A DNA toolbox for non-invasive genetic studies of sambar deer (*Rusa unicolor*).

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Abstract

Invasive sambar deer are having significant detrimental impacts on natural environments in south eastern Australia. Little, however, is known about their ecology, limiting evidence based management strategies directed at reducing deer impacts. Genetic data, generated from DNA isolated from deer scats, can be used to fill ecological knowledge gaps. This study outlines a non-invasive genetic sampling strategy by which good quality DNA from a single deer scat can be used to determine 1) species of origin, 2) sex and 3) a unique DNA profile. DNA from deer tissue and sambar deer scat samples were used to develop and optimise molecular methods to collect reliable genetic information. A DNA toolbox is presented which describes how to find, collect and store scat samples, isolate DNA and use molecular markers to generate informative genetic data. Generating genetic data using this approach will support studies aimed at acquiring ecological knowledge about sambar deer. Such knowledge will be critical for developing evidence-based recommendations to improve on-ground management decisions for sambar deer.

Introduction

Invasive species are a significant threat to biodiversity and a deep understanding of their ecology is required to develop effective management strategies and to reduce associated impacts. The detrimental impact of invasive species has been particularly evident on the Australian continent where they have been implicated in the majority of recent extinctions (Wayne *et al.* 2017). While invasive mammalian predators such as the red fox (*Vulpes vulpes*) and feral cat (*Felis catus*) have played an important and direct role in these extinctions (Doherty *et al.* 2017), habitat degradation caused by invasive herbivores has also resulted in significant biodiversity loss (Legge *et al.* 2011). Invasive herbivores can outcompete native animals for food and water (Davis *et al.* 2008), degrade vegetation (Edwards *et al.* 2010) and cause erosion (Bayne *et al.* 2004). Having established themselves throughout large areas of Australia, invasive deer species are now emerging as a significant threat to native ecosystems (Davis *et al.* 2016).

Deer were first introduced to Australia during the 19th and 20th centuries to provide game for hunting (Bentley 1957; Moriarty 2004). Of the 18 species of deer introduced to Australia, six have successfully established wild populations, including sambar (*Rusa unicolor,* Leslie 2011), fallow (*Dama dama*), red (*Cervus elaphus*), chital (*Axis axis*), rusa (*Cervus timorensis*) and hog deer (*Axis porcinus*) (Moriarty 2004). Wild populations of four deer species (sambar, fallow, red and hog) currently exist in the State of Victoria in south eastern Australia (Davis *et al.* 2016). Increasing numbers of applications to the State's Department of Environment, Land, Water and Planning (DELWP) to control deer on private land (Lindeman and Forsyth 2008) and rising harvest rates indicate that the distribution and abundance of these species are increasing across Victoria (GMA 2015). There is increasing concern about the potential for wild deer populations in south eastern Australia to cause considerable ecological damage and to act as reservoirs for agriculturally important diseases and parasites (Cripps *et al.* 2018).

Deer have been implicated in a broad range of detrimental impacts on ecosystems around the world (Côté *et al.* 2004). In Australia, deer damage native ecosystems through browsing, thrashing and rubbing of vegetation (Bilney 2013). Wild deer also compete with native fauna for forage (Davis *et al.* 2008), spread weeds (Davis *et al.* 2010) and alter habitats. In addition, deer carrion can provide a source of food for other invasive species, such as red foxes (Forsyth *et al.* 2014). In Victoria, reduction in the biodiversity of native vegetation by sambar deer is listed as a potentially threatening process (DSE 2010). Despite their significant and

increasing impact on the environment, resulting from their rapid population growth, sambar remain one of the least studied invasive mammal species in Australia.

There are important knowledge gaps associated with the ecology of all wild deer species in Victoria, including sambar deer (Davis *et al.* 2016). Analysis of harvest and capture rates per unit of effort have been used as indices of abundance for deer in Victoria (Forsyth *et al.* 2018), however, more accurate estimates of abundance using methods such as mark recapture have not been carried out for any of Victoria's wild deer species. Furthermore, there are very few studies describing home range, habitat use and dispersal patterns for invasive wild deer in Victoria (Davis *et al.* 2016). The effectiveness of deer control methods such as culling programs are also poorly understood (State of Victoria 2018). While addressing these knowledge gaps is a vital first step towards the effective management of deer, their cryptic nature makes this a formidable task.

Molecular ecology, where genetic data are used to address ecological questions, is an approach often used to obtain information about invasive species. For example, genetic data have been used widely in New Zealand to inform the management of invasive species including possums (Adams *et al.* 2014), stoats (Veale *et al.* 2014) and rats (Abdelkrim *et al.* 2010). In France and South Georgia, genetic data were used to determine the scale and feasibility of control operations by delineating population boundaries and assessing connectivity between populations of ship rat (*Rattus norvegicus*) (Abdelkrim *et al.* 2005; Robertson and Gemmell 2004). Genetic data can also be used to identify genetic bottlenecks after control efforts, in order to determine whether control programs have effectively reduced population size (Cowled *et al.* 2006). Furthermore, genetic studies that identify the sex of individuals can determine whether males or females display increased dispersal (Hansen *et al.* 2007), allowing control operations to target individuals of the sex with greater dispersal (Rollins *et al.* 2006).

Where invasive collection of DNA (e.g. tissue sampling) from wild animals is challenging, DNA can be sourced non-invasively through the collection of biological material discarded by an animal (e.g. scats). Scats are easily collected from the environment, which enables large areas to be searched and sampled without target animals having to be caught (Waits and Paetkau 2005). Scat collection can therefore, increase sample size, remove sampling bias and reduce sampling costs which are often associated with invasive sampling (Kohn and Wayne 1997). A number of studies have demonstrated how genetic data generated from scat DNA

can be used to improve the understanding of deer ecology (Brinkman and Hundertmark 2009; Valière *et al.* 2007).

Despite the many benefits of non-invasive sampling, the use of DNA sourced from discarded biological samples such as scats is associated with some difficulties. These are related to the rapid degradation of DNA following cell death (Alaeddini *et al.* 2010), a process which may be accelerated by environmental factors including rain, or by sample storage conditions (Agetsuma Yanagihara *et al.* 2017; Wedrowicz *et al.* 2013). Studies that utilise non-invasive genetic sampling are, therefore, often constrained by low DNA quantity (DNA amount) and quality (DNA integrity) which can cause errors in the genetic data obtained, leading to incorrect findings and conclusions (Bonin *et al.* 2004). It is therefore necessary to tailor sampling strategies, DNA isolation methods and molecular markers for the target species and the specific environment, to ensure the accuracy of data, knowledge and recommendations (Valière *et al.* 2007).

Here we present a method which can be used to source good quality DNA from a single sambar deer scat collected in a temperate environment. We describe how to find and collect scats suitable for genetic analysis, discuss methods for isolating target DNA from scats and present techniques for assessing DNA quantity and quality following DNA isolation. We evaluate a range of molecular markers for their application and performance in studies of deer, using DNA sourced non-invasively from scats. Finally, we identify a suite of molecular markers which can be used reliably with DNA isolated from scats to 1) confirm that the scat sample originated from sambar deer, 2) determine the sex of the individual and 3) provide a unique DNA profile that can identify individuals. Examples of how genetic data generated using this approach can be used to address ecological questions and inform management strategies of sambar deer in south eastern Australia are also provided.

Materials and Methods

Sample collection

Tissue samples (ear or liver) were collected from sambar (*n*=41), red (*n*=1), fallow (*n*=6) and hog (*n*=6) deer shot by park rangers and licenced recreational hunters during control operations in various parks and reserves in Victoria. These samples formed reference samples for our study and were stored in vials containing 20% dimethyl sulfoxide (DMSO) in a saturated salt solution and refrigerated at 4°C.

Deer trails in forested areas of west Gippsland were searched for sambar deer scats that appeared shiny with a visible mucus coating, indicating freshness. When scats were located, a single pellet was picked up with a single use toothpick. A sterile, rayon-tipped swab dipped in Longmire buffer was used to wipe the entire surface of the scat to sample intestinal epithelial cells. The head of the swab was cut off using scissors and stored in a 1.5 mL vial containing 0.5 mL of Longmire buffer. Following swabbing, scats were discarded in the field. All tubes containing swab heads and buffer were stored at 4°C until DNA isolation was undertaken.

DNA isolation

DNA was isolated from tissue samples using the Qiagen® DNeasy Blood and Tissue Kit according to the manufacturer's protocol. DNA was extracted from scat samples using the Qiagen® QIAamp DNA Mini kit following the manufacturer's protocol for DNA Purification from Blood or Body Fluids, with minor changes. Buffer AL (500µL) and Proteinase K (20 µL) were added directly to sample tubes (containing swab heads and Longmire buffer) and DNA was isolated from 400 µL of the resultant solution. DNA was eluted in 100 µL and stored at –20°C. DNA isolated from both tissues and scats were quantified using the Qubit® fluorometer (Invitrogen) following the manufacturer's protocol. In order to reduce the risk of contamination, all DNA extractions from scats were performed using separate equipment (pipettes and tube racks) in a dedicated facility.

Determining deer species

Scats from different deer species can be difficult to differentiate with a high degree of certainty based on appearance alone (Ramón-Laca *et al.* 2014). Therefore, an important first step when sampling scats, is to confirm the species from which the scat orginated. Here we amplified a stretch of the mitochondrial control region for 23 deer tissue samples (references for which species was know) from Victoria (sambar, n=10; fallow, n=6; red, n=1; and hog, n=6) and 16 scat samples thought to have originated from sambar deer. Polymerase Chain Reaction (PCR) assays consisted of 2 µL DNA template, 10 µL of GoTaq® Green Master Mix (Promega), 0.2 µM of primers; CervtPro and CervCRH (Balakrishnan *et al.* 2003) and 1 µg μL^{-1} of bovine serum albumin (BSA) made up to 20 µL with nuclease free water. DNA amplification was performed in a Veriti® thermal cycler with a 10 minute initial denaturation at 95°C, followed by 35 cycles of: 45 seconds at 95°C, 40 seconds at 54°C and 75 seconds at 72°C followed by a final extension of 10 minutes at 72°C. All PCR amplifications included positive and negative controls.

PCR products were visualised under ultra violet (UV) light on a 2% agarose gel stained with SYBR Safe™ DNA Stain (Invitrogen), and were subsequently purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). Sanger sequencing was performed by the Australian Genome Research Facility (AGRF), Melbourne, Australia. Sequence quality and base calls were assessed manually for all samples. DNA sequences were trimmed in Sequence Scanner 2.0 (Applied Biosystems) and aligned in MEGA7 (Kumar *et al.* 2016) using MUSCLE (Edgar 2004). Sequences derived from scat DNA were confirmed as sambar deer by comparison with deer sequences generated from reference tissue and BLAST analysis.

Determining sex

The amelogenin locus is found on both the X and Y chromosomes and has been used to genetically assign sex to ungulate DNA using a single primer pair, SE47 and SE48 (Ennis and Gallagher 1994). In North American ungulates, SE47 and SE48 produce X-linked and Y-linked amplicons of approximately 300 and 275 base pairs, respectively (Brinkman and Hundertmark 2009).

Tissue samples from two female and two male sambar deer were used to validate the ability of the primer pair SE47/SE48 to accurately infer sex. Serial dilutions (1:10) of DNA template from two validated females (16.6 ng/µL and 34.2 ng/µL) and two validated males (13.1 ng/µL and 23.0 ng/µL) were used to determine the sensitivity of the assay. The performance of the sex markers to amplify degraded DNA was finally tested by SE47/SE48 PCR amplification of scat DNA isolates (n=32) with concentrations ranging between 0.14 to 35.0 ng/µL.

PCR amplifications were performed in 10 µL volumes consisting of 5 µL GoTaq® Green Master Mix, 1 µg of BSA, 0.5 µM each of SE47 and SE48 primers and 1 µL of DNA template, adjusted to 10 µL with water. Thermal cycling began with an initial denaturation step of 15 minutes at 94°C, 35 cycles of 94°C for one minute, 60°C for 30 seconds and 72°C for one minute, followed by a final extension cycle at 72°C for 5 minutes. PCR products were separated on 2% agarose gel stained with SYBR Safe™ DNA Stain and visualised using UV light.

Identifying individual sambar deer

DNA isolated from sambar deer tissue samples were used to test the cross species amplification success of 17 microsatellite markers developed for ungulates: BL42, BM203, BM757, BM848, BMC1009, CSSM43, IDVGA55, INRA121, TGLA126, TGLA53, TGLA57, VH110 (Bonnet *et al.* 2002), Ca18, Ca43 (Gaur *et al.* 2003), CelJP38, OarFCB5 and RT7 (Pérez-Espona *et al.* 2008). All microsatellite loci had been previously used for multi-locus genotyping of Cervids including red deer (Pérez-Espona *et al.* 2008; Valière *et al.* 2007) and chital deer (Gaur *et al.* 2003). Genotyping was carried out on the Applied Biosystems 3730 DNA analyser and GENEMAPPER 3.7 software (Applied Biosystems) by AGRF, Melbourne, Australia.

Genotypic data were used to estimate the average number of alleles per locus (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), probability of identity (P_{ID}) and probability of identity among siblings (P_{SIBS}) using GENALEX version 6.503 (Peakall and Smouse 2006). GENEPOP version 4.2 (Rousset 2008) was used to test for linkage disequilibrium (LD) and departures from Hardy-Weinberg equilibrium (HWE) with Bonferroni correction.

Genotyping error rates

Amplification success is often lower and genotyping errors higher when amplifying DNA isolated from samples such as scats. Genotyping errors were estimated by genotyping DNA from a subset of scat samples (*n*=21) with eight microsatellite loci (BL42, BM757, BMC1009, IDVGA55, INRA121, TGLA126, TGLA53 and TGLA57). Error rates were calculated in GIMLET v 1.3.3 (Valière 2002b) using allele frequencies determined in GENALEX (Peakall and Smouse 2006). The threshold rule was applied when assigning consensus genotypes wherein a genotype had to appear at least twice to be accepted. Using the calculated error rates, the PCR repetition batch module of GEMINI v 1.3.0 (Valière 2002a) was used to estimate the minimum number of PCR replicates required to obtain reliable genotypes. Simulations were run (*n*=250) using hypothetical populations of 100 individuals, taking 50 samples on one sampling occasion.

Results

DNA quantification

DNA concentration was measured to quantify the amount of DNA isolated from tissue and scat samples. The mean DNA concentration for tissue isolates (*n*=54) was 24.6 ng/µL and ranged from 0.90 ng/µL to 75.6 ng/µL, whereas the mean DNA concentration for most scat

isolates (*n*=31) ranged from 0.14 ng/µL to 4.96 ng/µL with a mean of 1.68 ng/µL. One additional scat sample (collected immediately after a deer was observed defecating) had a high DNA concentration of 35.0 ng/µL.

Determining deer species

The mitochondrial control region was amplified for reference tissue samples for four species of deer which inhabit south east Australia (sambar, *n*=10; red, *n*=1; fallow, *n*=6 and hog deer, *n*=6). Agarose gel visualisation of the mtDNA control region amplicons (~560 bp) from reference samples showed a size difference between sambar deer amplicons and the amplicons of red, fallow and hog deer (Fig. 1). Sequence data confirmed an insertion of 78 bp within sambar deer DNA sequences compared to other deer species sampled for this study (Supplementary material). Aligned and trimmed sequences from reference samples were compared to those published on GenBank revealing six haplotypes for Victorian deer, one haplotype each for sambar, red and hog deer and three haplotypes for fallow deer. Haplotypes were provided with the following accession numbers on GenBank: MK473445 (sambar), MK473447 (red), MK473446 (hog), MK473448, MK473449 and MK473450 (fallow).

Comparison between sequences derived from scats (*n*=16) and deer reference sequences, confirmed that all scats originated from sambar deer. BLAST results also showed scat sequences had the highest similarity (based on maximum score and E value) with published sambar deer mitochondrial control region sequences (accession numbers: AF291884.1 and KY946815.1).

Determining deer sex

PCR products produced by the SE47/SE48 primer pair were homozygous for females and heterozygous for males. The observed size difference between the X and Y PCR products was ~50 bp, allowing separation of the two amplicons on a 2% agarose gel. Sex was assigned correctly to samples from individuals of known sex (two females and two males), where samples with a single band at ~290 bp were assigned as female (XX), whilst samples with two bands of ~290 bp and ~240 bp were assigned as male (XY) (Fig. 2A). Serial dilutions of sambar deer DNA consistently amplified to a minimum of 0.13 ng/µL (Table 1). Clear amplification and separation of X and Y PCR products was achieved for DNA isolated from scats, where 91% (29/32) of samples were successfully assigned sex (four individuals shown, Fig. 2B).

Identifying individual sambar deer

Genotyping of 17 microsatellite markers developed for ungulates demonstrated good cross species amplification success in sambar deer. All markers consistently amplified and were polymorphic except BM203 and CSSM43 (which had poor amplification success) and VH110 and BM848 (which were monomorphic) (Table 2). Significant LD was detected between one pair of loci (BMC1009 and OarFCB5).

The final suite of 11 microsatellite loci consistently amplified, showed no evidence of LD and did not deviate from HWE after Bonferroni correction. Allelic diversity among the 11 loci ranged from two to nine alleles per locus, with an average of 4.1 (Table 2). The mean observed (H_O) and expected (H_E) heterozygosity were 0.546 and 0.551, respectively (Table 2). The power of this marker suite to identify individual sambar deer was high, with a P_{ID} of 2.7×10^{-7} (~1 in 3.6 million) for unrelated individuals and 1.0×10^{-3} (~1 in 959) for full siblings, and are therefore suitable for identifying individuals and conducting population genetic studies of sambar deer in south eastern Australia.

Assessing genotyping performance for DNA isolated from scats

Genotyping error rates for DNA isolated from deer scats were low in this study (Table 3). From 1,344 amplifications the average proportion of positive PCR tests was 95% and varied between 88% and 99% among loci and 66% and 100% among samples. Allelic dropout (ADO) was estimated at 0.9% across loci and 1.6% across samples. No evidence of false alleles was detected. GEMINI simulations indicated that with two PCR replications per locus, the proportion of correct multi locus genotypes would be 93.48%, with three PCR replicates 99.98% and after four replicates 100%.

Discussion

There is an urgent need to gather information regarding populations of wild deer in south east Australia. Here, we have presented a non-invasive molecular approach that can be used to collect genetic information regarding contemporary sambar deer populations. The 'DNA toolbox', outlined in Fig. 3, describes how DNA isolated from a single scat can be reliably used for population genetic studies of sambar deer. Data generated using this approach will fill knowledge gaps regarding sambar deer ecology and provide critical information for the management of this invasive species.

Deer continuously deposit scats in the environment (Košnář and Rajnyšová 2012), therefore, if deer are present in an area their scats should be readily detected. Using a non-invasive sampling approach, genetic material from scats may be collected from protected areas such as national parks where hunting is prohibited. Scats can also be collected by citizen scientists, natural resource managers and other stakeholders, with fewer difficulties (compared with invasive sampling such as shooting or live capture) relating to requirements for the ethical destruction and handling of animals. However, sourcing DNA using a non-invasive approach is not a panacea for genetic studies and a number of significant issues associated with non-invasive sampling must be considered (Taberlet *et al.* 1999). DNA rapidly degrades after cell death, hence the quality and quantity of DNA recovered from a scat is typically lower than that obtained from tissue and blood samples (Bonin *et al.* 2004; Taberlet *et al.* 1999). Decreased DNA quantity and DNA degradation is also exacerbated by weathering, especially rainfall (Agetsuma Yanagihara *et al.* 2017). Consequently, the collection of scats for genetic studies should be conducted in dry conditions and during fine weather. Where possible, scats which appear fresh and undamaged should be sampled over those which appear dry and weathered.

Steps 1 through 3 of the DNA toolbox (Fig. 3) provide guidance relating to the collection of scats in order to improve the probability of obtaining good quality DNA from sambar deer scats. Step 1 describes environmental signs (e.g. rubbing of trees, footprints) that indicate recent deer activity, and identify areas in which fresh scats are likely to be found. To sample DNA from sambar deer scats, we recommend a swabbing method which collects epithelial cells shed from the gastrointestinal tract during defecation. These cells coat the outside surface of the scat (Ramón-Laca *et al.* 2015). The swabbing method reduces the amount of non-target DNA collected from other biological organisms that may be present in the scat (e.g. microorganisms, plants, fungi, invertebrates), and minimises the concentration of faecal contaminants (PCR inhibitors) which can reduce PCR success and increase genotyping errors (Waits and Paetkau 2005). The swabbing method yields higher quantities of target DNA (e.g. sambar deer), compared to other methods where slices or slurries of scats are used for DNA extraction (Ramón-Laca *et al.* 2015). Whole sample DNA extractions such as these, however, would enable additional data to be collected regarding an individual's diet and health (e.g. microbiome diversity, pathogen infection).

In step 2 of the DNA Toolkit (Fig. 3), we recommend the isolation of DNA using a commercially available DNA extraction kit. Such kits are widely used to isolate DNA from the scats of wild animals due to their simplicity and cost effectiveness (Brinkman *et al.* 2010; Lounsberry *et al.* 2015; Wedrowicz *et al.* 2013). During this study the Qiagen® QIAamp DNA Mini kit was effective at isolating DNA from scat swabs using an adapted blood and body fluid isolation protocol. In this study, the lowest measured DNA concentration was 0.14 ng/µL. It is important to note that, this figure refers to the total DNA present in a sample which may include considerable amounts of foreign DNA and low amounts of target (sambar) DNA. At DNA concentrations ranging from 0.01–0.03 ng/µL, amplification of the amelogenin locus failed, while concentrations above 0.13 ng/µL were successful (Table 1). We therefore suggest that scat samples with DNA concentrations less than 0.05 ng/µL are discarded because levels of target DNA are likely to be low. However, in some cases (e.g. when sample size is small) it may be preferable to carry such samples through to step 3 to confirm that sufficient sambar deer DNA has been isolated.

Distinguishing deer scats from other Australian mammals through visual cues is relativity easy for the trained eyed. However, assigning deer scats to specific sympatric deer species based on scat morphology alone is quite difficult, mainly due to variation in scat size within different deer age classes (Bowkett *et al.* 2013) and changes in the appearance of scats caused by differences in deer diet (Lunt and Mhlanga 2011). In step 3 of the DNA Toolkit (Fig. 3) DNA is amplified using a pair of deer specific molecular markers (CervtPro and CervCRH) which have been reported to specifically amplify the mitochondrial control region for deer and not for other non-deer species (Balakrishnan *et al.* 2003). Positive amplification of this primer pair can therefore be used to confirm that the scat sample is from deer. In addition, due to the presence of a 78 base pair insertion in the mitochondrial control region of sambar deer (Fig. 1), it is possible to distinguish sambar deer DNA from the DNA of other deer species (fallow, hog and red) using gel electrophoresis. Complete reliance on this approach to confirm the presence of sambar deer DNA is not ideal, however, as intraspecific variation could exist within the D-loop of the mitochondrial control region, resulting in incorrect species identification (Pun *et al.* 2009). Species identification using scat DNA should therefore be conducted by comparing sequences generated from scats with those from known reference samples (preferably with individuals from a similar region) and/or reference sequences which can be sourced from GenBank. Six haplotype sequences are reported here that can be used to genetically discriminate sambar, fallow, red and hog deer. Mitochondrial

sequence data can also be used for phylogenetic studies which can be useful for understanding invasion processes (Rollins *et al.* 2011) and inferring contemporary population structure of invasive species (Mora *et al.* 2018). If preferred, other genetic methods such as the approach taken by Furlan and Gleeson (2017) using quantitative PCR (qPCR), could be employed to identify species, negating the need for DNA sequencing.

Determining the sex of a sampled individual can be useful for understanding ecological and biological processes such as sex ratios, sex-biased dispersal and mating systems (Brinkman and Hundertmark 2009). In step 4 of the DNA Toolkit (Fig. 3), we recommend use of the primer pair SE47 and SE48 to assign sex to the sampled individual. When conducting genetic studies with degraded DNA it is important to choose or design molecular markers which amplify short pieces of DNA (generally <300 bp). This is because as DNA degrades, the DNA molecule itself is cleaved into shorter fragments. DNA regions targeted by particular markers may therefore be incomplete. Targeting shorter regions of DNA can thus increase amplification success (Waits and Paetkau 2005). The quality of DNA derived from sambar deer scats may be assessed by amplification with the SE47/SE48 pair in a similar fashion to that conducted by Hogan *et al.* (2008) for owl feathers and Wedrowicz *et al.* (2017) for koala scats.

Multi-locus genotyping with a suite of molecular markers will provide a DNA profile from which an individual can be identified. Here we tested 17 microsatellite markers, developed for Artiodactyls (even toed ungulates, including sheep and cattle), which have been applied in population genetics studies of Cervid species including red deer (Pérez-Espona *et al.* 2008; Valière *et al.* 2007) and chital deer (Gaur *et al.* 2003). Our results show that cross species amplification success is good for sambar deer with 15 of the 17 markers tested being polymorphic (Table 1). The final suite of 11 microsatellite markers identified had good resolution for the identification of individual sambar deer and it was estimated that the chance of unrelated deer having the same DNA profile was ~1 in 3.6 million. Genotyping error rates were found to be low (Table 2), showing that high quality DNA can be collected from scat samples. Our results show that using DNA sourced from scats, these markers have sufficient power for population genetic studies, however, there may be limitations for other applications, including the investigation of evolutionary lineages and/or fine scale dispersion processes.

This study demonstrates that DNA isolated from scats can be used to supplement invasive sampling methods. Such an approach is likely to be useful when collecting DNA from areas where hunting is not permitted and has the additional advantage of allowing large areas to be surveyed with minimal cost. Although scats are much easier to obtain than tissue samples, the methods for sampling, extracting and testing scat DNA are much more complex. Collection of poor samples (incorrect species, highly degraded) will result in increased costs in processing and analysing. It is therefore important to have measures in place to allow samples to be screened, ensuring that 1) DNA from the target is obtained and 2) DNA from the target is of sufficient quality to produce reliable genetic data.

Management implications

There is an urgent need to gather information regarding populations of wild deer in Victoria to inform their management and mitigate their ecological impacts. Genetic data can be used to provide evidence-based recommendations to improve on-ground management. We have demonstrated a toolbox of methods useful to confirm species, determine sex and genetically profile animals using DNA sourced non-invasively from deer scats. Application of the approach described here will facilitate the collection of large amounts of genetic data easily and quickly over large geographical areas. Once genetic data have been collected, contemporary population genetic analysis can detail aspects of deer ecology that have not yet been studied in Victoria, including population structure, the delineation of management units, abundance estimates, migration rates and dispersal characteristics. Furthermore, genetic identification of sambar deer scats will prevent misidentification of deer scats, improving species distribution models and the results of faecal pellet surveys. Conducting future studies on sambar deer using these approaches will allow evidence-based management of wild deer in Victoria and help assess the effectiveness of current control operations.

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Conflicts of Interest

The authors declare no conflicts of interest.

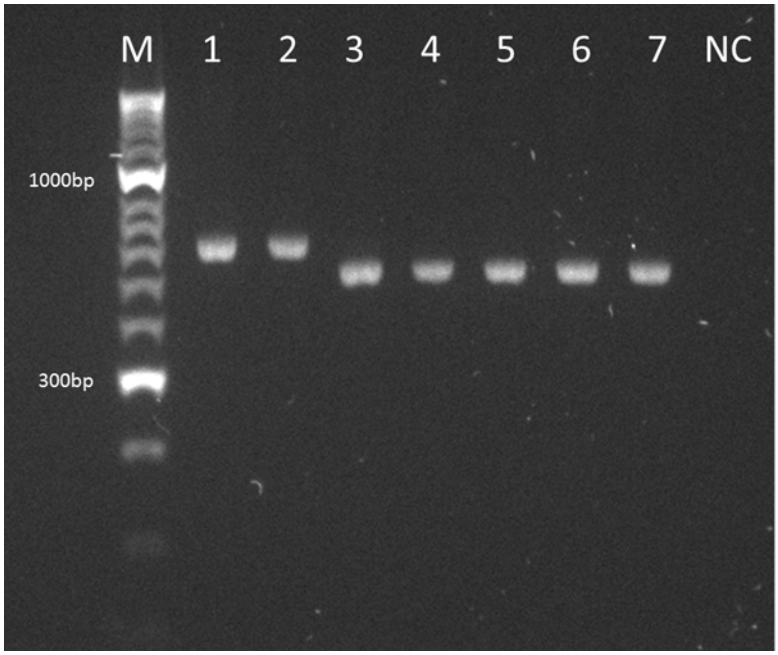

Fig. 1: Amplification of the mitochondrial control region using primers CervtPro and CervCRH (Balakrishnan *et al.* 2003) to differentiate sambar deer from other deer species. Lane M, Bioline Hyperladder II, Lanes 1-2: Sambar deer, Lane 3: Red deer, Lanes 4-5: Fallow deer, Lanes 6-7: Hog deer. NC: Negative control.

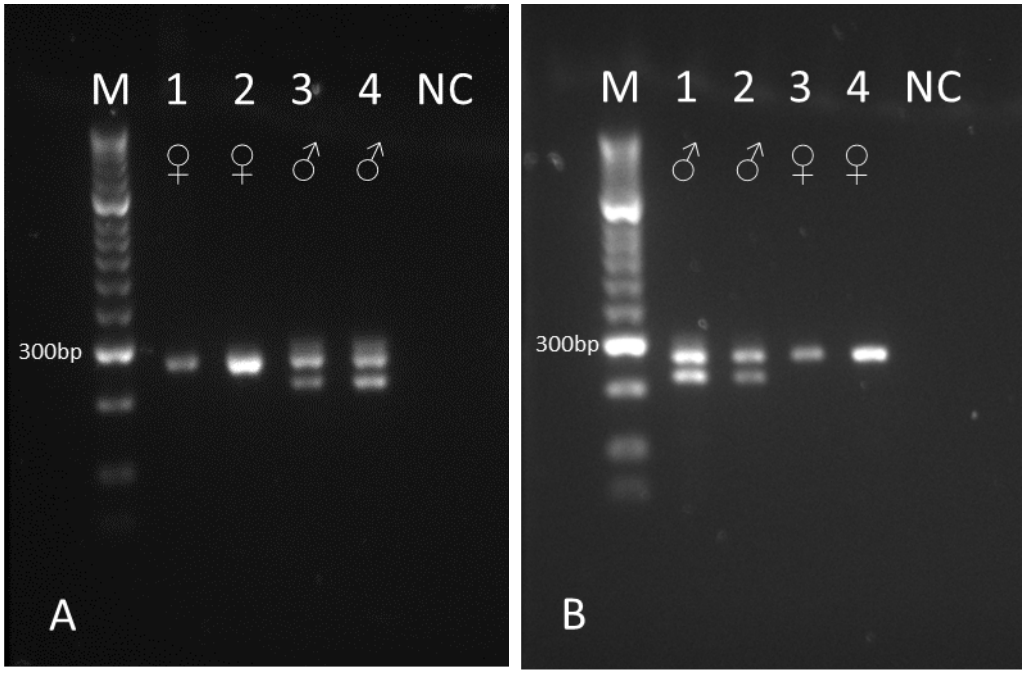

Fig. 2: Amplification of the amelogenin locus with SE47 and SE48 primers (Ennis and Gallagher 1994) to determine sambar deer sex. A) Lane M: Bioline Hyperladder II, Lanes 1-2: Female sambar deer DNA, Lanes 3-4: Male sambar deer DNA. B) Lane M: Bioline Hyperladder II, Lanes 1-2: Scat samples identified as male, Lanes 3-4: Sambar scat samples identified as female. NC: Negative control.

Table 1: Amplification success of sex markers (SE47 and SE48) for serial dilutions of sambar deer tissue DNA isolates. + denotes one band (homozygous) was observed for females and two bands (heterozygous) for males. – denotes that expected amplification was not observed.

Dilution	Sample 1 Female (DNA, ng/µL)	Sample 2 Female (DNA, ng/µL)	Sample 3 Male (DNA, ng/µL)	Sample 4 Male (DNA, ng/µL)
1:1	+ (16.6)	+ (34.2)	+ (13.1)	+ (23.0)
1:10	+ (1.66)	+ (3.42)	+ (1.31)	+ (2.30)
1:100	+ (0.16)	+ (0.34)	+ (0.13)	+ (0.23)
1:1000	- (0.02)	- (0.03)	- (0.01)	- (0.02)

Table 2: Characterisation of 17 Cervid microsatellite loci for sambar deer.

n – Number of individuals successfully genotyped, ns – Amplification success, N_A – Number of alleles, H_O – Observed heterozygosity, H_E – Expected heterozygosity. The 11 loci in bold are recommended for multi-locus genotyping.

Locus	*n*	ns (%)	N_A	H_O	H_E
BL42	**32**	**94**	**9**	**0.531**	**0.762**
BM757	**34**	**100**	**4**	**0.647**	**0.587**
Ca18	**33**	**97**	**4**	**0.636**	**0.562**
Ca43	**32**	**94**	**3**	**0.438**	**0.490**
CelJP38	**33**	**97**	**4**	**0.727**	**0.668**
IDVGA55	**34**	**100**	**5**	**0.441**	**0.483**
INRA121	**34**	**100**	**4**	**0.647**	**0.653**
OarFCB5	**34**	**100**	**3**	**0.588**	**0.554**
RT7	**34**	**100**	**4**	**0.735**	**0.652**
TGLA53	**34**	**100**	**3**	**0.471**	**0.515**
TGLA57	**34**	**100**	**2**	**0.147**	**0.136**
TGLA126	40	98	2	0.550	0.425
BMC1009	41	100	2	0.512	0.476
VH110	10	100	1	-	-
BM848	10	90	1	-	-
BM203	10	20	2	-	-
CSSM43	10	70	2	-	-

Table 3: Error rates for eight microsatellites based on eight replicates. PCR – Successful amplification rate, ADO – allelic dropout rate, FA – false allele rate.

Locus	PCR (%)	ADO (%)	FA (%)
BL42	88	0.0	0.0
BM757	96	2.5	0.0
BMC1009	92	0.0	0.0
IDVGA55	92	0.0	0.0
INRA121	95	4.8	0.0
TGLA126	97	0.0	0.0
TGLA53	99	0.0	0.0
TGLA57	98	0.0	0.0
Mean	95	1.0	0.0

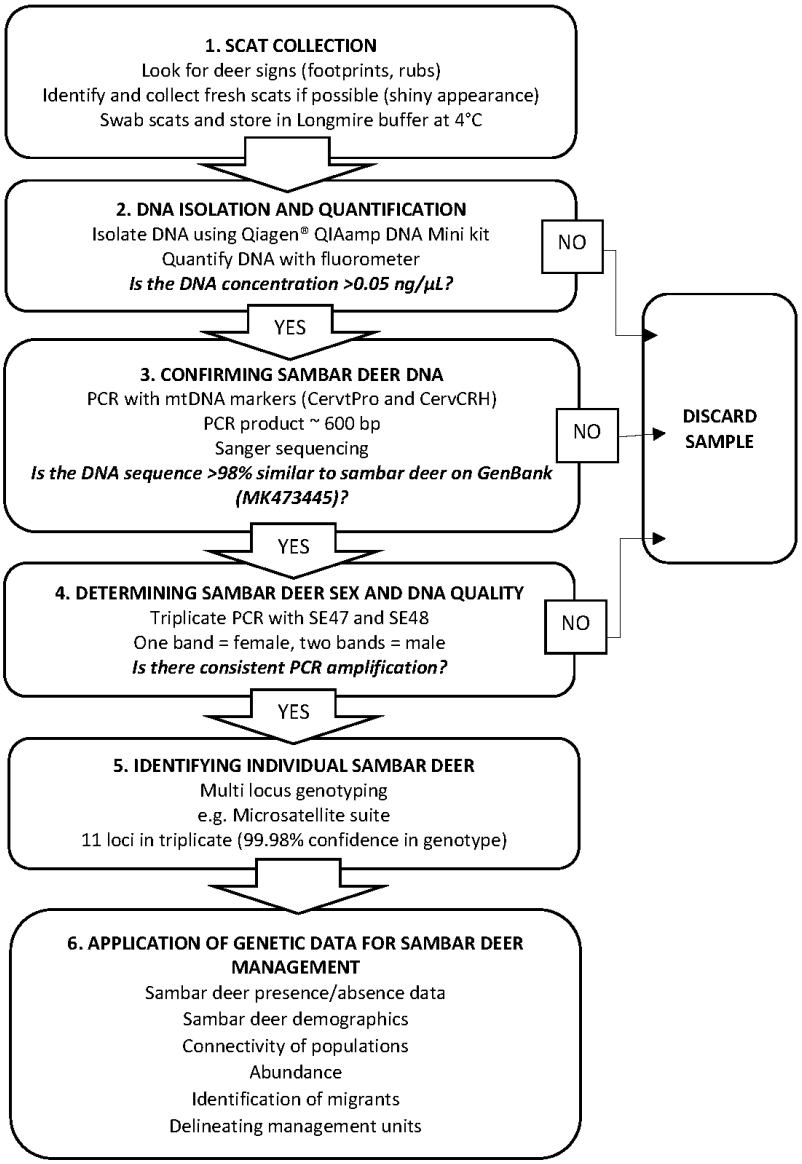

Fig. 3: 'DNA toolbox' for isolating good quality DNA from a single sambar deer scat to generate genetic data for applied sambar deer management.

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