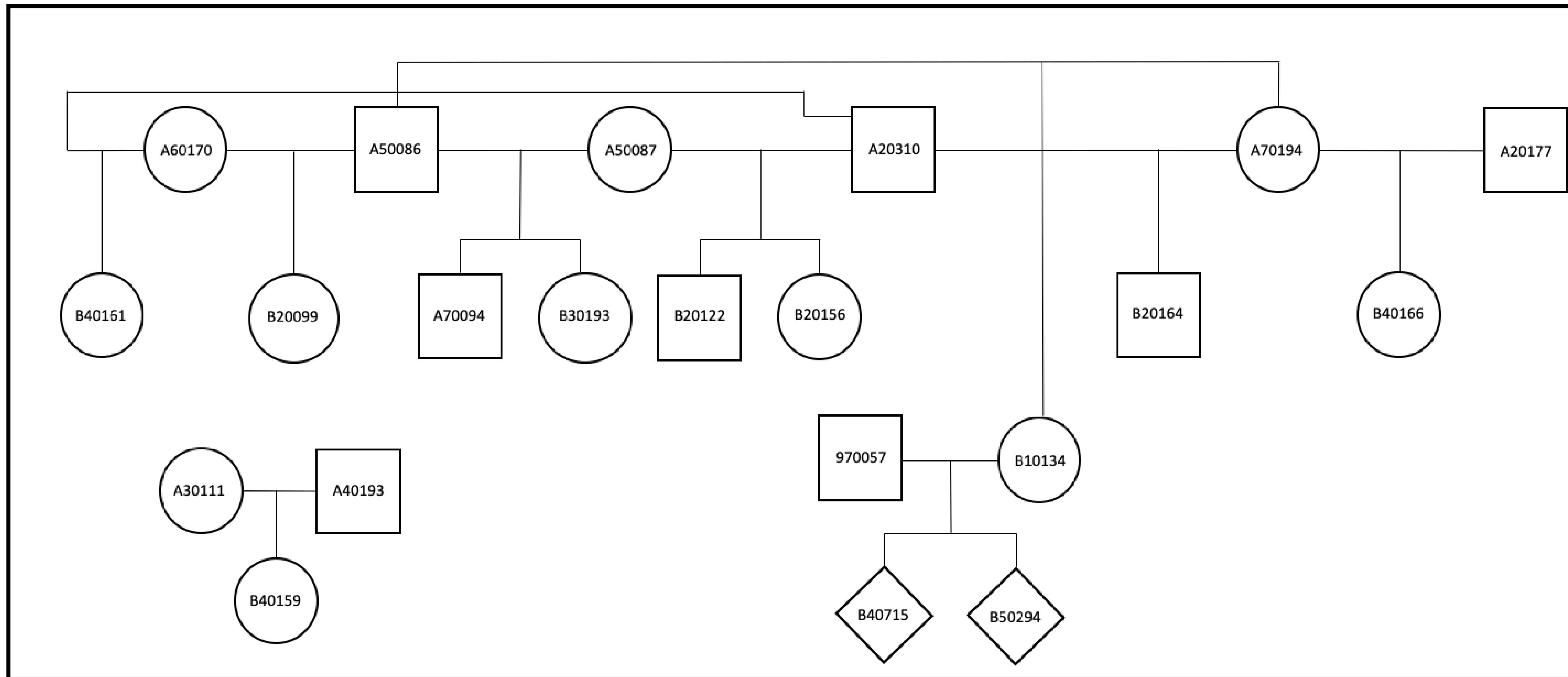

Figure 4.2 Reconstructed pedigree for subset of Dataset C, representing relationships in Currumbin Wildlife Sanctuary captive short beaked echidna population. Square = Male, Circle = Female, Diamond = Unknown sex.

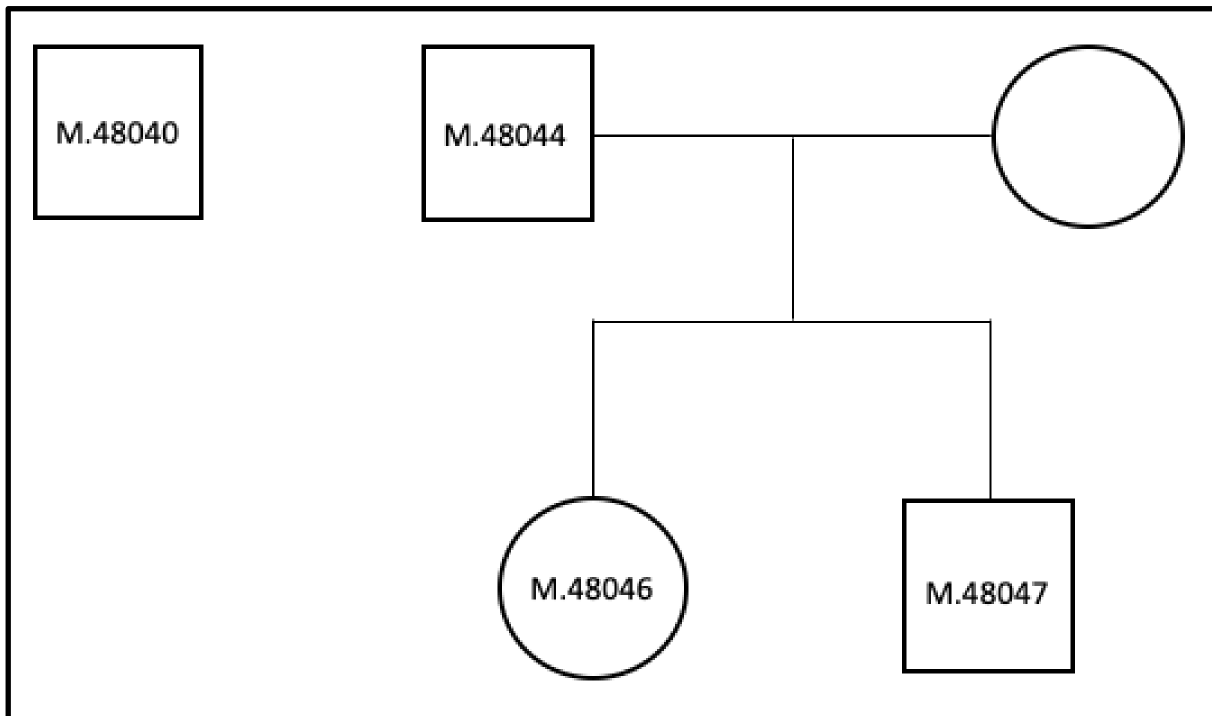

Figure 4.3 Reconstructed pedigree for subset of Dataset C, representing relationships in the Australia Zoo captive short beaked echidna population. Square = Male, Circle = Female.

Dataset D – Perth Zoo

Kinship coefficients were calculated using IBD, ML from Dataset D and then compared to the known pedigree samples. Figure 4.4 shows the summary of the known pedigree versus the observed IBD kinship values (raw values in Table A11). The graph shows similar results to Dataset C, with samples producing values only within two levels of relatedness; parent/offspring and full-siblings (k = 0.25), and second-order (k = 0.125). One undocumented parent/offspring relationship was illustrated in this analysis between M.48052 and M.48051, and corresponding second-order relationships (half-siblings) and these relationships can be seen as outliers in Figure 4.4. Figure 4.5 shows the reconstructed pedigree from the kinship values, demonstrating the relationships within this dataset.

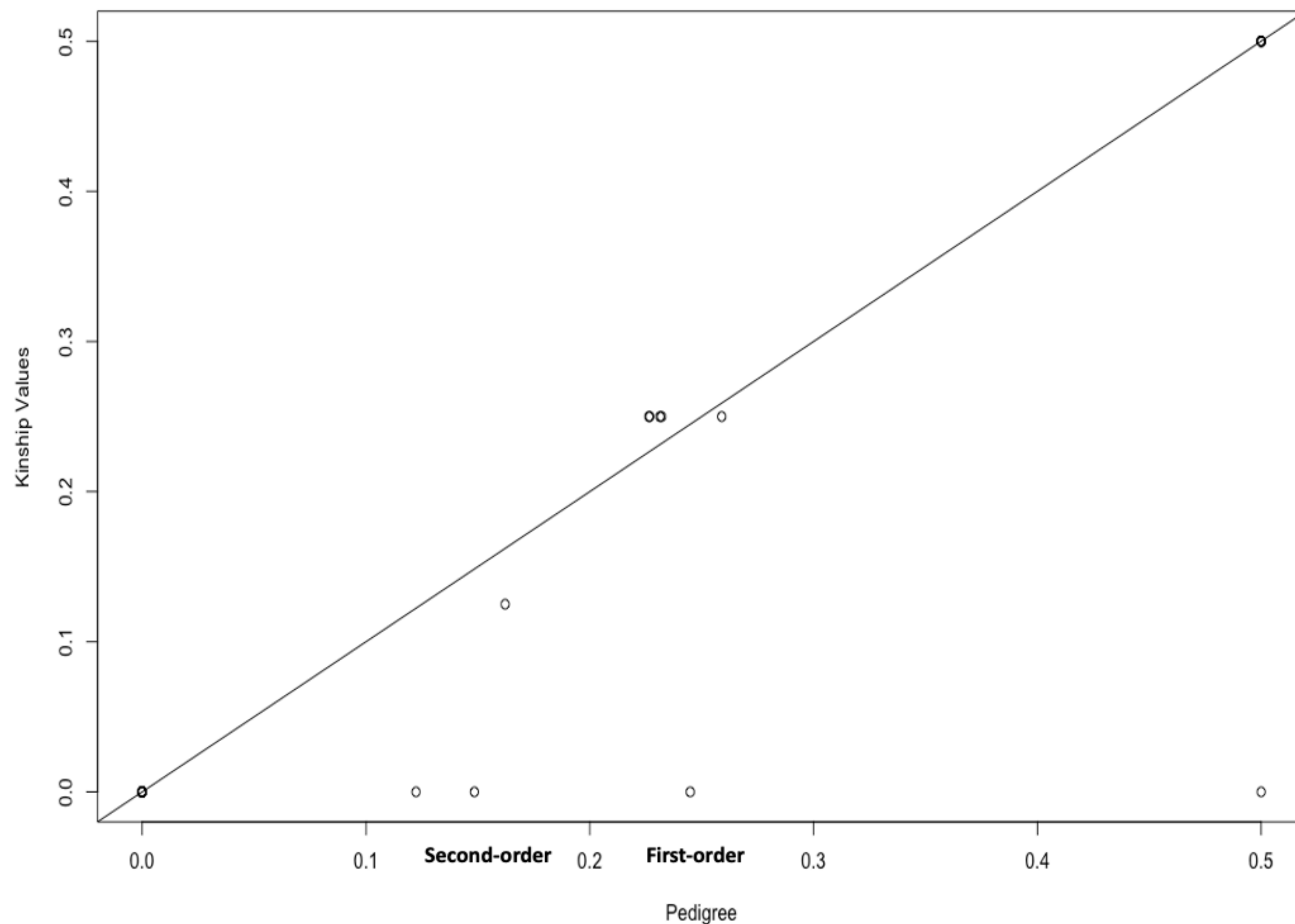

Figure 4.4 Comparison plot of short beaked echidna samples in Dataset D (Perth Zoo) kinship values obtained from IBD Maximum Likelihood Estimator (*SNPRelate*), compared to the expected values from the known pedigree. First-order: k = 0.25, Second-order: k = 0.125.

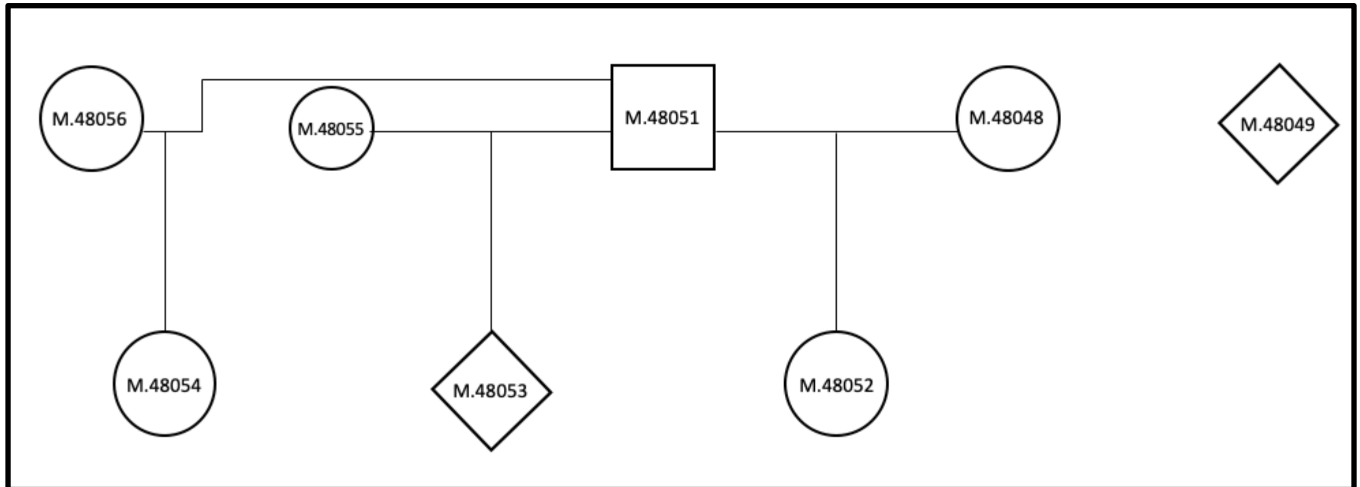

Figure 4.5 Reconstructed pedigree for Dataset D representing relationships within the Perth Zoo captive short beaked echidna population. Square = Male, Circle = Female, Diamond = Unknown sex.

Dataset E – Tasmania

The kinship values for the Tasmanian wild population are depicted in Figure 4.6. This figure shows the low number of relationship comparisons with kinship values of k = 0.25 (parent/offspring or full-sibling), and the increasing number having kinship at k = 0.125 (half-sibling or grandparent/grandoffspring). Twenty-five percent of compared relationships have kinship values < 0.1, indicating the large spread of different relationship levels within this wild population. When compared with the captive population of Currumbin Wildlife Sanctuary (Figure 4.7), the wild population has a higher proportion of distantly related (k < 0.1; cousins, second/third cousins) individuals compared to closely related individuals (k = 0.25; parent/offspring; full-siblings). Kinship values for all samples are presented in Table A12. Paternity of M.50841 was able to assigned using relatedness values with first-order relationship established with M.48072, and zero relatedness with the other two candidate males (M.48073 & M.48075).

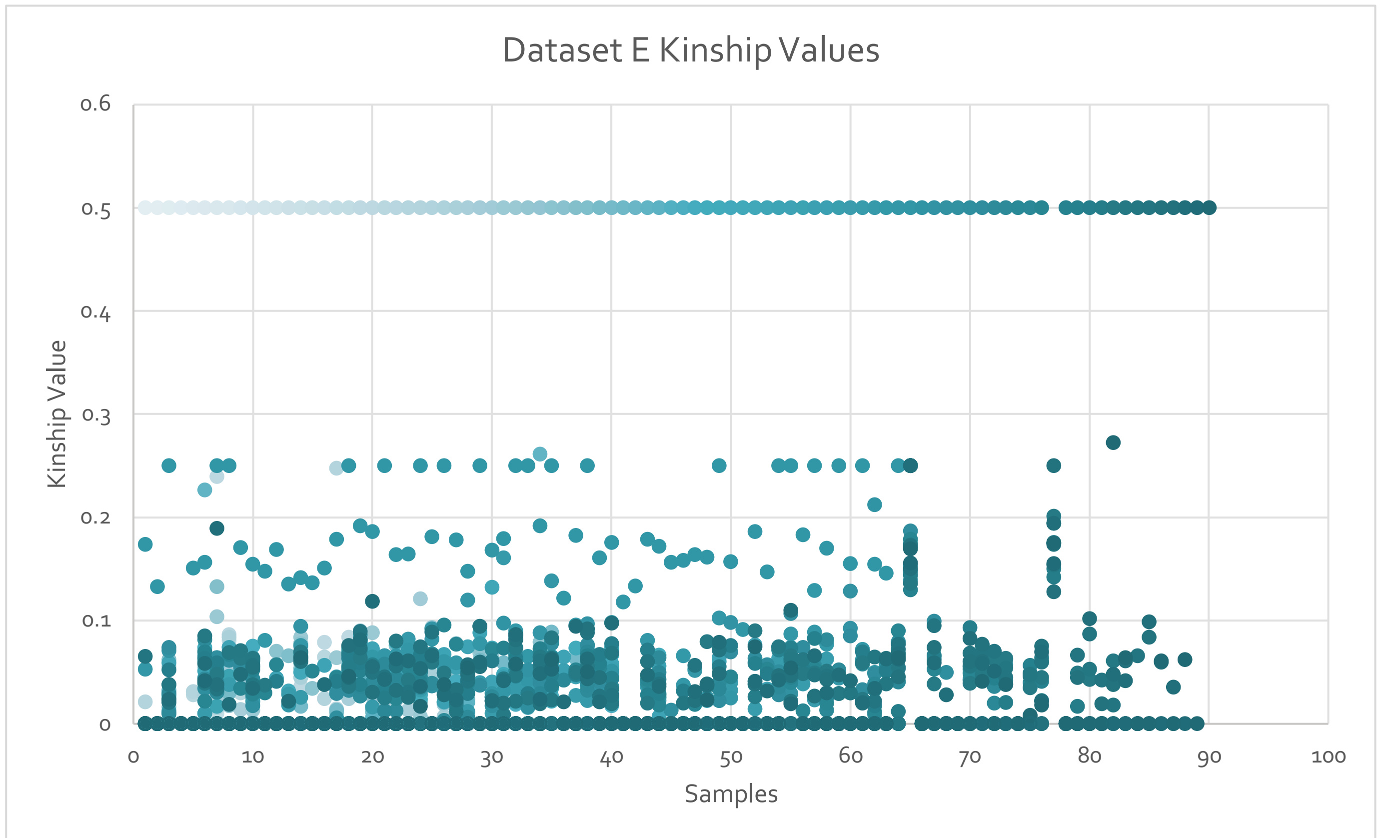

Figure 4.6 Kinship values from short beaked echidna samples (*n=88*) from Dataset E (Tasmania) using IBD Maximum Likelihood estimator (*SNPRelate*).

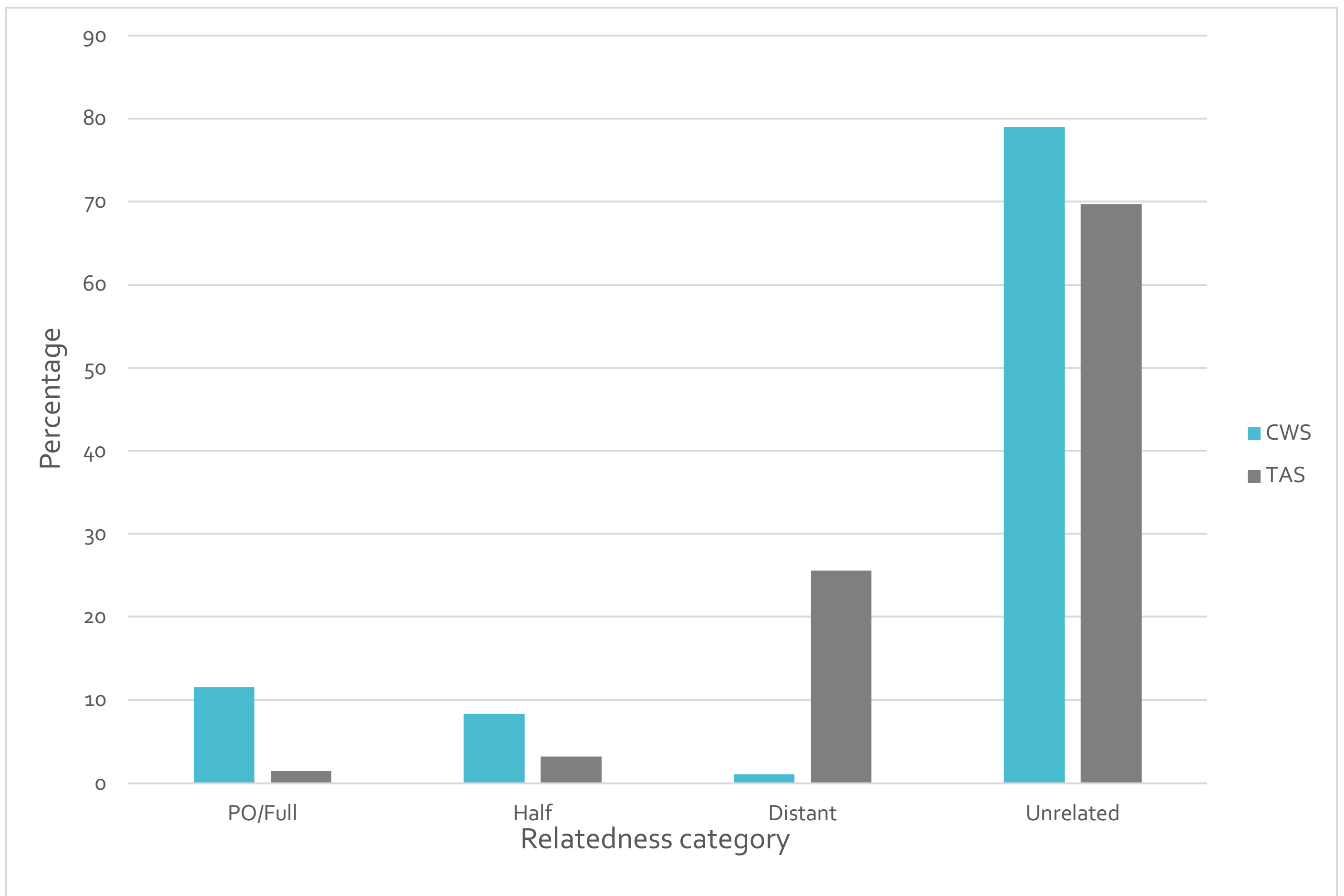

Figure 4.7 Comparison of the percentage of short beaked echidna relationships in wild Tasmania (TAS) population (grey) to the captive echidna population of Currumbin Wildlife Sanctuary (CWS) (blue). Relationships were categorised into Parent-Offspring/Full-siblings (k = ~0.25); Half-siblings (k = 0.10-0.18); Distant relatedness (k < 0.1); and Unrelated (k = 0).

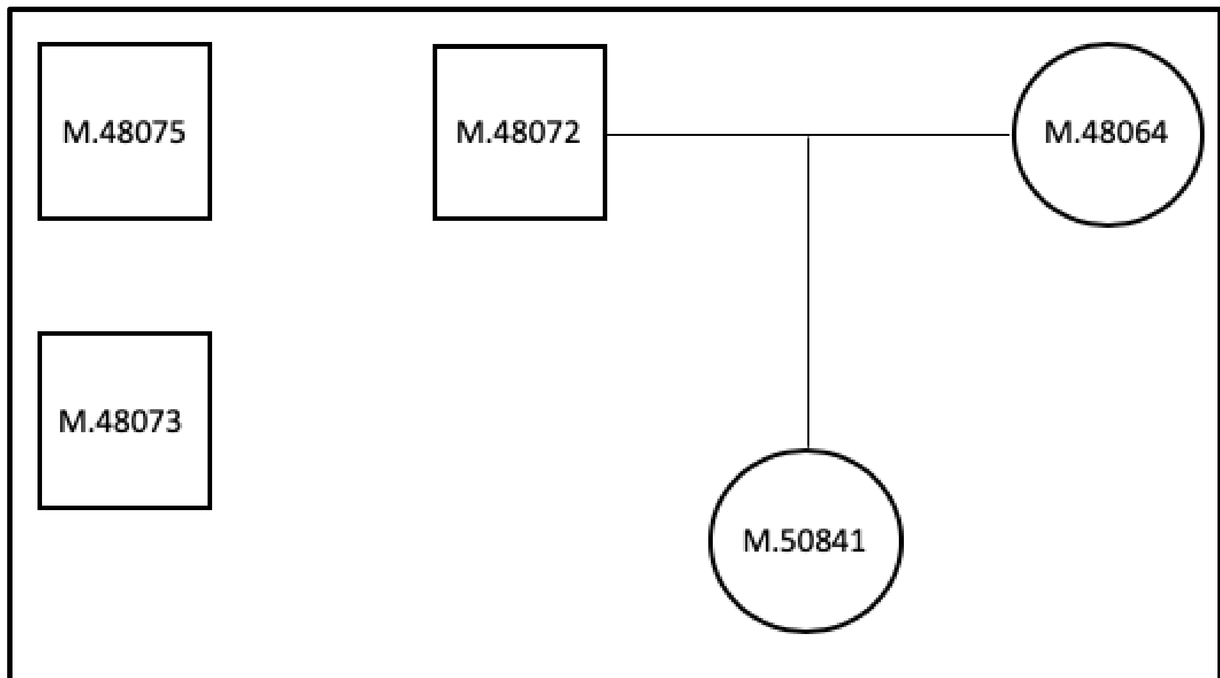

Figure 4.8 Reconstructed pedigree for short beaked echidna sample M.50841 based on IBD kinship values. M.48072 showed a kinship value representing a first-order relative, whereas M.48073 and M.48075 showed kinship values representing unrelated individual echidnas.

4.4.2 Probability of Identity

Probability of Identity is used for the purposes of determining the probability that two independent samples will have the same genotype. This can indicate how many SNPs are needed to get a low probability of two individuals randomly matching. After filtering for MAF of 0.4 or greater, 167 SNPs remained in the dataset (Table A13). Probability of identity (PI) for individuals and first-order relatives (PIsibs) was calculated using GENALEX (Figure 4.9). The PI decreased rapidly, reaching zero (PI <0.01) with the 15 most informative SNPs, and a combination of ~20 SNPs is enough to correctly identify/separate first-order relatives (PIsibs). The PI of the most common profile was 2.902×10^{-73} and the PI of the rarest profile was 3.462×10^{-112}. IBD testing was done on Dataset Bii (i.e. the subset of 167 SNPs) and demonstrated concordance with pedigree testing using the larger dataset. Identical pedigrees were able to be

reconstructed for each captive population, demonstrating all levels of relationships, as was found with the larger SNP datasets.

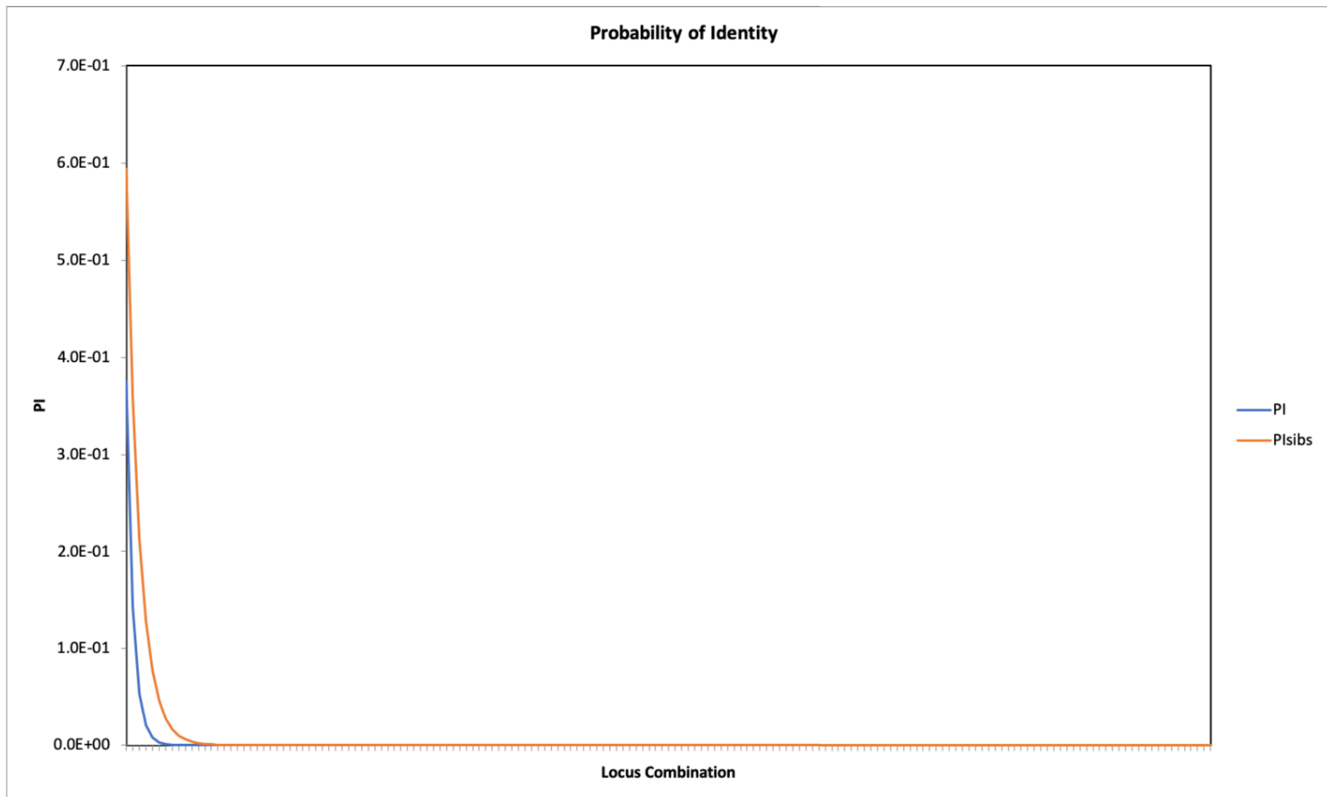

Figure 4.9 Probability of identity (PI) for short beaked echidna samples combining 167 SNPs calculated in GENALEX. The PI decreases rapidly with increasing number of SNPs and reaches zero within the first 15 informative SNPs. A combination of approximately 20 SNPs is sufficient to separate first-order relatives (PIsibs).

4.5 Discussion

This study presents the first set of highly variable SNP markers suitable to test relatedness in the short beaked echidna. I aimed to develop a robust set of markers that could determine relatedness between individuals and compare this to echidnas of known pedigree. From this pilot study, these markers can be incorporated into a wildlife forensic genetic toolbox that could be implemented when a short beaked echidna is traded. These SNP markers identified in this study, were shown to be of high resolution and produced reliable kinship information that defined individual relationships with a high level of statistical power making them relevant for casework applications. I found that 167 SNPs had a MAF of greater than or equal to 0.4, and that this smaller marker set could also determine relatedness between individuals. A combination of ~20 of the most informative SNPs would be appropriate to individualise unrelated echidnas, as well as distinguish first-order relatives, providing a cost and time efficient way of analysing samples. By analysing both captive and wild populations I was able to demonstrate patterns of relatedness for both scenarios which is useful background information for any investigation of suspect individuals. Additionally, these markers will also be of use for legitimate breeders, given the difficulties associated within Australian zoos, where paternity may often not be known due to the unique breeding behaviour in echidnas.

4.5.1 Relatedness analysis

The methods developed in these studies were able to verify the studbook pedigree of 34 out of 35 known samples from three captive populations of echidnas at Australian zoos; Currumbin Wildlife Sanctuary, Perth Zoo, and Australia Zoo, and reconstruct the pedigrees of these captive populations. Molecular data for one sample (M.48052)

from Perth Zoo did not conform with the studbook pedigree. The expected parents for this captive born echidna were M.48048 (mother), and M.48050 (father) (a sample that failed quality control and was not included in the final dataset), however due to values suggesting a first-order relationship, it is hypothesised that the parents are M.48048, and M.48051 which was also supported by second-order kinship values, or that there has been a sampling/labelling error that has been made prior to receiving the sample.

Currumbin Wildlife Sanctuary (CWS) had the largest captive population, with more than one generation represented. This allowed for a range of relationships to be examined and most fully demonstrated the usefulness of the markers developed effectively. The range of relationships represented (including half-siblings and grandparents) could mirror what is seen in a casework situation if suspect animals have actually been legitimately bred. Some samples had lower kinship values than predicted, particularly the half-siblings, however variation for IBD in siblings is expected (Visscher *et al.* 2006; Lopes *et al.* 2013; Städele and Vigilant 2016). A similar result was observed in a study on commercial pigs, where their half-sibling kinship coefficient value ranged from 0.02-0.28 when using IBD analysis to obtain kinship values (Lopes *et al.* 2013). Full-siblings are expected to have an average kinship of 0.25, but a study done on human full-siblings showed that this value can range from 0.19 to 0.41 (Visscher *et al.* 2006), and therefore it is usual to have such variances with half-siblings as well. Jones *et al.* (2010) reviewed various methods, including IBD, for parentage analysis and concluded that they all were suitable to be used for such analysis. However, they emphasised that a successful study should not depend solely on the method and should also include adequate sampling, and an

appropriate number of high-quality markers. It is crucial for studies where development of markers is taking place to have good sampling and a range of relationships to confirm the markers are robust, something which is demonstrated in this study. This is also important when developing markers for use in wildlife forensics to make sure that the tests are robust and suitable for use in unknown or varying scenarios.

4.5.2 Wild population analysis

The wild echidna population from Lovely Banks, Tasmania showed clear differences compared to the relatively closed captive populations, with a wide range of kinship values, many of which represent more distant relationships (e.g. cousins, second cousins) than that observed in the captive populations. This single Tasmanian population was studied for approximately twenty years, with a large amount of data collected about behaviour and relationships (e.g. Harris *et al.* 2012, Nicol and Andersen 2002, Morrow *et al.* 2009, Nicol *et al.* 2011), and several individual echidnas were monitored for multiple years. Research into the genetic variation of captive populations compared to wild populations is commonly done for conservation purposes, often to investigate inbreeding or species management (Coetzer *et al.* 2017; Svengren *et al.* 2017). The results here suggest that this wild population is more diverse than the captive population, however, there is still some level of close relatedness, given the number of first-order relationships within the wild population. Echidnas have the potential to travel large distances, though their home range has been suggested to be relatively small (~0.4-1.1km^2), particularly with females returning to their offspring (Abensperg-Traun 1991; Rismiller and McKelvey 1994, 2000), which could explain the number of close relationships in this wild population. Some studies have reported very limited amounts of IBD within wild populations

compared to captive (Knief *et al.* 2015), contrasting with what is observed here. To further understand the relatedness of both wild and captive echidnas, I would recommend broader sampling of wild populations, and multi-generational sampling of new echidnas born in captivity. This is will prove useful for construction of a database that has a wide and varied sampling of relationships.

When observing wild populations it is not always possible to witness mating events. Within the Tasmanian population there was an instance of unknown paternity, however three males were identified as being associated with the female at the time of conception, thus using the simple kinship values I was able to determine the father. Such an application of this test could also be used to provide information to zoos about their captive animals, especially if females are given mate choice through replicating mating trains (Wallage *et al.* 2015). This example also shows the benefits of having this test for exclusionary purposes. It can be used to eliminate potential relationships which can be key for providing intelligence to law enforcement. Within a forensic science context, exclusions of individuals or parent/offspring relationships can often be as important as not excluding a sample, and consequently should be reported in the same fashion as positive results (Butler 2009). In some casework situations, being able to exclude a level of relatedness may be sufficient in proving/disproving a claim or may be enough to warrant further investigation. It is also a common practice in agriculture (Sherman *et al.* 2004; Hayes 2011), as well as zoos (Ogden *et al.* 2007; Modesto *et al.* 2018; Norman *et al.* 2019) to exclude possible parentage and inform subsequent management of breeding programs. Caution in these scenarios should be taken with the possibility of identical twins as they will share the same DNA, and therefore two individuals cannot be excluded from

one DNA profile (Butler 2009). However there has been minimal evidence of short beaked echidnas producing more than one young per breeding season (Griffiths 1978; Pierce *et al.* 2007). A study by Pierce *et al.* (2007) recorded a female echidna successfully raising more than one young in a breeding season, though it was believed that they were not identical twins. A study by Griffiths *et al.* (1973) recorded a wild echidna producing twins, and Griffiths (1978) also lists two instances of observing more than one young but concludes that twins would be extremely rare.

4.5.3 Comparison of microsatellites and SNPs

This study showed the robustness of SNPs for documenting the detail of relatedness than can be generated from a comprehensive and informative dataset. Microsatellites were, and still are widely used for such pedigree and parentage testing (Butler *et al.* 2007; Ross *et al.* 2014; Coetzer *et al.* 2017), however SNPs have been used because they are more abundant, are representative, and are more stable (lower mutation rate) (Vignal *et al.* 2002; Amorim and Pereira 2005; Butler *et al.* 2007; Tokarska *et al.* 2009; Blåhed *et al.* 2018). In wildlife forensics, studies like what is presented here have been useful as the breadth of the trade emerges and the increase of live pet trade becomes more apparent. Many of these studies have been carried out using microsatellites (Ogden *et al.* 2008; Wesselink and Kuiper 2011; Frankham *et al.* 2015; Coetzer *et al.* 2017; Ciavaglia and Linacre 2018; Grela *et al.* 2019; Willows-Munro and Kleinhans 2019), with limited studies published using SNPs (Ogden *et al.* 2013; Dormontt *et al.* 2020). This study contributes to this growing field and can act as a guide for future research in wildlife forensics looking to develop a next-generation marker set.

Vanṕe *et al.* (2009) developed microsatellites for the short beaked echidna, though only 9 out of 43 tested were found to be reliable for use in genetic testing, and no research has been published using these markers since. When compared to this study, the SNPs developed here were able to produce high resolution genetic data, allowing for discrimination at the individual level. A study by Tokarska *et al.* (2009) conducted a comparative analysis of microsatellites and SNPs for parentage of European bison (*Bison bonasus*). Using microsatellites they were only able to confirm two paternities with 80% confidence using 17 loci, due to the low microsatellite diversity in this species. When using SNPs, they retrieved the most informative SNPs (i.e. ones with high heterozygosity), from this they deemed that 50-60 SNPs could be used to generate 95% confidence of paternity, without known mothers. A similar trend has been seen in the short beaked echidna data generated to date, due to the minimal suitable loci using microsatellites for this species, which was contrasted with the large number of SNPs that can be used for confirming individual relatedness in this study. As the microsatellites developed for the short beaked echidna have not been used for any published analysis of relatedness, it would be useful to follow this up with a comparative study to truly know the limits of both sets of markers. Many such comparative studies have been conducted in other species (Sellars *et al.* 2014; Deniskova *et al.* 2016; Garbe *et al.* 2016; Lemopoulos *et al.* 2019) and a similar study would be an important addition to knowledge of echidna genetics.

4.5.4 Application for the illegal wildlife trade

Currently short beaked echidnas are claimed to be legitimately bred in permitted breeding facilities and sold with only paperwork documentation (Beastall and Shepherd 2013). This test has provided the crucial first steps to the development of

SNP markers as part of the toolbox to investigate suspected short beaked echidna trade. With further optimisation these SNPs could be used to generate defendable evidence for enforcement as well as to build intelligence on this trade. The use of both captive and wild populations in this study provided the baseline for the expected relationships seen in each. This can then assist with identifying poached animals when individual genetic relatedness levels are calculated, and will in turn increase confidence and accuracy when compared with multiple other individuals, particularly when they have come from the same breeding facility.

It has been suggested that genetic relatedness data should be routinely integrated into studbooks or life history data kept for captive animals (Bömcke and Gengler 2009; Fienieg and Galbusera 2013) something which is supported by my data. Additionally, bio-banking genetic material of species should be considered when they are traded between reputable organisations or permitted to be traded, which can also be combined with zoo records (Hogg *et al.* 2018). When applying these in a casework situation it would be beneficial to work effectively with law enforcement and/or zoos to ensure results that are as accurate as possible. There is the possibility that intelligence may be limited or incorrect but when used in combination with genetic testing, this has potential to prove or disprove any claims. The use of the markers developed here should refined and become routine when echidnas are traded. This could also benefit legitimate zoos that breed echidnas by allowing them to definitively show true captive bred status. This could be widened with routine sample collection and should be an encouragement to zoos to sample future offspring. Similar results have also been found when pedigree and molecular data are used in conjunction and have provided useful results for management of populations, such as the Tasmanian

Devil (*Sarcophilus harrisii*) (McLennan *et al.* 2018), an endangered species which requires management to improve genetic diversity and limit inbreeding (Gooley *et al.* 2019). Additionally a study by Coetzer *et al.* (2017) on Cape Parrots (*Poicephalus robustus*), a South African species that is also found in the illegal pet trade, analysed captive bred populations in comparison to wild populations, as well as assigning parentage to captive bred animals with the use of microsatellites. Similar to this study, they were able to assign parentage with the use of known trios. They recommend the collaborative nature of a zoo's studbook, with genetic testing, and with increasing sample collection. Such an approach would also benefit the short beaked echidna, as continuously updating the information and sample set from captive animals can help ensure the legality of trade between zoos. Given results from Chapter 3 that demonstrated some structure between Tasmanian and the mainland samples, widespread sampling of the wildlife population, including known populations, could also further enhance what has been demonstrated in this chapter. These markers have the potential to be used with the other genetic tools developed, such as the mitochondrial DNA test (Chapter 2), or analysis of subspecies (Chapter 3) for the short beaked echidna to aid in stopping the illegal trade of this species.

4.5.5 Further work

The next key step for this research would be to validate my smaller informative marker set of 167 loci, having demonstrated (section 4.5.2) that it has the resolution to separate individuals as robustly as that based on thousands of SNPs. This should be done using robust validation criteria commonly used in forensic science such as accuracy, repeatability, sensitivity and specificity (Butler 2009; Linacre 2009; Linacre *et al.* 2011; SWGDAM 2016; SWFS Technical Working Group 2018). This should be

a goal for methods that are aimed to be used in a casework scenario, and would be appropriate for this test to align with the validated mitochondrial DNA test presented in Chapter 2 (Summerell *et al.* 2019). Given that my replicate samples produced expected values across three plates with low error, and repeatability is one of the common parameters used in such studies, this is a promising result that suggests these markers would be appropriate for validation.

I would recommend that the most informative SNPs be developed into a validated panel. SNP panels have been developed for many species such as plants and animals in agriculture (Matukumalli *et al.* 2009; Hayes 2011; Clarke *et al.* 2014; Bianco *et al.* 2016), conservation of wild populations (Kjeldsen *et al.* 2016; Kleinman-Ruiz *et al.* 2017; García-Fernández *et al.* 2018), and for captive populations and breeding programs (Lew *et al.* 2015; Norman *et al.* 2019). Additionally a number of commercial species panels (primarily microsatellites) developed for use in forensic science and breeding are now commonly used for species such as dogs (Dayton *et al.* 2009; Wictum *et al.* 2013), cats (Menotti-Raymond *et al.* 2005), and bovine (Van De Goor *et al.* 2011). Given that a combination of a relatively small number of SNPs (~20) can be used for individualisation of echidnas, and determine first-order relatives, such an assay would ideally be developed so that this can be implemented as a wildlife forensic technique to be used when the origin of an echidna is in question. It has been estimated that approximately greater than or equal to 100 SNPs will significantly reduce the probability of incorrect parental assignments associated with lower SNP frequencies (Hill *et al.* 2008). Therefore, as more distant relatives would also need to be taken into account, I recommend using the suite of 167 SNPs, as these

still have a high MAF and I found they were able to differentiate more complex relationships.

SNP panels allow for greater accuracy (Allendorf *et al.* 2010; Fienieg and Galbusera 2013) and should be a key development as studies such as this increase within wildlife forensics. A study by Dormontt *et al.* (2020) developed a panel of SNPs for individualisation of bigleaf maple (*Acer macrophyllum*), a timber that has a high profile in the illegal logging trade. They validated 131 SNPs for this species, which offer high discrimination and was one of the first of its kind for use in combatting this crime. They reported that their SNP panel also worked well with low concentrations of DNA, which has also been recorded in several other studies (Senge *et al.* 2011; Gettings *et al.* 2015; von Thaden *et al.* 2020). It is common in forensic science for samples to be of variable quality and often samples are degraded or contain PCR inhibitors (e.g. soil, dyes). SNPs, due to their smaller loci size are more likely to be amplified than microsatellites which can have longer amplicons and therefore high molecular weight loci can 'drop out' when a degraded sample is amplified (Butler 2009). This is particularly important for the short beaked echidna, as it would be ideal to have a SNP panel that works on DNA extracted from quills (to align with Chapter 2; Summerell *et al.* 2019) to provide a non-invasive way of determining genetic relatedness. A number of samples used in the SNP development did not pass quality control due to low concentration or likely degradation. A validation study on a smaller number of SNPs would further be able to test the limits of this, which is why I recommend this as the next stage of this research. This could potentially allow for some of the important low-quality samples to be added to the data already presented. Following validation of markers, investigation into the development of a population

database of echidnas could be conducted, similar to what is used in human forensics (Butler 2009) to increase the accuracy and reliability of the results.

My data demonstrate the possibility of marker development for any species within the illegal wildlife trade. I found that the key to obtaining quality results for a marker discovery project was to ensure that there were samples of known origin and relationships in the baseline dataset. This allowed me to verify that markers were performing as expected. Having trios from a number of different zoos was highly advantageous, allowing for trial and error testing within the dataset so I could understand the subset of SNPs was working efficiently after filtering. It also allowed comparison between captive bred animals and wild-caught animals. This is crucial in this trade, as breeders are claiming their animals have been bred legitimately, under similar circumstances as they would be in the zoos from which samples were collected. Once knowing what an 'expected' captive population looks like, which for echidnas (given their difficult breeding), does include some wild-caught (injured and rehabilitated) animals as well, in comparison to a wild population, parameters for analyses can be set. These can then be used in a casework situation to provide the evidence required for proving or disproving captive-bred status. While I recognise that known pedigree samples may be difficult to obtain in a number of species, I have demonstrated the benefit of using such samples.

4.6 Conclusion

This chapter presents the first set of SNP markers developed for the short beaked echidna that can determine relatedness between individuals. I was able to compare the kinship values produced by Identity by Descent analysis with expected values from

individuals of known pedigree. The analysis of both captive populations and wild populations demonstrated the key variances between the two, particularly demonstrating intergenerational mating in a wild population. The statistical power of the SNPs to separate individuals was high. The use of the informative SNPs is recommended to be validated and the SNPs made into a panel which can be used in a wildlife forensic context. This, in combination with the determination of source region test (Chapter 2) can be put in place for circumstances where the legality of a short beaked echidna is under question and can be used to combat the illegal trade of this iconic and important species.

4.7 References

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Chapter Five: Investigation of a sex determination test for the short beaked echidna (*Tachyglossus aculeatus*) using real-time PCR – the challenges of validating in wildlife forensic science

5.1 Abstract

Wildlife forensics is a sub-discipline of forensic science that is dedicated to providing expertise to investigate and prosecute wildlife crime. It follows current best practice for the forensic sciences, therefore tests within this field should be validated where possible. Best practice is key for presenting defensible evidence for the prosecution of the illegal wildlife trade in court. In recent years, there has been a reported increase of short beaked echidnas (*Tachyglossus aculeatus*) being trafficked illegally from the wild, claiming they have been legitimately bred in captivity. The well documented difficulties of breeding short beaked echidnas in captivity have likely contributed to its rarity and desirability. Within wildlife forensics, having a well-rounded set of tools that provide evidence and intelligence can be important to ensure accuracy and confidence in results presented. Depending on the trade, multiple analyses may also be employed to confirm species, origin, or sex. Determining the sex of echidnas, especially juvenile animals, is useful knowledge for understanding mating behaviour and improving captive breeding success. A lack of sexual dimorphism in echidnas means an invasive internal examination is required to assign sex, which often means echidnas need to be anesthetised for this to be carried out. The aim of this chapter is to investigate the utility of a recently published PCR based sex determination protocol for forensic application. It explores the development of a rapid protocol using real-time PCR and melt curve analysis of two amplicons: a male specific product, and a product with size variation between males and females. A validation study was carried out to test these primers against the criteria of *accuracy, repeatability, limit of detection,* and *specificity* to add to the genetic toolbox I have developed. Results were inconsistent across three criteria: *accuracy*, *limit of detection,* and *specificity,* thus the

validation was not successful. The results presented here explore the source of the conflicting results obtained and the limitations of this method and provide recommendations for future analysis.

5.2 Introduction

Validation of methods in forensic science is a crucial aspect to ensure that tests, reagents, equipment, and research is standardised and consistent both within and between laboratories (Buckleton *et al.* 2016). Validation studies usually test parameters such as accuracy, sensitivity, limit of detection, specificity, reproducibility, and robustness (Tobe *et al.* 2009; Johnson *et al.* 2014; Buckleton *et al.* 2016). These variables are important to quantify so that they are understood when deciding on a test to use, or interpreting the results of a particular test. The recent National Academy of Science (NAS) (National Research Council 2009) and Presidents Council of Advisors of Science and Technology (PCAST) (PCAST 2016) reports, focused on forensic science in the United States and were highly critical of fields presenting methods in court that were not validated or standardised. They emphasised these studies needed to be carried out to avoid misunderstandings, incorrect evidence, and potential miscarriages of justice. While wildlife forensic was not specifically mentioned in either of these reports, as a mature forensic discipline these recommendations are highly relevant (Linacre 2009; Johnson *et al.* 2014).

Wildlife forensics is a sub-discipline of forensic science, and there are potentially thousands of species that may be encountered in testing. These tests often need to be developed specifically for species; therefore, development and validation can be more complex in comparison to human forensics. It is currently not feasible to have every

developed test validated. However, if it is in the scope of the laboratory, it is ideal to validate any tests that are being developed for the numerous species that are found in the illegal wildlife trade, especially those that are used on a frequent basis (Linacre 2009). This is particularly important in wildlife forensic genetics, where tests may be used to confirm the species, geographic origin, parentage, or sex of the animal in question (Johnson 2010; Johnson *et al.* 2014; Moore and Frazier 2019). If any investigation is to proceed, the need for valid and consistent testing could be crucial to the case, particularly for the acceptance of evidence into court proceedings. As the illegal wildlife trade increases, the breadth of wildlife forensics is also expanding, and the increase in validation should be encouraged (Linacre *et al.* 2011; Johnson *et al.* 2014; Burnham-Curtis *et al.* 2015).

There are relatively few published studies reporting a validated genetic test targeted for use in wildlife forensic genetics (Dawnay *et al.* 2007, 2009; Ogden *et al.* 2008; Dubey *et al.* 2011; Kitpipit *et al.* 2012; Aarnes *et al.* 2015; Ciavaglia *et al.* 2015; Van Hoppe *et al.* 2016; Dicks *et al.* 2017; Ewart *et al.* 2018; Summerell *et al.* 2019), highlighting the importance of validation within and between laboratories (Linacre *et al.* 2011). Anecdotally, some laboratories validate tests internally, but do not publish them in the literature. Given that this is a global trade, it would greatly assist the wildlife forensic community for these to be published for other laboratories to use. To date this has only been done in a handful of cases. A study by Ewart *et al.* (2018) validated a species identification test for rhinoceros horn. This should be seen as a model example, where validation was carried out across five different labs and four different countries, demonstrating the robustness of this test that can now be implemented in casework of one of the most high profile species in the illegal wildlife

trade. Like the test validated by Ewart *et al.* (2018), most published validated wildlife forensic tests are for the purposes of determining species identity or geographic origin (Dawnay *et al.* 2007; Tobe and Linacre 2008; Kitpipit *et al.* 2012; Ciavaglia *et al.* 2015; Alves *et al.* 2017; Dicks *et al.* 2017; Summerell *et al.* 2019). There are very minimal sex determination tests that have been validated for use in a wildlife forensic context (Gupta *et al.* 2006; Bidon *et al.* 2013; Joshi *et al.* 2019). This is likely because that knowing the sex of individuals is only required in specific circumstances. For example, there are increased penalties when a females of certain species are involved; such as female parrots, which are often highly sought after for breeding purposes (Piper 2015). Additionally, hunting regulations in the USA include a range of rules and penalties that can depend on the sex of the species. For example, some jurisdictions may only allow males to be legally hunted (or vice versa), and hunting licences may not be permitted for females with offspring (Burnham-Curtis *et al.* 2015; Piper 2015; Moore and Frazier 2019). Knowing the sex is also useful for pedigree studies, particularly for species that do not exhibit sexual dimorphism, and can be incorporated into a genetic marker panel and used for further individualisation (Dicks *et al.* 2017; Blåhed *et al.* 2018). The validation of sex determination tests can be useful in not only wildlife forensics, but other areas such as captive breeding in zoos or conservation research.

The use of genetic testing to determine the sex of animals has been used with increasing frequency over the past twenty years. Genetic tests have become more accurate and straightforward to develop, with increased accessibility and reduced cost of genetic and genomic technologies (Griffiths *et al.* 1998; Miyaki *et al.* 1998; Fridolfsson and Ellegren 1999; Rosel 2003; Shaw *et al.* 2003; Mucci *et al.* 2016).

Zoos, conservation programs, and breeders often use genetic sex determination tests as tools to aid with breeding programs or population management, particularly when there is no sexual dimorphism present (Griffiths *et al.* 1998; Fridolfsson and Ellegren 1999; Griffiths 2000; Mucci *et al.* 2016). This is common in bird species, especially as juveniles, and thus a large number of sex determination tests have been focused on birds (Griffiths *et al.* 1998; Griffiths 2000; Chang *et al.* 2008a; Han *et al.* 2009; Ghorpade *et al.* 2012). Determining the sex of newly hatched chicks allows decisions to be made about housing to maximise their future survival (e.g. avoid competition between males) and breeding success. It can be particularly useful for advising breeding programs as well, as they can receive results rapidly and avoid invasive exams for live animals.

Genetic sex determination has been carried out through the use of sex-linked primers, amplifying regions that will either vary in size between the sex chromosomes of males and females, or are only present on the hemizygous chromosome (Akane *et al.* 1991; Mannucci *et al.* 1994; Griffiths *et al.* 1998; Robertson and Gemmell 2006; Joshi *et al.* 2019; Perry *et al.* 2019). The PCR amplicons can then be sized via electrophoresis and visualised under UV light to determine sex using either a size difference between the sexes (Fridolfsson and Ellegren 1999; Shaw *et al.* 2003; Perry *et al.* 2019) or amplicon presence/absence (Miyaki *et al.* 1998; Dawson *et al.* 2001; Perry *et al.* 2019).

The use of real-time PCR (RT-PCR) as an efficient method for detection and quantification of DNA targets in real time has increased significantly in recent years (Rebrikov and Trofimov 2006). RT-PCR has been used extensively across various

fields including forensic science, diagnostic medicine, and conservation biology (Rebrikov and Trofimov 2006; Mackay 2007) and can be adapted to produce a quick and simple test that negates the need for using separate steps for PCR and gel electrophoresis. This makes it ideal for applications such as molecular sex determination (Chen *et al.* 2012; Angles d'Auriac *et al.* 2014; Faux *et al.* 2014). In particular, RT-PCR can resolve issues that can arise from using gel electrophoresis such as poor discrimination between closely sized bands, or difficulties determining if there has been PCR failure when there is an absence of bands (Dawson *et al.* 2001; Robertson and Gemmell 2006; Faux *et al.* 2014; Mucci *et al.* 2016). RT-PCR can also be adapted for high sample throughput, producing rapid results. Such techniques have been developed for a range of economically or agriculturally important species including chickens, salmon, and pigs (Chen *et al.* 2012; Morinha *et al.* 2012; Ballester *et al.* 2013; Angles d'Auriac *et al.* 2014; Faux *et al.* 2014).

The short beaked echidna (*Tachyglossus aculeatus*) is one of only three monotremes, along with the platypus (*Ornithorhynchus anatinus)* and the long beaked echidna (*Zaglossus spp.)* (Griffiths 1978; Augee *et al.* 2006). Monotremes in general have historically been very difficult to breed in captivity (Temple-Smith and Grant 2001; Hawkins and Battaglia 2009; Morrow *et al.* 2009). Most of the successful echidna breeding events in Australia have occurred in the last 5-10 years (Beard and Grigg 2000; Temple-Smith and Grant 2001; Morrow *et al.* 2009; Wallage *et al.* 2015). One of the main challenges of captive breeding has been re-creating the complex mating behaviour echidnas have in the wild, where male echidnas (up to ten) will follow a female echidna in what is known as an 'echidna train' when she is in oestrus, for up to two weeks (Augee *et al.* 2006; Nicol and Andersen 2007; Morrow *et al.* 2009). This,

along with other improvements to captive breeding environments e.g. the introduction of burrow boxes and heat lamps (Wallage *et al.* 2015), has resulted in several zoos recording successful breeding events in multiple years (Rismiller and McKelvey 2000; Morrow *et al.* 2009; Wallage *et al.* 2015). Echidnas also generally lay one egg per breeding season (Augee *et al.* 2006; Nicol and Andersen 2007; Morrow *et al.* 2009). Monotremes typically do not exhibit sexual dimorphism and sex can be challenging to determine even outside of breeding season (Griffiths 1978; Nicol and Andersen 2007). While adult female monotremes technically have a pouch, it is not a true pouch as seen in marsupial mammals. Instead monotreme 'pouches' resemble more a fold of skin; these can be difficult to identify when females aren't lactating, and can often be confused with a contraction of abdominal muscles that also occurs in males (Rismiller and McKelvey 2000). Male monotremes have a spur on their hind legs (likely used for breeding purposes (Wong *et al.* 2013)); however, in echidnas, spurs have also been recorded in females, and some males can lose their spur, adding to the difficulties of determining sex (Griffiths 1978; Nicol and Andersen 2007). Unlike Eutherians and Metatherians which have X and Y sex chromosomes, Monotremes have extremely complex sex chromosomes; females have 10 X chromosomes, male platypuses have 5 X and 5 Y, and male echidnas have 5 X and 4 Y (Grützner *et al.* 2003; Rens *et al.* 2004, 2007). It appears that in the male echidna genome, the Y_3 and the Y_5 chromosomes have fused, or potentially have undergone fission in the male platypus genome (Grützner *et al.* 2003; Rens *et al.* 2004, 2007).

A genetic test to determine the sex of short beaked echidnas was developed by Perry *et al.* (2019) using two genes: a male specific gene, Mediator complex subunit 26 Y gametolog (*CRSPY)*, and the anti-Müllerian hormone gene (*AMH)*, a sex-linked gene

found on both the X and Y chromosomes with known size variations between male and female monotremes. Primers were designed using the platypus as the closest published extant genome for reference. This test determines sex by analysing the *CRSPY* gene, which is present in male echidnas but absent in females, combined with analysing the size of the *AMH* gene which has a 70 bp difference between males and females (*AMHY* - 350 bp, *AMHX* - 280 bp) (Perry *et al.* 2019). Such a test would be extremely useful to aid breeding programs, and for application in the illegal wildlife trade. Given that short beaked echidnas are suspected to be in the illegal wildlife trade, the importance of validation within forensic science, and the lack of many validated sex determination tests within wildlife forensics, I aimed to investigate this test in a forensic context, using RT-PCR melt curve analysis to make it a rapid and high-throughput test.

5.3 Methods

5.3.1 Sample collection and extraction

A total of 28 short-beaked echidna samples of known sex were used, sourced from a number of Australian zoos and museums (see Table A14 for sample list). The sex of the animal had been previously determined either by internal exams, evidence of breeding, or museum records. Samples used consisted of blood and tissue (muscle or liver), as well as DNA previously extracted from the Currumbin Wildlife Sanctuary samples by the University of Queensland which were stored at -20°C upon arrival. DNA extractions were carried out using either the ISOLATE II Genomic DNA Kit using the animal tissue protocol (Bioline, Australia), or a salting out technique (Sunnucks and Hales 1996). DNA extractions were quantified using the Qubit 2.0 Fluorometer High Sensitivity Assay (Thermo Fisher Scientific, USA). All samples

were collected under UTS Animal Ethics 2015000040 and Taronga Zoo Animal Ethics 3b/06/16.

5.3.2 Melt curve analysis

Reaction volumes of 20μL were made, consisting of 10μL of either QIAGEN QuantiTect SYBR® Green MasterMix (QIAGEN, Germany) or Applied Biosystems SYBR™ Green MasterMix (Thermo Fisher Scientific, Australia), 1μL of 10μM forward and reverse primers, and 1ng of genomic DNA. Primers used were: *Crspy* fwd-ACCAGTAAATGCTGTGAAACCTC, *Crspy* rev-TTCTTTTTATTGGCTGGTTCTGA and *AMH* fwd- 5'-ACAGGGTCCACGGGTCAGTT -3, *AMH* rev - 5'-CCAAAAGCAGCAACAGGTCC -3' (Perry *et al.* 2019). RT-PCR was carried out on a Thermo Fisher QuantStudio 3 under the following conditions: denaturation at 95°C for 15 mins, followed by 40 cycles of: 95°C (15s), 56°C (30s) 72°C, followed by amplicon elongation step at 72°C (7min). The melt curve analysis was performed at 95°C (15s), 50°C (1min), then one cycle of an increase of 0.05°C steps up to 95°C.

5.3.3 Validation parameters

Validation was carried out based on the following characteristics: 1) *accuracy* 2) *repeatability* 3) *limit of detection* and 4) s*pecificity.*

To validate *accuracy*, 28 samples of known sex (12 female, 16 male), plus negative controls, were amplified using both RT-PCR and conventional PCR (using protocols in Perry *et al.* (2019)) followed by a gel electrophoresis using Invitrogen E-gel. Both

primer sets were used and compared to the known sex confirmed by the zoo or museum.

Repeatability was tested using both *intra-run* replicates; triplicates of six samples (three female, three male); and *inter-run* replicates; the same repeats of the six samples run on two different RT-PCR runs on separate days.

Limit of detection was tested using 1 in 10 (1ng), 1 in 100 (0.1ng), 1 in 1000 (0.01ng) and 1 in 10000 (0.001ng) serial dilutions of four (two male, two female) DNA samples, previously quantified using the Qubit 2.0 Fluorometer High Sensitivity Assay (Thermo Fisher Scientific, USA). The dilutions were also tested on two separate RT-PCR runs on separate laboratory days.

Species *specificity* was analysed using the following species (Table A15) from the Australian Museum's Mammal, Bird, Herpetology, and Fish collections. All but one of these samples were of unknown sex: female Western long beaked echidna *(Zaglossus bruijnii)* (M.47975), Platypus *(Ornithorhynchus anatinus)* (M.35614), Bilby *(Macrotis lagotis)* (WGM118-186), Eastern crevice skink *(Egernia mcpheei)* (R.150174), and Queensland grouper (*Epinephelus lanceolatus)* (I.39681). These samples were chosen either because they were closely related species, or to represent a range of genera and potential contaminants for my laboratory. They were run on two separate plates with the regular RT-PCR conditions.

Results were exported and analysed using Microsoft Excel. Samples that did not produce a peak above 5000 fluorescence, or over 50°C melting temperature (T_m) were deemed to be failed results.

5.4 Results

5.4.1 RT-PCR amplification

The sex of 28 individuals was tested using RT-PCR melt curve analysis. Both the *AMH* and *CRSPY* genes were successfully amplified using the RT-PCR assay using 1ng of DNA. The *AMH* gene consistently produced a melting temperature (T_m) peak at ~81-82°C in all known males; and either produced failed peaks, multiple peaks, or a peak indistinguishable from males for known females. The *CRSPY* gene produced a peak at 79°C for all males, and either 69°C or a failed result for females. No peaks passing quality control standards were produced in the negative controls.

5.4.2 Validation

Inconsistent and non-specific results were obtained across three of the validation criteria tested (*accuracy, limit of detection, specificity*) and thus I was unable to definitively identify the sex of all of the individuals tested.

Accuracy (Sex determination): In 21 (13 male; 8 female) out of 28 known samples the sex was confirmed via the published PCR and agarose gel electrophoresis protocols from Perry *et al.* (2019). 7 samples either failed to produce a result for one or both genes or were ambiguous in one or both genes (i.e. multiple non-specific bands, or unable to determine size) using the published protocol. Figure A1 displays agarose gels with conventional PCR amplicons.

Using the RT-PCR method, amplification of the *AMH* gene generated peaks at different melting points. The *AMH* peak for all males was averaged at T_m 82.2°C ± 0.366 and the peak for females varied widely between samples (range 76-86°C). The peaks for females were either a single peak at ~81-82°C (*n=6*), or three peaks at 76, 81, and 85°C (*n=3*), or some simply did not amplify (*n=3*) (see Table A16 for all melting temperatures)

Within the *CRSPY* gene, known male samples produced a peak with a melting temperature average of 79.4°C ± 0.408; and six male samples also generated a peak at 69.0°C ± 0.263. Four out of 12 female samples displayed no amplification (expected); however, eight out of 12 generated a single peak at 69.0°C ± 0.158 (unexpected) (see Table A16 for all melting temperatures).

Discrepancies with dye: The *AMH* gene was not amplified in eight out of 12 female samples when using the QIAGEN SYBR Green as an intercalating dye. Samples with no amplified alleles using QIAGEN SYBR Green produced one peak or multiple peaks using Applied Biosystems SYBR Green. Table 5.1 shows the common trends in melting temperature for the known male and female samples, comparing the two brands of dye.

Table 5.1 Average T_m of peaks for short beaked echidna samples amplified with AMH and CRSPY genes, comparing QIAGEN and Applied Biosystems SYBR Green.

	AMH		***CRSPY***	
	Known Male (T_m°C ± SD)	Known Female (T_m°C ± SD)	Known Male (T_m°C ± SD)	Known Female (T_m°C ± SD)
QIAGEN SYBR Green	82.397 ± 0.15	Inconclusive	79.474 ± 0.10	69.570 ± 0.20
Applied Biosystems SYBR green	81.634 ± 0.077	81.743 76.444 85.248	78.420 ± 0.1707 68.896 ± 0.150	68.943 ± 0.185

Repeatability: Of the six samples that were run in triplicate, five were successfully amplified in both *inter-run* tests. All six gave similar T_m within the *intra-run* tests with low standard error (Table 5.2), with one sample failing consistently in both plates. Table A17 shows raw values for all samples across both plates.

Table 5.2 Average melting temperature (T_m) (± SD) and standard error for six short beaked echidna samples used in *repeatability* validation study, using both *AMH* and *CRSPY* genes. Melting temperatures were averaged from six replicates across two plates.

	AMH	***Average T_m (°C)***	***±SD***	***Error***
M	A50345	81.653	0.681	0.278
	M.48051	82.055	0.441	0.180
	M.48073	81.614	0.520	0.212
F	M.32567	81.701	0.337	0.137
	M.46626	81.618	0.376	0.153
	A70194	50.484	0.575	0.235
	CRSPY	***Average T_m (°C)***	***±SD***	***Error***
M	A50345	79.130	0.372	0.152
	M.48051	78.976	0.590	0.241
	M.48073	78.844	0.748	0.305
F	M.32567	69.005	0.319	0.130
	M.46626	68.986	0.207	0.085
	A70194	50.188	0.298	0.122

Limit of detection: The melting temperatures varied considerably depending on the amount of input DNA as seen in Table 5.3 (10ng-0.001ng). For 10 samples (5 males, 5 females), reducing the input DNA concentration gave inconsistent results via altering the T_m of the main peak. The 10ng and 1ng dilutions were successfully amplified in 9 out of 16 and 10 out 16 of samples respectively. Input of 0.1ng of DNA showed weaker and inconsistent results, with only 6 out of 16 amplifying as expected. The 0.01ng and 0.001ng dilutions did not amplify in the majority of samples (0.01 = 9 out of 16 failed; 0.001 = 10 out of 16 failed).

Table 5.3 *Limit of detection* serial gDNA dilution results for two genes amplified for short beaked echidna samples of known sex.

Green = expected result; Red = failed results; Yellow = alternate/inconsistent results.

NB: for the *CRSPY* known female sample the expected result was that it would fail to amplify due to the primers being designed to only amplify the Y-chromosome.

Gene/Sex	Sample	T_m (°C)	T_m (°C)	T_m (°C)	T_m (°C)	T_m (°C)
***AMH* Known Male**	1	81.928	81.928	81.979	81.979	50.001
	2	82.438	82.285	50.968	51.477	50.255
	3	50.205	80.952	81.159	81.005	81.253
	4	82.438	82.285	50.968	51.477	50.255
***AMH* Known Female**	5	81.519	81.571	76.839	76.737	76.685
	6	85.323	76.942	76.633	76.414	79.189
		81.724		80.320		76.517
		76.634				
	7	50.612	52.496	50.204	50.714	51.630
	8	50.001	51.070	51.121	51.223	51.121
***CRSPY* Known Male**	1	79.229	79.280	50.153	69.453	51.274
	2	79.688	53.260	50.917	50.255	69.554
	3	68.561	68.664	49.999	50.487	50.924
	4	78.430	78.485	50.977	69.075	68.870
		68.782	68.973			
***CRSPY* Known Female**	5	68.664	68.870	50.000	50.000	50.669
	6	69.076	50.878	69.230	68.860	50.000
	7	68.791	69.249	50.001	50.408	50.001
	8	50.714	50.204	50.001	51.579	69.504

Specificity: Of the five species tested, three (*Zaglossus bruijnii, Ornithorhyncus anatinus, Macrotis lagotis*) showed cross-reactivity with these protocols designed for the short beaked echidna (Table 5.4). The western long beaked echidna *AMH* gene produced results that varied from the short beaked echidna samples for *AMH* (~84°C) but were identical in the *CRSPY* gene (~80°C). The platypus samples had similar results to the short beaked echidna in both genes (*AMH*: ~82°C, *CRSPY*: ~80°C). The bilby sample produced a peak in amplification for the *AMH* (~76°C), which could clearly be distinguished from the echidna results based on melting temperature; however, the *CRSPY* result reflects what has been seen in female short beaked echidnas (~69°C). The Eastern crevice skink and the Queensland grouper failed to produce any successful amplification.

Table 5.4 Species *specificity* results averaged over two RT-PCR runs.

Species	***AMH*** **T_m (^{o}C) ± SD**	***CRSPY*** **T_m (^{o}C) ± SD**
Western long beaked echidna (Female)	84.144 ± 0.611	80.077 ± 0.373
Platypus	82.237 ± 0.067	80.471 ± 0.112
Bilby	76.577 ± 0.582	69.028 ± 0.384
Eastern crevice skink	No amplification	No amplification
Queensland grouper	No amplification	No amplification

5.5 Discussion

This chapter outlines the steps taken to attempt to replicate a previously published DNA based sex determination test for the short beaked echidna that used a standard PCR method and gel electrophoresis (Perry *et al.* 2019). Based on previous success using real-time PCR (RT-PCR) on birds and mammals for species ID and sex determination (Berry and Sarre 2007; Chen *et al.* 2012; Morinha *et al.* 2012; Ballester *et al.* 2013; Faux *et al.* 2014; Powell *et al.* 2019), I aimed to take these published protocols and develop a rapid genetic test, and validate it for use with RT-PCR. There are two main applications for a validated sex determination test for echidnas, 1) to assist with captive breeding programs, and 2) for use in wildlife forensic applications, as echidnas are known to be trafficked (Beastall and Shepherd 2013).

The validation was performed to investigate if this test was reproducible and robust, and particularly to explore the limits of this test for lower template samples. Using these methods with RT-PCR was investigated due to the closely sized fragments of the *AMH* gene (*AMHY* - 350 bp, *AMHX* - 280 bp), which are used to distinguish between sexes on an agarose gel, which could sometimes be ambiguous and difficult to differentiate, particularly for low template samples. Additionally a RT-PCR method can make it more feasible to have a high-throughput of samples (Schmittgen *et al.* 2008). Understanding the experimental parameters for which a test will work is crucial when dealing with forensic samples (Linacre 2009). Ideally this test would be used in circumstances where the legitimacy of a traded short beaked echidna is in question and knowing the sex could aid in distinguishing captive bred status by giving weight to parent/offspring claims, as well as provide further individualisation. However, despite going through all the requisite processes, the protocol could not be

validated, with inconsistent results obtained across three (*accuracy, limit of detection*, and *specificity)* out of four validation criteria tested. The major inconsistencies arose when testing the *accurac*y and *limit of detection* of the test. When testing *accuracy* two inconsistencies were observed; firstly, a non-specific band appeared in the *CRSPY* result for known female samples, and secondly, large variances in the melting temperatures were obtained for the known females in the *AMH* gene. When testing *limit of detection*, low template samples were found to produce melting temperatures that differed to those produced at higher concentrations. These are discussed further below.

5.5.1 Validation

The majority of known male and female samples did produce amplification patterns and melt curves consistent with expected outcomes; however, there was some variation which could complicate sex determination in unknown samples. The samples were successful in the *repeatability* study, but without consistency in other criteria, more work is needed for this test to be validated.

The similar melting temperature that was observed for some female samples and male samples for the *AMH* gene made it difficult to be certain of the sex of an unknown sample using this gene with RT-PCR. Additionally, there were also some samples that produced three peaks rather than one. Given previous studies that separated males and females with less than 70 bp size difference in genes using SYBR green (Chang *et al.* 2008a; Robertson *et al.* 2018), it was hypothesised that this should be sufficient for the *AMH* gene in this study. Given the complex nature of echidna sex chromosomes (Rens *et al.* 2007), and the use of the *AMH* gene, which is not commonly used for sexing of other species, further optimisation of tests using the *AMH* gene is required

once an echidna genome is available, in order to produce an accurate test. The dye used also may be the issue and should be investigated (discussed further below). Given that even some of the conventional PCR results - particularly the low template samples - produced ambiguous results for known sex animals (Figure A1), I encourage further exploration into this. An echidna genome would provide further insight into the sex chromosomes and could potentially lend information about more specific locations for primer binding to optimise and ensure differentiation between the sexes, as well as improve specificity if required.

Another aspect of the *accuracy* test that was of concern was the non-specific peak observed for the *CRSPY* gene that was identified in known females, given these primers were designed to only amplify a male-specific gene. While sequencing of these non-specific peaks was attempted it was unsuccessful (data not shown), thus I am not able to tell what is being amplified to produce this non-specific peak at 69°C at this stage, though further work is recommended, including attempting to re-sequence these amplicons.

While studies report that the *CRSPX* gene in platypuses is reasonably divergent from the *CRSPY* gene (Tsend-Ayush *et al.* 2012), without an echidna genome it is difficult to know if the same applies to short beaked echidnas. PCR primers in the original study were designed based on the platypus genome, and determination of sex was based on the presence/absence of amplified *CRSPY* product when run out on an agarose gel (Perry *et al.* 2019). RT-PCR, however, is known to have higher sensitivity than conventional PCR with agarose gel electrophoresis (Morinha *et al.* 2012; Faux *et al.* 2014; Powell *et al.* 2019) and thus, based on my results, it seems to be amplifying

non-specific products that were not visible using standard PCR and gel electrophoresis in the original study. Furthermore, it has been demonstrated the X and Y chromosomes of the platypus and echidna vary slightly (Rens *et al.* 2007). Female platypuses and echidnas both have 10 X chromosomes, however male platypuses have 5 X and 5 Y chromosomes, and male echidnas have 5 X and 4 Y chromosomes (Grützner *et al.* 2003; Rens *et al.* 2004, 2007; Ferguson-Smith and Rens 2010). It is possible then that the primers are amplifying different regions in the echidna and platypus. Due to this complex nature of monotreme chromosomes, it would be useful to have more information on the echidna's sex chromosomes, ideally via an echidna genome, such as has been done for the platypus to conduct an in-depth study (Warren *et al.* 2008). This would then allow primers to be designed specifically for the short beaked echidna.

When validating the *specificity* it was found that the samples from the long beaked echidna and the platypus had either identical or very similar melting temperatures to the short beaked echidna. This is not unexpected as the primers were designed from the platypus genome (Perry *et al.* 2019). Even though there was some overlap in the results, in the event a species identification was required, the long beaked and short beaked echidnas could be resolved morphologically. In the event a whole specimen was not available (i.e. parts of a specimen, or suspected contamination), mitochondrial DNA sequencing is suitable to distinguish between species (Summerell *et al.* 2019; Chapter 2). The sample from the bilby produced peaks in both genes; however only the *CRSPY* overlapped with expected results for the short beaked echidna. As this result was the 69°C peak that has been seen in a large proportion of samples, this again raises questions about the true origins of this peak. The *specificity*

test produces further evidence that encourages investigation into both the dye used and the difference between the platypus and echidna sex chromosomes. It is recommended that this be further investigated in male and female samples of both the platypus and long beaked echidna; thorough testing of the expected results for those variables will provide a baseline if close contaminants emerge as an issue.

For the *limit of detection* study, 0.1ng appears the limit at which results become ambiguous (i.e. low amplification or change in T_m) or fail to amplify. When observing the decrease in input DNA for some samples, it appeared that this altered the melting temperature of the amplicon. Table 5.2 shows a known female *AMH* sample (Sample 5) change from a strong peak at ~81ºC at both 10ng and 1ng, to a peak of ~76ºC at 0.1, 0.01, and 0.001ng. These results suggest blind testing would be unreliable, therefore I cannot conclusively use this gene to determine sex using melt curve analysis, particularly for low template samples. This study demonstrates the importance of thoroughly understanding the limits of tests, as has been stressed widely in the forensic sciences. Samples in both human and wildlife forensics are extremely varied and are often of less than ideal quantity and quality, and priorities will need to be made (Linacre 2009). Cases in human forensics where the parameters of a test were not clearly known, were misunderstood, or were not competently peer reviewed, have had serious impacts on cases, some leading to miscarriages of justice (Huff and Killias 2013; Roach 2014; Turvey and Cooley 2014).

5.5.2 Recommendations and future directions

Ideally this test will be further investigated in the future to better understand the limitations in the current test. Given the successful validation study presented in Chapter 2 (Summerell *et al.* 2019), and the benefits that this can provide for the trade

in short beaked echidnas, it should be a goal to reach similar standards with the protocols in this chapter.

Based on the results of the validation study presented here, two potential reasons for the failed validation were identified and will need to be further investigated. These are 1) the impact of the dye used and 2) potential amplification of non-target regions using these primers. To investigate the impact of the dyes used, I would recommend use of a High Resolution Melt analysis (HRM). SYBR green was chosen initially due to several other published studies that utilised it in several bird species (Chang *et al.* 2008a; Chen *et al.* 2012) and mammals (Berry and Sarre 2007; Moran *et al.* 2008; Powell *et al.* 2019), as well as the ease of use and low cost advantages. It has been noted in a number of studies, however, that SYBR green can often inhibit samples, compared to similar intercalating dyes such as EvaGreen and SYTO9 used for the same purposes (Monis *et al.* 2005; Gudnason *et al.* 2007; Eischeid 2011). An HRM analysis could potentially be preferable for resolving such issues, as the use of a saturating intercalating dye does not inhibit polymerase and will bind permanently to the desired peaks. Research has shown that such dyes are able to be used for species tests using SNPs and therefore would be a good avenue to investigate for this purpose (Venables *et al.* 2014; Mehta *et al.* 2017), and could potentially resolve some ambiguity such as the *AMH* variations in females. HRM has been shown to separate sex in birds with size differences of just 2 bp (Morinha *et al.* 2013), so could potentially be useful for this purpose, with a size difference of 70 bp when using the *AMH* primers.

Alternatively, a number of studies have used molecular probes such as TaqMan® probes for species ID and sex determination (Chang *et al.* 2008b; Ghorpade *et al.* 2012; Wozney and Wilson 2012; O'Neill *et al.* 2013; Eysturskarð *et al.* 2017), which could be an alternative route for this assay. This has downsides of being more costly and will involve the labour of designing species specific probes, but would ensure accuracy and also align with many human forensics quantification tests (Green *et al.* 2005; Vernarecci *et al.* 2015). These tests are known to be precise, sensitive, and suited to low template samples. This should be in conjunction with further sequencing of all non-specific bands to resolve the questionable results produced.

If either method can resolve the issues with non-specific and ambiguous peaks, then a new validation study with similar parameters should be conducted, making sure the limits of the methods are known to provide a robust forensic test.

5.6 Conclusion

This chapter outlines why validation and maintaining quality is crucial in forensic science. Due to several issues (such as non-specific bands and ambiguous results at low template), this test was deemed to be not fit for purpose in its current form. These issues resulted in inconclusive results for the determination of sex for the short beaked echidna. There are some trends present, particularly in male samples, and aspects of the validation such as *repeatability* and some *specificity* testing did produce expected results. However, overall it does not meet the requirements to be able to be used in a forensic context, which is what was aimed. I would recommend continuing with standard PCR and agarose gel electrophoresis at this stage, though caution with results from low template samples must also be taken. There is still scope to continue this research and I recommend that further studies (such as HRM and further

sequencing of amplicons) are carried out, so that sex determination can be a trustworthy and suitable tool used when a short beaked echidna is traded.

5.7 References

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Chapter Six: General Discussion

6.1 Thesis overview

This thesis presents the first comprehensive genetic toolbox designed to be used in a wildlife forensic context to combat the illegal trade of the short beaked echidna (*Tachyglossus aculeatus*). This toolbox was required after information emerged suggesting that the trade in captive bred echidnas is likely to include wild-caught individuals to maintain the numbers being sold (Beastall and Shepherd 2013). This assumption is further supported by the common knowledge that legitimate zoos have encountered significant challenges breeding this species in captivity (Ferguson and Turner 2012; Wallage *et al.* 2015). This trade is a threat to the integrity of captive populations around the world and, if unchecked, could pose a threat to the conservation of wild populations. Poaching is suspected to be occurring in South East Asia, where facilities are producing quotas of captive bred animals that cannot realistically be met by genuine captive breeding (Janssen and Chng 2017). There is very little in the literature about the genetics of the short beaked echidna, and what has been published does not provide detailed phylogeographic or population structure information that can be used in a wildlife forensic context to help understand this trade (Griffiths 1978; Augee *et al.* 2006; Vanṕe *et al.* 2009).

One of the main challenges to wildlife forensics science is that new genetic markers are developed for each species impacted in the illegal wildlife trade. When new species emerge in the trade, the development of genetic tests to determine source region and relatedness between individuals can not only provide the evidence and intelligence needed to aid law enforcement and prosecutors of illegal trade, but also

benefit zoos and legitimate breeding facilities in maintaining the integrity of their collections.

This research aimed to develop a set of both mitochondrial DNA and nuclear DNA genetic markers for the short beaked echidna that can be used to provide information on source region, subspecies, and relatedness between individuals. Additionally, it aimed to validate a sex determination test for use with real-time PCR (RT-PCR) methods. In combination, these tests can form a toolbox needed in circumstances where a wild short beaked echidna is suspected to have been trafficked, and provide a sound understanding of the development of novel tests for species that would benefit from similar attention.

The major outcomes of each research chapter of this thesis are summarised below:

Chapter 2

- Developed a test using a mitochondrial DNA marker that can discriminate between subspecies of short beaked echidnas from Australia and New Guinea.
- Developed and tested non-invasive DNA sampling methods using short beaked echidna quills.
- Validated the primers used so that these tests can be applied in a wildlife forensic context.

Chapter 3

- Characterised a robust set of several thousand single nucleotide polymorphisms (SNPs) that can be used for the short beaked echidna.

- Investigated the applicability of these SNPs to discriminate between the five short beaked echidna subspecies to aid with determination of geographic location for a wildlife forensic context.
- Provided additional molecular data to support the recommendation that the subspecies designations be retained for the foreseeable future.
- Identified issues regarding sampling coverage needed to truly analyse short beaked echidna subspecies.
- Generated the first published phylogeographic dataset for the short beaked echidna

Chapter 4

- Developed and successfully blind-tested a technique to confirm relatedness between individuals of known pedigree using SNPs.
- Analysed and compared relatedness levels of both captive populations and wild populations.
- Identified relatedness between individuals of unknown pedigree using Identity by Descent (IBD).
- Determined the minimum number of informative SNPs to be able to individualise an echidna and distinguish first-order relatives.

Chapter 5

- Tested primers previously developed for a short beaked echidna sex determination test using RT-PCR methods on samples of known sex.
- Used standard validation parameters to test the utility of the primers.

- Identified limitations with the primers, particularly with resultant non-specific PCR products, that would prevent applicability in a wildlife forensic context.

6.2 Non-invasive sampling

Utilising non-invasive sampling methods for collection of genetic material is advantageous for the processing of seized animals in the illegal wildlife trade (Waits and Paetkau 2005). Such techniques are often necessary within the illegal wildlife trade due to limited access of veterinary expertise, issues with legality, and harm reduction to the animal (Speller *et al.* 2011; du Toit *et al.* 2016; Tawichasri *et al.* 2017). While blood or tissue biopsy samples from live animals provide high quality and reliable samples for testing, they require specialised training (e.g. formal veterinary training) to collect, whereas non-invasive samples such as quills can be taken by enforcement officers with minimal training. In the live animal trade, the animal in question could potentially be in a poor condition and suffering from some form of trauma, so developing techniques that can reduce impact is favourable. The research in Chapter 2 presents the first published methods of extracting DNA from echidna quills and amplifying informative mitochondrial sequences of those both plucked and shed from a live echidna, in addition to quills from a deceased echidna. Given that a large proportion of non-invasive sampling research has been completed on feathers (Sefc *et al.* 2003; Waits and Paetkau 2005; Hogan *et al.* 2008; Speller *et al.* 2011) and hair (Suenaga and Nakamura 2005; Mccafferty and Saccheri 2006; Henry *et al.* 2011), this study highlights the advantages and options of using non-invasive sampling for quilled mammals. A study by Perry *et al.* (2019) used non-invasive sampling from echidna fur, though at least ten hairs were needed to be

plucked to extract sufficient DNA. The advantage of obtaining DNA from shed quills is that one could completely avoid handling the animal and potentially retrieve the sample even after the animal has left the enclosure. Shed feathers, fur, and skin have been demonstrated as useable samples in other studies (Sloane *et al.* 2000; Rudnick *et al.* 2005; Swanson *et al.* 2006; Hogan *et al.* 2008). One limitation of the shed quills used in this study is the unknown age of the quills. It is presumed they are likely of 'recently shed' status as they were collected from a zoo enclosure from echidnas that are regularly monitored, but I recommend further testing to better understand the time window during which DNA of suitable quality can be obtained. Additionally, for future work, it would be ideal to test the SNPs described in Chapter 4 on DNA extracted from quills. SNPs are known to work well with degraded or low quantities of DNA (Senge *et al.* 2011; Dormontt *et al.* 2020), such as the quantities produced when extracting from quills in this study. Studies using less than 200 SNPs have been successfully applied for non-invasive samples collected from species such as wolves, mountain bongo, and Iberian lynx (Kraus *et al.* 2015; Kleinman-Ruiz *et al.* 2017; Svengren *et al.* 2017). Having a panel of informative SNPs, such as the 167 subjected to preliminary analysis in Chapter 4, could then allow for an in-depth genetic analysis from non-invasive samples and allow for more ability to combine the tools developed in this thesis.

6.3 Determination of geographic origin

Determining the geographic origin of an animal or animal part is a tool used increasingly in wildlife forensics as evidence of new species targeted by the illegal trade continues to emerge (Ogden and Linacre 2015; Moore and Frazier 2019). This can be especially important for species that have distributions across different jurisdictions where protections may differ and a simple species identification will not

be sufficient to provide key details such as poaching hotspots and trade routes (Moore and Frazier 2019). Such tests have been developed for a number of species to determine origin for a wildlife forensic purpose, including elephants, pangolins, deer, and leopards (Frantz *et al.* 2006; Mondol *et al.* 2015; Mwale *et al.* 2017; Zhao *et al.* 2019). The results from Chapters 2 and 3 now make this possible for the short beaked echidna. There are limitations and benefits to both nuclear and mitochondrial (mtDNA) markers. It is therefore recommended that this be approached on a case-by-case basis for the species of interest.

One key question that I was able to answer was 'can we differentiate between a New Guinea echidna (*T.a. lawesii*) and the other four Australian subspecies (*T.a. aculeatus, T.a. acanthion, T.a. setosus, T.a. multiaculeatus*)?' from a simple mtDNA test. Chapter 2 presents the first published phylogeographic test for the short beaked echidna, and the first validated test for this species. Like many routine mtDNA tests, this can be done relatively quickly and at a lower cost (in comparison to next-generation sequencing) (Budowle *et al.* 2003; Gupta *et al.* 2013; Johnson *et al.* 2014). This success allows for a single mtDNA test to provide information about source region to the zoo or organisation that is questioning the origin of their animal. Additionally, it has the advantage of using opportunistically collected quills, reducing the time and expertise needed to take a sample. This test represents the first stage of the toolbox and provides much needed genetic knowledge about the difference between the two main regions of this species.

The mtDNA test did not have the power to discriminate between the subspecies within Australia, whereas my SNP marker set, developed using a reduced

representation methodology, successfully provided more detail into the source region. At present there is only anecdotal evidence of trade in echidna parts being reported, in Australia, though this is not thought to be widespread (Lavelle, S, personal communication, June 2020). To provide zoos with peace of mind, however, or to confirm source region determined from the mtDNA test, it may be important to know specifically where an echidna has originated. The use of two sets of markers (mtDNA and nuclear DNA) to determine whether the echidna is from New Guinea or Australia is also beneficial and provides additional evidence if needed.

The analyses presented here are the first to use a next-generation marker set to investigate molecular support for subspecies in the short beaked echidna and make recommendations regarding future sampling and analysis. When my samples were analysed with the high resolution SNP markers I developed, the population clusters that were observed coincided with the four recognised subspecies (*T.a. aculeatus, T.a. acanthion, T.a. setosus, T.a. lawesii*). In particular, distinct division of clusters between New Guinea and Australia was apparent, as was the case for the mtDNA data. As Chapter 3 outlines, it is important to recognise that there is the possibility of sampling bias. While samples for echidnas through central Australia do exist in Museum collections, they were not accessible due to another echidna research group who had planned on publishing a phylogeography of the species, thus my sample collection was primarily focused on obtaining known pedigree samples (as seen in Chapter 4) so as not to overlap with that group, however to date this phylogeography has not be published. In particular, caveats should be made with regard to conclusions about the *T.a. setosus* (Tasmania) and *T.a. acanthion* (Western Australia) subspecies; though the analyses may suggest they form a distinct cluster, at this stage I am

cautious of definitively supporting the taxonomic validity of these subspecies. While Tasmanian populations of many species with trans-Bassian distributions are genetically distinct subspecies (Firestone *et al.* 1999; Norgate *et al.* 2009; Frankham *et al.* 2016; Cooper *et al.* 2019) majority of samples in my study were from a single population. Thus, more sampling from across Tasmania would therefore be ideal to confirm the differentiation I observed. I also recommend more extensive sampling from animals across central Australia to span the sampling gap in my data between *T.a. acanthion* and *T.a. aculeatus.* Currently there is debate about where these two subspecies meet, or if they overlap across central Australia (Griffiths 1978; Augee *et al.* 2006). Analysis of central Australian populations will be required to determine whether *T.a. aculeatus* and *T.a. acanthion* are truly distinct subspecies separated by specific biogeographic barriers, such as those seen in the grey kangaroo (Neaves *et al.* 2009) and the fat-tailed dunnart (Cooper *et al.* 2000), or if the patterns of isolation by distance seen north-south along the east coast of Australia is also seen east-west across the short beaked echidna distribution. I recommend further sample collection and analysis to conduct a full phylogeographic study to ensure that the source region of an Australian short beaked echidna can be determined.

A lack of samples from certain locations is a common challenge among non-model species and posed a limitation throughout this project. As the trade is likely originating from South East Asia, it would have been desirable to have a larger number of samples from the New Guinea region. It can be logistically very difficult to obtain these samples, and the samples obtained from this region were from the Australian Museum Mammal collection. Some samples from that region were also very old (e.g. between 90-100 years old), making the extraction and amplification of

DNA a technically difficult process, and in two cases I was unable to recover any usable DNA.

Despite the sampling limitations, the results presented here provide no indication that the subspecies should be revised at this stage. Additionally, for use in my wildlife forensic toolbox, I am satisfied that both the mitochondrial test and SNP analyses were able to clearly differentiate the New Guinea and Australian samples used in this study. This indicates that there is sufficient phylogenetic difference to support the subspecies naming, and additionally provides the most crucial test for point of origin that can provide intelligence to law enforcement agencies.

6.4 Marker development and individualisation

For development of markers for the short beaked echidna, it was crucial that I chose a method that would be highly informative and work for a range of applications. Within both human and wildlife forensics, microsatellites have been the method of choice for carrying out individualisation, population, or relatedness studies (Butler *et al.* 2007; Butler 2009; Johnson *et al.* 2014; Ogden and Linacre 2015; Moore and Frazier 2019). However SNPs have shown several advantages, particularly with reproducibility and use for relatedness studies (Tokarska *et al.* 2009; Huisman 2017).

Microsatellites were previously developed for the short beaked echidna; however, that study had several limitations (e.g. minimal number of markers and less representation of the genome) (Vanṕe *et al.* 2009). To circumvent these limitations, SNPs were chosen for this study. This brought its own challenges due to the lack of a published genome for the short beaked echidna, but my results from Chapter 3 and 4 show that

the reduced representation method of SNP discovery was appropriate and produced sufficient informative SNPs. Similar studies have developed robust marker sets for use in wildlife forensics for a range of species including both plants and animals (Ogden *et al.* 2013; DeHaan *et al.* 2014; Blanc-Jolivet *et al.* 2019; Ewart *et al.* 2019; Dormontt *et al.* 2020). The marker set I have developed here is the first of its kind for the short beaked echidna and adds to the increasing wealth of information that is broadening the wildlife forensics field. It also improves upon the limited genetic information that was provided by the microsatellite markers. Ideally, I would also recommend that both microsatellite and SNP markers should be compared and contrasted in a separate study to provide a thorough analysis and recommendations for future work.

The dataset presented here confirmed the relatedness of 34 individuals from samples provided to me by zoos with known studbook pedigree. These markers also revealed clear differences between wild and captive populations in terms of patterns of relationships. There have been no previous investigations into relatedness analyses for the short beaked echidna making this a crucial finding of my research. If echidnas are traded and claimed to be bred in captivity, knowing the relatedness levels of individuals could be key in providing evidence to law enforcement. Relatedness studies have been increasingly important for providing new evidence and intelligence for the illegal wildlife trade, such as the microsatellite set to aid with prevention of the broad-headed snake trade (Frankham *et al.* 2015), or using paternity testing to solve tortoise theft (Mucci *et al.* 2014). I demonstrated here, using newly developed markers, the ability to resolve the relatedness of short beaked echidnas using Identity by Descent (IBD) analysis. Additionally, this is one of the few studies within wildlife

forensics that has used SNPs to resolve such questions (Ogden *et al.* 2013; Ewart *et al.* 2019) and represents an additional next-generation study for the field.

By investigating the probability of identity, ~20 of the most informative SNPs (i.e. high minor allele frequency) have sufficient resolution to separate individuals and first-order relatives. Profiles using a combination of 167 SNPs gave extremely rare match probabilities. Ideally the next stage of this research, would be to perform a rigorous validation study on such a set of markers. Such a study could allow for development of a standardised SNP genotyping assay (e.g., KASP™; © LGC Biosearch Technologies, 2020), which could be crucial for the law enforcement agencies that require intelligence on the echidna trade. The panel would align with other small informative marker sets (both microsatellites and SNPs) that have been developed for use in a wildlife forensic context for species such as carpet pythons (Ciavaglia and Linacre 2018), cape parrots (Coetzer *et al.* 2017), bigleaf maple (Dormontt *et al.* 2020), and hen harrier (Van Hoppe *et al.* 2016). Given the limited resources and large suite of animals in the illegal wildlife trade, it is generally the high profile iconic species, such as elephants (Gupta *et al.* 2006; Wasser *et al.* 2008; Wozney and Wilson 2012; Zhao *et al.* 2019), rhinoceroses (Hsieh *et al.* 2003; Peppin *et al.* 2010; Ewart *et al.* 2018), or pangolins (Zhang *et al.* 2015; du Toit *et al.* 2016; Mwale *et al.* 2017) that have had a range of wildlife forensics tests developed for them. This study presents a suite of newly developed tests that can be used for a species that is not listed on the Convention on International Trade in Endangered Species of wild flora and fauna (CITES), nor is it currently endangered. This serves to bring awareness of the trade and provides tools to detect it early, prior to the species

coming under significant threat. I hope that this will encourage the development of more studies on other species facing comparable challenges.

6.5 Validation in wildlife forensic science

Frequently in wildlife forensics, samples of unknown quality are presented for testing, however, beyond species ID, there may be limited suitable tests available for analysis (Butler 2009). It is therefore imperative for practitioners to understand the limitations of any test, which is why validation can be extremely valuable (Linacre 2009; SWGDAM 2016). Scientists must be able to determine if a sample provided will be able to produce quality results that can be peer-reviewed and stand up in court (Butler 2009). This saves time and money in a casework context, and such information can then be used to direct the examination of the case and triage what evidence has been provided, reducing the chance of misleading results. Validation is limited in wildlife forensics due to the vast number of species (as opposed to human forensics where there is one species of concern), and the associated increase in time and cost to cover each individual species (Moore and Frazier 2019). Validation and standardisation are recommended where possible, and therefore I aimed to do so for two methods in this study.

As outlined in Chapter 2, my research successfully validated primers used for the mitochondrial test to determine source region of a short beaked echidna. Published validation studies are not yet as common in wildlife forensics as in human forensics, but is recommended in the Society for Wildlife Forensic Science (SWFS) standards and guidelines (SWFS Technical Working Group 2018). This makes my test an important contribution to the field as it is the first published validated test for the short

beaked echidna. I have clearly demonstrated the specificity and limit of detection of this test, finding it to be reliable and robust. Other studies that have validated a wildlife forensic technique (Dawnay *et al.* 2007, 2009; Ogden *et al.* 2008; Meganathan *et al.* 2011; Kitpipit *et al.* 2012; Aarnes *et al.* 2015; Ciavaglia *et al.* 2015; Ewart *et al.* 2018) highlight the key validation criteria and benefits of doing so, particularly with commonly used tests such as the Cytochrome Oxidase 1 barcoding marker. I chose to validate this test to contribute to this field of study, with the intention that this test could become routine in the investigation of the short beaked echidna trade. A study such as Ewart *et al.* (2018), where a species ID test for rhinoceroses was validated within and between laboratories and countries, would be an ideal next stage of my source region test to allow for validation across different labs and jurisdictions to test further reproducibility. The success of this mitochondrial test validation contrasted with Chapter 5, where the validation of the sex determination test produced several issues that warrant further investigation.

Sex determination tests are routinely incorporated into human forensic testing, as they aid the individualisation process (Mannucci *et al.* 1994). They are also commonly used in wildlife and conservation genetics, particularly in zoos for species where no sexual dimorphism exists (Griffiths *et al.* 1998; Costantini *et al.* 2008; Chen *et al.* 2012). The majority of these tests involve birds, especially when they are young and the zoos cannot determine the sex of the individual animal (Griffiths *et al.* 1998; Han *et al.* 2009; Smith 2010). In wildlife forensics, sex determination tests are much less common, mostly due to the fact that sex determination is not often a factor in ascertaining if a law has been broken. As the field moves towards individualisation, there is an increased likelihood that such techniques may become regularly utilised,

depending on the requirements for the species. Sex determination tests in wildlife forensics could be favoured when there are different penalties for different sexes e.g., in some species, illegal trade in female birds attracts a higher penalty, and laws surrounding hunting can often vary based on sex of the animal (Burnham-Curtis *et al.* 2015; Piper 2015; Moore and Frazier 2019). Although the echidna is not targeted in the trade based on sex, such a test would also be very useful for legitimate zoos, especially when pairing of animals is required (which is currently done by behaviour or previous life-history knowledge).

While a test for determining sex had been developed for the short beaked echidna (Perry *et al.* 2019), validation attempts were unsuccessful and showed it was at times ambiguous and needed high quality DNA to produce a reliable result. A validated rapid and clear sex determination test would be advantageous to add to the toolbox for the short beaked echidna.

My validation tests of RT-PCR methods using the previously published primers gave insight into why it is imperative to validate and standardise methods, particularly when a result may be presented in court. Issues arose with the presence of a non-specific band, particularly in known female samples amplifying with male specific primers. Additionally, the study on the *limit of detection* generated further complications when it produced ambiguous results at low quantities of DNA. There have been studies that have produced quality sex determination tests that involved low template DNA (Durnin *et al.* 2007; Brinkman and Hundertmark 2009; Tawichasri *et al.* 2017), and it should be a goal for all studies to test such parameters and ensure the margins of a test are known. While this study did not provide the results I

hypothesised, it provides a compelling case in support of why many researchers in this field recommend that wildlife forensics emulate human forensics where appropriate, and if possible, implement standardisation across this field (Ogden *et al.* 2009, 2016; Alacs *et al.* 2010; Johnson *et al.* 2014; Moore and Frazier 2019). I do, however, provide clear recommendations for further testing that could improve upon this method and successfully validate the sex determination test. Additionally, to merge all tools developed in this study as much as possible, I would recommend investigating the SNPs even further to determine if any are sex-linked and could be developed into a new SNP-based sex determination test. This would allow for sex information to be conjoined with pedigree or subspecies testing once a SNP panel is developed. For example, a study by Blåhed *et al.* (2018) was conducted on moose which aimed to help with population monitoring. They carried out SNP marker discovery using reduced representation methods to analyse population structure, as well as pedigree. They also included the use of a sex determination test within the SNP discovery, which can aid with pedigree analysis and sex ratios. A similar test would be appropriate for the toolbox I have developed and would assist in providing as much information as possible.

6.6 Future directions and recommendations

The findings of this thesis will significantly improve future investigation and management of the illegal trade of the short beaked echidna. However, there are questions that remain to be answered and areas that require further testing in order to provide an enhanced basis for future genetic research on this species.

One limitation in this study was the inclusion of only one wild population (other samples were from museum collections covering a wide geographic area). It would be extremely beneficial to increase not just the number of captive animals within this dataset, but also include more wild animals to represent additional populations spanning a broader geographic range than was available for this study. This would strengthen my findings regarding the variations between captive and wild-caught echidnas. Additionally, to understand the true breadth and potential overlap of the subspecies, and whether the genetic data backs up this assignment, samples should be collected from: The Northern Territory; North and Centre of Western Australia; Tasmania (outside of Hobart region); South Australia; and Kangaroo Island. This highlights the importance of close collaboration with not just zoos and museums, but with protected areas and sanctuaries (especially national parks) and any other researchers with collections. With the use of the non-invasive sampling methods that I have tested, the collection of such samples should be more feasible in the future and will allow for constant additions to the sample set. The collection of feathers for genetic testing is a routine practice in many zoos (Costantini *et al.* 2008; Hogan *et al.* 2008; Moran *et al.* 2008), and with appropriate communication, a similar practice could be implemented with quills.

Some limitations of this test will of course arise in casework scenarios where only one animal is seized for testing. If the results show no relatedness to any other echidnas, and population of origin is not able to be inferred, then little information can be derived that would assist an investigation. As is the case in human forensic science, absence of a result (i.e. not being able to include or exclude) does not mean that there is no relatedness, but rather there is insufficient data (Butler 2009). This is why zoos

and sanctuaries in Australia and around the world should be strongly encouraged to continuously collect and archive samples for this purpose. This will increase my dataset and statistical power to provide sufficient information about any traded echidnas, and by its presence, discourage illegal trade. It should also encourage the development of a robust population database using the allele frequencies of the most informative SNPs, similar to what is seen in human forensics (Ayres *et al.* 2002; Monson *et al.* 2002; Steele and Balding 2014). Furthermore, other genomic tools such the use of long haplotypes, or microhaplotypes could be investigated to allow for robust data analysis and further investigation into individualisation and population history. It is also important that zoos collect data on the origins of the animal where possible; this can then help law enforcement, in conjunction with genetic testing, to pinpoint animals that are coming from specific breeders. From this, relatedness information can then be provided. Genetic testing is crucial, but is most effective when data is shared, both by combining genetic data from other studies, and merging with studbook information. Such collaboration is important not only in wildlife forensics, but in also more broadly in wildlife genetics (Fienieg and Galbusera 2013; Schmidt *et al.* 2015; Coetzer *et al.* 2017).

The development of these markers is crucial in wildlife forensic science, due to the vast array of species, and therefore the need to perform such pilot studies each time a new species is involved is key. There is unfortunately an extremely large number of species caught up in a trade similar to the short beaked echidna, including a large number of birds and reptiles (Nijman and Shepherd 2009), as well as the endangered long beaked echidna (*Zaglossus spp*) (Shepherd & Sy 2017). One limitation of wildlife forensic genetic analysis is that new tests are required for each species, which

can be time and cost consuming (Moore and Frazier 2019). It is hoped that the recommendations and results presented in this thesis can provide guidance to any future studies of a similar nature.

If the development of a forensic toolbox is being considered for other species in the illegal wildlife trade, the following points should be taken into consideration:

- A large sample set, incorporating both wild and captive bred animals should be aimed for. It is crucial to demonstrate that the markers are working and are informative down to the individual level, rather than just at a higher population level.
- If developing a similar novel set of markers, it is ideal to include samples of known relatedness as in the present study, particularly with more than one group of trios (parents/offspring). Other studies that successfully inferred relatedness have included trios and other relatives for species such as the Tasmanian devil, European bison, and pigs (Tokarska *et al.* 2009; Lopes *et al.* 2013; McLennan *et al.* 2018).
- If samples are taken opportunistically, it is recommended that storage conditions yield sufficient DNA.
- Non-invasive samples should be taken where possible (e.g. is it possible to collect a buccal swab, hair, feather, or scat if appropriate for the animal), but it is important to be aware that they may not always produce DNA of sufficient quality.
- High quality samples should be used for reference data, but any developed methods should be tested on lower quality samples. This will likely mimic a

casework situation which can be variable and will often involve samples of diminished integrity (Butler 2009; Linacre 2009). Due to the fact that some quills were able to produce DNA suitable for markers, this has potential to be an option for short or long beaked echidnas, but as the quills were highly variable this should not be something that should be relied upon until further validation on a smaller SNP panel has taken place.

- It is recommended that zoos collaborate closely for sample collection and share data where possible about the specific animals to improve the reference data. While this may be difficult in some localities, it benefits both the researcher and the zoo. In particular, collaboration with local museums for storage of samples should be prioritised, in order to better utilise the facilities of the museum. It would also be recommended that governments and agencies, such as the World Association of Zoo and Aquariums, and the Zoo and Aquarium Association Australasia, mandate sample collection to maintain the integrity of the legitimate zoo industry.

6.7 Conclusion

This study has provided a set of genetic tools developed to aid and deliver crucial information to law enforcement and zoos in order to help stop the illegal trade in short beaked echidnas. This trade is one of many that involves the trafficking of animals claimed as 'captive bred', with no genetic data to support this claim. The toolbox presented in this thesis is the first to develop such a range of tests for the short beaked echidna. Methods developed in this study can distinguish between New Guinea and Australian echidnas using both a validated mitochondrial marker, as well as a robust nuclear marker set. This nuclear SNP marker set can also resolve the relatedness between individuals, a crucial step in determining the status of a traded echidna.

Lastly, deeper investigation of existing published tests provide compelling evidence as to why the wildlife forensic community should be encouraged to validate any new tests developed. These methods provide a framework for further research within this field, both for the short beaked echidna, as well as any other species with evidence of a similar trade. There is scope for these tests to be developed further and used effectively to stop the illegal trade of the short beaked echidna, one of the Australasian regions' iconic species.

6.8 References

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APPENDICES

Table A1 List of echidna (*Tachyglossus aculeatus* and *Zaglossus bruijnii*) samples used for analyses in Chapter 2.

QLD = Queensland; NSW = New South Wales, SA = South Australia, WA = Western Australia, TAS = Tasmania, ACT = Australian Capital Territory, PNG = Papua New Guinea, AM = Australian Museum, ANWC = Australian National Wildlife Collection, CWS = Currumbin Wildlife Sanctuary, MV = Museum Victoria, QM = Queensland Museum, UTAS = University of Tasmania.

Registration Number	***Donor***	***Tissue type***	***Country***	***Location***
M.48041.001	Australia Zoo	Quill	Australia	Beerwah, QLD
M.48041.002	Australia Zoo	Blood	Australia	Beerwah, QLD
M.48044.002	Australia Zoo	Blood	Australia	Beerwah, QLD
M.48045.001	Australia Zoo	Quill	Australia	Kuranda, QLD
M.30518.001	AM	Tissue	Australia	Yathong Nature Reserve, NSW
M.30757.001	AM	Tissue	Australia	Kangaroo Island, SA
M.30758.001	AM	Tissue	Australia	Kangaroo Island, SA
M.42826.002	AM	Tissue	Australia	Cobar, NSW
M.43654.001	AM	Tissue	Australia	Warringah, NSW
M.43685.001	AM	Tissue	Australia	Griffith, NSW
M.45058.001	AM	Tissue	Australia	Menangle, NSW
M.46626.001	AM	Tissue	Australia	Castlereagh, NSW
M.46902.001	AM	Tissue	Australia	Coffs Harbour, NSW
M.47764.004	AM	Quill	Australia	NSW
M.48130.001	AM	Quill	Australia	Sydney, NSW
M.48138.001	AM	Quill	Australia	NSW
M.48534.001	AM	Tissue	PNG	Semebedeben

M.48536.001	AM	Tissue	PNG	Serki
M.48537.001	AM	Tissue	PNG	Serki
M4594	AM	Skin	Australia	Moa Island, Torres Strait
M5966	AM	Skin	PNG	Port Moresby
M24799	ANWC	Tissue	Australia	Canberra, ACT
M24799	ANWC	Tissue	Australia	Canberra, ACT
M29902	ANWC	Tissue	Australia	Cooma, NSW
M29959	ANWC	Tissue	Australia	Bookham, NSW
M29960	ANWC	Tissue	Australia	Bookham, NSW
A50086	CWS	DNA	Australia	Coffs Harbour, NSW
A60170	CWS	DNA	Australia	Coffs Harbour, NSW
M.48008.001	Los Angeles Zoo	DNA	Australia	NSW
M.48009.001	Los Angeles Zoo	DNA	Australia	NSW
MV384	MV	Tissue	Australia	Great Victoria Desert, WA
M.48052.001	Perth Zoo	Blood	Australia	Perth, WA
M.48056.001	Perth Zoo	Blood	Australia	Badginbgarra, WA
M.48057.001	Perth Zoo	Blood	Australia	Mundijong, WA
A003256	QM	Tissue	Australia	Deception Bay, QLD
A003258	QM	Tissue	Australia	Greenbank, QLD

A009485	QM	Tissue	Australia	Buccan, QLD
A011501	QM	Tissue	Australia	Bowen, QLD
M.48010.001	San Diego Zoo	DNA	Australia	Melbourne Zoo
M.48012.001	San Diego Zoo	DNA	Australia	Sydney, NSW
M.48011.001	San Diego Zoo	DNA	Indonesia/PNG	
970139	Taronga Zoo	Blood	Australia	Cooma, NSW
A50176	Taronga Zoo	Blood	Australia	Frenchs Forest, NSW
A50345	Taronga Zoo	Blood	Australia	Beacon Hill, NSW
B30171	Taronga Zoo	Blood	Australia	Manly Vale, NSW
B50077	Taronga Zoo	Blood	Australia	Tooraweenah, NSW
B60344	Taronga Zoo	Blood	Australia	Bob's Farm, NSW
M.48059.001	UTAS	Tissue	Australia	Kingston, TAS
M.48061.001	UTAS	Tissue	Australia	Bonnet Hill, TAS
M.48063.001	UTAS	Tissue	Australia	Acton Park, TAS
M.48069.001	UTAS	Tissue	Australia	Lovely Banks, TAS
TM0013	WA Museum	Tissue	Australia	Kingsway, WA
TM0014	WA Museum	Tissue	Australia	Toodyay, WA
TM0235	WA Museum	Tissue	Australia	Ilkurlka, WA
M.47975.001 (Zaglossus bruijnii)	AM	Tissue	New Guinea	

Table A2 List of species used in *specificity* validation testing in Chapter 2. All samples donated by the Australian Museum.

Registration	***Species***	***Sample type***
M.47975.001	Western long beaked echidna (*Zaglossus bruijnii*)	Tissue
M.35614.001	Platypus (*Ornithorhynchus anatinus*)	Tissue
WGM118-186	Bilby (*Macrotis lagotis*)	Tissue
B10002	Little Penguin (*Eudyptula minor*)	Tissue
AM205-7	Agile Antechinus (*Antechinus agilis*)	Tissue
R.150174.001	Eastern Crevice Skink (*Egernia mcpheei*)	Tissue
O.71701.001	White-browed Woodswallow (*Artamus superciliosus*)	Tissue
I.39681.003	Queensland Grouper (*Epinephelus lanceolatus*)	Tissue
I.31253.130	Rock Cod (*Lotella rhacina*)	Tissue

Table A3 List of short beaked echidna samples (*n=192*) used for analyses Chapter 3.

* = failed DArTseq quality control; ▲ = Removed from dataset due to individual call rate < 80%

AU = Australia, QLD = Queensland; NSW = New South Wales, SA = South Australia, WA = Western Australia, TAS = Tasmania, ACT = Australian Capital Territory, PNG = Papua New Guinea, AM = Australian Museum, ANWC = Australian National Wildlife Collection, CWS = Currumbin Wildlife Sanctuary, MV = Museum Victoria, QM = Queensland Museum, UTAS = University of Tasmania.

Registration	*Donor*	*Sample Type*	*Location*	*Subspecies (based on location)*
M.48043.002	Australia Zoo	Blood	Beerwah, QLD, AU	*T.a. aculeatus*
M.48044.002	Australia Zoo	Blood	Beerwah, QLD, AU	*T.a. aculeatus*
M.30517.002	AM	Tissue	Lismore, NSW, AU	*T.a. aculeatus*
*M.30757.001**	AM	Tissue	Kangaroo Island, SA, AU	*T.a. multiaculeatus*
*M.30758.001**	AM	Tissue	Kangaroo Island, SA, AU	*T.a. multiaculeatus*
M.32567.001	AM	Tissue	Fitzroy Falls, NSW, AU	*T.a. aculeatus*
M.37231.002	AM	Tissue	Wondecla, QLD, AU	*T.a. aculeatus*
M.37233.001	AM	Tissue	Yungaburra, QLD, AU	*T.a. aculeatus*
M.39210.001	AM	Tissue	Coffs Harbour, NSW, AU	*T.a. aculeatus*
M.42783.001	AM	Tissue	Scheyville NSW, AU	*T.a. aculeatus*
M.42826.002	AM	Tissue	Cobar, NSW, AU	*T.a. aculeatus*
M.43205.001	AM	Tissue	Cobar, NSW, AU	*T.a. aculeatus*
M.43654.001	AM	Tissue	Warringah, NSW, AU	*T.a. aculeatus*
M.43684.001	AM	Tissue	Brigalow Park, NSW, AU	*T.a. aculeatus*
M.43685.001	AM	Tissue	Griffith, NSW, AU	*T.a. aculeatus*
M.43686.001	AM	Tissue	Illawarra, NSW, AU	*T.a. aculeatus*
M.43689.001	AM	Tissue	Menangle, NSW, AU	*T.a. aculeatus*
M.44950.001	AM	Tissue	Seaforth, NSW, AU	*T.a. aculeatus*
M.45058.001	AM	Tissue	Menangle, NSW, AU	*T.a. aculeatus*
M.45790.001	AM	Tissue	Somersby, NSW, AU	*T.a. aculeatus*
M.45839.001	AM	Tissue	Mosman, NSW, AU	*T.a. aculeatus*
M.45949.001	AM	Tissue	Mosman, NSW, AU	*T.a. aculeatus*
M.46902.001	AM	Tissue	Coffs Harbour, NSW, AU	*T.a. aculeatus*
M.47764.004	AM	Quill	NSW, AU	*T.a. aculeatus*
M.48130.001	AM	Quill	Sydney, NSW, AU	*T.a. aculeatus*
M.48534.001	AM	Tissue	Sembedeben, PNG	*T.a. lawesii*
M.48536.001	AM	Tissue	Serki, PNG	*T.a. lawesii*

Registration	*Donor*	*Sample Type*	*Location*	*Subspecies (based on location)*
M.48537.001	AM	Tissue	Serki, PNG	*T.a. lawesii*
*M4594**	AM	Skin	Moa Island, Torres Strait	*T.a. lawesii*
*M5966**	AM	Skin	Port Moresby, PNG	*T.a. lawesii*
M24799	ANWC	Tissue	O'Connor, ACT, AU	*T.a. aculeatus*
M29902	ANWC	Tissue	Cooma, NSW, AU	*T.a. aculeatus*
M29959	ANWC	Tissue	Bookham, NSW, AU	*T.a. aculeatus*
M29960	ANWC	Tissue	Bookham, NSW, AU	*T.a. aculeatus*
M29989	ANWC	Tissue	Oberon, NSW, AU	*T.a. aculeatus*
A20177	CWS	DNA	Gatton, QLD, AU	*T.a. aculeatus*
A20310	CWS	DNA	Currumbin, QLD, AU	*T.a. aculeatus*
A20358	CWS	DNA	Gatton, QLD, AU	*T.a. aculeatus*
A30111	CWS	DNA	Tweed Heads, NSW, AU	*T.a. aculeatus*
A40193	CWS	DNA	Currumbin, QLD, AU	*T.a. aculeatus*
A60170	CWS	DNA	Lismore, NSW, AU	*T.a. aculeatus*
A70194	CWS	DNA	Currumbin, QLD, AU	*T.a. aculeatus*
M.48008.001	Los Angeles Zoo	DNA	Sydney, NSW, AU	*T.a. aculeatus*
M.48009.001	Los Angeles Zoo	DNA	Sydney, NSW, AU	*T.a. aculeatus*
11034	MV	Tissue	Healesville, VIC, AU	*T.a. aculeatus*
21586	MV	Tissue	Romsey, VIC, AU	*T.a. aculeatus*
MV384	MV	Tissue	Great Victoria Desert, WA, AU	*T.a. acanthion*
M.48048.001	Perth Zoo	Blood	Mundijong, WA, AU	*T.a. acanthion*
M.48049.001	Perth Zoo	Blood	Jurien Bay, WA, AU	*T.a. acanthion*
M.48050.001	Perth Zoo	Blood	Mardella, WA, AU	*T.a. acanthion*
M.48051.001	Perth Zoo	Blood	Gin Gin, WA, AU	*T.a. acanthion*
M.48056.001	Perth Zoo	Blood	Badgingarra, WA, AU	*T.a. acanthion*
A003256	QM	Tissue	Deception Bay, QLD, AU	*T.a. aculeatus*
A003258	QM	Tissue	Greenbank, QLD, AU	*T.a. aculeatus*
A009485	QM	Tissue	Buccan, QLD, AU	*T.a. aculeatus*
A009533	QM	Tissue	Stradbroke Island, QLD, AU	*T.a. aculeatus*
A010298	QM	Tissue	Brisbane, QLD, AU	*T.a. aculeatus*
A011501	QM	Tissue	Bowen, QLD, AU	*T.a. aculeatus*
A013193	QM	Tissue	Belbowrie, QLD, AU	*T.a. aculeatus*
M.48010.001	San Diego Zoo	DNA	Melbourne, VIC, AU	*T.a. aculeatus*

Registration	***Donor***	***Sample Type***	***Location***	***Subspecies (based on location)***
M.48011.001	San Diego Zoo	DNA	Indonesia/PNG	*T.a. lawesii*
970139	Taronga Zoo	Blood	Cooma, NSW, AU	*T.a. aculeatus*
980292[▲]	Taronga Zoo	Blood	Haberfield, NSW, AU	*T.a. aculeatus*
A20450[*]	Taronga Zoo	Blood	Belfield, NSW, AU	*T.a. aculeatus*
A20451	Taronga Zoo	Blood	Narrabeen, NSW, AU	*T.a. aculeatus*
A40624	Taronga Zoo	Blood	Ingleside, NSW, AU	*T.a. aculeatus*
A50176	Taronga Zoo	Blood	Frenchs Forest, NSW, AU	*T.a. aculeatus*
A50345	Taronga Zoo	Blood	Beacon Hill, NSW, AU	*T.a. aculeatus*
B30126	Taronga Zoo	Blood	Greta, NSW, AU	*T.a. aculeatus*
B30171	Taronga Zoo	Blood	Manly Vale, NSW, AU	*T.a. aculeatus*
B50077[▲]	Taronga Zoo	Blood	Tooraweenah, NSW, AU	*T.a. aculeatus*
B60344	Taronga Zoo	Blood	Bob's Farm, NSW, AU	*T.a. aculeatus*
M.48059.001	UTAS	Tissue	Kingston, TAS, AU	*T.a. setosus*
*M.48060.001**	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.48061.001	UTAS	Tissue	Bonnet Hill, TAS, AU	*T.a. setosus*
M.48062.001	UTAS	Tissue	South Hobart, TAS, AU	*T.a. setosus*
M.48063.001	UTAS	Tissue	Acton Park, TAS, AU	*T.a. setosus*
M.48066.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.48067.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.48068.001[▲]	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.48069.002	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.48070.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.48076.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.48077.002	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50696.002	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
*M.50706.001**	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
*M.50707.001**	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50708.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50713.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50714.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50726.001[▲]	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50727.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50736.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*

Registration	*Donor*	*Sample Type*	*Location*	*Subspecies (based on location)*
M.50737.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50738.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50739.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50740.001[*]	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50742.002	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50746.002	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50748.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50749.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50750.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50751.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50752.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50753.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50755.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50757.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50758.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50759.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50760.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50761.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50762.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50763.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50765.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50767.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50769.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50772.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50774.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50777.001[▲]	UTAS	Tissue	Hamilton, TAS, AU	*T.a. setosus*
M.50781.001	UTAS	Tissue	Flagstaff, TAS, AU	*T.a. setosus*
M.50782.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50783.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50785.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50786.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50787.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50790.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50791.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*

Registration	*Donor*	*Sample Type*	*Location*	*Subspecies (based on location)*
M.50792.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50795.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50796.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50798.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50799.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50800.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50803.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
*M.50804.001**	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50814.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50819.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50820.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50821.001	UTAS	Tissue	Campbell Town, TAS, AU	*T.a. setosus*
M.50822.001	UTAS	Tissue	Forestier Peninsula, TAS, AU	*T.a. setosus*
M.50823.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50825.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50828.001	UTAS	Tissue	Fern Tree, TAS, AU	*T.a. setosus*
M.50829.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50830.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50833.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50835.001	UTAS	Tissue	Bonnett Hill, TAS, AU	*T.a. setosus*
M.50838.001	UTAS	Tissue	Hamilton, TAS, AU	*T.a. setosus*
M.50839.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50840.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50841.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50842.003	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50844.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50845.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50846.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50848.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50850.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50852.001	UTAS	Tissue	Bruny Island, TAS, AU	*T.a. setosus*
M.50853.001	UTAS	Tissue	Cambridge, TAS, AU	*T.a. setosus*
M.50854.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50855.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*

Registration	***Donor***	***Sample Type***	***Location***	***Subspecies (based on location)***
M.50856.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50857.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50858.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50859.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50860.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50861.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50863.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50865.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50867.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50868.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50870.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
*M.50871.001**	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50873.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50875.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50877.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50878.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50881.001[▲]	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50883.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50884.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50904.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50906.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M.50924.001	UTAS	Tissue	Lovely Banks, TAS, AU	*T.a. setosus*
M48499	WA Museum	Tissue	Ravensthorpe, WA, AU	*T.a. acanthion*
TM0012	WA Museum	Tissue	Worsley, WA, AU	*T.a. acanthion*
*TM0013**	WA Museum	Tissue	Kingsway, WA, AU	*T.a. acanthion*
*TM0014**	WA Museum	Tissue	Toodyay, WA, AU	*T.a. acanthion*
TM0070	WA Museum	Tissue	Brookton, WA, AU	*T.a. acanthion*
TM0235	WA Museum	Tissue	Beadell, WA, AU	*T.a. acanthion*
*M52299**	WA Museum	Tissue	Ajana, WA, AU	*T.a. acanthion*

Table A4 List of extraction methods and DNA concentrations for short beaked echidna (*Tachyglossus aculeatus*) samples used in Chapter 3.

Bioline = Bioline ISOLATE II Genomic DNA Kit using animal tissue protocol

Registration Number	*Extraction Method*	*DNA Concentration (ng/µL)*
M.48043.002	Salting out	2.03
M.48044.002	Salting out	22.8
M.30517.002	Bioline	18.1
M.30757.001	Bioline	3.47
M.30758.001	Bioline	0.026
M.32567.001	Bioline	9.05
M.37231.002	Bioline	18.3
M.37233.001	Bioline	13.3
M.39210.001	Bioline	17.6
M.42783.001	Bioline	1.44
M.42826.002	Bioline	3.12
M.43205.001	Bioline	15.4
M.43654.001	Bioline	4.13
M.43684.001	Bioline	30.03
M.43685.001	Bioline	97.7
M.43686.001	Bioline	185.59
M.43689.001	Bioline	11.1
M.44950.001	Bioline	13.2
M.45058.001	Bioline	80.64
M.45790.001	Bioline	8.86
M.45839.001	Bioline	5.97
M.45949.001	Bioline	15.8
M.46902.001	Bioline	14.12
M.47764.004	QIAGEN QIAamp Investigator Kit Hair and Nails Protocol	6.45
M.48130.001	QIAGEN QIAamp Investigator Kit Hair and Nails Protocol	4.43
M.48534.001	Bioline	9.97
M.48536.001	Bioline	32.6
M.48537.001	Bioline	6.85
M4594	Bioline	2.64
M5966	Bioline	1.12
M24799	Bioline	204
M29902	Bioline	48.8
M29959	Bioline	35.90
M29960	Bioline	9.82

Registration Number	*Extraction Method*	*DNA Concentration (ng/µL)*
M29989	Bioline	4.56
A20177	Extracted prior to this study	19.4
A20310	Extracted prior to this study	14.7
A20358	Extracted prior to this study	28.5
A30111	Extracted prior to this study	20.3
A40193	Extracted prior to this study	13.1
A60170	Extracted prior to this study	12.4
A70194	Extracted prior to this study	14.9
M.48008.001	Extracted prior to this study	1.91
M.48009.001	Extracted prior to this study	2.19
11034	Bioline	12.4
21586	Bioline	24.3
MV384	Bioline	126.00
M.48048.001	Salting out	64.28
M.48049.001	Salting out	13.53
M.48050.001	Salting out	0.982
M.48051.001	Salting out	8.41
M.48056.001	Salting out	9.20
A003256	Bioline	32.90
A003258	Bioline	15.20
A009485	Bioline	70.70
A009533	Bioline	64.60
A010298	Bioline	560.00
A011501	Bioline	32.7
A013193	Bioline	137
M.48010.001	Extracted prior to this study	2.92
M.48011.001	Extracted prior to this study	1.60
970139	Salting out	1.30
980292	Salting out	0.211
A20450	Salting out	0.296
A20451	Salting out	10.87
A40624	Salting out	26.77
A50176	Salting out	0.39
A50345	Salting out	1.94
B30126	Salting out	7.28
B30171	Salting out	1.04
B50077	Salting out	0.772
B60344	Salting out	1.76

Registration Number	*Extraction Method*	*DNA Concentration (ng/µL)*
M.48059.001	Bioline	20.90
M.48060.001	Bioline	2.1
M.48061.001	Bioline	6.62
M.48062.001	Bioline	34.2
M.48063.001	Bioline	1.01
M.48066.001	Bioline	34.3
M.48067.001	Bioline	32
M.48068.001	Bioline	37.2
M.48069.002	Bioline	4.01
M.48070.001	Bioline	26.8
M.48076.001	Bioline	0.5
M.48077.002	Bioline	5.67
M.50696.002	Bioline	9.27
M.50706.001	Bioline	0.277
M.50707.001	Bioline	0.071
M.50708.001	Bioline	0.222
M.50713.001	Bioline	3
M.50714.001	Bioline	6.68
M.50726.001	Bioline	0.846
M.50727.001	Bioline	2.48
M.50736.001	Bioline	0.194
M.50737.001	Bioline	2.31
M.50738.001	Bioline	1.65
M.50739.001	Bioline	1.38
M.50740.001	Bioline	0.531
M.50742.002	Bioline	3.67
M.50746.002	Bioline	4.12
M.50748.001	Bioline	8.73
M.50749.001	Bioline	6.86
M.50750.001	Bioline	17.1
M.50751.001	Bioline	2.84
M.50752.001	Bioline	2.16
M.50753.001	Bioline	7.29
M.50755.001	Bioline	9.43
M.50757.001	Bioline	3.72
M.50758.001	Bioline	3.5
M.50759.001	Bioline	5.21
M.50760.001	Bioline	2.61

Registration Number	*Extraction Method*	*DNA Concentration (ng/µL)*
M.50761.001	Bioline	5.17
M.50762.001	Bioline	2.37
M.50763.001	Bioline	4.04
M.50765.001	Bioline	2.69
M.50767.001	Bioline	2.43
M.50769.001	Bioline	1.72
M.50772.001	Bioline	21.2
M.50774.001	Bioline	9.04
M.50777.001	Bioline	2.32
M.50781.001	Bioline	18.6
M.50782.001	Bioline	9.17
M.50783.001	Bioline	3.35
M.50785.001	Bioline	9.96
M.50786.001	Bioline	7.14
M.50787.001	Bioline	1.97
M.50790.001	Bioline	6.03
M.50791.001	Bioline	4.13
M.50792.001	Bioline	4.73
M.50795.001	Bioline	6.38
M.50796.001	Bioline	3.08
M.50798.001	Bioline	1.23
M.50799.001	Bioline	6.87
M.50800.001	Bioline	2.01
M.50803.001	Bioline	1.94
M.50804.001	Bioline	1.76
M.50814.001	Bioline	9.69
M.50819.001	Bioline	4.02
M.50820.001	Bioline	2.68
M.50821.001	Bioline	44.6
M.50822.001	Bioline	45.4
M.50823.001	Bioline	34.4
M.50825.001	Bioline	2.68
M.50828.001	Bioline	25.9
M.50829.001	Bioline	30.9
M.50830.001	Bioline	18.4
M.50833.001	Bioline	15.7
M.50835.001	Bioline	10.6
M.50838.001	Bioline	13.1

M.50839.001	Bioline	32.6
M.50840.001	Bioline	25.2
M.50841.001	Bioline	30.6
M.50842.003	Bioline	10.8
M.50844.001	Bioline	1.47
M.50845.001	Bioline	31.4
M.50846.001	Bioline	29.7
M.50848.001	Bioline	21
M.50850.001	Bioline	6.13
M.50852.001	Bioline	15.2
M.50853.001	Bioline	22.2
M.50854.001	Bioline	3.64
M.50855.001	Bioline	37.2
M.50856.001	Bioline	28.2
M.50857.001	Bioline	15
M.50858.001	Bioline	0.754
M.50859.001	Bioline	15.8
M.50860.001	Bioline	33.1
M.50861.001	Bioline	17.2
M.50863.001	Bioline	18.9
M.50865.001	Bioline	3.27
M.50867.001	Bioline	29.2
M.50868.001	Bioline	38.5
M.50870.001	Bioline	0.054
M.50871.001	Bioline	6.7
M.50873.001	Bioline	11.5
M.50875.001	Bioline	2.3
M.50877.001	Bioline	17.7
M.50878.001	Bioline	17.1
M.50881.001	Bioline	3.7
M.50883.001	Bioline	9.43
M.50884.001	Bioline	1.06
M.50904.001	Bioline	3.61
M.50906.001	Bioline	22.2
M.50924.001	Bioline	2.25
M48499	Bioline	11
TM0012	Bioline	1.01
TM0013	Bioline	0.365
TM0014	Bioline	3.57
TM0070	Bioline	10.3

TM0235	Bioline	19.8
M52299	Bioline	2.1

Table A5 Filtering applied on SNP dataset used in Chapter 3 using the *dartR* R package.

	Number of SNPs	***Missing data (%)***
No filtering	27,258	31.1
Call rate (0.95)	3782	3.64
Reproducibility Average (1)	2560	3.60
Secondaries	2406	2.76
Individual call rate (6 samples removed)	2391	1.00
Recalculation and monomorphs removed	2388	0.90

Table A6 Short beaked echidna (*Tachyglossus aculeatus*) samples used for analyses in Chapter 4, including corresponding method of extraction and DNA concentration.

* = failed DArTseq quality control; ▲ = Removed from dataset due to individual call rate < 80%; ♦ = Replicate sample.

CWS = Currumbin Wildlife Sanctuary, UTAS = University of Tasmania.

Bioline = Bioline ISOLATE II Genomic DNA Kit using animal tissue protocol

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
M.48040.002	Australia Zoo	Blood	Salting out	18.3
M.48041.002*	Australia Zoo	Blood	Salting out	8.24
M.48042.002*	Australia Zoo	Blood	Salting out	2.28
M.48043.002*	Australia Zoo	Blood	Salting out	2.03
M.48044.002	Australia Zoo	Blood	Salting out	22.8
M.48045.002*	Australia Zoo	Blood	Salting out	29.71
M.48046.001*	Australia Zoo	Blood	Salting out	2.82
M.48046.001B	Australia Zoo	Blood	Salting out	29.79
M.48047.001	Australia Zoo	Blood	Salting out	57
970057	CWS	DNA	Extracted prior to study	16.1
A20177	CWS	DNA	Extracted prior to study	19.4
Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)

A20310	CWS	DNA	Extracted prior to study	14.7
A20310♦	CWS	DNA	Extracted prior to study	14.7
A20358	CWS	DNA	Extracted prior to study	28.5
A30111	CWS	DNA	Extracted prior to study	20.3
A30111♦	CWS	DNA	Extracted prior to study	20.3
A40193	CWS	DNA	Extracted prior to study	13.1
A40193♦	CWS	DNA	Extracted prior to study	13.1
A50086	CWS	DNA	Extracted prior to study	10.7
A50087	CWS	DNA	Extracted prior to study	14.2
A50087♦	CWS	DNA	Extracted prior to study	14.2
A60170	CWS	DNA	Extracted prior to study	33.0
A60170♦	CWS	DNA	Extracted prior to study	33.0
A60170♦	CWS	DNA	Extracted prior to study	33.0
A60170♦	CWS	DNA	Extracted prior to study	33.0

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
A70094	CWS	DNA	Extracted prior to study	28.3
A70194	CWS	DNA	Extracted prior to study	14.9
B10134	CWS	DNA	Extracted prior to study	34

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
B20099	CWS	DNA	Extracted prior to study	25.3
B20122	CWS	DNA	Extracted prior to study	24.9
B20156	CWS	DNA	Extracted prior to study	20.6
B20164	CWS	DNA	Extracted prior to study	6.42
B30193	CWS	DNA	Extracted prior to study	30.2
B40159	CWS	DNA	Extracted prior to study	27.1
B40159[♦]	CWS	DNA	Extracted prior to study	27.1
B40161	CWS	DNA	Extracted prior to study	18.8
B40166	CWS	DNA	Extracted prior to study	39.6
B40715	CWS	DNA	Extracted prior to study	14.3
B50294	CWS	DNA	Extracted prior to study	1.96
CWS-33[▲]	CWS	DNA	Extracted prior to study	29
CWS-34	CWS	DNA	Extracted prior to study	18.3
CWS-35	CWS	DNA	Extracted prior to study	6.3
M.48048.001	Perth Zoo	Blood	Salting out	64.28
M.48048.001[♦]	Perth Zoo	Blood	Salting out	64.28
M.48048.001[♦]	Perth Zoo	Blood	Salting out	64.28

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
M.48049.001	Perth Zoo	Blood	Salting out	13.53
M.48050.001*	Perth Zoo	Blood	Salting out	0.982
M.48051.001	Perth Zoo	Blood	Salting out	8.41
M.48051.001[◆]	Perth Zoo	Blood	Salting out	8.41
M.48052.001	Perth Zoo	Blood	Salting out	3.48
M.48053.001	Perth Zoo	Blood	Salting out	16.1
M.48054.001	Perth Zoo	Blood	Salting out	2.15
M.48055.001	Perth Zoo	Blood	Salting out	9.84
M.48056.001	Perth Zoo	Blood	Salting out	9.2
M.48057.001*	Perth Zoo	Blood	Salting out	4.24
970139	Taronga Zoo	Blood	Salting out	1.3
980292[▲]	Taronga Zoo	Blood	Salting out	0.211
A20450*	Taronga Zoo	Blood	Salting out	0.296
A20451	Taronga Zoo	Blood	Salting out	0.452
A30404*	Taronga Zoo	Blood	Salting out	0.301
A40624	Taronga Zoo	Blood	Salting out	0.384
A50176	Taronga Zoo	Blood	Salting out	0.39

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
A50345*	Taronga Zoo	Blood	Salting out	1.94
A50345	Taronga Zoo	Blood	Salting out	1.94
B10546*	Taronga Zoo	Blood	Salting out	1.09
B30126	Taronga Zoo	Blood	Salting out	0.54
B30171	Taronga Zoo	Blood	Salting out	10.4
B50077*	Taronga Zoo	Blood	Salting out	0.772
B60344	Taronga Zoo	Blood	Salting out	1.76
B60344◆	Taronga Zoo	Blood	Salting out	1.76
TZ-14*	Taronga Zoo	Blood	Salting out	0.8
M.48064.001▲	UTAS	Tissue	Bioline	23.9
M.48064.001◆	UTAS	Tissue	Bioline	23.9
M.48065.001	UTAS	Tissue	Bioline	33.5
M.48065.001◆	UTAS	Tissue	Bioline	33.5
M.48066.001	UTAS	Tissue	Bioline	34.3
M.48066.001◆	UTAS	Tissue	Bioline	34.3
M.48066.002◆	UTAS	Tissue	Bioline	80.36
M.48066.002◆	UTAS	Tissue	Bioline	80.36

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
M.48066.003◆	UTAS	Tissue	Bioline	16
M.48067.001	UTAS	Tissue	Bioline	32
M.48067.001◆	UTAS	Tissue	Bioline	32
M.48067.002◆	UTAS	Tissue	Bioline	5.84
M.48067.003◆	UTAS	Tissue	Bioline	4.18
M.48068.001	UTAS	Tissue	Bioline	37.2
M.48068.001◆	UTAS	Tissue	Bioline	37.20
M.48069.001▲	UTAS	Tissue	Bioline	4.01
M.48069.002◆	UTAS	Tissue	Bioline	1.06
M.48070.001	UTAS	Tissue	Bioline	26.8
M.48070.002◆	UTAS	Tissue	Bioline	5.8
M.48071.001	UTAS	Tissue	Bioline	9.33
M.48071.002◆	UTAS	Tissue	Bioline	4.26
M.48072.001	UTAS	Tissue	Bioline	4.8
M.48072.002◆	UTAS	Tissue	Bioline	1.06
M.48073.001	UTAS	Tissue	Bioline	11.8

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
M.48073.001◆	UTAS	Tissue	Bioline	11.8
M.48074.001	UTAS	Tissue	Bioline	12.7
M.48074.002◆	UTAS	Tissue	Bioline	2.3
M.48075.001	UTAS	Tissue	Bioline	8.65
M.48075.002◆	UTAS	Tissue	Bioline	4.62
M.48076.001	UTAS	Tissue	Bioline	0.5
M.48076.001◆	UTAS	Tissue	Bioline	0.5
M.48076.002◆	UTAS	Tissue	Bioline	12.7
M.48077.001	UTAS	Tissue	Bioline	5.67
M.48077.002◆	UTAS	Tissue	Bioline	7.22
M.50696.002	UTAS	Tissue	Bioline	9.27
M.50697.001*	UTAS	Tissue	Bioline	0.435
M.50698.001*	UTAS	Tissue	Bioline	0.469
M.50699.001*	UTAS	Tissue	Bioline	0.65
M.50700.001*	UTAS	Tissue	Bioline	0.535
Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
M.50701.001*	UTAS	Tissue	Bioline	0.215

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
M.50706.001*	UTAS	Tissue	Bioline	0.277
M.50707.001*	UTAS	Tissue	Bioline	0.071
M.50708.001	UTAS	Tissue	Bioline	0.222
M.50711.001*	UTAS	Tissue	Bioline	0.086
M.50712.001*	UTAS	Tissue	Bioline	0.3
M.50713.001	UTAS	Tissue	Bioline	3
M.50714.001	UTAS	Tissue	Bioline	6.68
M.50716.001*	UTAS	Tissue	Bioline	0.339
M.50717.001*	UTAS	Tissue	Bioline	0.563
M.50720.002*	UTAS	Tissue	Bioline	1.53
M.50724.001*	UTAS	Tissue	Bioline	0.514
M.50726.001*	UTAS	Tissue	Bioline	0.846
M.50727.001	UTAS	Tissue	Bioline	2.48
M.50731.001*	UTAS	Tissue	Bioline	2.36
M.50736.001	UTAS	Tissue	Bioline	0.194
M.50737.001	UTAS	Tissue	Bioline	2.31
M.50738.001	UTAS	Tissue	Bioline	1.65

M.50739.001	UTAS	Tissue	Bioline	1.38
M.50740.001*	UTAS	Tissue	Bioline	0.531
M.50742.002	UTAS	Tissue	Bioline	3.67
M.50743.001*	UTAS	Tissue	Bioline	0.19
M.50747.001*	UTAS	Tissue	Bioline	3.64
M.50748.001	UTAS	Tissue	Bioline	8.73
M.50749.001	UTAS	Tissue	Bioline	6.86
M.50750.001	UTAS	Tissue	Bioline	17.1
M.50751.001	UTAS	Tissue	Bioline	2.84
M.50752.001	UTAS	Tissue	Bioline	2.16
M.50753.001	UTAS	Tissue	Bioline	7.29
M.50755.001	UTAS	Tissue	Bioline	9.43
Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
M.50756.001*	UTAS	Tissue	Bioline	4.76
M.50757.001	UTAS	Tissue	Bioline	3.72
M.50758.001	UTAS	Tissue	Bioline	3.5
M.50760.001	UTAS	Tissue	Bioline	2.61
M.50762.001	UTAS	Tissue	Bioline	2.37

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
M.50763.001	UTAS	Tissue	Bioline	4.04
M.50765.001	UTAS	Tissue	Bioline	2.69
M.50766.001*	UTAS	Tissue	Bioline	5.8
M.50767.001	UTAS	Tissue	Bioline	2.43
M.50769.001	UTAS	Tissue	Bioline	1.72
M.50770.001*	UTAS	Tissue	Bioline	11.5
M.50772.001	UTAS	Tissue	Bioline	21.2
M.50772.001[♦]	UTAS	Tissue	Bioline	21.2
M.50774.001	UTAS	Tissue	Bioline	9.04
M.50775.001*	UTAS	Tissue	Bioline	1.61
M.50776.001*	UTAS	Tissue	Bioline	8.45
M.50778.001*	UTAS	Tissue	Bioline	1.22
M.50780.001*	UTAS	Tissue	Bioline	3.06
M.50782.001	UTAS	Tissue	Bioline	9.17
M.50783.001	UTAS	Tissue	Bioline	3.35
M.50784.001*	UTAS	Tissue	Bioline	1.11
M.50785.001	UTAS	Tissue	Bioline	9.96

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
M.50786.001	UTAS	Tissue	Bioline	7.14
M.50787.001	UTAS	Tissue	Bioline	1.97
M.50788.001*	UTAS	Tissue	Bioline	3.16
M.50790.001	UTAS	Tissue	Bioline	6.03
M.50791.001	UTAS	Tissue	Bioline	4.13
M.50792.001	UTAS	Tissue	Bioline	4.73
M.50795.001	UTAS	Tissue	Bioline	6.38
M.50796.001	UTAS	Tissue	Bioline	3.08
M.50798.001	UTAS	Tissue	Bioline	1.23
M.50799.001	UTAS	Tissue	Bioline	6.87
M.50800.001	UTAS	Tissue	Bioline	2.01
M.50803.001	UTAS	Tissue	Bioline	1.94
M.50804.001*	UTAS	Tissue	Bioline	1.76
M.50814.001	UTAS	Tissue	Bioline	9.69
M.50819.001	UTAS	Tissue	Bioline	4.02
M.50820.001	UTAS	Tissue	Bioline	2.68
M.50823.001	UTAS	Tissue	Bioline	34.4

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
M.50825.001	UTAS	Tissue	Bioline	2.08
M.50826.001*	UTAS	Tissue	Bioline	11.2
M.50829.001	UTAS	Tissue	Bioline	30.9
M.50830.001	UTAS	Tissue	Bioline	18.4
M.50830.001♦	UTAS	Tissue	Bioline	18.4
M.50833.001	UTAS	Tissue	Bioline	15.7
M.50837.001*	UTAS	Tissue	Bioline	31.3
M.50839.001	UTAS	Tissue	Bioline	32.6
M.50840.001	UTAS	Tissue	Bioline	25.2
M.50841.001	UTAS	Tissue	Bioline	30.6
M.50842.003	UTAS	Tissue	Bioline	10.8
M.50843.001*	UTAS	Tissue	Bioline	28.1
M.50844.001	UTAS	Tissue	Bioline	1.47
M.50845.001	UTAS	Tissue	Bioline	31.4
M.50846.001	UTAS	Tissue	Bioline	29.7
M.50848.001	UTAS	Tissue	Bioline	21
M.50850.001	UTAS	Tissue	Bioline	6.13

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
M.50851.001*	UTAS	Tissue	Bioline	22.3
M.50854.001	UTAS	Tissue	Bioline	3.64
M.50855.001	UTAS	Tissue	Bioline	37.2
M.50856.001	UTAS	Tissue	Bioline	28.2
M.50856.001◆	UTAS	Tissue	Bioline	28.2
M.50857.001	UTAS	Tissue	Bioline	15
M.50857.001◆	UTAS	Tissue	Bioline	15
M.50858.001	UTAS	Tissue	Bioline	0.754
M.50859.001	UTAS	Tissue	Bioline	15.8
M.50859.001◆	UTAS	Tissue	Bioline	15.8
M.50860.001	UTAS	Tissue	Bioline	33.1
M.50861.001	UTAS	Tissue	Bioline	17.2
M.50862.001*	UTAS	Tissue	Bioline	11.7
M.50863.001	UTAS	Tissue	Bioline	18.9
M.50864.001*	UTAS	Tissue	Bioline	13.9
M.50865.001	UTAS	Tissue	Bioline	3.27
M.50866.001*	UTAS	Tissue	Bioline	9.78

Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)
M.50867.001	UTAS	Tissue	Bioline	29.2
M.50868.001	UTAS	Tissue	Bioline	38.5
M.50870.001	UTAS	Tissue	Bioline	0.054
M.50870.001♦	UTAS	Tissue	Bioline	0.054
M.50871.001*	UTAS	Tissue	Bioline	6.7
M.50873.001	UTAS	Tissue	Bioline	11.5
M.50874.001	UTAS	Tissue	Bioline	0.921
M.50877.001	UTAS	Tissue	Bioline	17.7
M.50878.001	UTAS	Tissue	Bioline	17.1
M.50880.001*	UTAS	Tissue	Bioline	22.2
M.50881.001*	UTAS	Tissue	Bioline	3.7
M.50883.001	UTAS	Tissue	Bioline	9.43
M.50884.001	UTAS	Tissue	Bioline	1.06
M.50888.001*	UTAS	Tissue	Bioline	0.184
M.50890.001*	UTAS	Tissue	Bioline	0.359
M.50898.001*	UTAS	Tissue	Bioline	0.23
Sample ID	Donor	Tissue Type	Extraction Method	DNA concentration (ng/µl)

M.50904.001	UTAS	Tissue	Bioline	3.61
M.50904.001*	UTAS	Tissue	Bioline	3.61
M.50906.001	UTAS	Tissue	Bioline	22.2
M.50924.001	UTAS	Tissue	Bioline	2.25

Table A7 Filtering applied on SNP dataset used in Chapter 4 using the *dartR* R package.

	Number of SNPs	***Missing data (%)***
No filtering	27,258	31.1
Call rate (0.95)	3813	3.04
Reproducibility average (1)	2591	2.99
Secondaries	2430	2.98
Individual call rate (4 samples removed)	2417	1.03
Recalculation and monomorphs removed	2406	0.99

Table A8 List of kinship values (k) for replicates used in Chapter 4. Observed value represents value obtained when replicate sample was compared to original sample of the same name.

Registration Number	*Expected (k)*	*Observed (k)*	*±SD*
A20310	0.5	0.499	0.001
A30111	0.5	0.497	0.002
A40193	0.5	0.498	0.001
A50087	0.5	0.497	0.002
A60170	0.5	0.465	0.025
A60170	0.5	0.499	0.001
A60170	0.5	0.480	0.014
B40159	0.5	0.498	0.001
B60344	0.5	0.452	0.034
M.48048.001	0.5	0.496	0.003
M.48048.001	0.5	0.498	0.001
M.48051.001	0.5	0.495	0.003
M.48064.001	0.5	0.495	0.004
M.48065.001	0.5	0.494	0.004
M.48066.001	0.5	0.499	0.001
M.48066.001	0.5	0.491	0.007
M.48066.001	0.5	0.473	0.019
M.48066.001	0.5	0.483	0.012
M.48067.002	0.5	0.496	0.003
M.48067.002	0.5	0.462	0.027
M.48067.002	0.5	0.493	0.005
M.48067.002	0.5	0.5	0
M.48068.001	0.5	0.493	0.005
M.48070.001	0.5	0.478	0.016
M.48071.001	0.5	0.497	0.002
M.48072.001	0.5	0.496	0.003
M.48073.001	0.5	0.496	0.003
M.48074.001	0.5	0.483	0.012
M.48075.001	0.5	0.495	0.003
M.48076.002	0.5	0.5	0
M.48076.002	0.5	0.486	0.010
M.48077.001	0.5	0.471	0.020
M.50772.001	0.5	0.497	0.002
M.50830.001	0.5	0.497	0.002
M.50856.001	0.5	0.495	0.003
M.50857.001	0.5	0.495	0.004
M.50859.001	0.5	0.496	0.003
M.50870.001	0.5	0.499	0.000
M.50904.001	0.5	0.496	0.003

Table A9 Subset from Dataset C: Kinship values for short beaked echidna (*Tachyglossus aculeatus*) samples from Australia Zoo (*n=4*), calculated using Identity by Descent Maximum Likelihood estimator in the *SNPRelate* R package.

	M.48044.002	*M.48040.002*	*M.48047.001*	*M.48046.001*
M.48044.002	0.5			
M.48040.002	0	0.5		
M.48047.001	0.263	0	0.5	
M.48046.001	0.246	0	0.246	0.5

Table A10 Subset of Dataset C: Kinship values for short beaked echidna (*Tachyglossus aculeatus*) samples from Currumbin Wildlife Sanctuary (*n=24*); calculated using Identity by Descent Maximum Likelihood estimator in *SNPRelate* R package.

	A60170	*970057*	*A20358*	*B40161*	*B40715*	*A70094*	*A30111*	*B20099*	*B20122*	*B30193*	*B20164*	*A20310*	*A50087*	*B40166*	*A20177*	*A70194*	*B40159*	*B10134*	*B20156*	*A50086*	*A40193*	*B50294*	*CWS34*	*CWS35*
A60170	0.5																							
970057	0	0.5																						
A20358	0	0	0.5																					
B40161	0.239	0	0	0.5																				
B40715	0	0.187	0	0	0.5																			
A70094	0	0	0	0	0	0.5																		
A30111	0	0	0	0	0	0	0.5																	
B20099	0.234	0	0	0.101	0	0.084	0	0.5																
B20122	0	0	0	0.066	0	0.100	0	0	0.5															
B30193	0	0	0	0	0.061	0.242	0	0.117	0.104	0.5														
B20164	0	0	0	0	0	0	0	0	0.095	0	0.5													
A20310	0	0	0	0.181	0	0	0	0	0.239	0	0.186	0.5												
A50087	0	0	0	0	0	0.221	0	0	0.230	0.251	0	0	0.5											
B40166	0	0	0	0	0	0	0	0	0	0	0.113	0	0	0.5										
A20177	0	0	0	0	0	0	0	0	0	0	0	0	0	0.237	0.5									
A70194	0	0	0	0	0.127	0	0	0	0	0	0.237	0	0	0.234	0	0.5								
B40159	0	0	0	0	0	0	0.243	0	0	0	0	0	0	0	0	0	0.5							
B10134	0	0	0	0	0.230	0	0	0.077	0	0.092	0.096	0	0	0.113	0	0.226	0	0.5						
B20156	0	0	0	0.060	0	0.092	0	0	0.239	0.123	0.109	0.221	0.232	0	0	0	0	0	0.5					
A50086	0	0	0	0	0.133	0.230	0	0.238	0	0.229	0	0	0	0	0	0	0	0.215	0	0.5				
A40193	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.240	0	0	0	0.5			
B50294	0	0.230	0	0	0.221	0	0	0	0	0.074	0	0	0	0	0	0.109	0	0.215	0	0.114	0	0.5		
CWS34	0	0	0	0	0	0	0	0	0	0	0	0	0	0.191	0.174	0.191	0	0	0	0	0	0	0.5	
CWS35	0	0	0	0	0	0	0	0	0	0	0.123	0	0	0.187	0.167	0.241	0	0.098	0	0	0	0	0.186	0.5

Table A11 Dataset D: Kinship values for short beaked echidna samples (*Tachyglossus aculeatus*) from Perth Zoo (*n=8*), calculated using Identity by Descent; Maximum Likelihood estimator in *SNPRelate* R package.

	M.48048.001	*M.48049.001*	*M.48051.001*	*M.48052.001*	*M.48053.001*	*M.48054.001*	*M.48055.001*	*M.48056.001*
M.48048.001	0.5							
M.48049.001	0	0.5						
M.48051.001	0	0	0.5					
M.48052.001	0.231	0	0.249	0.5				
M.48053.001	0	0	0.259	0.148	0.5			
M.48054.001	0	0	0	0.122	0.162	0.5		
M.48055.001	0	0	0	0	0.226	0	0.5	
M.48056.001	0	0	0	0	0	0.232	0	0.5

Table A12 Dataset E: Kinship values for the short beaked echidna (*Tachyglossus aculeatus*) population from Lovely Banks, Tasmania (*n=88*), calculated using Identity by Descent; Maximum Likelihood estimator in *SNPRelate* R package.

	M.48069	*M.48070*	*M.48076*	*M.48067*	*M.48066*	*M.50750*	*M.50924*	*M.50783*	*M.50792*	*M.50828*	*M.50838*	*M.50846*	*M.50856*
M.48069	0.500												
M.48070	0	0.500											
M.48076	0	0	0.500										
M.48067	0	0.032	0.025	0.500									
M.48066	0	0	0	0.021	0.500								
M.50750	0	0	0	0	0	0.500							
M.50924	0	0	0	0.047	0	0	0.500						
M.50783	0	0	0	0	0	0	0	0.500					
M.50792	0	0	0.054	0	0	0	0	0	0.500				
M.50828	0	0	0	0	0	0	0	0	0	0.500			
M.50838	0	0	0	0.019	0	0	0	0	0	0	0.500		
M.50846	0	0	0	0.010	0	0	0	0	0	0	0	0.500	
M.50856	0	0	0	0.042	0	0	0	0	0	0	0	0	0.500
M.50867	0	0	0.044	0.047	0.062	0	0	0	0.078	0	0.079	0	0.084
M.50713	0	0	0	0.239	0	0	0	0	0.036	0	0	0	0.047
M.50749	0	0	0	0.053	0.030	0	0.058	0	0	0	0.065	0.042	0.030
M.48074	0	0.031	0	0	0	0	0	0	0.075	0	0	0	0.039
M.50795	0	0	0.064	0	0	0	0	0	0	0	0	0	0
M.50829	0	0	0	0.063	0	0	0	0	0	0	0.025	0.248	0.067
M.50839	0	0	0	0	0	0	0	0	0.073	0	0	0	0.048
M.50857	0	0	0.029	0.037	0	0	0.044	0	0	0	0	0.014	0
M.50868	0	0	0.028	0.016	0	0	0	0	0.045	0	0	0	0.044
M.50727	0	0	0.040	0.054	0.014	0	0	0	0	0	0	0.001	0.024
M.50751	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50760	0	0.056	0	0.023	0	0	0	0	0	0	0	0	0
M.50772	0	0	0	0	0.041	0	0	0	0	0	0	0	0.059
M.50785	0	0	0.058	0.104	0.049	0	0	0	0.084	0	0	0.064	0.055
M.50796	0	0	0	0	0	0	0	0	0	0	0	0	0
M.48064	0	0.033	0.056	0.033	0.042	0	0	0	0.059	0	0	0.037	0.035
M.50840	0	0.016	0.060	0	0	0	0.070	0	0.078	0.034	0	0.011	0.055
M.50848	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50859	0	0.062	0	0.133	0.053	0	0	0	0	0	0	0	0
M.50873	0	0	0.045	0.037	0	0	0	0.066	0.053	0	0	0	0

M.50752	0	0.021	0	0	0	0	0	0	0	0	0	0	0
M.50781	0	0.028	0.071	0.064	0	0	0	0	0.017	0	0	0.013	0.071
M.50786	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50798	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50841	0	0	0.226	0.031	0	0	0	0	0	0	0	0	0
M.50860	0	0.032	0	0	0	0	0	0	0	0	0	0	0.034
M.50696	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50753	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50762	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50821	0	0	0.010	0	0	0	0	0	0	0	0	0	0
M.50787	0	0.027	0.074	0	0	0	0	0	0	0	0	0	0
M.50799	0	0	0	0.049	0	0	0	0	0.065	0	0	0	0
M.50852	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50861	0	0	0	0	0	0	0	0.032	0.050	0	0	0	0
M.50878	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50737	0	0	0.051	0	0	0	0	0	0	0	0	0.034	0.074
M.50763	0	0.059	0.044	0.056	0.057	0	0.045	0	0.069	0	0.057	0.046	0.042
M.50822	0	0.024	0	0.017	0	0	0	0	0	0	0	0	0.032
M.50800	0	0	0	0.059	0	0	0	0	0	0	0	0	0.056
M.48073	0	0	0.061	0	0	0	0	0	0	0	0	0	0
M.50853	0	0	0	0.038	0	0	0	0	0	0	0	0	0
M.50738	0	0	0	0.061	0	0	0	0	0	0	0	0	0
M.50755	0	0.013	0.010	0	0	0	0	0.029	0	0	0	0	0
M.50765	0	0	0	0	0	0	0	0	0.026	0	0	0	0.073
M.50823	0	0	0	0	0.059	0.081	0	0	0	0	0	0	0
M.50790	0	0	0.066	0.035	0.055	0	0	0.019	0.076	0	0	0.001	0
M.48072	0.133	0.250	0.157	0.250	0.171	0.148	0.169	0.135	0.142	0.136	0.151	0.179	0.250
M.50739	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50757	0	0.071	0	0	0	0	0	0	0.095	0	0	0.042	0.067
M.50782	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50791	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50845	0	0.011	0.034	0.064	0	0	0	0	0	0	0	0.006	0
M.50842	0	0.074	0.044	0.041	0.057	0	0	0	0.065	0	0	0.029	0.033
M.50906	0	0	0.082	0.048	0	0	0	0	0	0	0	0	0.069
M.50748	0	0.010	0.040	0	0	0	0	0	0	0	0	0	0.042
M.48077	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50769	0	0.029	0	0.031	0	0	0	0	0	0	0	0	0.052

M.50814	0	0.038	0.022	0.051	0.034	0.030	0.057	0	0	0.051	0	0.045	0
M.50708	0.123	0.178	0.170	0.180	0.168	0.143	0.152	0.142	0.158	0.188	0.147	0.187	0.155
M.50865	0	0	0.070	0	0	0	0	0	0	0	0	0	0
M.50803	0	0	0	0	0	0	0	0	0.063	0	0	0	0.064
M.50820	0	0.052	0.064	0.060	0	0	0.041	0	0	0	0	0.041	0.067
M.50742	0	0	0	0	0	0	0	0	0	0	0	0	0.055
M.50870	0	0	0.039	0.048	0.064	0.037	0	0	0.070	0	0	0	0.040
M.50904	0	0	0.085	0	0.071	0	0	0	0	0	0	0	0.036
M.50825	0	0.038	0	0	0	0	0	0	0	0	0	0	0
M.50767	0	0.021	0.041	0.034	0	0	0	0	0.061	0	0	0	0
M.50844	0	0	0	0	0	0	0	0	0.060	0	0	0	0
M.50874	0	0	0	0.038	0	0	0	0	0	0	0	0	0.076
M.50819	0	0.024	0.059	0.189	0.048	0	0	0.022	0	0	0.038	0	0.045
M.50850	0	0	0.042	0	0	0	0	0	0	0	0	0	0
M.50883	0	0	0	0	0	0	0	0	0.064	0	0	0	0.046
M.50858	0	0	0	0.040	0	0	0	0	0.055	0	0	0	0
M.50884	0	0	0.111	0	0	0	0	0	0	0	0	0	0.040
M.50714	0	0.073	0	0.014	0	0	0	0	0	0	0	0	0.058
	M.50867	*M.50713.001*	*M.50749*	*M.48074*	*M.50795*	*M.50829*	*M.50839*	*M.50857*	*M.50868*	*M.50727*	*M.50751*	*M.50760*	*M.50772*
M.50867	0.500												
M.50713	0	0.500											
M.50749	0.081	0.026	0.500										
M.48074	0.085	0.061	0.045	0.500									
M.50795	0	0	0	0.057	0.500								
M.50829	0.043	0.042	0	0.023	0	0.500							
M.50839	0.082	0.058	0	0	0	0.045	0.500						
M.50857	0.031	0.037	0.057	0.061	0	0.027	0	0.500					
M.50868	0	0	0.065	0.061	0	0.038	0.047	0.007	0.500				
M.50727	0.044	0.037	0.014	0	0.013	0.057	0	0	0	0.500			
M.50751	0.062	0	0.039	0	0	0.050	0	0.051	0	0	0.500		
M.50760	0.064	0.059	0	0	0.040	0.121	0	0.019	0	0	0	0.500	
M.50772	0.046	0.064	0.060	0.047	0.048	0.007	0	0.030	0.034	0.010	0	0.030	0.500
M.50785	0.090	0.088	0	0	0.046	0.045	0	0.059	0	0	0.068	0.069	0
M.50796	0	0	0	0	0	0	0	0	0	0	0	0	0
M.48064	0.049	0.028	0.052	0	0.052	0.053	0.094	0.063	0.078	0	0	0	0
M.50840	0.072	0.043	0.033	0.043	0.031	0.031	0.059	0	0.043	0.036	0.073	0.062	0.031
M.50848	0.075	0.038	0	0	0	0	0	0	0	0	0	0	0

M.50859	0	0.069	0	0	0	0.032	0.068	0.045	0	0	0	0	0
M.50873	0.084	0	0.059	0.076	0.045	0.021	0	0.034	0	0	0	0	0
M.50752	0	0.012	0.043	0	0	0	0	0	0	0	0.062	0	0
M.50781	0.044	0.071	0	0.056	0.044	0.044	0.061	0.021	0.031	0.024	0	0	0.060
M.50786	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50798	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50841	0.045	0	0.035	0	0.047	0	0	0.033	0.041	0	0	0	0
M.50860	0.058	0.006	0	0	0	0	0	0	0	0.021	0	0	0
M.50696	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50753	0	0	0	0	0	0.035	0	0	0	0	0	0	0
M.50762	0	0	0	0	0	0	0	0	0.041	0	0	0	0
M.50821	0	0	0	0	0	0.028	0.037	0	0	0.047	0	0	0
M.50787	0.079	0	0.048	0	0.029	0.062	0	0.031	0	0.040	0	0	0.048
M.50799	0	0.065	0	0	0	0	0	0	0	0	0	0	0
M.50852	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50861	0.061	0	0	0	0	0.024	0	0.037	0	0.041	0	0	0
M.50878	0	0	0	0	0	0	0	0	0	0	0	0.132	0
M.50737	0	0.073	0.042	0.051	0.052	0.066	0	0.067	0	0	0.088	0.074	0
M.50763	0.074	0.070	0.038	0.065	0.053	0.054	0.067	0.034	0.055	0.075	0.059	0.040	0.045
M.50822	0	0	0	0.079	0.029	0.033	0	0	0	0	0	0	0.019
M.50800	0.077	0	0.044	0	0	0.038	0.065	0	0	0	0	0	0.045
M.48073	0.082	0	0	0	0	0.052	0	0.021	0.032	0	0.078	0	0
M.50853	0.057	0	0	0	0	0.038	0	0	0	0	0	0	0
M.50738	0	0	0	0	0	0	0	0	0	0.031	0	0	0
M.50755	0.071	0	0	0	0	0.033	0	0	0	0.041	0.040	0.010	0
M.50765	0.036	0.016	0	0.036	0.082	0	0	0	0	0.012	0	0	0.161
M.50823	0.079	0.065	0.066	0	0	0	0	0.055	0	0	0	0	0
M.50790	0.086	0	0.066	0.066	0.072	0.050	0.082	0.060	0.059	0.120	0.078	0.029	0.032
M.48072	0.192	0.187	0.250	0.164	0.165	0.250	0.181	0.250	0.179	0.148	0.250	0.168	0.180
M.50739	0	0	0	0	0	0	0	0	0	0	0	0	0.098
M.50757	0	0	0	0	0.028	0.055	0.092	0	0	0	0	0	0
M.50782	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50791	0	0	0	0	0	0.068	0	0	0	0	0	0	0
M.50845	0.070	0	0.056	0	0	0.035	0.037	0	0	0.003	0	0.018	0
M.50842	0.063	0.038	0	0.060	0.044	0.061	0	0.038	0	0.043	0.037	0.026	0.042
M.50906	0	0.040	0	0	0	0	0.039	0	0	0.006	0	0	0
M.50748	0.027	0	0.058	0.013	0.034	0.051	0	0.003	0	0	0.060	0.027	0

M.48077	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50769	0.036	0.055	0.013	0.044	0.038	0.046	0.068	0	0	0.047	0	0	0
M.50814	0.072	0.056	0.036	0.024	0.054	0.050	0	0.096	0.077	0.040	0.057	0.072	0
M.50708	0.161	0.149	0.157	0.166	0.187	0.190	0.172	0.250	0.185	0.250	0.168	0.172	0.168
M.50865	0.052	0	0	0	0.028	0.040	0	0	0	0	0	0	0
M.50803	0.038	0.057	0.031	0	0	0.064	0	0	0.018	0.043	0	0	0.074
M.50820	0.059	0.054	0.044	0	0.061	0.066	0	0	0	0.029	0.094	0.061	0
M.50742	0	0	0.025	0	0	0	0	0	0	0	0	0	0
M.50870	0.064	0	0.043	0	0.043	0	0.060	0.049	0.021	0.051	0	0	0.009
M.50904	0	0	0	0.081	0	0	0.067	0	0	0.042	0	0	0
M.50825	0	0	0	0	0	0	0	0	0.012	0	0	0.022	0.044
M.50767	0.090	0.050	0.046	0.056	0.031	0.075	0.089	0	0	0.042	0.095	0	0
M.50844	0.074	0.031	0	0.033	0.026	0	0	0	0.033	0.048	0.055	0	0.020
M.50874	0	0.056	0	0	0	0	0	0	0	0	0	0	0
M.50819	0.082	0.119	0.043	0	0.061	0.054	0.066	0.028	0.023	0.057	0.060	0.023	0.042
M.50850	0	0	0	0.063	0	0.036	0	0	0	0	0	0	0
M.50883	0	0	0	0	0	0.017	0	0.038	0	0.054	0	0	0
M.50858	0	0	0.035	0.033	0	0.012	0	0	0	0	0	0	0
M.50884	0	0.015	0	0	0	0	0	0.049	0	0	0	0	0
M.50714	0.066	0.025	0.029	0	0	0	0	0.037	0	0	0	0	0

	M.50785	*M.50796*	*M.48064*	*M.50840*	*M.50848*	*M.50859*	*M.50873*	*M.50752*	*M.50781*	*M.50786*	*M.50798*	*M.50841*	*M.50860*
M.50785	0.500												
M.50796	0	0.500											
M.48064	0.042	0.034	0.500										
M.50840	0.066	0	0.079	0.500									
M.50848	0	0	0	0	0.500								
M.50859	0	0	0.047	0	0	0.500							
M.50873	0.068	0.040	0.054	0.055	0.048	0	0.500						
M.50752	0.031	0	0	0.035	0	0	0	0.500					
M.50781	0.034	0.040	0.024	0.089	0.042	0.063	0.064	0.023	0.500				
M.50786	0	0	0	0	0	0	0	0	0	0.500			
M.50798	0	0	0	0	0	0	0	0	0	0	0.500		
M.50841	0.040	0	0.261	0.043	0	0	0.042	0	0	0	0	0.500	
M.50860	0.022	0.016	0	0.022	0	0	0	0.042	0	0	0	0	0.500
M.50696	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50753	0.019	0.022	0.022	0.027	0	0	0	0	0.018	0	0	0	0
M.50762	0	0	0	0.061	0	0	0	0	0	0	0	0	0

M.50821	0	0	0	0.022	0	0	0	0.042	0	0	0	0	0
M.50787	0.087	0	0.050	0.055	0	0.074	0.076	0	0.055	0	0	0	0.066
M.50799	0	0	0	0	0	0	0	0	0.066	0	0	0	0
M.50852	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50861	0.021	0	0.042	0.022	0	0	0.040	0	0.074	0	0	0	0
M.50878	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50737	0	0	0.030	0.065	0	0	0.065	0.058	0.046	0	0	0	0.025
M.50763	0.074	0	0.069	0.059	0.065	0.045	0.075	0.053	0.058	0	0	0.036	0.007
M.50822	0.052	0	0.041	0.044	0	0	0	0.029	0	0	0	0.039	0
M.50800	0.083	0	0	0.042	0	0	0	0	0.054	0	0	0	0
M.48073	0.039	0.034	0	0.034	0.052	0	0.063	0	0.071	0	0	0	0.056
M.50853	0	0	0.036	0	0	0	0	0	0.046	0	0	0	0
M.50738	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50755	0.034	0	0.036	0	0	0.028	0.038	0.031	0.028	0	0	0.036	0.022
M.50765	0.034	0	0	0.040	0	0	0.023	0	0.044	0	0	0	0
M.50823	0.090	0.065	0.039	0.080	0	0.096	0.071	0	0.072	0	0	0	0
M.50790	0.086	0.042	0.088	0.139	0	0.050	0.092	0.036	0.099	0	0	0.081	0.028
M.48072	0.250	0.250	0.192	0.250	0.122	0.183	0.250	0.161	0.176	0.118	0.134	0.179	0.172
M.50739	0	0	0	0	0	0	0	0	0.051	0	0	0	0
M.50757	0	0.052	0.066	0	0	0	0.097	0	0	0	0	0	0
M.50782	0	0	0	0	0	0	0	0	0.028	0	0	0	0
M.50791	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50845	0.052	0.017	0.044	0.054	0	0	0	0	0.036	0	0	0	0
M.50842	0.067	0	0.040	0.078	0	0.048	0.077	0.040	0.068	0	0	0	0
M.50906	0	0	0	0.053	0	0	0.033	0	0.044	0	0	0	0.064
M.50748	0	0	0	0.047	0	0	0.038	0	0.024	0	0	0.039	0.027
M.48077	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50769	0.046	0	0.030	0	0.043	0.049	0.070	0	0	0	0	0	0.050
M.50814	0.057	0	0.068	0.065	0	0.052	0.061	0.054	0.025	0	0	0.043	0.019
M.50708	0.170	0.133	0.189	0.178	0.156	0.181	0.187	0.184	0.166	0.087	0.106	0.178	0.173
M.50865	0.056	0	0	0	0	0	0.054	0	0	0	0	0	0
M.50803	0.086	0	0.073	0.048	0	0	0.066	0.058	0.045	0	0	0.028	0.029
M.50820	0.064	0.030	0.050	0.037	0	0.048	0.062	0.067	0.078	0	0	0.071	0.043
M.50742	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50870	0.074	0.025	0.057	0.061	0	0.050	0.055	0	0.034	0	0	0.020	0
M.50904	0.047	0	0.065	0.083	0	0	0	0	0.020	0	0	0	0
M.50825	0.022	0	0	0.023	0	0	0	0	0.068	0	0	0	0

M.50767	0.086	0	0.061	0	0	0	0.088	0.027	0.028	0	0	0.061	0.039
M.50844	0.082	0	0.020	0.064	0	0	0.092	0.045	0	0	0	0	0
M.50874	0	0	0	0	0	0	0.044	0	0	0	0	0	0
M.50819	0.086	0	0.049	0.053	0.021	0.094	0.063	0.022	0.097	0	0	0.047	0.036
M.50850	0	0	0.024	0.053	0	0	0	0	0	0	0	0	0
M.50883	0.058	0	0	0.062	0	0	0.051	0	0	0	0	0	0
M.50858	0.056	0.026	0.052	0.069	0	0	0.073	0	0.018	0	0	0.042	0
M.50884	0.054	0	0	0	0	0	0.070	0	0.076	0	0	0.066	0
M.50714	0.064	0	0	0.048	0	0	0	0	0.057	0	0	0	0.213

	M.50696	*M.50753*	*M.50762*	*M.50821*	*M.50787*	*M.50799*	*M.50852*	*M.50861*	*M.50878*	*M.50737*	*M.50763*	*M.50822*	*M.50800*
M.50696	0.500												
M.50753	0	0.500											
M.50762	0	0	0.500										
M.50821	0	0	0	0.500									
M.50787	0	0	0	0	0.500								
M.50799	0	0	0	0	0	0.500							
M.50852	0	0	0	0	0	0	0.500						
M.50861	0	0	0	0	0	0	0	0.500					
M.50878	0	0	0	0	0	0	0	0	0.500				
M.50737	0	0	0	0.022	0	0	0	0	0	0.500			
M.50763	0	0	0.053	0	0.060	0	0	0.076	0.034	0.045	0.500		
M.50822	0	0	0	0	0	0	0	0	0	0	0	0.500	
M.50800	0	0	0	0	0	0	0	0	0	0.064	0.055	0	0.500
M.48073	0	0	0	0	0.064	0	0	0	0	0.074	0.040	0	0
M.50853	0	0	0	0	0	0	0	0	0	0	0	0	0.083
M.50738	0	0	0	0	0	0	0	0	0	0	0.060	0	0
M.50755	0	0	0	0	0.022	0.040	0	0.014	0	0.059	0.074	0.039	0.023
M.50765	0	0	0	0	0	0.033	0	0	0	0	0.047	0	0
M.50823	0	0.066	0	0	0	0	0	0	0	0	0.074	0	0.129
M.50790	0	0.037	0.052	0.025	0.103	0.025	0	0.060	0	0.036	0.087	0	0.089
M.48072	0.157	0.159	0.164	0.162	0.250	0.157	0.091	0.186	0.147	0.250	0.250	0.183	0.250
M.50739	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50757	0	0	0	0	0	0.098	0	0	0	0	0.070	0	0
M.50782	0	0	0	0	0	0	0	0	0	0	0.030	0	0
M.50791	0	0	0	0	0	0	0	0	0	0	0	0	0
M.50845	0.013	0	0	0	0	0	0	0.038	0	0.051	0	0	0

M.50842	0	0	0.022	0	0.023	0.070	0	0.063	0.057	0.028	0.107	0.074	0.057
M.50906	0	0	0	0	0.076	0	0	0	0	0	0.050	0	0.053
M.50748	0	0	0	0	0.040	0	0	0	0	0.035	0.046	0	0.054
M.48077	0	0	0	0	0	0	0	0	0	0	0.040	0	0
M.50769	0	0	0	0	0.034	0	0	0	0	0	0.054	0	0.065
M.50814	0	0	0.019	0	0	0.045	0	0.041	0.053	0.046	0.060	0.013	0.053
M.50708	0.148	0.150	0.132	0.151	0.165	0.160	0.049	0.171	0.171	0.196	0.170	0.171	0.182
M.50865	0	0	0	0	0	0	0	0	0	0.071	0.045	0	0
M.50803	0	0	0	0	0.049	0	0	0.059	0	0	0.074	0	0.083
M.50820	0	0.032	0	0	0.055	0.076	0	0	0.049	0.045	0	0.053	0.049
M.50742	0	0	0	0	0	0	0	0.026	0	0	0	0	0
M.50870	0	0	0	0.079	0	0	0	0.090	0	0	0.066	0.049	0.050
M.50904	0	0	0	0.039	0.079	0	0	0	0	0.075	0.063	0.062	0
M.50825	0	0.020	0	0	0	0	0	0	0	0	0.022	0	0
M.50767	0	0	0.057	0.037	0.072	0	0	0	0	0	0.049	0	0.047
M.50844	0	0	0.023	0	0	0	0	0.039	0	0.047	0.047	0	0.027
M.50874	0	0	0	0	0.048	0	0	0	0	0	0	0	0
M.50819	0	0	0.031	0.038	0.079	0	0	0.041	0.032	0.042	0.110	0	0.066
M.50850	0	0	0	0	0	0	0	0	0	0	0.020	0	0
M.50883	0	0	0	0.023	0	0	0	0.075	0	0	0.054	0	0
M.50858	0	0	0	0	0.037	0	0	0	0	0	0.029	0	0
M.50884	0	0	0	0.036	0	0	0	0	0	0	0.042	0	0.036
M.50714	0	0.019	0	0	0.057	0	0	0	0	0.047	0.034	0	0

	M.48073	*M.50853*	*M.50738*	*M.50755*	*M.50765*	*M.50823*	*M.50790*	*M.48072*	*M.50739*	*M.50757*	*M.50782*	*M.50791*	*M.50845*
M.48073	0.500												
M.50853	0	0.500											
M.50738	0	0	0.500										
M.50755	0.063	0	0	0.500									
M.50765	0	0	0	0	0.500								
M.50823	0.077	0	0	0.072	0	0.500							
M.50790	0.045	0.044	0	0.062	0.050	0.055	0.500						
M.48072	0.170	0.250	0.156	0.250	0.155	0.146	0.250	0.500					
M.50739	0	0	0	0	0.212	0	0	0.174	0.500				
M.50757	0	0	0.092	0	0	0	0.067	0.139	0	0.500			
M.50782	0	0	0.129	0	0	0	0	0.147	0	0	0.500		
M.50791	0	0	0	0	0	0	0	0.154	0	0	0	0.500	
M.50845	0.025	0	0	0	0	0.057	0.041	0.250	0	0	0.050	0	0.500

M.50842	0.028	0	0.086	0.033	0.048	0	0.080	0.187	0	0.099	0	0	0.053
M.50906	0.082	0	0	0	0	0	0.068	0.148	0	0	0	0	0
M.50748	0.045	0.052	0.030	0.058	0.009	0	0.064	0.250	0	0.066	0	0	0.043
M.48077	0	0	0	0	0	0	0	0.149	0	0	0	0	0
M.50769	0.013	0	0	0	0.039	0.051	0.040	0.250	0	0	0	0	0.043
M.50814	0.020	0.030	0	0	0.012	0.052	0.090	0.179	0	0.053	0	0	0.049
M.50708	0.191	0.163	0.095	0.161	0.250	0.151	0.177	0.250	0.143	0.155	0.140	0.147	0.181
M.50865	0	0	0	0	0	0	0	0.130	0	0	0	0	0
M.50803	0	0	0	0.022	0.033	0	0.062	0.250	0	0.066	0	0	0.055
M.50820	0.045	0	0	0.069	0.018	0.052	0.054	0.250	0	0.062	0	0	0.093
M.50742	0	0	0	0	0	0	0.012	0.144	0	0	0	0	0
M.50870	0.051	0	0	0.024	0	0.064	0.072	0.250	0	0.039	0	0	0.040
M.50904	0	0.030	0	0.059	0	0	0.055	0.136	0	0	0	0	0
M.50825	0	0	0.030	0	0.065	0.038	0	0.174	0	0	0	0	0.056
M.50767	0.046	0	0	0	0.022	0	0.077	0.171	0	0.095	0	0	0.068
M.50844	0.043	0	0	0.021	0	0	0.045	0.172	0	0.074	0	0	0.060
M.50874	0	0	0	0	0	0	0.068	0.151	0	0	0	0	0
M.50819	0.030	0.048	0.042	0.020	0.034	0.063	0.063	0.250	0	0.059	0.028	0	0.083
M.50850	0	0	0	0	0	0	0.063	0.156	0	0	0	0	0
M.50883	0	0	0	0	0	0	0.064	0.170	0	0	0	0	0
M.50858	0	0	0	0.026	0	0	0.048	0.186	0	0	0	0	0
M.50884	0.043	0	0	0	0.028	0.135	0.054	0.250	0	0	0.009	0	0.018
M.50714	0.076	0	0	0	0	0.079	0.063	0.185	0	0	0	0	0.048

	M.50842	*M.50906*	*M.50748*	*M.48077*	*M.50769*	*M.50814*	*M.50708*	*M.50865*	*M.50803*	*M.50820*	*M.50742*	*M.50870*	*M.50904*
M.50842	0.500												
M.50906	0	0.500											
M.50748	0.058	0.036	0.500										
M.48077	0	0	0	0.500									
M.50769	0.053	0.020	0.057	0	0.500								
M.50814	0.048	0	0.042	0	0.040	0.500							
M.50708	0.162	0.149	0.185	0.119	0.190	0.352	0.500						
M.50865	0	0.066	0	0	0	0	0.194	0.500					
M.50803	0.077	0.044	0	0	0.057	0.041	0.176	0	0.500				
M.50820	0.064	0.064	0.062	0	0.050	0.064	0.151	0	0	0.500			
M.50742	0	0	0.021	0	0	0.023	0.142	0	0.017	0	0.500		
M.50870	0.049	0.063	0.064	0	0.035	0.060	0.201	0	0.049	0.043	0	0.500	
M.50904	0.046	0	0	0	0.046	0.045	0.175	0	0	0	0	0.061	0.500

M.50825	0.059	0	0.054	0	0.048	0	0.154	0	0	0.053	0	0	0
M.50767	0.069	0.071	0.045	0	0	0.061	0.174	0	0.067	0.087	0.043	0.018	0
M.50844	0.077	0	0.039	0	0	0.075	0.250	0	0.045	0	0.019	0.038	0
M.50874	0.060	0	0	0	0	0	0.128	0	0	0	0	0	0.061
M.50819	0.059	0	0	0	0.009	0.070	0.195	0	0	0.102	0	0.048	0.042
M.50850	0	0.051	0	0	0	0.022	0.156	0	0	0	0	0	0
M.50883	0.041	0	0.038	0	0.008	0.019	0.175	0	0	0	0	0.272	0.064
M.50858	0	0	0	0	0	0	0.167	0	0.043	0.028	0	0	0.085
M.50884	0.080	0.059	0.016	0	0	0	0.190	0	0.027	0	0	0.041	0.053
M.50714	0.038	0	0.024	0	0	0	0.149	0	0	0.052	0	0.037	0

	M.50825	*M.50767*	*M.50844*	*M.50874*	*M.50819*	*M.50850*	*M.50883*	*M.50858*	*M.50884*	*M.50714*
M.50825	0.500									
M.50767	0.066	0.500								
M.50844	0	0.084	0.500							
M.50874	0	0	0	0.500						
M.50819	0	0.099	0.060	0.035	0.500					
M.50850	0	0	0	0	0	0.500				
M.50883	0	0	0.061	0	0.062	0	0.500			
M.50858	0	0	0	0	0.017	0	0	0.500		
M.50884	0	0	0	0	0.058	0	0.011	0	0.500	
M.50714	0.044	0.042	0.010	0	0.058	0	0	0	0	0.500

Table A13 List of SNPs (*n=167*) with minor allele frequency >0.4, used for probability of identity calculations in Chapter 4.

SNP ID	*SNP*	*Call rate*
19347805|F|0—34	C/A	0.975694
19347921|F|0—26	G/T	0.96875
19347930|F|0—15	C/T	0.979167
19347967|F|0—64	C/G	0.982639
19348321|F|0—28	C/T	0.954861
19348355|F|0—16	G/A	0.972222
19348574|F|0—44	G/C	0.954861
19348645|F|0—20	A/G	0.961806
19348841|F|0—8	A/G	0.954861
19349020|F|0—6	G/C	0.965278
19349262|F|0—28	A/G	0.965278
19349539|F|0—58	C/T	0.958333
19349584|F|0—32	T/G	0.961806
19349846|F|0—29	T/C	0.965278
19349852|F|0—10	A/G	0.975694
19350004|F|0—14	T/G	0.986111
19350177|F|0—35	C/A	0.972222
19350236|F|0—6	T/C	0.979167
19350569|F|0—17	G/A	0.975694
19350705|F|0—45	C/T	0.979167
19350822|F|0—64	G/A	0.965278
19350876|F|0—26	C/T	0.972222
19351036|F|0—44	C/T	0.982639
19351115|F|0—50	G/A	0.975694
19351171|F|0—15	G/A	0.972222
19354043|F|0—16	C/T	0.96875
19354337|F|0—28	A/T	0.965278
19354510|F|0—49	A/G	0.986111
19354567|F|0—12	T/C	0.958333

SNP ID	***SNP***	***Call rate***
19354622|F|0—36	G/A	0.965278
19354671|F|0—12	G/A	0.972222
19354799|F|0—28	A/G	0.961806
19355108|F|0—19	C/A	0.975694
19356223|F|0—6	T/C	0.975694
19356227|F|0—19	G/A	0.965278
19356259|F|0—43	T/C	0.972222
19356306|F|0—55	A/G	0.958333
19356364|F|0—43	C/A	0.975694
19356504|F|0—36	G/T	0.982639
19356761|F|0—54	T/C	0.961806
19357050|F|0—60	G/A	0.96875
19357300|F|0—42	T/C	0.961806
19357426|F|0—7	G/A	0.958333
19357465|F|0—20	A/T	0.979167
19357514|F|0—27	T/G	0.958333
19357579|F|0—60	A/G	0.979167
19357649|F|0—17	T/C	0.96875
19357688|F|0—42	A/G	0.982639
19357690|F|0—58	C/G	0.961806
19357750|F|0—12	A/G	0.972222
19357830|F|0—35	C/T	0.972222
19357845|F|0—67	C/G	0.958333
19357931|F|0—13	C/A	0.982639
19357935|F|0—23	G/A	0.975694
19357936|F|0—30	A/G	0.96875
19358018|F|0—51	A/G	0.965278
19358038|F|0—58	A/G	0.961806
19358432|F|0—23	C/T	0.961806
19358435|F|0—11	A/T	0.975694
19358557|F|0—5	G/C	0.979167
19358624|F|0—54	A/G	0.961806

SNP ID	*SNP*	*Call rate*
19358781|F|0—46	G/A	0.979167
19358979|F|0—34	T/C	0.972222
19359177|F|0—20	T/C	0.954861
19359495|F|0—22	A/G	0.972222
19359606|F|0—38	C/T	0.958333
19359616|F|0—8	G/T	0.958333
19359750|F|0—59	A/G	0.975694
19360918|F|0—30	C/T	0.972222
19361320|F|0—26	A/G	0.979167
19361432|F|0—26	C/A	0.979167
19361532|F|0—16	T/C	0.979167
19361550|F|0—21	C/G	0.975694
19361573|F|0—5	C/G	0.972222
19361601|F|0—9	T/A	0.96875
19361735|F|0—31	G/A	0.96875
19361762|F|0—37	G/A	0.965278
19361818|F|0—27	G/A	0.975694
19361828|F|0—6	C/G	0.986111
19362584|F|0—47	C/T	0.972222
19363019|F|0—12	G/T	0.982639
19363089|F|0—20	A/C	0.958333
19363212|F|0—14	T/C	0.979167
19363704|F|0—38	G/A	0.972222
19363779|F|0—30	G/A	0.986111
19364135|F|0—16	T/C	0.965278
19364217|F|0—23	C/G	0.979167
19364347|F|0—55	G/A	0.982639
19364357|F|0—27	G/A	0.96875
19364534|F|0—25	G/A	0.979167
19364555|F|0—31	A/T	0.979167
19364659|F|0—15	A/G	0.986111
19364751|F|0—19	C/T	0.972222

SNP ID	*SNP*	*Call rate*
19364776|F|0—22	G/A	0.965278
19364802|F|0—25	G/A	0.951389
19365845|F|0—36	C/T	0.975694
19366050|F|0—14	G/T	0.965278
19366089|F|0—22	G/A	0.975694
19366098|F|0—20	C/T	0.958333
19366161|F|0—21	A/G	0.965278
19366177|F|0—36	A/G	0.961806
19366282|F|0—19	G/A	0.96875
19366506|F|0—25	C/G	0.965278
19366555|F|0—14	T/C	0.954861
19366567|F|0—7	C/A	0.982639
19366581|F|0—31	G/C	0.979167
19367140|F|0—59	A/T	0.965278
19367157|F|0—11	A/T	0.972222
19367343|F|0—14	G/A	0.979167
19367381|F|0—61	A/T	0.979167
19367573|F|0—37	A/G	0.986111
19367914|F|0—11	C/T	0.986111
19367992|F|0—20	T/C	0.972222
19368177|F|0—61	C/G	0.96875
19369292|F|0—44	A/G	0.982639
19369309|F|0—47	C/T	0.972222
19369314|F|0—14	C/G	0.979167
19369491|F|0—6	G/A	0.96875
19369520|F|0—20	G/A	0.96875
19369525|F|0—15	A/G	0.972222
19369638|F|0—32	C/T	0.96875
19369667|F|0—19	C/G	0.954861
19369688|F|0—19	G/T	0.979167
19369791|F|0--6	G/A	0.975694
19370001|F|0—27	C/A	0.958333

SNP ID	***SNP***	***Call rate***
19370527|F|0—24	A/T	0.979167
19370748|F|0—9	T/G	0.982639
19371196|F|0—65	T/C	0.982639
19371275|F|0—67	C/T	0.979167
19371507|F|0—9	T/C	0.958333
19371516|F|0—13	G/A	0.986111
19371595|F|0—14	G/C	0.982639
19371598|F|0—26	A/G	0.972222
19371604|F|0—8	T/C	0.965278
19371666|F|0—31	C/T	0.982639
19371670|F|0—7	T/C	0.986111
19371748|F|0—26	T/A	0.982639
19371770|F|0—14	C/T	0.972222
19372131|F|0—58	T/G	0.972222
19372393|F|0—53	C/T	0.979167
19372695|F|0—22	A/G	0.965278
19372744|F|0—9	A/G	0.961806
19372751|F|0—8	A/G	0.979167
19372792|F|0—27	C/G	0.972222
19372861|F|0—9	C/A	0.954861
19372881|F|0—18	T/A	0.982639
19373149|F|0—31	A/C	0.982639
19373191|F|0—14	A/G	0.975694
19373445|F|0—28	G/A	0.975694
19373482|F|0—35	G/A	0.986111
19373990|F|0—39	A/G	0.979167
19374259|F|0—20	T/C	0.989583
19374486|F|0—16	A/G	0.979167
19374677|F|0—6	G/A	0.986111
19374683|F|0—6	G/A	0.989583
19374999|F|0—8	A/G	0.979167
19375066|F|0—12	G/C	0.989583

SNP ID	***SNP***	***Call rate***
19375458|F|0—43	A/C	0.982639
19376353|F|0—10	G/A	0.96875
19376403|F|0—11	G/A	0.96875
23890509|F|0—21	C/A	0.961806
24481069|F|0—30	G/T	0.986111

Table A14 List of short beaked echidna (*Tachyglossus aculeatus*) samples used for analyses in Chapter 5.

AM = Australian Museum, ANWC = Australian National Wildlife Collection, CWS = Currumbin Wildlife Sanctuary, MV = Museum Victoria, UTAS = University of Tasmania

Registration Number	***Donor***	***Tissue Type***	***Sex***
M.48040.002	Australia Zoo	Blood	Male
M.48041.002	Australia Zoo	Blood	Male
M.48042.002	Australia Zoo	Blood	Male
M.48045.002	Australia Zoo	Blood	Female
M.48046.001	Australia Zoo	Blood	Female
M.30518.001	AM	Tissue	Male
M.32567.001	AM	Tissue	Female
M.43684.001	AM	Tissue	Female
M.45790.001	AM	Tissue	Male
M.46626.001	AM	Tissue	Female
M29902	ANWC	Tissue	Female
A20310	CWS	DNA	Male
A50087	CWS	DNA	Female
A70094	CWS	DNA	Male
A70194	CWS	DNA	Female
B20122	CWS	DNA	Male
B30193	CWS	DNA	Female
21603	MV	Tissue	Male
M.48048.001	Perth Zoo	Blood	Female
M.48050.001	Perth Zoo	Blood	Male
M.48051.001	Perth Zoo	Blood	Male
M.48057.001	Perth Zoo	Blood	Male
A20450	Taronga Zoo	Blood	Male
A30404	Taronga Zoo	Blood	Female
A50345	Taronga Zoo	Blood	Male
M.48068.001	UTAS	Tissue	Female
M.48075.001	UTAS	Tissue	Male
M.48077.001	UTAS	Tissue	Male

Table A15 List of species used in *specificity* validation testing in Chapter 5. All samples donated by the Australian Museum.

Registration	***Species***	***Sample type***
M.47975.001	Western long beaked echidna (*Zaglossus bruijnii*) (Female)	Tissue
M.35614.001	Platypus (*Ornithorhynchus anatinus*)	Tissue
WGM118-186	Bilby (*Macrotis lagotis*)	Tissue
R.150174.001	Eastern Crevice Skink (*Egernia mcpheei*)	Tissue
I.39681.003	Queensland Grouper (*Epinephelus lanceolatus*)	Tissue

Table A16 Melting temperature (T_m°C) and fluorescence for short beaked echidna (*Tachyglossus aculeatus*) samples used in *accuracy* validation study in Chapter 5.

Registration Number	*Sex*	*AMH T_m (°C)*	*AMH fluorescence*	*CRSPY T_m (°C)*	*CRSPY fluorescence*
21603	M	82.489	237,186.938	79.434	28,459.574
A20310	M	81.979	46,589.168	79.987	38,581.867
A20450	M	82.987	184,212.578	79.857	12,732.962
A30404	F	81.571	28,785.570	50.714	3,318.646
A50087	F	75.862 85.169 80.490	51,992.570 36,269.336 59,603.320	68.870	96,451.797
A50345	M	82.132	71,670.313	79.535	31,570.178
A70094	M	81.724	53,170.855	78.588 68.922	88,187.734 65,259.164
A70194	F	56.213	2,941.792	50.714	3,691.389
B20122	M	82.336	411,081.844	79.586	11,264.087
B30193	F	82.385	55,071.211	51.070	3,640.628
M.30518.001	M	81.928	58,223.172	79.280	62,857.027
M.32567.001	F	81.612	203,079.641	69.193	89,122.000
M.43684.001	F	81.560	44,000.008	69.039	10,822.309
M.46626.001	F	81.724 76.120 85.323	45,722.930 44,300.016 10,290.868	69.076	49,399.492
M.48040.002	M	82.183	110,154.008	79.382	28,527.412
M.48041.002	M	81.569	206,578.016	69.075 78.330	61,366.543 13,672.291
M.48042.002	M	82.285	118,692.555	78.485	57,682.398
M.48045.002	F	85.427	69,392.094	68.870	80,880.539
M.48046.001	F	50.153	4,170.838	50.001	4,527.035
M.48048.001	F	51.630	3,725.594	50.001	3,492.234
M.48050.001	M	81.673	236,929.188	78.536 68.870	93,574.414 58,882.961
M.48051.001	M	82.54	152,445.875	79.539	44,858.086
M.48057.001	M	82.438	114,108.930	79.433	58,075.754
M.48068.001	F	81.261	209,808.047	68.715	65,259.164
M.48075.001	M	82.439	121,068.852	78.829	21,976.695
M.48077.001	M	82.030	114,024.109	79.280	51,263.488
M.45790.001	M	82.147	123,968.227	79.869	22,614.330
M29902	F	80.988	17,488.627	68.808	65,288.992

Table A17 Melting temperature (T_m°C), Average T_m, SD and standard error for all short beaked echidna samples (*Tachyglossus aculeatus*) used in *repeatability* study in Chapter 5.

Plate 1

		AMH T_m(°C)					
		1.1	*1.2*	*1.3*	*Average*	*±SD*	*Std Error*
	A50345	82.132	82.336	82.336	82.268	0.118	0.068
M	*M.48051*	82.488	82.438	82.438	82.454	0.029	0.017
	M.48073	82.132	82.081	82.030	82.081	0.051	0.029
	M.32567	81.958	81.758	81.958	81.892	0.115	0.067
F	*M.46626*	81.058	81.239	81.932	81.410	0.461	0.266
	A70194	50.153	51.426	50.968	50.849	0.645	0.372

		CRSPY T_m(°C)					
		1.1	1.2	1.3	*Average*	*±SD*	*Std Error*
	A50345	79.535	79.586	79.229	79.450	0.193	0.111
M	*M.48051*	79.484	79.586	79.433	79.501	0.078	0.045
	M.48073	79.494	79.593	79.459	79.515	0.069	0.040
	M.32567	68.892	69.402	69.402	69.232	0.294	0.170
F	*M.46626*	69.127	68.808	68.654	68.863	0.241	0.139
	A70194	50.001	50.001	50.204	50.069	0.118	0.068

Plate 2

		AMH T_m(°C)					
		2.1	2.2	2.3	*Average*	*±SD*	*Std Error*
M	*A50345*	80.952	81.159	81.005	81.039	0.107	0.062
	M.48051	81.673	81.724	81.569	81.656	0.079	0.046
	M.48073	81.210	80.988	81.245	81.147	0.139	0.080
F	*M.32567*	81.758	81.047	81.724	81.510	0.401	0.231
	M.46626	81.724	81.929	81.827	81.827	0.103	0.059
	A70194	50.153	50.204	50.001	50.120	0.106	0.061

		CRSPY T_m(°C)					
		2.1	2.2	2.3	*Average*	*±SD*	*Std Error*
	A50345	78.789	78.851	78.789	78.810	0.036	0.021
M	*M.48051*	78.536	78.588	78.227	78.451	0.195	0.113
	M.48073	78.109	78.009	78.397	78.172	0.202	0.116
	M.32567	68.808	68.654	68.870	68.777	0.111	0.064
F	*M.46626*	69.076	69.178	69.075	69.110	0.059	0.034
	A70194	50.153	50.000	50.770	50.308	0.407	0.235

Figure A1 Amplicons from conventional PCR amplification of 28 short beaked echidna (*Tachyglossus aculeatus*) samples used in *accuracy* validation study in Chapter 5. Figures a) and b) are *AMH* amplicons, Figures c) and d) are *CRSPY* amplicons. Samples in red = known female samples; Samples in blue = known male samples; NTC = Negative template control. Wells with no label are samples not used in validation study.