CENTRE FOR
INVASIVE SPECIES SOLUTIONS

THE ROLE OF WILD DEER IN THE TRANSMISSION OF DISEASES OF LIVESTOCK

FINAL REPORT FOR PROJECT P01-L-002

AUTHORS

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We acknowledge the Traditional Custodians of the lands on which we meet and work and pay our respects to Elders — past, present and emerging.

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THE ROLE OF WILD DEER IN THE TRANSMISSION OF DISEASES OF LIVESTOCK

FINAL PROJECT REPORT FOR PO1-L-002

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INTRODUCTION

Wildlife populations are arguably the most significant source of emergent infectious diseases that impact human health, biodiversity and agriculture (Morner et al. 2002). As a result, pathogen transmission between wildlife and livestock is a global concern in agriculture (Rhyan and Spraker 2010).

Since deer are ruminants and closely related to economically important livestock species such as cattle, sheep and goats, it is not surprising that they share many pathogens, including several of major agricultural significance. The role of wild deer as a source of infection for livestock has been mostly reviewed and documented in Europe and North America (Böhm et al. 2007; Conner et al. 2008; Martin et al. 2011; Ruiz-Fons et al. 2014), where there is a higher need for monitoring wildlife diseases given the increased wildlife–livestock interaction.

Deer are not native to Australia and were introduced at different times in the early nineteenth century from Europe and Asia (Bentley 1998; Hall and Gill 2005). Six species – chital (*Axis axis*), fallow (*Dama dama*), hog (*Axis porcinus*), red (*Cervus elaphus*), rusa (*Rusa timorensis*) and sambar (*Rusa unicolor*) deer – have established self-sustaining wild herds in Australian habitats (Bentley 1998; Moriarty 2004; Hall and Gill 2005). Wild deer populations continue to increase in number and distribution throughout mainland Australia, and are currently scattered throughout all states and territories (Moriarty 2004; Davis et al. 2016), particularly in south-eastern Australia, where few areas are left unoccupied by deer (Davis et al. 2016). Interestingly, bioclimatic modelling – which matches animal species' requirements to suitable environments – suggests that deer currently occupy only a fraction of their potential distribution in Australia and could expand beyond their current distribution (Moriarty 2004; Davis et al. 2016).

Wild deer pose a biosecurity threat to the livestock industry as deer population density increases, because they commonly feed on agricultural landscapes, and the deer–livestock contact rate increases. Pathogen transmission between deer and livestock may hamper current biosecurity preventive measures. However, data about the infection status of Australian wild deer populations are sparse, and have been predominantly collected with a focus on viral and parasitic infections relevant to human health or the livestock industry. This data was first collected across a handful of limited surveys performed in small geographic areas in the 1960s and 1980s (Munday 1966; Munday 1972; Presidente and Westbury 1979; Slee and Presidente 1981; English 1982; McKenzie et al. 1985). A small number of larger studies were performed between 2014 and 2021 across a larger geographical area (Davies 2014; Koehler et al. 2016; Panozzo 2018; Jenkins et al. 2020; Lamb et al. 2021).

To successfully mitigate these risks, it is important to improve our understanding of deer ecology, with a particular focus on deer landscape-use and movements, deer density and their interaction with livestock species.

This report was prepared by the core team of the PO1-L-002 project: The role of wild deer in the transmission of diseases of livestock. We provide a summary of the approach, results to date and their implications for management and policymakers. Detailed descriptions of the methods, results and, most importantly, acknowledgment of the numerous collaborators are provided in the drafted manuscripts and specific reports attached as appendices to this document.

OBJECTIVE: ASSESS THE RISK OF DEER TRANSMITTING DISEASES TO LIVESTOCK

This project collected data that aimed to assess the likely risk of disease transmission between deer and livestock by (i) evaluating deer densities at the forest–farm interface, (ii) assessing the level of interactions between wild deer and domestic livestock, and (iii) quantifying the degree of connectivity between deer populations.

We also conducted a deer disease-status assessment, which provided data on the presence and distribution of diseases in deer that are relevant to livestock. These data helped quantify the posed

biosecurity risks posed by deer – we intended to use the molecular data generated by the disease screening to estimate likely cross-species infection between deer and livestock species.

Ultimately, we aimed to develop an epidemiological model to evaluate the potential disease dynamics in the case of an incursion of an exotic disease in either deer or livestock.

METHOD

We conducted a disease investigation in wild deer populations by testing blood or serum samples with serological and molecular diagnostic tools (see Appendix I). Our screening included several viruses and parasites known to co-infect deer and livestock species.

Knowledge of deer densities adjacent to farms is important because it quantifies the potential for interactions between deer and livestock at the forest–farm interface. We estimated the densities of deer in forested habitats adjacent to farms by monitoring three sites where sambar and fallow deer occur. At each site, faecal-pellet transects were established and non-invasive genetic samples were collected to carry out a spatially explicit capture–mark–recapture study. Molecular techniques were used to identify individuals by extracting deer DNA from faecal material and genotyping each sample with a multilocus panel. Sampling at each site occurred over three years in autumn and summer. These surveys were replicated in the paddocks of selected properties (beef-cattle farms) adjacent to the forest blocks that were monitored. The new laboratory protocols that we developed are described in detail in a manuscript which is now ready to be submitted for publication (Appendix II).

We also deployed camera arrays in the forest. We used the camera data from the forested habitat to estimate density. Together with the data obtained from the DNA extracted from the faecal pellet samples, we used these data to quantify the number of deer that enter the paddocks where livestock is concurrently present as a function of deer density within each season.

Estimating the level of connectivity between different deer populations is important because it defines the dispersal potential for intraspecies spread of disease. We estimated the connectivity between local deer populations using population genetic analyses of sambar, rusa and fallow deer. Tissue samples were collected by collaborating with deer-culling programs and recreational hunters. We genotyped > 10,000 genome-wide single nucleotide polymorphisms (SNPs) (see Appendix III and IV).

Information on deer densities, distribution, connectivity (dispersal) and deer–livestock interactions (contact rates) obtained during the project were used to develop a prototype for a spatially explicit model of the population dynamics, geographic spread and disease transmission between wild deer species and livestock (Appendix V). While initially we had proposed to use the Australian Animal Disease Spread Model as a platform to develop the model, access to the platform was prevented due to the lack of a confidentiality agreement between Victorian government and the Australian Department of Agriculture, Fisheries and Forestry – hence we used a standalone, individual-based model. Using the foot and mouth disease virus as a case study to investigate what it may happen in the event of an exotic disease incursion, we modelled possible emergency responses including deer culling, livestock vaccination and deer-exclusion fencing and predicted the most cost-effective strategies to achieve disease eradication or to limit the impact on the agricultural industry.

RESULTS AND DISCUSSION

At the beginning of this project, very limited information was available on the pathogens present in Australian wild deer populations. This project generated very important baseline data that will enable the assessment of deer populations' pathogen presence and distribution changes in the future. It highlighted the differences between species and geographical location. We also established a biobank of samples that will be suitable for future publications. A detailed review of our findings from the disease investigation and their implications is included in the manuscript submitted for publication in Appendix I.

Collecting ecological data on deer populations is critical to a better understanding of deer population dynamics that are fundamental not only for the epidemiological modelling that we are undertaking, but also for control operations, which can be aimed at the mitigation of biosecurity risks, biodiversity conservation or protection of primary industry production. Indeed, any modelling and successful management programs are underpinned by fundamental ecological knowledge.

The techniques that we developed in this project enabled the estimation of deer densities as well as other important population parameters (e.g. recruitments), which are currently lacking. These protocols are being already used to support control and eradication efforts in at least three states (Vic, Qld and NSW) by several programs. This information is so vital that it was used before some of our results were finalised, and we are only beginning to distribute them. Information has been distributed through participation at workshops, at conferences, our scientific publications, meetings within government organisations and word of mouth.

We now have a better understanding of deer's landscape use, population structure and dispersal distances. Our genetic work also highlighted the potential role that deer farms play, as well as human-assisted deer movements that maintain or establish new populations. Further details on this work and its implications are captured in Appendix III and IV.

Estimates of deer densities were broadly consistent between camera and deer-faecal-pellet data, and ranged between 2 and 14 deer per km^2. Deer densities did not seem to have changed after a severe bushfire, and our data clearly demonstrated that deer readily use farming paddocks as foraging grounds in proximity to livestock. There was a strong indication of a seasonal effect on deer behaviour, where they appeared to move across a wider area in warmer months. Deer movements detected with scat data were highly consistent with the estimates obtained from our population genetic analysis, giving us a high confidence in the results.

Our epidemiological modelling suggested that if early detection is not achieved, deer populations can be responsible for disease outbreaks in livestock. Where livestock vaccines exist, these would appear to be a convenient means to limit the impact of infectious diseases transmitted by deer, and probably more convenient than deer fencing or culling. The latter did not cause a substantial reduction in the number of outbreaks at the rate we implemented in our model (10%). Further details on the camera/scat data analysis, and the epidemiological modelling are provided in Appendix V.

So far, the project has generated 12 manuscripts that are either published or submitted to peer-reviewed journals.

RECOMMENDATIONS

Climate patterns can significantly change pathogen transmission and interactions between wildlife hosts, vectors and even humans. Our sampling timing (which was dictated by culling-program operational restrictions) limited our capacity to sample vector-borne pathogens, such as bluetongue virus, Akabane virus, bovine ephemeral fever virus, epizootic haemorrhagic disease virus, *Babesia*, *Theileria*, *Trypanosoma* and *Plasmodium*; and while our study partially screened for these pathogens, this should be the focus of future research. This should go alongside the integration of a One Health approach to disease prevention because diseases in humans, livestock and wildlife are strictly interlaced – as has been demonstrated by recent events and thoroughly evaluated in the scientific literature.

Furthermore, we recommend developing a passive surveillance system through the Australian deer-hunter community. Training hunters to identify, record macroscopic lesions, and collect specimens could be provided as part of their licensing process, and hunters may be of critical help in monitoring the presence and distribution of infectious diseases. The investigations conducted in the last five years provided vital baseline data for future research. Monitoring changes in the disease status will give us a more comprehensive view of the dynamics of infectious diseases to minimise the risk of impacts on humans, livestock and wild animals.

Our population genetic analysis detected a relatively large number of deer hybrids. Deer hybrids can present as larger in body size than their parental species, and this phenomenon is often encouraged in the deer-farming industry to promote larger meat yields (Pearse 1992; Tate et al. 1997; Tuckwell 1998). Hybridisation in ungulates has also been shown to increase disease resistance (Grossen et al. 2014; Barbato et al. 2017), growth rate and body weight (Asher et al. 1996; Ismail and Saidi 2009; Senn et al. 2010), and is suggested to increase population growth and dispersal (Goedbloed et al. 2013; Manunza et al. 2016; Iacolina et al. 2019). These characteristics may lead to an increase in negative impacts associated with deer in areas where sambar, rusa and their hybrids co-occur. Further research is necessary to understand the full scale of the negative impacts likely to be associated with the presence of rusa × sambar hybrids in the Australian landscape. Related to this, it is important to understand the implication of deer-farming practices (e.g. deer escapees) and the illegal release of deer for hunting purposes. Indeed, while our results do not provide definitive evidence, they do suggest an important role of deer farming in the dynamics of wild deer populations. We consider it highly likely that mitigating these risks would require substantial changes in current policies.

We identified a clear signal of ‘demographically separated’ population structure between the Victoria/south NSW and the north NSW sambar populations. However, the lack of genetic structure observed in sambar deer in Victoria/south NSW suggests a relatively high level of deer movements within and across Gippsland and south NSW. This presents a challenge for management, because any effort to control locally the deer numbers will be quickly counteracted by re-invasion from nearby uncontrolled areas. The only exception to this high degree of movement within the sambar population in Gippsland might be the Melbourne peri-urban area, where some indication of substructuring (i.e. marginal differentiation within the population) has been identified. This is being further investigated by a follow-up project funded by the Victorian government.

Genetic data suggest that the majority of deer-dispersal events occur within distances of 20 km and very rarely over 50 km. This pattern appears to be consistent in both sambar and rusa deer. These results are highly relevant for management and biosecurity programs. Indeed, a buffer zone of 20–50 km should be considered when establishing a program to protect assets (such as a biodiversity hotspot or high-production agricultural land) or a biosecurity exclusion zone to prevent pathogen transmission. The actual extent of the buffer zone will depend on the degree of risk that can be tolerated should an incursion occur.

The data collected from our intensive monitoring sites (i.e. camera and faecal-pellet data) demonstrate that deer can reach high densities at the interface between agricultural land and forested habitats, and it is highly likely that transmission between deer and livestock can readily occur. We did not model mitigation actions that implement an asset-protection approach. Such spatially heterogeneous management actions (e.g. fencing of high-production areas or intensive culling in strategic locations) should be the focus of further studies. Similarly, we did not use our deer-monitoring data to quantify the economic impact of deer on the primary industry by limiting resources (e.g. use of pasture otherwise dedicated to livestock) or infrastructure damage (e.g. fencing) because we instead focused on the biosecurity risk posed by deer to the livestock industry. However, the data we collected are suitable for this use, and it would be beneficial for the industry to undertake such studies and provide guidance on the possible return on investment of different management strategies.

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APPENDICES

APPENDIX 1. TECHNICAL REPORT – THE ROLE OF WILD DEER IN THE TRANSMISSION OF DISEASES OF LIVESTOCK

The role of wild deer in the transmission of diseases of livestock

C. Pacioni, E. Hill, L. Woodford, J. Hampton, N. Murphy, and D. Ramsey

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Arthur Rylah Institute for Environmental Research
Unpublish Client Report

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Acknowledgment

We acknowledge and respect Victorian Traditional Owners as the original custodians of Victoria's land and waters, their unique ability to care for Country and deep spiritual connection to it. We honour Elders past and present whose knowledge and wisdom has ensured the continuation of culture and traditional practices.

We are committed to genuinely partner, and meaningfully engage, with Victoria's Traditional Owners and Aboriginal communities to support the protection of Country, the maintenance of spiritual and cultural practices and their broader aspirations in the 21st century and beyond.

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Front cover photo: A male sambar deer caught on a camera trap while jumping a livestock fence (ARI).

The role of wild deer in the transmission of diseases of livestock

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Context:

Wildlife populations are the most significant source of emergent infectious diseases that impact human health, biodiversity, and agriculture. As a result, pathogen transmission between wildlife and livestock is a global concern in agriculture. Since deer are ruminants and closely related to economically important livestock species such as cattle, sheep, and goats, it is not surprising that they share many pathogens, including several of major agricultural significance. The role of wild deer as a source of infections for livestock has been mostly reviewed and documented in Europe and North America, while understanding of these dynamics and the efficacy of possible mitigation actions in Australia is limited.

Aims:

This project aimed to assess the likely risk of disease transmission between deer and livestock by (i) evaluating deer densities at the forest/farm interface (ii) assessing the level of interactions between wild deer and domestic livestock and (iii) developing an epidemiological model to evaluate the potential disease dynamics in the case of an incursion of an exotic disease.

Methods:

We estimated the densities of deer in forested habitats adjacent to farms by monitoring three sites where sambar (*Cervus unicolor*),) and fallow deer (*Dama dama*) occur using camera trap arrays and non-invasive genetics.

Information on deer densities, distribution, connectivity and deer/livestock interactions (contact rates) obtained during the project was used to develop a spatially explicit model of the population dynamics, geographic spread and disease transmission between wild deer and livestock. We used Foot and Mouth Disease Virus as a case study. An epidemiological model was used to assess emergency responses and predict the most cost-effective strategies to achieve disease control/eradication in the event of exotic disease incursion by assessing the efficacy of various interventions including deer culling, livestock vaccination and exclusion.

Results:

Estimates of deer densities were broadly consistent between camera and deer faecal pellet index data and ranged between 2 and 14 deer/km^2. Deer densities do not seem to have been significantly changed after a severe bushfire at one of the monitored sites. Our data clearly demonstrate that deer readily use farm paddocks as foraging grounds in close proximity to livestock species. There was a strong indication of a seasonal effect on deer movement with larger ranging areas apparent in the warmer months. Despite modelling a pathogen with a low transmission rate in deer, our results suggest that if early detection is not achieved, deer populations can be responsible for outbreaks in livestock. Based on our modelling results, where vaccines exist, these would appear to be a convenient means to limit the impact of infectious diseases transmitted by deer. On the contrary, additional removal of deer did not cause a substantial reduction in the number of outbreaks at the rate we implemented in our model (10%).

Conclusions and implications:

Our results demonstrate that deer can reach high densities at the interface between agricultural land and forested habitats. It is highly likely that transmission between deer and livestock can readily occur. Even if the transmission rate of the infectious disease in question is relatively low, deer can be responsible for outbreaks in livestock that can have a substantial economic impact. In the instances where livestock vaccination can prevent infections, this is likely to be a more efficient option to minimise economic losses than deer-proof fencing or culling. We acknowledge that we did not model mitigation actions that implement an asset protection approach. Such spatially heterogeneous management actions (e.g. fencing of high production areas or intensive culling in strategic locations) should be the focus of further studies. Similarly, we did not use our deer monitoring data to quantify the economic impact of deer on the primary industry by limiting resources (e.g. use of pasture otherwise dedicated to livestock) or infrastructure damage (e.g. fencing), because we focused on the biosecurity risk posed by deer to the livestock industry. However, the data we collected are suitable for this use and it would be beneficial for the industry to undertake such studies and provide guidance on the possible return on investment of different management strategies.

1 Introduction

Wildlife populations are arguably the most significant source of emergent infectious diseases that impact human health, biodiversity and agriculture (Morner *et al.* 2002). As a result, pathogen transmission between wildlife and livestock is a global concern in agriculture (Rhyan and Spraker 2010).

Since deer are ruminants and closely related to economically important livestock species such as cattle, sheep and goats, it is not surprising that they share many pathogens, including several of major agricultural significance (Cripps *et al.* 2019). The role of wild deer as a source of infection for livestock has been mostly reviewed and documented in Europe and North America (Böhm *et al.* 2007; Conner *et al.* 2008; Martin *et al.* 2011; Ruiz-Fons *et al.* 2014), where the increasing wildlife-livestock interaction due to human development and changes in land uses raises the need for monitoring wildlife diseases.

Deer are not native to Australia and were introduced at different times in the early nineteenth century from Europe and Asia (Bentley 1998; Hall and Gill 2005). Six species – chital (*Axis axis*), fallow (*Dama dama*), hog (*Axis porcinus*), red (*Cervus elaphus*), rusa (*Cervus timorensis*), and sambar (*Cervus unicolor*) deer – have established self-sustaining wild herds in Australian habitats (Bentley 1998; Hall and Gill 2005; Moriarty 2004b). Wild deer populations continue to increase in number and distribution throughout mainland Australia and are currently found in all states and territories (Davis *et al.* 2016; Moriarty 2004b), particularly in south-eastern Australia (Davis *et al.* 2016). Interestingly, bioclimatic modelling, which matches animal species' requirements to suitable environments, suggests that deer currently occupy only a fraction of their potential distribution in Australia (Davis *et al.* 2016; Moriarty 2004b).

Wild deer are likely susceptible to endemic and exotic diseases that may affect other animal species, including humans and livestock and opportunities to acquire or transmit these pathogens occur when using improved pastures and livestock water resources (Cripps *et al.* 2019). Red deer in south-eastern Queensland have shown serological evidence of exposure to several endemic livestock diseases, including leptospirosis and Akabane virus, and they carry several species of parasitic helminths (McKenzie *et al.* 1985). Additionally, deer in Australia have also been shown to display serological evidence of exposure to Q fever (*Coxiella burnetii*), *Neospora caninum*, bovine ephemeral fever virus, *Pestivirus*, and ticks (*Ixodes* spp.) (Huaman *et al.* In press; Huaman *et al.* 2020; Moriarty 2004a; Voss L. 2022). The potential introduction of exotic animal diseases such as foot-and-mouth disease (FMD) or surra (*Trypanosoma evansi*) is of particular concern. The cost of an outbreak of FMD in Australia has been estimated at AU$0.6–5.2 × 10^{10} (Buetre 2013). Our ability to effectively contain an emergency disease in wild deer populations is unknown, but it is likely to be a substantial challenge considering their high mobility, cryptic behaviour and the use of inaccessible terrain, which would make control difficult (Animal Health Australia 2011). As a result, wild deer pose a growing biosecurity threat to the livestock industry because they commonly feed in agricultural landscapes, and deer-livestock contact rates are likely to increase as deer population densities rise. Pathogen transmission between deer and livestock may hamper current preventive measures aimed at mitigating economic impacts of livestock infections. However, data about these dynamics in Australian wild deer populations are sparse. In order to successfully mitigate the biosecurity risks that deer pose to livestock, it is important to improve our understanding of deer ecology, with a particular focus on deer landscape use, movements, density and the likelihood of their interaction with livestock species.

Aims

We aimed to assess the likely risk of disease transmission between deer and livestock by (i) evaluating deer densities at the forest/farm interface (ii) assessing the level of interactions between wild deer and domestic livestock and (iii) developing an epidemiological model to evaluate the potential disease dynamics in the case of an incursion of an exotic disease.

2 Methods

2.1 Site selection

Study sites were selected across eastern Victoria. These sites satisfied the criteria that they were livestock farming properties adjacent to forested public land, farmers had reported deer entering their paddocks from the adjacent public land, and site access was available all year round. Three locations were selected near the towns of Kinglake, Gembrook and Willow Grove (Fig 1). All three farm locations were separated from the public forested land by means of a standard cattle fence (~1.4 meters high).

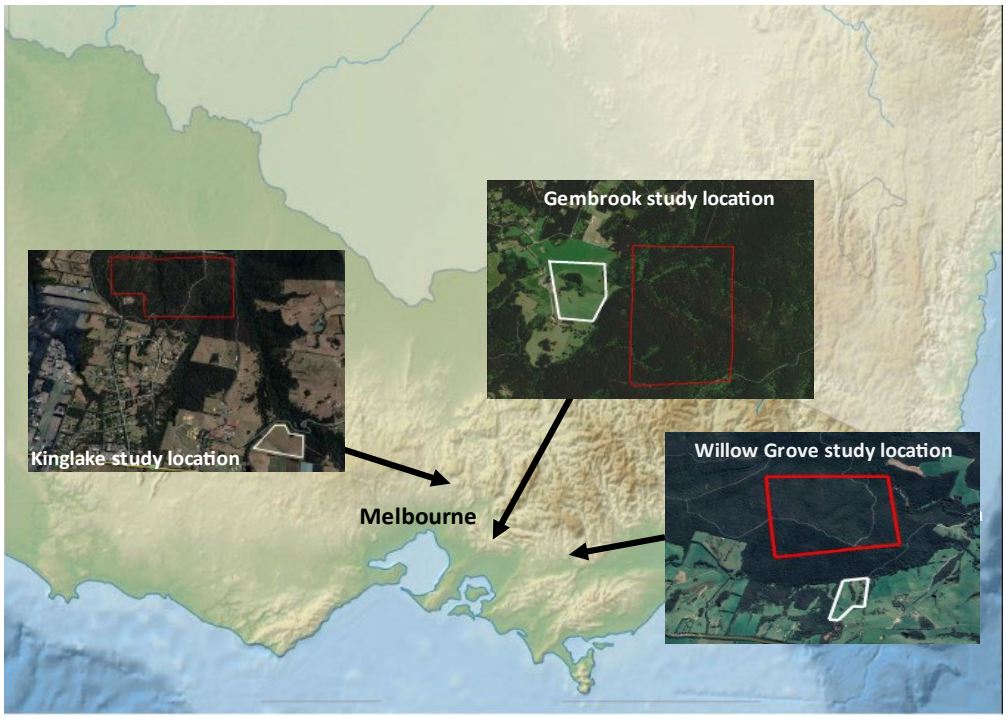

Figure 1. Map showing the study sites for assessing wild deer and livestock interactions.

The study sites at three locations in eastern Victoria. The red polygons outline the forested public land study zones, and the white polygons outline the livestock farm study zones.

Each location had a polygon overlayed on both the public land (approximately 2 km²) and the livestock paddocks (appropriate to the paddock area available) with both forest and paddocks being roughly adjacent to each other. The final selection of site locations depended on the land characteristics such as topography and ease of access. For each site on public land, 6–7 transects, each 1.5–2 km long (approximately 12 km in total) and spaced 300 meters from each other were established in the forest and used to guide the sampling of faecal pellets to collect DNA and deploying cameras (see below). Similarly, transects were also established in the livestock paddocks, however these were 10–50 m from each other depending on the terrain. An example of transect locations can be seen in Figure 2. These sites were visited twice a year, in Spring and in Autumn from 2018 until Autumn 2020, resulting in five sampling seasons (except for Willow grove where there was no sampling in Autumn 2020). For each season, three separate visits were made three–four weeks apart.

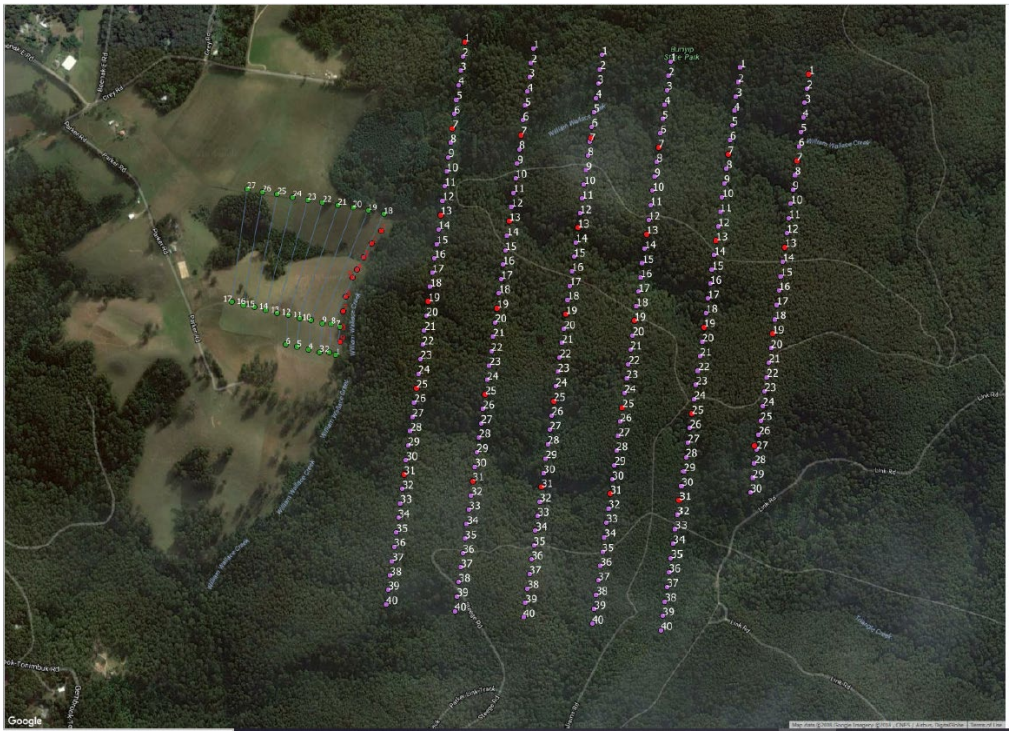

Figure 2. Example of study site set up for assessing wild deer and livestock interactions.

Deer pellet sampling transects and numbered plots in forested public land near Gembrook, adjacent to farm livestock paddocks. The red circles show the location of the infrared cameras.

2.2 Deer detection methods

2.2.1 Camera monitoring

We deployed Reconyx remote infrared cameras (Reconyx Hyperfire© Wisconsin, USA) in the forested public land at 300 m intervals along the transects, resulting in approximately 30 cameras per site. These were deployed on the first of the three visits during each season and removed on the third visit. Cameras were mounted on trees at 1.2 – 1.5 m above the ground and faced south to minimise sun glare. At a subset of 10 of the cameras, four reflective markers mounted on stakes were placed in the camera field of view at 2.5, 5, 7.5 and 10 meters from the camera location. These markers were subsequently used to tag images of deer and place them in one of five distance categories (i.e.: 0 – 2.5, 2.5 – 5.0, 5.0 – 7.5, 7.5 – 10, 10+) indicating a distance interval (in meters) from the camera (Figure 3). Camera traps were not lured. The ground vegetation was trimmed between the camera and the stakes and around the mounting tree, to identify taxa more easily and minimise false triggers.

Cameras were set to take five images whenever a motion was detected, at the maximum speed allowed by the camera (i.e.: hyperfire mode) with no sleep period between detections. On each visit, the SD cards containing the image data and the batteries for each camera were replaced.

Figure 3. Image of a typical camera field of view showing the distance markers in forested public land.

2.2.2 Deer faecal pellet monitoring

Non-invasive genetic samples were collected from deer faecal pellets along each transect. All samples were labelled with a site-specific letter code (e.g., KL for Kinglake), a progressive number, the coordinates, and the date. Once sampled, the remaining pellets in a group were scattered to avoid sampling them again in successive visits. Pale pellets, or pellets with a hard crust, fissures or mould growing on them were not sampled as they were deemed too old to recover usable genetic material. Extra searches were also conducted between the transects and following animal trails, creeks and drainage lines to increase the likelihood of encountering fresh pellets suitable for DNA sampling. These were undertaken by using the track mode on hand-held GPS units to enable repeat visits. The samples were labelled and stored at room temperature but were kept away from direct sunlight while out in the field, and then frozen at -20°C upon return until DNA extractions were performed.

DNA sampling followed the methods in Davies *et al.* (2019). Briefly, the surface of the pellets was swabbed with a sterile Rayon tipped swab that was dipped in a DNA preservative (Longmires Solution), whilst holding the pellet with a toothpick. The swab tip was then cut from the swab shaft and stored in an Eppendorf vial containing approximately 500 mL of the Longmires DNA preservative solution, and extracted using a modified DNeasy Blood and Tissue Kit (Qiagen) protocol.

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2.3 Data analysis

2.3.1 Camera data

Images containing deer from each camera were given metadata tags using DigiKam software. Metadata tags included the species, the number of individuals in the image, the age class, sex (if determinable), and, if distance markers were used, the distance category of the closest individual was recorded. The deer images were distributed in a hierarchical folder label that included the site location, ‘farm’ or ‘forest’, the species and then the tagged images. Data were extracted with a customised version of the R package camtrapR (Niedballa *et al.* 2016). The main modifications of the package included the capacity to correctly handle multiple detections within the same images and their metadata, creating a record table with one line for each detection and being able to subset these data based on time intervals between the images (https://github.com/carlopacioni/camtrapRdeluxe). The distance data obtained from the images for each detected deer were used to estimate a detection function, which describes how the probability of deer detection declines with increasing distance from the camera (Howe *et al.* (2017). We considered two possible detection functions (half-normal and hazard rate) either with or without cosine adjustments (Buckland 1992). When adjustments were used, either the second or third order adjustments were fitted. Model comparison was carried out following the two-step approach recommended by Howe *et al.* (2019) to

account for overdispersion usually present in distance sampling data from non-independent observations. The most supported detection function was then applied to the image data from the forest to estimate deer density for each site separately.

2.3.2 Deer faecal pellet data

Deer DNA was extracted from each deer pellet and sequenced using an amplicon panel according to Hill *et al.* (in prep). The sequencing data were then used to identify and genotype 194 SNP loci as described in Hill *et al*. (in prep). The SNP dataset was imported into *R* with PopGenReport (Adamack and Gruber 2014) and the package *poppr* (Kamvar *et al.* 2014) was used to filter for samples with more than 75% missing data, loci with more than 15% missing data, monomorphic loci, and loci whose minor allele was observed less than 5 times. Individuals were identified using unique multi-locus genotypes while allowing missing data to match any other genotype using a 3.5% error rate. Retained genotypes thus represent information on the individual ID and location of detected deer.

To estimate deer density from the pellet DNA data, a detection history was constructed for each genotype ID (i.e., 1 – detected; 0 – not detected) for visit for each season. Since the detection histories also included locations, these were analysed using a discrete space, spatial capture-recapture model (Royle *et al.* 2013). This model involves partitioning the area where the deer pellet searches were conducted into a grid of hexagons (each of 100-m in diameter) and determining the number of detections within each cell. The probability of detection is expressed as a function of distance from the search path (which is expressed from the centre of the cell to the transect or GPS search path). We used a Jolly-Seber open population model, which also allows estimation of the survival (φ), per capita recruitment (f) and the rate of increase (or decrease; λ) between the time interval of primary sampling occasions (where primary sampling occasions are each season-year combination), as well as density (D) at the time of each primary session (Efford and Schofield 2020). It is important to note that this modelling approach cannot distinguish between death or emigration, and between recruitment or immigration and parameters that are affected by these dynamics (survival, and recruitment) should be interpreted carefully.

We used the R package openCR (Efford and Schofield 2020) to fit the model with a conditional likelihood (in which density is a derived parameter) because the convergence of these models is typically easier than full likelihood models. Confidence intervals of derived parameters were obtained by running a full likelihood model, with the primary parameters' estimates from the conditional likelihood model as starting values, without maximising the likelihood (i.e., only the variance of the parameters is computed).

We considered progressively more complex models including: the simplest model (where only the intercept was included), a model where the season (i.e., spring or autumn) of sampling would influence the spatial scale parameter (i.e., home range activity scale), movement of the home range centre location (see details below) and whether the detection rate was different between paddocks and forest and the combination of these two. We also considered two detection functions (half-normal or hazard rate), and three animal movement models. We modelled animals' primary activity centre as static (i.e., no movement between sessions, the default), independently in each primary sampling session, or as potential dispersal between primary sessions with movement distance defined by a bivariate normal kernel density function. The best model for each site was selected with the Akaike's Information Criterion corrected for small sample size (AICc) (Anderson and Burnham 2002). Models with unidentifiable parameters (i.e., where the rank of the Hessian matrix was less than the number of parameters), were not considered as we presumed there was insufficient information in the data for those models.

2.4 Epidemiological modelling

A published spatially explicit, individual-based, SIR (Susceptible, Infectious, Recovered/Removed) epidemiological model (Ramsey *et al.* 2016; Ramsey *et al.* 2014) was re-parameterised based on our results. The model is stochastic and simulates life events and behaviour (e.g., birth, death, dispersal) of individual deer in two-dimensional space. Individuals can be in one of three states: susceptible (S), infected (I), or removed and immune (R). Individuals in state R are considered "removed" and are not part of the susceptible population for the length of their individual immunity (Ramsey *et al.* 2014). As a case study, we focused on the Gippsland region in Victoria as this represents a large area of continuous habitat with an established deer population surrounded by farmland (Hill *et al.* In review). We considered a hypothetical incursion of an exotic disease, drawing available information from the literature on epidemiological

characteristics of the foot and mouth disease virus (FMDV) in deer. The model was modified as described in the sections below.

Deer habitat layer

The spatial model requires a description of variation in deer carry-capacity across the landscape as a raster layer. Accordingly, we constructed a GIS raster layer of likely habitat associations of sambar deer as a surrogate for biological carrying capacity. The spatial extent was limited to eastern Victoria and the resolution of the raster (i.e., grid cell size) was set to 1 km^2. Predictions of habitat suitability for sambar deer were informed by Sotorra *et al.* (2021) and generated by multiplying tree density (scaled between zero and one) with distance to water (again rescaled to be between zero and one). Known observations of sambar deer were plotted over the predictions and indicated good correspondence.

The maximum number of individuals that could occupy a grid cell (i.e., patch) was determined by the predicted habitat suitability for that cell. For the highest suitability values, an upper limit of 5 sambar deer per square kilometre was applied. The relationship between habitat suitability and carrying capacity was described by a logistic function that allowed more individuals in higher quality habitat than in lower quality habitat (Figure 4).

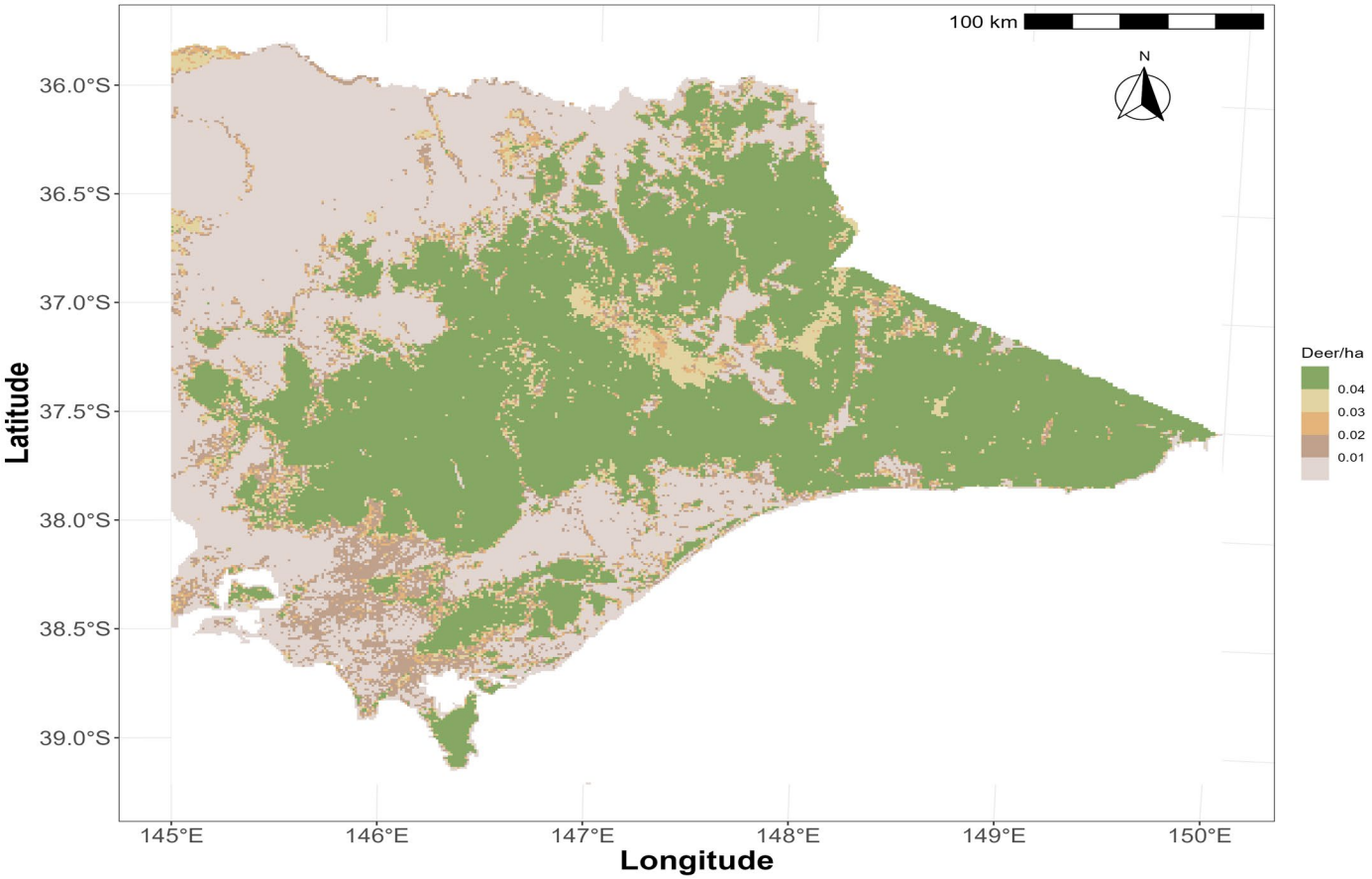

Figure 4. Raster spatial layer describing sambar deer carrying capacity across eastern Victoria. Maximum carrying capacity was set at 5 deer/km^2 (0.05 deer/ha).

Cattle Layer

The model also requires information on cattle numbers. These were derived from spatial layers obtained from Agriculture Victoria on the numbers of beef and dairy herds and their locations, across Victoria. We rasterized this layer using the same 1 km^2 spatial resolution as used for the deer carrying capacity layer with the cell values being equal to the number of beef and dairy cows at that location (Figure 5).

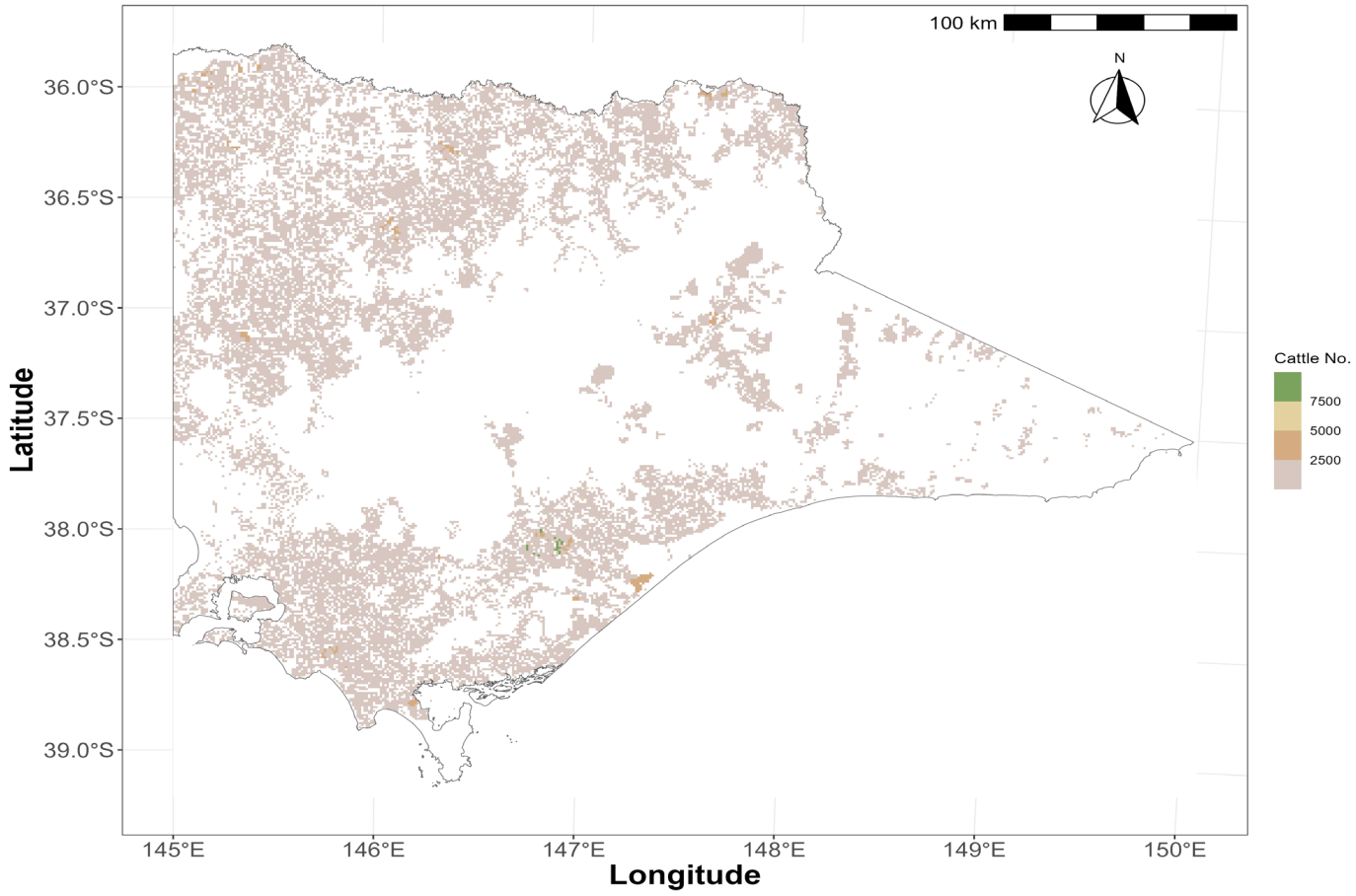

Figure 5. Raster spatial layer describing cattle carrying capacity across eastern Victoria based on data obtained from Agriculture Victoria.

Deer dispersal and FMD epidemiology

The model uses a gamma distribution to simulate dispersal distances. We used dispersal distances obtained from genetic data (Hill *et al.* In review) from the Gippsland population to compute the distribution parameters (mean and shape parameters) using the R package fitdistrplus (Delignette-Muller and Dutang 2015). Similarly, the distance of inter-seasonal movements was modelled by estimating the parameters for a gamma distribution using the data obtained by the estimated animal movements based on the spatially explicit capture recapture analysis described above. Because the model only accepts integer values for these parameters, these were then adjusted accordingly. The maximum life span was set to 20 years (https://www.iucnredlist.org/), and no vertical transmission was assumed.

Forman *et al.* (1974) investigated the shedding of FMDV by infected red, fallow and roe deer (*Capreolus capreolus*) and found that this occurred for a variable time starting shortly after infection, with about half of the fallow deer monitored still shedding the virus 63 days post-infection (mean=40.6). These authors did not detect any statistical differences between the three species. We used these data to estimate the removal rate, α (i.e. in the model, infected deer are removed with a yearly rate α and do not return to be susceptible), by conducting a survival analysis with an exponential model using the R package survival (Therneau and Grambsch 2000), following Ramsey and Cowan (2003).

The model also requires a transmission rate from deer to cattle, β_c. In absence of data on this parameter, we arbitrarily assumed this would be 1 and then scaled this by the probability of at least one encounter between deer and cattle based on the estimated mean of the Poisson distribution from the spatially explicit capture recapture analysis described above (λ_0) using the equation (Royle *et al.* 2013):

$$1 - e^{-\lambda_0}$$

It is important to also note that the model scales this parameter by the 'intensity' of space used by infected individuals, which is determined by the home-range location of infected individuals. Hence, transmission from deer to cattle is governed by the product of the transmission rate defined above, the intensity of home range use by deer in proximity to farmland locations, as well as the cattle stocking rate (see Ramsey *et al.* 2016 for details; Ramsey and Efford 2010). The model assumed a stable home range throughout the simulated years, which is parameterised by providing the standard deviation of a half normal distribution, except if dispersal occurs. We could not find in the literature any quantitative information on home range size in sambar deer in eastern Australia. Within the native distribution, sambar deer showed a highly variable home range size, from 1.8 to 43 km^2 (Chatterjee *et al.* 2014; Kamil *et al.* 2001), with males' home ranges typically larger than those of females. However, our limited data (see Results below) suggested that these may be smaller in Gippsland, a trend that has also been observed in other introduced deer species (Amos *et al.* 2022; Santosa *et al.* 2015; Spaggiari and de Garine-Wichatitsky 2006; Taylor 1971). In fact, our estimates of the area of activity are several fold (10-50) smaller than the home ranges of deer tracked for a similar amount of time (~2 months) (Kamil *et al.* 2001). Because of these considerations and the fact that the model assumed a stable home range throughout the simulated years, we retained the default settings of the model, which were devised for white tail deer (*Odocoileus virginianus*), of home ranges of approximately 0.6 and 1.5 km^2 for females and males, respectively.

Lastly, we modified the deer-to-deer transmission rate, β_d. This parameter was estimated by calibrating the model using the following rationale: deer are susceptible to FMDV, including red, sambar and fallow deer, which are commonly found in the Gippsland area (Cripps *et al.* 2019 and within). For example, after two hours of exposure to infected cows, fallow and red deer acquired the infection and transmitted it to their conspecifics (Gibbs *et al.* 1975). These species produced a concentration of virus comparable to cattle, although they had mild clinical signs (Forman and Gibbs 1974; Gibbs *et al.* 1975). Fallow and red deer continued to excrete FMDV for up two months, similarly to other ungulate species (Forman *et al.* 1974). Furthermore, a roe deer population showed a prevalence of 4.4% (95% CI= 1.5 – 15%, n=68), following an outbreak in wild boar (Alexandrov *et al.* 2013). These studies suggest that the transmission rate of FMDV in deer is not zero. However, the lack of endemic FMD in deer populations or detection during large outbreaks in UK in 2001 (Elbers *et al.* 2003; Keeling *et al.* 2001; Lawman *et al.* 1978; Simpson 2002) suggests that, due to behavioural attributes or low susceptibility, their transmission rates are likely to be lower than in cattle (Brooksby 1968; Gibbs *et al.* 1975). Based on these considerations, we calibrated the values of β_d and selected values that produced a simulated mean prevalence, computed over the last 10 years of 60-year simulations, that included the range 1.5-4.4% (that is, between the lower confidence interval and the mean found by Alexandrov *et al.* (2013)) within their 5^{th} and 95^{th} percentile. This is because we hypothesise that, in agreement with the literature (Gibbs *et al.* 1975; Simpson 2002), deer are not likely to be important hosts for the maintenance of FMDV in the wild (hence the conservative deer transmission rates), but they can readily acquire the infection and are still likely to transmit FMDV before the outbreak dies off within the deer population (Cripps *et al.* 2019; Dhollander *et al.* 2016; Weaver *et al.* 2013). This is particularly relevant in Australia where we demonstrated the intense interaction between deer and cattle. To account for the uncertainty in the transmission rates in our analysis, further simulations to test the baseline and control scenarios were set up by randomly selecting five values within the selected range of β_d combining the results for analysis.

2.4.1 Baseline scenarios – no intervention

Cattle will shed the virus before showing clinical signs when they are infected with FMDV (for up to 8.4 days, Orsel *et al.* 2009). Hence it is likely that deer can acquire the infection before authorities become aware of the FMDV outbreak and any removal of infected cattle could occur. Once the outbreak is identified in cattle, we assumed that authorities' management actions will be prompt and efficient and no further infection of cattle-to-deer will occur. Under these scenarios, we assumed that a variable number of deer (on average: 1, 6, 30, 140, 700, 3560 deer, which is equivalent to about 0.0025%, 0.0125%, 0.063%, 0.31%, 1.56% of the total deer population) will become infected depending on the seriousness of the initial outbreak in cattle and time needed to respond. These scenarios were run for 20 years and constituted our baseline scenarios (i.e. no intervention) that we used as a term of comparison with scenarios where management actions were put in place to specifically reduce the FMDV spillback (deer to cattle).

2.4.2 Management scenarios – deer mitigation actions

The use of deer specific (e.g., 1.9 m) exclusion fences is often considered to mitigate the impact of deer and other invasive species. This tool is effective also within the context being investigated here because it would limit the contact rate between deer and livestock species. We explored the possible effects that deploying deer exclusion fences would have by progressively decreasing the proportion of successful contacts between deer and cattle using a relative cattle contact rate factor (CRF), changing its value from 1 (no reduction in relative contact rate – used in the baseline scenarios) to a range between 0.8 and 0.1 (i.e., relative contact rate reduced between 20% and 90%). Using a similar approach, it is also possible to evaluate the effect of a vaccine. The efficiency of FMDV vaccines has been evaluated to be relatively high in preventing the disease (70%) and infection (63%) (Knight-Jones *et al.* 2014) while serological responses to vaccination have been close to 100% (Park *et al.* 2021).

Lastly, we considered whether the additional removal of 10% of the deer population (for example through increased hunting pressure or culling) will mitigate the impact of an FMDV incursion.

Over the 20 simulated years, we monitored the prevalence of FMDV in the deer population and the number of cells in the landscape that had at least one positive cattle FMD case in each time step (referred to as a breakdown) to infer the potential impact of FMD on the livestock industry.

3 Results

A bushfire occurred during the late summer/early Autumn of 2019 in and around the Gembrook study site, preventing access to the public forested land for this sampling period. However, we still sampled the farmland during this period. We were also prevented from entering the farmland at Willow Grove during the sampling period of Autumn 2020 due to budget restrictions.

3.1 Camera data

There were 24,852 deer detections in the camera data, across the three sites (Fig 6). Of these, the majority were sambar deer (24,588, 98.9%) and 220 (0.9%) were fallow deer. There were also 44 (0.2%) detections where the species could not be confidently determined. About a third of the detections (7,900) had an associated distance class and these were used to compute the detection function. Inspection of the raw detection data suggested a seasonal pattern in which detections in spring were generally higher. Overall, Gembrook had the highest number of detections (11,737, 47%) while Kinglake and Willow Grove had a similar total number of detections (6,919 and 6,196, respectively), although the number of detections varied greatly between seasons and sites. Daily activity patterns did not differ greatly between autumn and spring, displaying a typical crepuscular behaviour (Fig. 7).

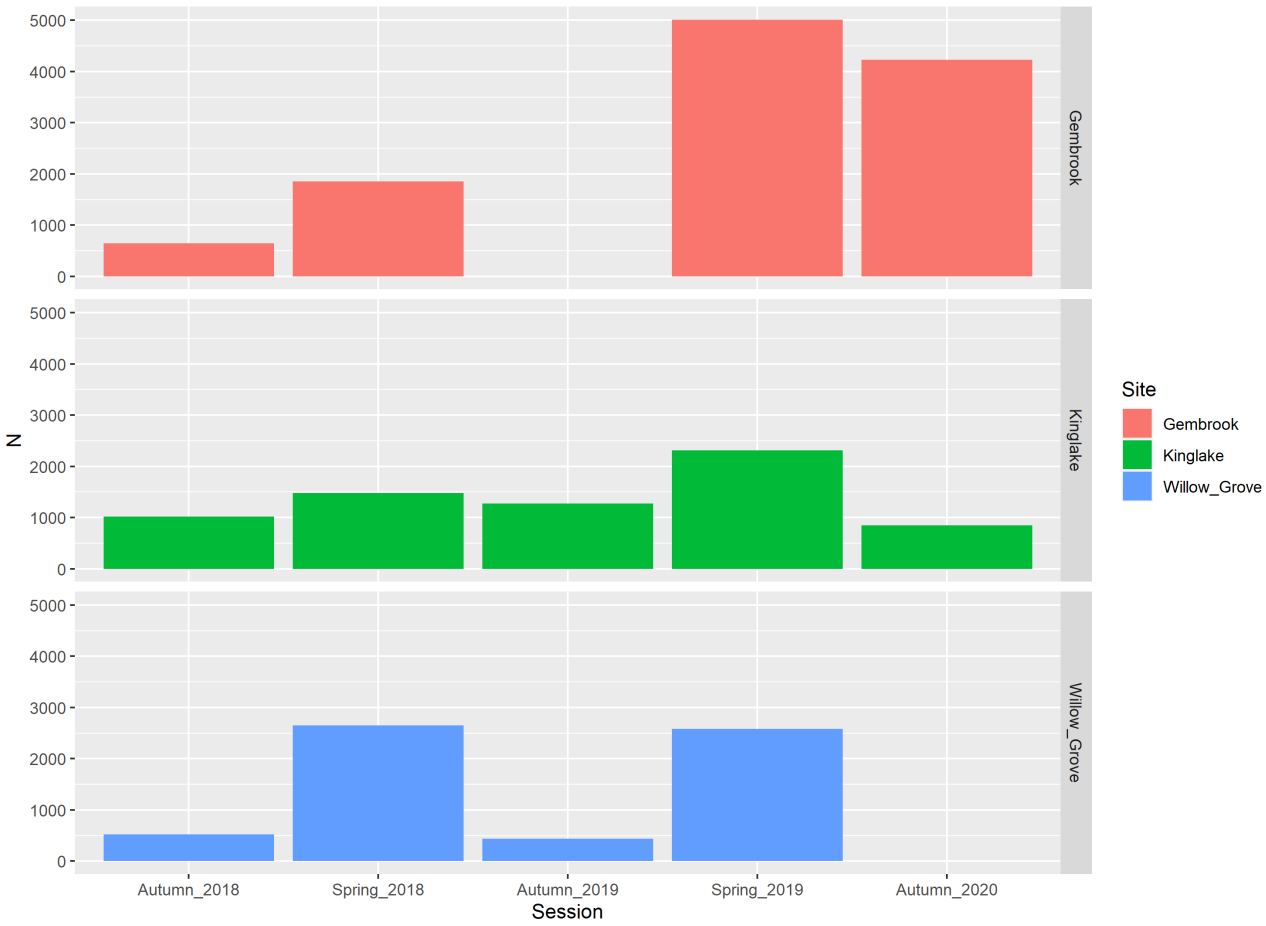

Figure 6. Number of deer detection across the three monitored sites (rows) within each session (y-axis is the sum of the detections over the three survey visits, each about three weeks apart).

No data could be collected in Gembrook in Autumn 2019 due to a bushfire and Autumn 2020 in Willow Grove.

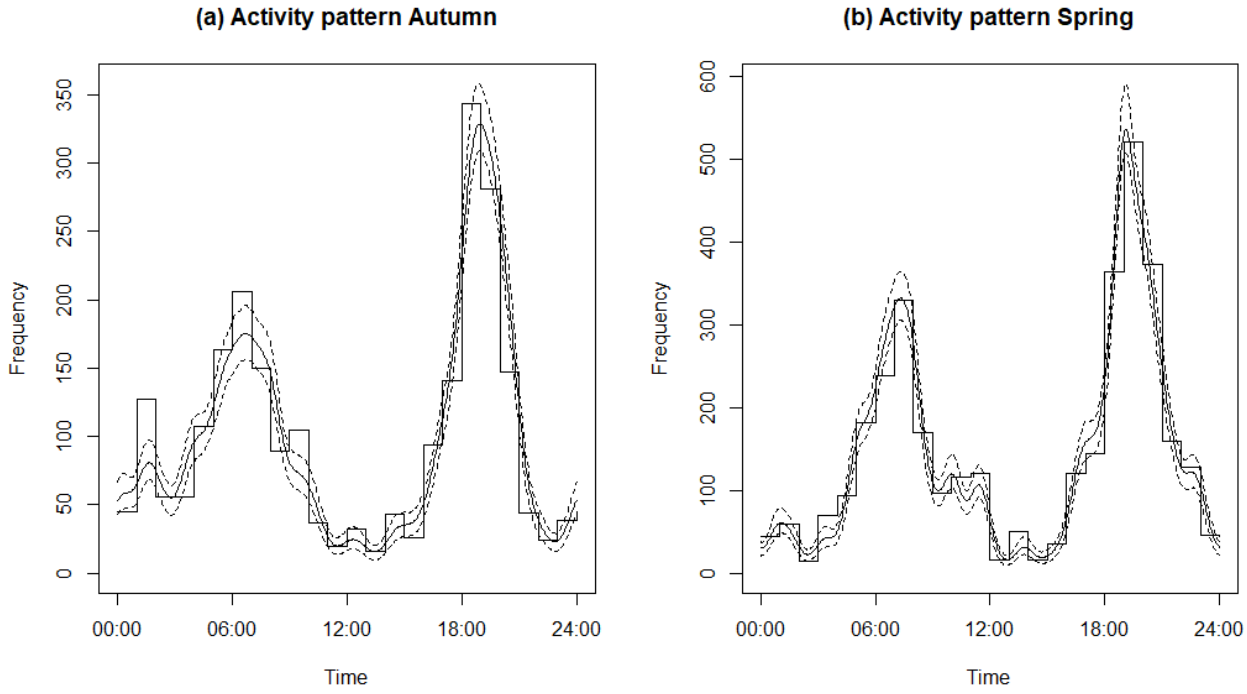

Figure 7. Number of deer detection as a function of time.

The model with a hazard-rate detection function with a third order cosine adjustment was the most supported model and the goodness of fit test indicated a good fit of this model to the data (p=0.335). Density estimates varied between 2 and 14 deer/km^2. As suggested by the raw detection numbers, density estimates for Gembrook were higher than Kinglake and Willow Grove, which in turn were comparable (although Kinglake had marginally lower estimates) (Figure 9).

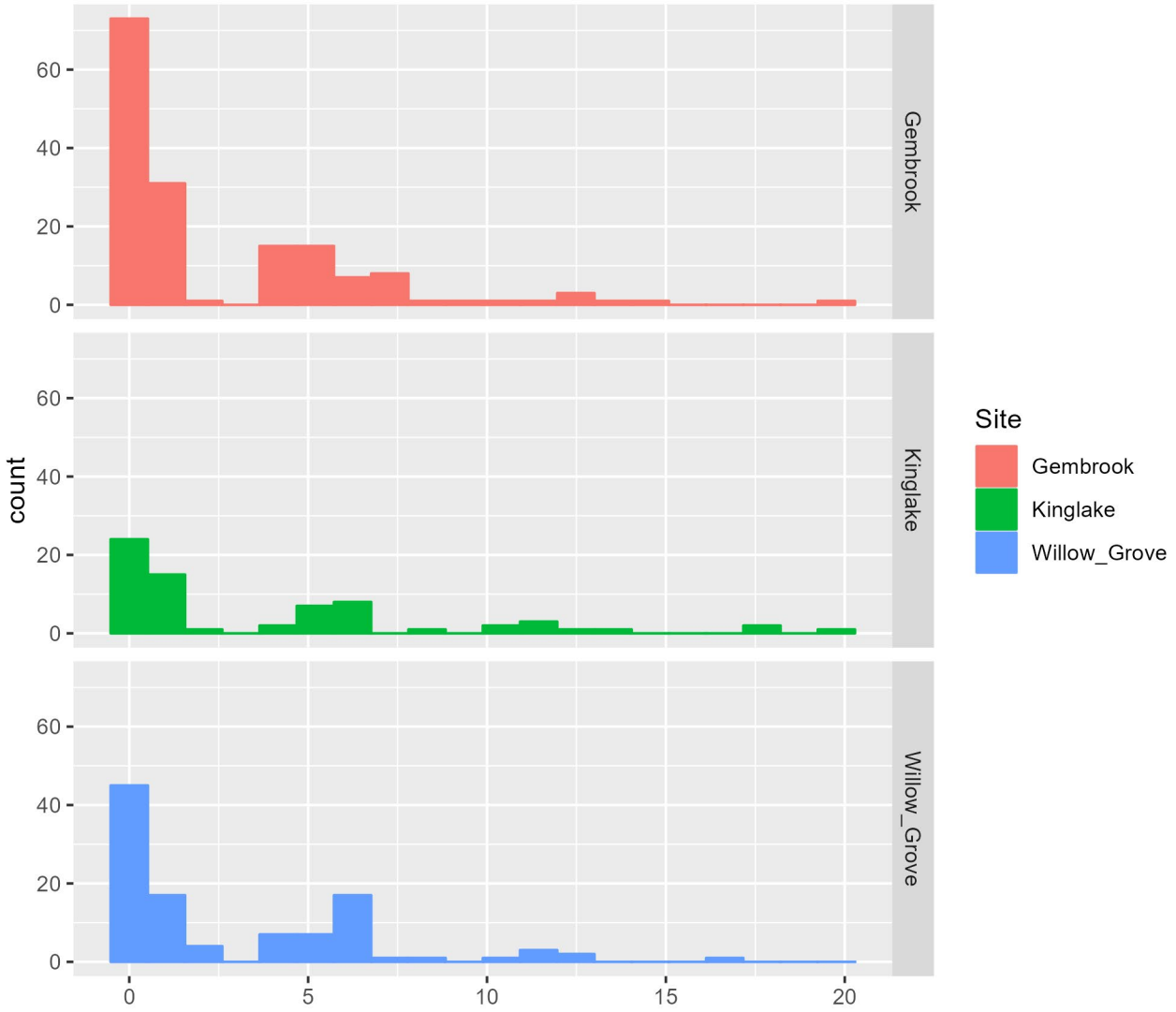

Figure 8. Frequencies of recapture intervals (in months) for the three sites.

3.2 Deer faecal pellet data

Of 1184 samples, we obtained genotypes from 1006 samples and 194 loci (i.e., four loci failed to generate any data). After the filtering steps (described above), we retained 694 samples and 56 loci for analysis. Gembrook was the site with the highest number of successfully genotyped samples, n=292, of which 154 were collected from the forest. On the contrary, Kinglake was the site with the lowest sample count, n=184. Samples were roughly equally distributed between the forest and the paddocks (93 and 91, respectively). We genotyped 218 samples from Willow Grove and, in this site, most of the samples were collected in the forest (n=147). From these data, we identified 269 unique individuals (assuming a 3.5% error rate) across all three sites. We detected nine individuals (3.4%) moving across sites during the study. These movements always involved movements between Willow Grove and Gembrook or Gembrook and Kinglake (approximate range 40 to 60 km). The shortest time interval between detections at different sites was 7-8 weeks apart. These detections involved two individuals that were first identified in Gembrook in Autumn 2019. Both animals were then detected in Kinglake (~60 km). The next shortest time interval was 4-5 months, where two further individuals were first detected in Willow Grove and then in Gembrook. All other movements (n=5) had >11 months time interval (up to 25 months as maximum) and all were from Willow Grove to Gembrook but one that was from Kinglake to Gembrook.

There were a total of 425 recaptures that involved 137 individuals. The overall mean recapture rate was 2.6 (range=1 – 18). The distribution of time intervals between recaptures within the same location was similar across the three sites (Fig. 8). The majority of the recaptures were within the same session and only 79 individuals were recaptured in different sessions. The majority of these were in successive sessions (i.e. approximately six months apart), with only a few with longer intervals, up to a maximum of 20 months. About 17% of individuals were detected in both forest and paddocks in the same site, 58% only in the forest and 25% only in the paddocks (note that individuals detected in multiple sites were counted multiple times).

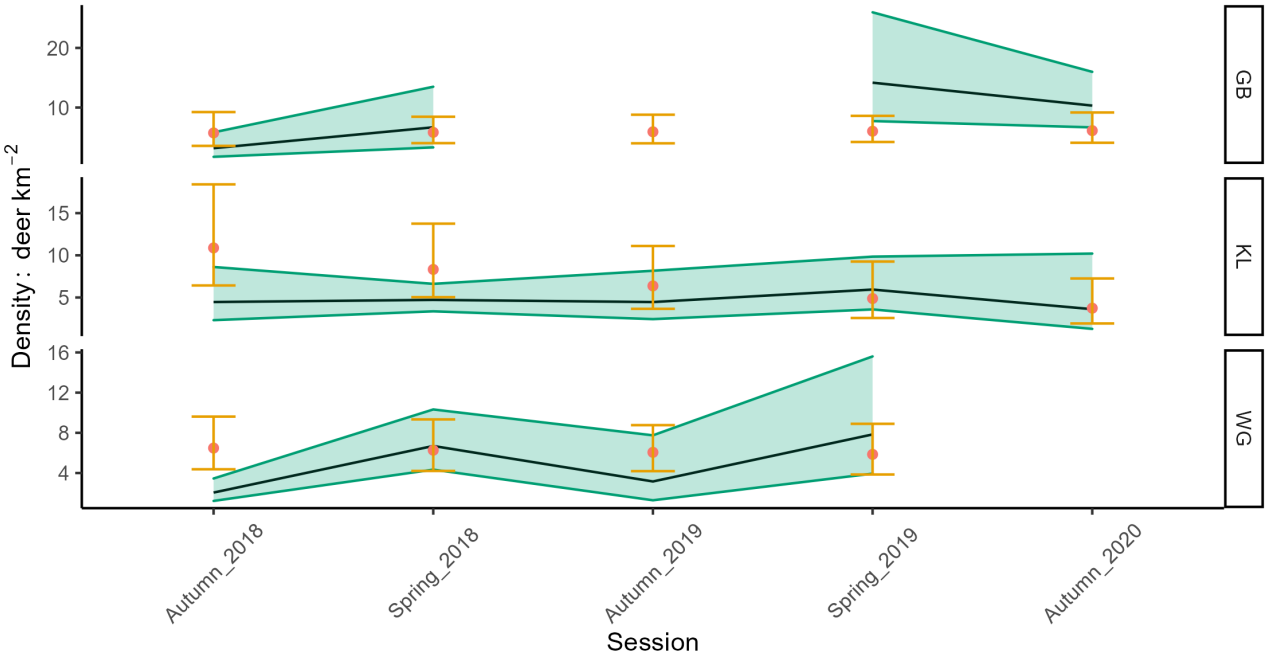

Figure 9. Density estimates for each site (rows) for each session based on camera data (lines showing the mean and shaded areas the 95% confidence intervals) and deer faecal pellet sampling (dot showing the mean and error bar the 95% confidence intervals). GB=Gembrook, KL=Kinglake and WG=Willow Grove.

When the different statistical models were compared, consistently across all sites, the most supported model was with a hazard rate detection function, where the detection rate was different between the paddock and forest and with a bivariate normal kernel density function to model animal dispersal. Season was retained as a covariate on the spatial parameter and the bivariate density function for animal movements.

Density estimates were between 4 and 10 deer/km^2 (Fig 9, Table 1). Within each site, differences between seasons in density estimates were not as accentuated as observed in the camera data. A clear declining trend in density was evident in Kinglake, which was also supported by the fact that this site was the only one where the growth rate (λ) was <1 and its confidence intervals did not include 1 (Table 2). The density estimates for 2018 for this site were very high, at about 10 deer/km^2, but towards the end of the study, it was close to the lower end of the range estimated for the other sites. On the other hand, the other two sites had comparable density estimates and, interestingly, density estimates in Gembrook in autumn 2019 were not different from other sessions, even though, in that session, a bushfire prevented access to the forest and the samples could only be collected in the paddock. The mean detection rate was generally higher in the paddocks, although the lower limit of the confidence intervals for the estimates in the paddocks marginally overlapped with the upper limit in the forest (Table 2 estimates). The only exception was Willow Grove where it seems that, on average, the detection rate was greater in the paddock. However, the confidence intervals around the detection rate on this site were very wide. Depending on the site, there was between 20 to 35% probability of encountering a deer in the paddock once, and between 5 to 10% twice (Fig. 10). The parameters f, φ and z were consistently estimated across sites. Similarly, the spatial parameter, δ, was comparable across sites and seasons (although it was marginally greater during spring in Gembrook) (Table 3). The parameter of the bivariate normal distribution governing deer movements, α, was generally larger in

spring in all sites, and in Gembrook the mean estimates were higher than in other sites, although confidence intervals overlapped (Table 3). From this parameter, it is possible to compute the 95% upper limit of deer movements, which was in the range of 0.5 – 1.5 km (Fig. 11).

Table 1. Summary of density estimates (95% confidence intervals)/km^2, number of individual deer detected (n) in each session and number of recaptures (m).

Site	Session	Season	n	m	Density
Gembrook	Autumn_2018	A	26	0	5.74 (3.57 - 9.24)
Gembrook	Spring_2018	S	39	19	5.84 (4.03 - 8.46)
Gembrook	Autumn_2019	A	33	8	5.93 (4 - 8.8)
Gembrook	Spring_2019	S	35	17	6.03 (4.22 - 8.6)
Gembrook	Autumn_2020	A	27	11	6.13 (4.09 - 9.17)
Kinglake	Autumn_2018	A	30	0	10.88 (6.43 - 18.41)
Kinglake	Spring_2018	S	29	5	8.33 (5.04 - 13.75)
Kinglake	Autumn_2019	A	33	10	6.37 (3.66 - 11.11)
Kinglake	Spring_2019	S	19	9	4.88 (2.57 - 9.26)
Kinglake	Autumn_2020	A	5	3	3.73 (1.92 - 7.26)
Willow Grove	Autumn_2018	A	34	0	6.48 (4.37 - 9.61)
Willow Grove	Spring_2018	S	31	14	6.27 (4.21 - 9.34)
Willow Grove	Autumn_2019	A	32	12	6.06 (4.19 - 8.77)
Willow Grove	Spring_2019	S	26	11	5.86 (3.85 - 8.9)

A=Autumn; S=Spring

Table 2. Parameter estimates (95% confidence intervals) for each sampling site.

Site	Gembrook	Kinglake	Willow Grove
λ_0(forest)	0.11 (0.06 - 0.19)	0.2 (0.09 - 0.46)	0.6 (0.25 - 1.41)
λ_0(paddock)	0.3 (0.17 - 0.53)	0.76 (0.34 - 1.68)	0.38 (0.14 - 1.02)
f	0.26 (0.15 - 0.46)	0.17 (0.06 - 0.47)	0.27 (0.15 - 0.49)
λ	1.02 (0.91 - 1.14)	0.77 (0.67 - 0.87)	0.97 (0.83 - 1.12)
φ	0.76 (0.61 - 0.86)	0.6 (0.42 - 0.75)	0.7 (0.54 - 0.82)
z	1.86 (1.64 - 2.11)	1.9 (1.65 - 2.19)	2.03 (1.79 - 2.31)

λ_0=Expected deer detection (encounter) rate at distance 0 (in the forest and in the paddock); f=per capita recruitment rate; λ=growth rate; φ =survival; z=shape parameter of the hazard rate detection function.

Table 3. Parameter estimates (95% confidence intervals) for each sampling site for each season.

Parameter	Season	Gembrook	Kinglake	Willow Grove
δ	Autumn	46.71 (29.97 - 72.78)	33.5 (17.64 - 63.62)	41.98 (21.88 - 80.56)
δ	Spring	81.99 (54.15 - 124.14)	31.24 (16.5 - 59.15)	29.88 (15.05 - 59.33)
α	Autumn	377.43 (263.36 - 540.89)	95.4 (18.71 - 486.35)	95.4 (18.71 - 486.35)
α	Spring	764.67 (477.31 - 1225.03)	221.82 (70.52 - 697.73)	228.89 (107.36 - 487.99)

δ=spatial parameter; α=Movement parameter for the bivariate normal distribution of animal movements

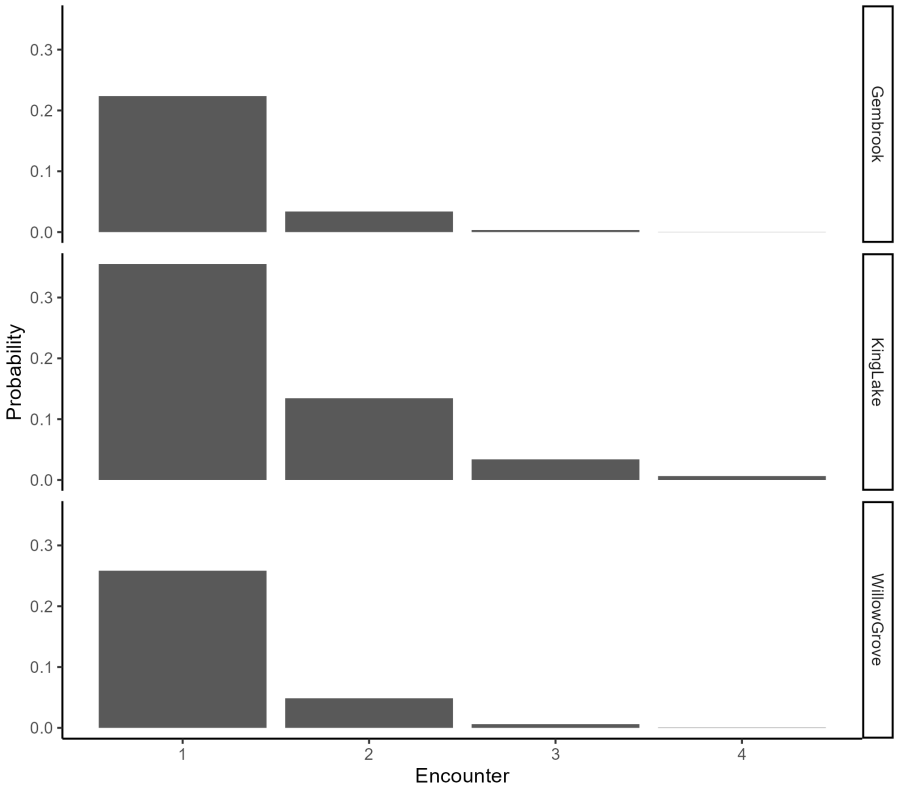

Figure 10. Probability of encountering an individual deer in the paddock 1 to 4 times at each site, in each session.

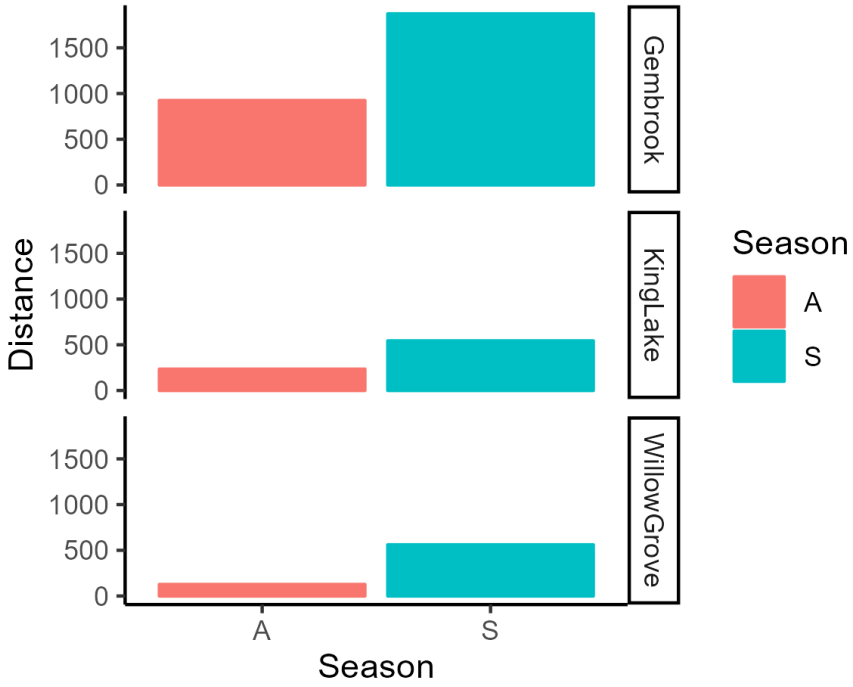

Figure 11. Plot of the 95% upper limit of deer distance (in m) moved between sessions (approximately six months) based on the assumed bivariate normal animal movement model.

3.3 Epidemiological modelling

Calibration of the model indicated 0.1-0.112 as a suitable range of values for the deer-to-deer transmission rate parameter, β_d (Fig 12) and this range was used to randomly draw values for further simulations.

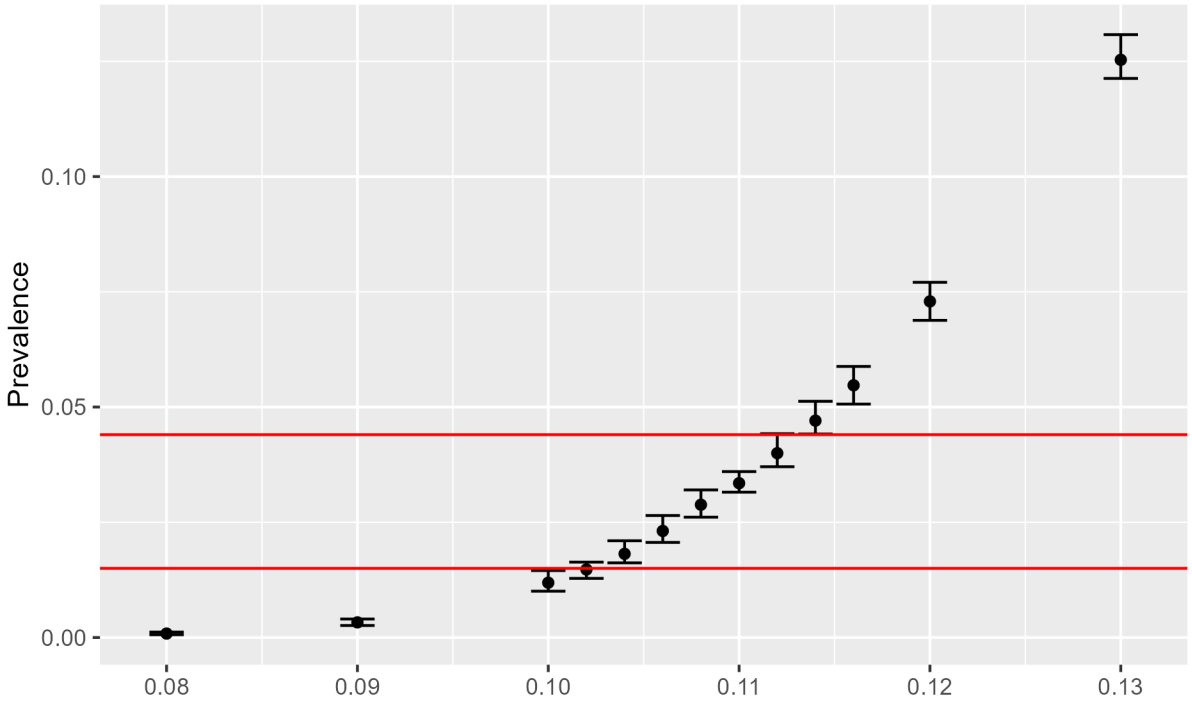

Figure 12. Plot of the deer-to-deer transmission parameter (β_d, x-axis) values used to calibrate the epidemiological model and corresponding prevalence estimated by the model (y-axis).

3.3.1 Baseline scenarios – no intervention

When the simulations were initialised with a relatively low number of deer, the outbreak within the deer population was relatively negligible (Fig. 13). While the mean prevalence in deer was relatively stable, there was a high level of uncertainty around these estimates, and only when the initial number of deer infected was 700 or more, we considered the outbreak in the deer population serious enough to deserve attention. In fact, in these conditions, there could be >200 breakdowns per year (Fig 14).

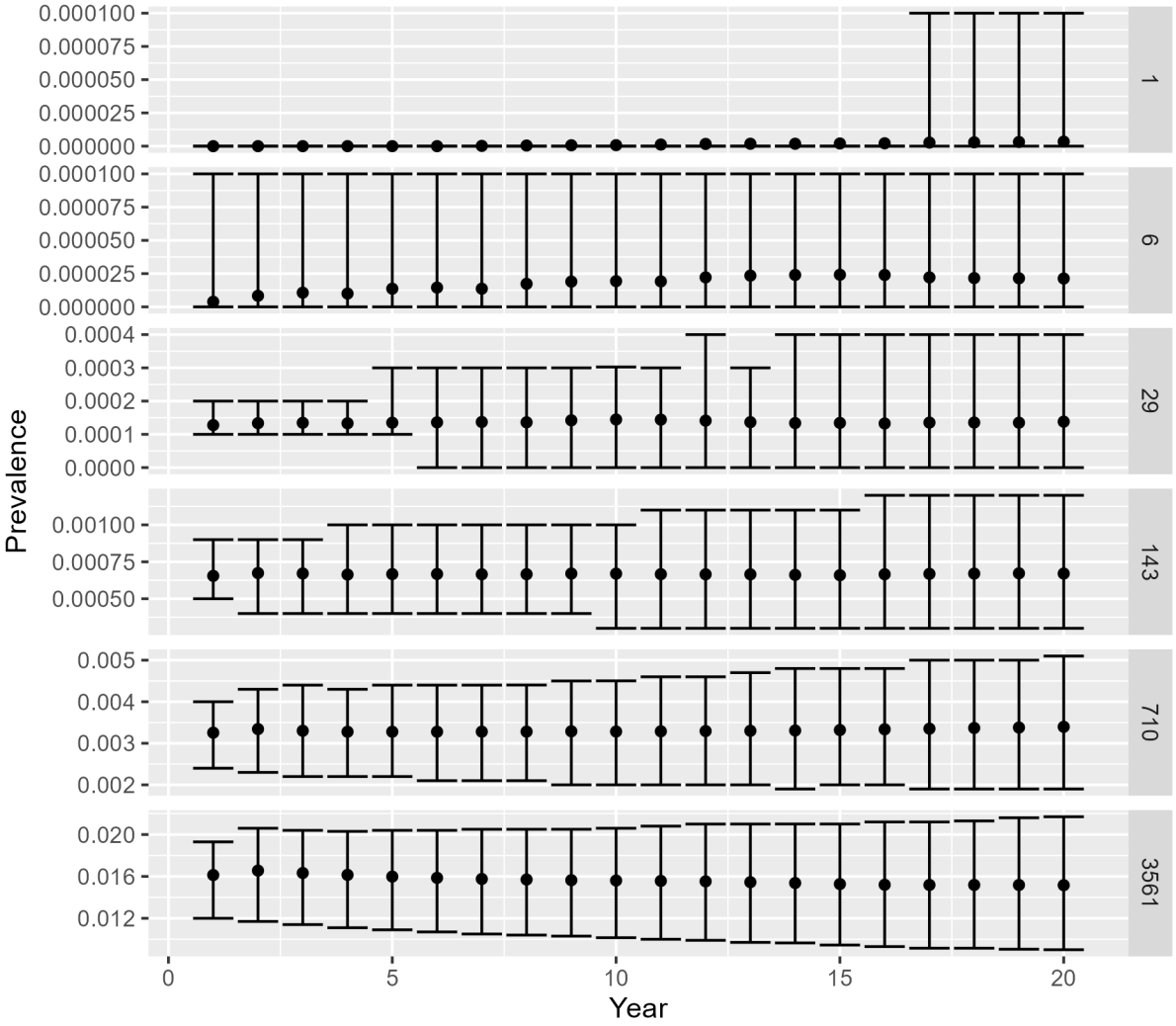

Figure 13. Plots of mean prevalence in deer population (y-axis, note the different scale between plots), with 95% CI, as a function of time (Year). Each row was initialised with a different initial number of infected deer (summarised in the labels on the right-hand side).

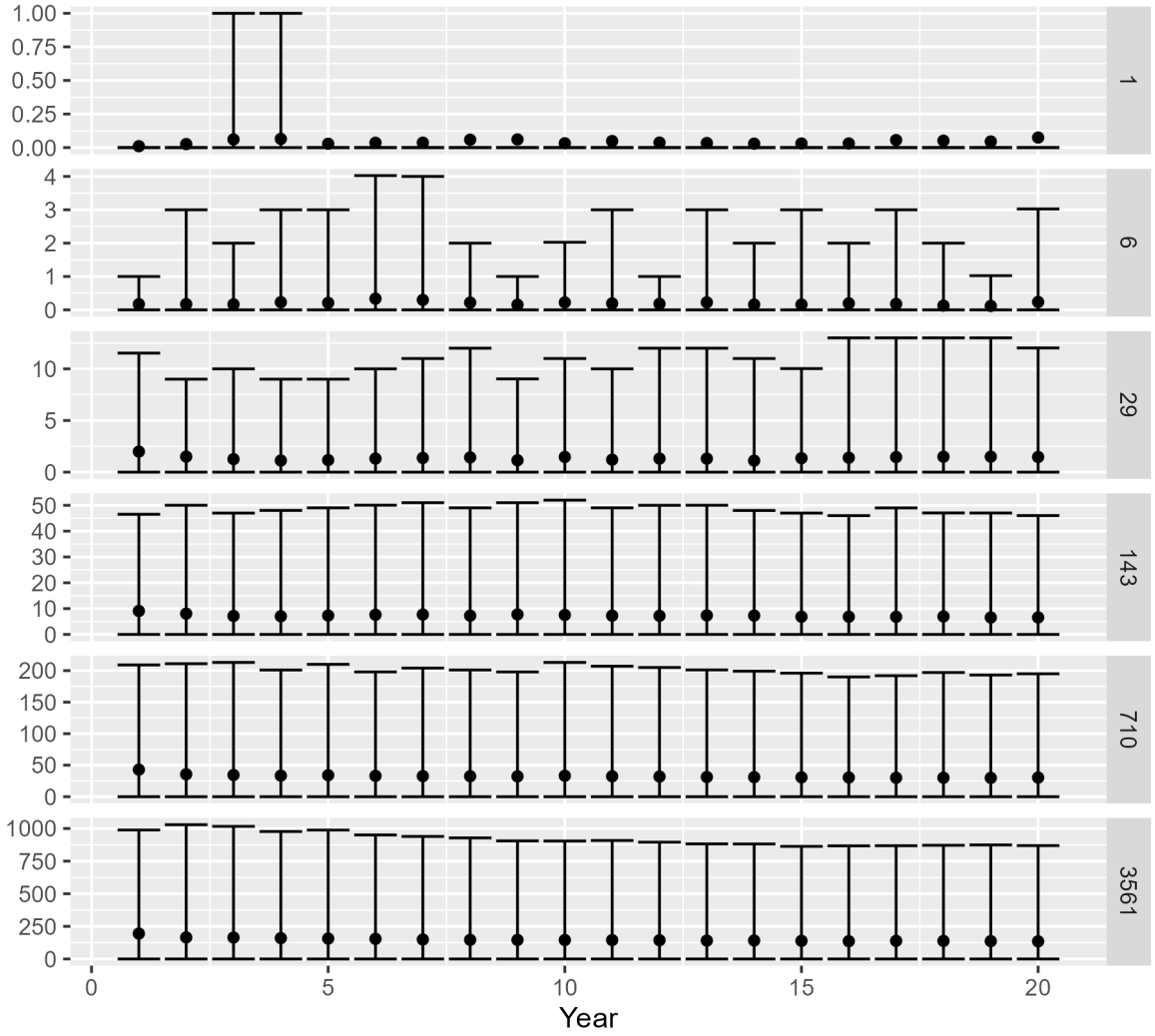

Figure 14. Plots of the mean number of breakdowns (y-axis, note the different scale between plots), with 95% CI as a function of time (Year). Each row was initialised with a different initial number of deer infected (summarised in the labels on the right-hand side).

3.3.2 Management scenarios – deer mitigation actions

In our simulations, there is an almost linear relationship between the relative reduction in the contact rate between cattle and deer (CRF) and the number of breakdowns (Fig 15), with a reduction of about 50% of the latter when CRF=0.5.

Additional removal of deer through culling or hunting did not appreciably change either the prevalence of FMD in the deer population or the number of breakdowns (data not shown).

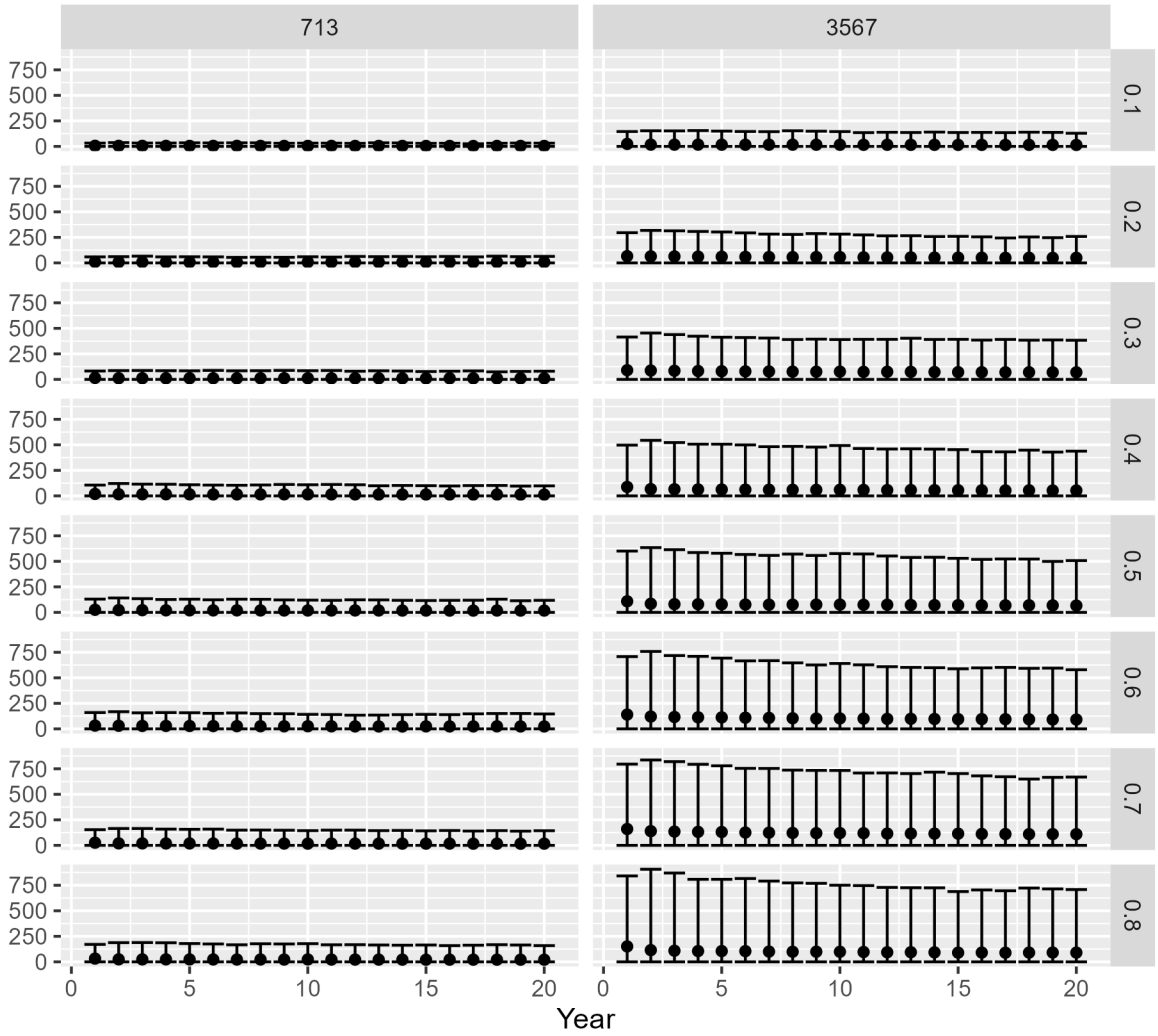

Figure 15. Plots of the mean number of breakdowns (y-axis), with 95% CI as a function of time (Year). Each panels shows simulations initialised with a different initial number of deer infected (summarised in the labels at the top) and each row different proportional reduction of the Contact Rate Factor (summarised in the labels on the right-hand side).

4 Discussion

Estimates of deer densities were broadly consistent between camera and deer faecal pellet data, with only minor differences. The faecal pellet data identified a decline in deer density at the Kinglake site, which was not apparent in the camera data. The reasons for this are currently unknown, but it is possible that local disturbance (e.g., fire tracks maintenance with heavy machinery or activity in the neighbouring timber plantation) or recreational hunting (a few carcasses were observed in the field towards the end of the study) coupled with control activities in nearby areas may have played a role. The only density estimate where the confidence intervals between the camera and the capture mark-recapture estimates did not overlap was in autumn 2018 in Willow Grove. Interestingly, the severe bushfire near Gembrook did not appear to have influenced deer activity as density estimates immediately after the fire (autumn 2019) and about six months later were not different from the densities in the year before and after. Camera data could have suggested a marginal increase post-fire, but this may depend on an increase in deer activity in the forest as it recovers from the bushfire. It is likely that deer were easily able to move away from the fire as they are highly mobile and connectivity to large areas of bushland habitat is high around this site. They may also have concentrated in the unburnt areas after the fire, but their overall density remained unchanged.

In addition to the deer density, most of the other model parameter estimates were similar across sites including deer survival, per capita recruitment and growth rate. The bivariate normal distribution model was also consistently the most supported as the animal movement model across all sites, and similarly, the spatial and movement parameters were higher in spring in all sites suggesting a seasonal effect on deer behaviour. These results are consistent with previous work that demonstrated a shift of habitat use between seasons in Victoria (Comte *et al.* 2022; Ramsey 2022). This is relevant also from a biosecurity risk standpoint because, in case of an outbreak during the warming months, it is likely that deer will be able to spread the disease across a wider geographical area.

The deer detection rate was higher in the paddock in all sites except for Willow Grove. This is probably related to the fact that deer pellets were more visible on pasture than in the forest understorey. Our results clearly demonstrated that deer readily used paddocks as foraging ground and there was a high probability of interaction with livestock species. In fact, the probability that an individual deer would be detected in the paddock was between 26% and 53%.

Activity pattens of sambar deer were highest in the late evening, with another spike in activity in the early morning. This is in accordance with other studies (Comte *et al.* 2022; Davies *et al.* 2020) but provides little effective information for mitigating cattle to deer interactions, other than the unlikely possibility of moving cattle away from the farm/forest boundary during these times.

Deer movements between sites are perfectly in line with those estimated using genetic analysis. In fact, Hill *et al.* (In review) estimated that the majority of long distance movements would occur within 20 km and, based on the fitted model on sambar deer distances in Gippsland, only about 3% of individuals would be expected to move beyond 40 km (1.2% between 40 and 60 km). The minimal distance between our sampling sites was about 40 km, so we could not quantify the proportion of movements that occurred at a shorter distance, but the alignment with the results of Hill *et al.* (In review) for the proportion of individuals that moved >40 km (3.4%) was remarkable.

In our epidemiological model, the prevalence of the simulated FMDV infection in the deer population in the baseline scenarios was at very low levels. However, as the simulations were initialised with a higher number of infected deer, a substantial number of breakdowns was recorded, demonstrating how, even with such a low transmission rate, deer can be important in the epidemiology of this disease within the Australian context. While we focused our attention here on a disease that is mostly transmitted from cattle, and that would likely be promptly detected by the livestock industry, our results also highlight the importance of early detection of infections in deer when the relevant pathogen may become endemic in wild deer populations.

Our results showed that fences offer limited protection at the landscape scale unless they can guarantee a high level of exclusion. It is possible that these may be effective at a local scale where they can be maintained and the initial investment is reduced. However, we argue that livestock vaccines may be more efficient in that they provide a reduction of infections of about 70%. This would generate a comparable reduction in the number of breakdowns to that obtained by a deer-specific exclusion fencing but with a much more attainable initial investment and no ongoing maintenance costs. Where an outbreak occurs despite vaccination, a proportion of these would be expected to be asymptomatic, and therefore cause minimal impact on production unless authorities implement an eradication strategy that requires the destruction of infected individuals regardless of the clinical status. Hunting or further deer culling does not seem to provide an adequate reduction of breakdowns at the rate tested here. While it seems unlikely that a much higher removal rate can be achieved on private land at such a large scale, we acknowledge that we did not test explicitly for spatially strategic asset protection management actions. Similarly, we did not explore the possible source-sink effect that could occur on private land when deer are removed from public land (e.g., selected national parks) nor the removal rate that would be required for such an effect to be detected. These additional strategies should be the focus of further studies.

4.1 Conclusion

Our results demonstrate that deer readily use pasture and interact with livestock in areas where deer distributions and agricultural sites overlap, posing a biosecurity risk for the primary industry. This risk is higher in warmer months when movement patterns seem to be across larger areas and therefore likely to increase the geographical spread of infectious diseases. Even with a limited transmission rate, as is likely the case for FMDV implemented in our modelling, deer may play a role in the epidemiology of this disease and have an impact on the livestock industry. For diseases that have a higher transmission rate within deer and between deer and livestock species, these impacts are likely to be much higher than that showed here. In the instances where livestock vaccination can prevent infections, this is likely to be a more efficient option to minimise economic losses than deer-proof fencing, but in absence of these, high intensity control or extensive fencing would be required to limit infection in livestock. Low rates of deer removal (e.g., through

recreational hunting) do not appear to be sufficient to limit the spread of outbreaks and should not be relied upon to prevent disease spread.

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APPENDIX 2. VIRAL AND PARASITIC INFECTIONS OF WILD DEER IN AUSTRALIA – A REVIEW AND UPDATE

SUMMARY

We review the information on the infectious status of deer populations in Australia, comparing recent studies to previous research conducted about 40 years ago and considering them in a broader international context. Finally, we identify the areas of future research and describe what potential role deer may have in a changing environment under the One Health approach.

VIRAL AND PARASITIC INFECTIONS OF WILD DEER IN AUSTRALIA – A REVIEW AND UPDATE

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ABSTRACT

Wild animals harbour a wide pathogen diversity. Research in Europe and North America has demonstrated that deer can act as reservoirs for viral, prion, bacterial and parasitic infections. Wild deer often feed on agricultural land, therefore representing a biosecurity risk due to their potential ability to transmit diseases to livestock. Many studies on the infection status of wild deer in Australia date back 40 years, but deer populations have increased significantly in abundance and distribution in recent decades. These studies are not without their limitations in terms of sample size, deer species sampled, and the detection methods utilised. However, collectively, they represent an important data source for understanding the pathogens carried by wild deer in Australia. Recent investigations using ELISA, PCR-based assays and next-generation sequencing have substantially increased our understanding of viral and parasitic infections in Australian deer. These studies indicate that deer may act as reservoirs for pathogens such as Pestivirus, *Neospora caninum and Entamoeba bovis.* The use of next-generation sequencing has led to the discovery of novel viruses such as Picobirnavirus and a novel species of the genus *Bopivirus*, both with transmission risks for domestic animals. Recent research confirms that deer could be a future source of viral infections for domestic livestock and other wildlife species.

Keywords: One Health, infectious disease, invasive species, pest control, wildlife management.

INTRODUCTION

Several infectious diseases have emerged or re-emerged in recent decades, raising questions about their pathogenesis and epidemiology (Barroso *et al.* 2021; McClymont *et al.* 2022; Rhyan and Spraker 2010). Wildlife populations are the most significant source of emergent infectious diseases that impact human health, biodiversity, and agriculture (Morner *et al.* 2002). As a result, pathogen transmission between wildlife and livestock is a global concern (Rhyan and Spraker 2010). Of particular concern in wildlife health are pathogens that do not exhibit host specificity or can infect host species across different taxa. In this context, it is estimated that 77% of pathogens detected in livestock can infect multiple wild and domestic species (Cleaveland *et al.* 2001). Wild animals typically carry more pathogens than are present in domestic animals (Walker *et al.* 2017), and these pathogens do not need to persist for an extended period within a wildlife population for transmission to livestock to occur (Morgan *et al.* 2006).

Due to altered landscapes or the introduction of non-native species, changes in wildlife demographics lead to new interfaces between livestock and wildlife, potentially exacerbating processes that promote pathogen transmission (Miller *et al.* 2013; Rhyan and Spraker 2010). Since deer are ruminants and closely related to economically important livestock species such as cattle, sheep, and goats, it is not surprising that they share many pathogens, including several of major agricultural significance. The role of wild deer as a source of infection for livestock has been mostly documented and reviewed in Europe and North America (Böhm *et al.* 2007; Conner *et al.* 2008; Martin *et al.* 2011; Ruiz-Fons *et al.* 2014), where increasing wildlife-livestock interaction raises the need for monitoring wildlife diseases.

Deer are not native to Australia and were introduced at different time points in the early nineteenth century from Europe and Asia (Bentley 1998; Hall and Gill 2005). Six species – chital (*Axis axis*), fallow (*Dama dama*), hog (*Axis porcinus*), red (*Cervus elaphus*), rusa (*Rusa timorensis*), and sambar (*Cervus unicolor*) deer – have established self-sustaining wild herds in Australian habitats (Bentley 1998; Moriarty 2004b). Wild deer populations continue to increase in number and distribution in mainland Australia and Tasmania (Cunningham *et al.* 2022; Davis *et al.* 2016; Moriarty 2004b). Wild deer are particularly widespread and abundant in south-eastern Australia, where population densities of up to 39 per km^2 occur in large agricultural regions (Bengsen *et al.* 2022). Bioclimatic modelling, which matches animal species' requirements to suitable environments, suggests that deer currently occupy only a fraction of their potential distribution in Australia (Davis *et al.* 2016; Moriarty 2004b).

Australia is currently free of many animal pathogens detected worldwide, and exotic diseases remain a major threat to Australia's livestock industry as well as to human and wildlife health. Therefore, monitoring the presence of pathogens in wildlife is crucial to identifying potential reservoirs of infectious diseases and preventing future disease outbreaks. As reviewed elsewhere (Böhm *et al.* 2007; Conner *et al.* 2008; Cripps *et al.* 2019; Martin *et al.* 2011; Ruiz-Fons *et al.* 2014), wild deer can act as reservoirs for various livestock diseases. Wild deer pose a biosecurity threat to the livestock industry as deer populations rise because they commonly feed on agricultural landscapes, and the deer-livestock contact rate increases (Fig. 1). This fact may hamper current preventive measures. However, data about the infection status of Australian wild deer populations are sparse and have been predominantly collected with a focus on viral and parasitic infections. These data were first collected in a handful of limited surveys performed in small geographic areas in the 1960s and 1980s (English 1982; McKenzie *et al.* 1985; Munday 1966; Munday 1972; Presidente and Westbury 1979; Slee and Presidente 1981), with a small number of larger studies being performed in the last few years across a larger geographical area (Davies 2014; Huaman *et al.* 2020; Huaman *et al.* 2022a; Huaman *et al.* 2021a; Huaman *et al.* 2022b; Huaman *et al.* 2021b; Huaman *et al.* 2021c; Jenkins *et al.* 2020; Koehler *et al.* 2016; Lamb *et al.* 2021; Panozzo 2018).

Here, we summarise the data available for the prevalence of both viral and parasitic infections in wild deer in Australia. We also highlight how advanced genomic techniques have increased our understanding of wild deer diseases. The present review aimed to i) review current knowledge of deer infection in Australia; ii) examine how this compares to deer worldwide/outside Australia, and iii) provide implications for wild and domestic animal populations and humans.

VIRAL DISEASES IN DEER

There are many important viral infections of livestock in Australia, and viral infection of deer has been examined across multiple decades in many studies across Australia. All six deer species have been assessed for various viral infections; however, most data are available for fallow deer. The first study performed on Australia's wild deer was published in 1966 (Munday 1966) and assessed wild fallow deer in Tasmania for the presence of Bovine Herpesvirus (BoHV-1) and *Pestivirus*. The study demonstrated a complete absence of BoHV-1, which was later confirmed in other small geographical studies, as well as a more recent study across large areas of eastern Australia (Table 1, (English 1982; Huaman *et al.* 2020; McKenzie *et al.* 1985; Munday 1966; Munday 1972; Presidente and Westbury 1979)). These findings perhaps indicate that Australian deer are not carriers of BoHV-1, despite the high levels of this virus in cattle in Australia and the close contact between cattle and wild deer in many locations across Australia (Gu and Kirkland 2008). Interestingly, BoHV-1 has been confirmed to experimentally infect multiple deer species (Chow and Davis 1964; Mollema *et al.* 2005) and has also been detected serologically in deer in Poland with prevalence rates below 5%, as well as in deer in Hungary with much higher prevalence rates ranging from 12% to 47% via PCR detection and subsequent sequencing (Fabisiak *et al.* 2018; Kalman and Egyed 2005). All studies involving Australian deer were conducted using serological tests, and it may be that this detection method in deer is less sensitive than PCR; however, further work is needed to confirm this.

Pestivirus infection is widespread in Australian cattle (Reichel 2000; Scharnbock *et al.* 2018), and past studies of exposure in different deer species in Australia have been conducted across small geographical areas, with more recent studies encompassing larger land areas (Table 1). All these studies have found varying but consistently low prevalence. Seropositive fallow deer were first reported in a small study in Tasmania with a prevalence of 14.5% (Munday 1966), and a decade later in another small study in NSW with a prevalence of 1.2% (English 1982). A larger study assessing *Pestivirus* prevalence in red deer sourced from 20 localities in eastern Australia in 1985 found a 4% prevalence, and a recent (2018 − 2022) study sourcing five deer species from locations in eastern Australia reported a similar prevalence of 3% for *Pestivirus* antibodies (Huaman *et al.* 2020). These findings are consistent with the low seroprevalence of deer species globally for *Pestivirus* (reviewed in (Passler *et al.* 2016)). Coupled with the high seropositive rates in Australian cattle and the low ability of experimentally infected deer with *Pestivirus* to shed the virus or display clinical symptoms (reviewed in (Passler *et al.* 2016)), this is consistent with deer acting as an accidental spillover host for *Pestivirus*.

Many significant viral infections of Australian livestock are carried by mosquitoes, termed arboviruses. As they are vector-borne viruses, their occurrence is mainly driven by the effects of temperature and rainfall, with distinct geographical areas showing changes in the seasonal incidence of important livestock-associated arboviruses (Geoghegan *et al.* 2014). The ability of wild deer in Australia to carry varying arboviruses has been assessed across multiple studies and locations over the last four decades, with some evidence that deer may become infected with agriculturally significant arboviruses including Bluetongue virus, Bovine Akabane (A Simbu group virus), Ephemeral Fever Virus (BEFV) and Epizootic Haemorrhagic Disease Virus (EHDV) (McKenzie *et al.* 1985; Moriarty 2004a). These observations have mainly come from one large study conducted across Queensland on captured red deer in 1985; where 43% of deer were seropositive for BEFV; 19 − 50% seropositive for one of five strains of EHDV, and 13% seropositive for Akabane virus (McKenzie *et al.* 1985). These results were supported again in 2004 in a small study from NSW, where seropositive outcomes were seen in rusa deer for Akabane virus and EHDV. More recently, a large-scale PCR-based screening study of deer blood samples collected from eastern Australia revealed no acute infection for a range of vector-borne diseases (Huaman *et al.* 2020; Huaman *et al.* 2021a). These include three viruses (BEFV, EHDV and Akabane). However, considering i) endemicity of these pathogens in eastern Australia, ii) the presence of suitable vectors, and iii) expected increases in the distribution and abundance of deer (in the absence of substantial control efforts) (Cunningham *et al.* 2022; Davis *et al.* 2016), the possibility that deer species could be a future source of infection of these pathogens cannot be ruled out.

Through next-generation sequencing (NGS), novel viruses were identified and characterised in deer serum, plasma, and faecal samples in recent studies (Huaman *et al.* 2022a; Huaman *et al.* 2021b; Huaman *et al.* 2021c). These studies illustrate how novel molecular techniques can provide a new understanding of viral epidemiology and evolution. Picobirnaviruses (PBVs) were detected in serum and plasma (Huaman *et al.* 2021c), with subsequent molecular screening being performed in a range of specimens collected from wild deer as well as in faecal samples from farmed cattle. PBV has been detected in several animal species worldwide, mostly in faecal samples; however, it has also been detected in blood and respiratory tract samples (Malik *et al.* 2014; Smits *et al.* 2011). High prevalence was obtained in deer and cattle sampled in southeastern Australia (Huaman *et al.* 2021c), with a predominance of genogroup I and simultaneous genogroups I and II detection in these host species. Moreover, the detection of identical sequences in the trachea and nasal swabs of the same animal, with no amplification in the lung, suggests active replication and infection of PBVs in the upper respiratory tract, expanding our knowledge of picobirnavirus tropism.

Genomic and phylogenetic analyses of NGS data revealed the presence of a new member of the genus *Bopivirus*, proposed as '*Bopivirus* C' (Huaman *et al.* 2021b). Further epidemiological investigation of fallow deer, sambar deer, red deer and cattle faecal samples showed an overall prevalence of 8% in deer but no detection in sambar deer and cattle. In addition, phylogenetic and sequence analyses indicate that the same genotype is circulating in south-eastern Australia. To our knowledge, this study reports for the first time a deer-origin bopivirus and the presence of a member of the genus *Bopivirus* in Australia.

A nearly complete genome of an endogenous betaretrovirus was characterised in fallow deer (Huaman *et al.* 2022a). Further genomic analysis showed that this provirus, tentatively named cervid endogenous betaretrovirus 1 (CERV β1), has typical betaretroviral genome features (*gag-pro-pol-env*). In addition, CERV β1 pol sequences were detected by PCR in the six non-native deer species with wild populations in Australia. Phylogenetic analyses suggested that CERV β1 endogenisation occurred between 3.3 and 5 million years ago (Huaman *et al.* 2022a). Although this provirus does not appear to constitute a current risk for livestock, these results provide important insights into the evolution of betaretroviruses in cervids.

PARASITIC DISEASES IN DEER

Unlike viral infections, research in parasitic infections on Australian deer populations was mostly conducted in the last 20 years and has focused on detecting gastrointestinal parasites and helminths. However, exposure to four vector-borne parasites, *Sarcocystis*, *Entamoeba*, and *Neospora caninum,* was recently assessed in PCR-based assays in wild deer from eastern Australia (Table 2).

PCR-based screening of deer serum and blood samples revealed no acute infection for *Sarcocystis* and a range of vector-borne infections (*Babesia, Theileria, Trypanosoma*, and *Plasmodium*) relevant for the livestock industry (Huaman et al. 2020; Huaman et al. 2021a). With the endemicity of these pathogens in livestock, the presence of suitable vectors, and the increasing distribution and abundance of deer (Davis *et al.* 2016), it cannot be ruled out that deer species could serve as sources of infection in the future. This survey represents the first large-scale molecular study of its type in Australian deer and provides important baseline information about the infection status of wild deer in eastern Australia.

Following a similar research project on detecting *Neospora caninum* parasites in wild dogs (Davidson *et al.* 2021) and given the current gaps in knowledge about the sylvatic life cycle of this parasite, the seroprevalence of *N. caninum* was investigated in wild deer (*unpublished data*). Detecting *N. caninum* antibodies in wildlife species represents a significant challenge due to the lack of validated serological tools. However, competitive ELISA (cELISA) has emerged as a trusted approach because it can theoretically be used to test samples of a different animal species than the one they have been initially designed for (Knowles and Gorham 1993). After evaluating two commercial competitive ELISA kits using a Bayesian approach to determine their ability to detect *N caninum* antibodies in deer samples, a seroprevalence of 4% was obtained in deer serum samples collected in south-eastern Australia. This outcome suggests that wild deer contribute to the sylvatic cycle of this parasite in Australia. To our knowledge, this is the first reported detection of *N. caninum* antibodies in Australian

wild deer and highlights the usefulness of cELISA for assessing serological assays in wildlife populations.

The cross-species infection was evaluated by estimating the time to a most recent common ancestor (TMCRA) of *Entamoeba bovis* sequences of wild deer and cattle origin (Huaman *et al.* 2022b). This parasite was detected worldwide in farm and wild ruminants, but their epidemiology and distribution remain largely unexplored (Stensvold *et al.* 2010). The TMCRA in this study was estimated to have existed >200 years ago (before cattle and deer were introduced to Australia), providing no evidence of *E. bovis* transmission between wild deer and cattle in Australia. This finding is somewhat unexpected, but it is possible that since wild deer populations have only recently increased to densities at which widespread control has been deemed necessary (Bengsen *et al.* 2022), they did not play an important role in the transmission of these parasites thus far.

CURRENT AND FUTURE DIRECTIONS

Reviews from North America (Conner *et al.* 2008) and the United Kingdom (Böhm *et al.* 2007) have covered shared deer-livestock infectious diseases in detail but focused on their local implications. Since most pathogens studied in Australian deer recently are the causative agents of vector-borne diseases, weather can limit or enhance their transmission or influence their future emergence. Thus, climate patterns can significantly impact pathogen transmission and interactions between wildlife hosts, vectors, and even humans. In addition, climate patterns are increasingly recognised or predicted to play a major role in the emergence of vector-borne diseases in humans and wildlife (Fouque and Reeder 2019; Rocklov and Dubrow 2020). Temperature, precipitation, and humidity can affect vectors' development and survival, affecting their abundance in the environment.

Moreover, weather patterns can affect arthropod vector distribution and feeding behaviours. Therefore, long-term changes, especially warming temperatures, could significantly alter the distribution and prevalence of vector-borne diseases in wildlife populations (Fouque and Reeder 2019). Even though the current data suggest a low risk of transmission from deer to livestock, this scenario should not be dismissed under climate change scenarios where the distribution of vectors may shift dramatically, altering current epidemiological patterns.

Advances in high-throughput sequencing and bioinformatics have dramatically increased our understanding and capacity to identify novel microorganisms. For example, in the last decade, a novel virus (Schmallenberg virus - SBV) was described in European cattle, constituting an emerging threat to the livestock industry in Europe and worldwide (Endalew *et al.* 2019; Hoffmann *et al.* 2012). Since its first description in 2011, high seroprevalence of SBV has been detected in European deer, indicating that deer could act as a source of this virus for livestock (Garcia-Bocanegra *et al.* 2017; Jimenez-Ruiz *et al.* 2021; Mouchantat *et al.* 2015). Interestingly, one study identified a novel *Bopivirus* in fallow and rusa deer from south-eastern Australia using next-generation sequencing, the first description of this virus in deer in Australia (Huaman *et al.* 2021b). The authors hypothesised that this virus, as other enteric picornaviruses, is transmitted via the faecal-oral route. However, further work is required to determine its distribution in other deer species (e.g., chital, rusa, hog) and livestock species other than cattle to increase our understanding of the potential for cross-transmission between wild deer and livestock. Moreover, additional study locations would help detect prevalence and infection intensity fluctuations.

As detailed and discussed previously, deer are infected and susceptible to a long list of diseases, and many of them are zoonotic, meaning they can also affect humans. Increasing the focus on wildlife disease surveillance to detect emerging infectious diseases and integrate wildlife and environmental health into One Health policies is crucial to prepare Australia to better recognise and manage the adverse impacts of zoonotic diseases. Indeed, high deer densities may cause concern for human health via the transmission of infectious agents through direct contact, the consumption of venison or contamination of the environment (particularly water) with faeces or urine.

Recently, several studies have suggested that deer could be a reservoir of the SARS-CoV-2 virus, which causes COVID-19 in humans. High seroprevalence in free-ranging white-tailed deer (*Odocoileus virginianus*) was found in the USA, revealing high susceptibility to infection of this deer

species and active deer-to-deer transmission (Hale *et al.* 2022; Murphy and Ly 2021; Palmer *et al.* 2021). Moreover, the identification of different SARS-CoV-2 variants suggests human-to-deer infections and that wild deer can sustain transmission (Hale *et al.* 2022). In contrast, no evidence of infection was found in common European deer species (Holding *et al.* 2022; Moreira-Soto *et al.* 2022). These studies indicate that deer should be considered when identifying potential reservoir and intermediate hosts of emerging zoonotic diseases. They also highlight the importance of wildlife disease surveillance as part of a One Health approach to disease prevention.

Finally, we recommend developing a passive surveillance system through the Australian deer hunter community. Training in identifying, recording macroscopic lesions, and collecting specimens could be provided to hunters as part of the licensing process, and hunters could be of critical help in monitoring the presence and distribution of infectious diseases. The investigations conducted in the last five years provided vital baseline data for future research. Monitoring changes in the disease status would allow researchers to gain a more comprehensive view of the dynamics of infectious diseases to minimise the risk of impacts on humans, livestock, and wild animals. These findings extended our knowledge of known and novel viruses and parasites associated with Australian deer.

CONFLICTS OF INTEREST

David Forsyth and Anthony Pople were guest Associate Editors for this special issue. Despite this relationship, they did not at any stage have editor-level access to this manuscript while in peer review, as is the standard practice when handling manuscripts submitted by an editor to this journal. *Wildlife Research* encourages its editors to publish in the journal, and they are kept separate from their manuscripts' decision-making process. The authors have no further conflicts of interest to declare.

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DATA AVAILABILITY

Not applicable

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FIGURES

Figure 1. A paddock in a farm in Victoria, Australia. Note the presence of deer scats on the foreground, while domestic animals (horses) are on the background.

TABLES

Table 1. Viral infections investigated in wild deer in Australian

Pathogen	Prevalence (%)	Deer species	Diagnostic method	State or territory	Reference
Akabane virus	13	Red	VNT	QLD	(McKenzie et al. 1985)
	ND	Rusa	ND (antibodies)	NSW	(Moriarty 2004a)
	0	Chital, fallow, rusa, sambar	PCR	ACT, NSW, QLD, Victoria	(Huaman et al. 2020)
BEFV	43	Red	VNT	QLD	(McKenzie et al. 1985)
	ND	Rusa	ND (antibodies)	NSW	(Moriarty 2004a)
	0	Chital, fallow, rusa, sambar	PCR	ACT, NSW, QLD, Victoria	(Huaman et al. 2020)
	0	Chital, fallow, rusa, sambar	PCR	ACT, NSW, QLD, Victoria	(Huaman et al. 2020)
Bopivirus	7.7	Fallow, red, sambar	PCR	NSW, Victoria	(Huaman et al. 2021b)
Bovine herpesvirus	0	Fallow	VNT	Tasmania	(Munday 1966; Munday 1972)
	0	Fallow, rusa	ND (antibodies)	Victoria	(Presidente and Westbury 1979)
	0	Fallow	VNT	NSW	(English 1982)
	0	Red	VNT	QLD	(McKenzie et al. 1985)
	0	Chital, fallow, rusa, sambar	ELISA	ACT, NSW, QLD, Victoria	(Huaman et al. 2020)
Bluetongue	13	Red	VNT	QLD	(McKenzie et al. 1985)
EHDV	19 - 50	Red	VNT	QLD	(McKenzie et al. 1985)
	0	Chital, fallow, rusa, sambar	PCR	ACT, NSW, QLD, Victoria	(Huaman et al. 2020)
Palyam group	86	Red	VNT	QLD	(McKenzie et al. 1985)
Parainfluenza-3	0	Fallow	VNT	Tasmania	(Munday 1972)
Pestivirus	14.5	Fallow	VNT	Tasmania	(Munday 1966; Munday 1972)
	1	Sambar	VNT	Victoria	(Slee and Presidente 1981)
	1.2	Fallow	VNT	NSW	(English 1982)
	4	Red	VNT	QLD	(McKenzie et al. 1985)

	3	Chital, fallow, rusa, sambar	ELISA	ACT, NSW, QLD, Victoria	(Huaman et al. 2020)
Picobirnavirus	50.7	Fallow, sambar	PCR	NSW, Victoria	(Huaman et al. 2021c)
Other arboviruses	ND	Fallow, rusa, sambar	VNT	Victoria, NSW	(English 1982; Presidente and Westbury 1979; Slee and Presidente 1981)

ND: no data, QLD: Queensland, NSW: New South Wales, ACT: Australian Capital Territory, VNT: Virus neutralisation test, NGS: next-generation sequencing, ELISA: Enzyme-Linked Immunosorbent Assay.

Table 2. Parasitic infections investigated in wild deer in Australia

Pathogen (genera or species)	Prevalence (%)	Deer species	Diagnostic method	State or territory	Reference
Babesia	0	Chital, fallow, rusa, sambar	PCR/IFAT	ACT, NSW, QLD, Victoria	(Huaman et al. 2021a)
Cryptosporidium	1−9	Fallow, red, sambar	PCR	Victoria	(Cinque et al. 2008; Koehler et al. 2016; Nolan et al. 2013)
	0.7	ND	PCR	NSW	(Ng et al. 2011)
Entamoeba bovis	82	Fallow, sambar	PCR	NSW, Victoria	(Huaman et al. 2022b)
Fasciola hepatica	1	Red	M.E	QLD	(McKenzie et al. 1985)
	53	Fallow	M.E	NSW	(Jenkins et al. 2020)
	0	Fallow	M.E	ACT	
	0	Sambar	M.E	Victoria	
	45	Fallow	M.E	NSW	(Lamb et al. 2021)
	15	Hog	M.E	Victoria	(Game Management Authority 2006)
Giardia	21.2	ND	PCR	NSW	(Ng et al. 2011)
	0−14	Fallow, red, and sambar	PCR	Victoria	(Koehler et al. 2016; Nolan et al. 2013)
Neospora caninum	4	Fallow, red, sambar	ELISA	NSW, Victoria	Huaman et al. 2022 (in review)
Plasmodium	0	Chital, fallow, rusa, sambar	PCR	ACT, NSW, QLD, Victoria	(Huaman et al. 2021a)
Sarcocystis	0	Chital, fallow, rusa, sambar	PCR	ACT, NSW, QLD, Victoria	(Huaman et al. 2021a)

Theileria	0	Chital, fallow, rusa, sambar	PCR	ACT, NSW, QLD, Victoria	(Huaman et al. 2021a)
Toxoplasma	0	Fallow	IFAT	Tasmania	(Munday 1972)
Trypanosoma	0	Chital, fallow, rusa, sambar	PCR	ACT, NSW, QLD, Victoria	(Huaman et al. 2021a)
Other gastrointestinal helminths	2−18	Red	M.E	QLD	(McKenzie et al. 1985)
	ND	Fallow, rusa	M.E	NSW	(Moriarty 2004a; Mylrea et al. 1991)
	15-60	Fallow, sambar, hog	M.E, PCR, NGS	Victoria	(Davies 2014; Panozzo 2018)

ND: no data, QLD: Queensland, NSW: New South Wales, ACT: Australian Capital Territory, VNT: Virus neutralisation test, IFAT: Immunofluorescence antibody test, M.E: microscopic examination, NGS: next-generation sequencing.

APPENDIX 3. MINING PUBLICLY AVAILABLE GENOMIC DATASETS TO DESIGN XGEN™ CUSTOM AMPLICON PANELS FOR NON-INVASIVE WILDLIFE MONITORING

MINING PUBLICLY AVAILABLE GENOMIC DATASETS TO DESIGN AMPLICON PANELS FOR NON-INVASIVE WILDLIFE MONITORING

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ABSTRACT

Non-invasive genetic sampling is utilised to monitor rare and invasive species however many issues can arise with the quality and quantity of DNA present in these samples. Single nucleotide polymorphism (SNP) marker panels are becoming more common in non-invasive monitoring of wildlife however costs can be high when SNP panels have not yet been characterised and require initial sequencing for SNP identification. In this study, we demonstrate how this initial sequencing step can be skipped by utilising publicly accessible reduced representation sequencing data generated through previous research and develop SNP amplicon panels to assist in invasive deer species identification and distinguishing individuals present in the Australian landscape. Sequence data from several deer species was downloaded from GenBank, and primer pairs designed for amplicons where polymorphic SNPs could be identified. Amplicons were then sequenced across four deer species present in Australia, and panels designed that showed fixed SNP differences between all four species, and polymorphisms in sambar deer (*Cervus unicolor*) and the closely related rusa deer (*Cervus timorensis*). The latter SNP panel was then sequenced in conjunction with a microsatellite panel in 92 sambar deer scat samples to compare the performance of both SNP and STR panels for individual identification. Results of clone inference and probability of identity showed that the SNP panel outperformed the STR panel, demonstrating the advantages of implementing SNP amplicon panels for non-invasive monitoring of wildlife.

INTRODUCTION

Non-invasive genetic sampling is a common method to monitor rare and elusive species for conservation purposes, and more recently as a tool to monitor invasive species (Karssene *et al.* 2019; Kleemann *et al.* 2022; McKelvey *et al.* 2006; Rodrigues *et al.* 2020). This typically involves collecting hair or scat samples and is advantageous as the target species does not need to be captured in order to obtain a viable genetic sample (Taberlet *et al.* 1999). However, issues often arise with the quality and quantity of DNA present in these types of genetic samples and methods of DNA extraction and genotyping therefore need to be robust to ensure viable genetic datasets can be generated (Andrews *et al.* 2018; Waits and Paetkau 2005). This is especially true for studies that utilise scat samples as DNA inhibitors and non-target DNA is often co-extracted and amplified, including dietary items, bacteria, and viruses (Eggert *et al.* 2005; Rutledge *et al.* 2009). Despite these challenges, scat samples have been successfully utilised in a range of studies for species identification, connectivity

and dispersal, mark-recapture, and population genetics (Davies *et al.* 2021; Mondol *et al.* 2009; Parker *et al.* 2021; Valière *et al.* 2003; Walker *et al.* 2016).

Microsatellites have commonly been utilised for non-invasive genetic studies to address population genetic research questions and estimate species abundance. These markers are hypervariable with multiple possible alleles at a given locus, and many markers already exist for a range of taxa although they may not always be species specific, allowing amplification in other closely related species (McKelvey and Schwartz 2004). Despite these advantages, many challenges exist with utilising microsatellite markers for amplification in non-invasive genetic samples. High rates of genotyping errors (e.g. allelic dropout) are common, and samples often need to be genotyped multiple times to reach a consensus for each microsatellite marker, increasing processing times and lab costs (Lukacs and Burnham 2005; Rutledge *et al.* 2009). Shorter fragment lengths are also more desirable to increase amplification success (Broquet *et al.* 2007), however this may not always be feasible when relying on previously published microsatellite marker panels. Introduced and invasive populations are generally less genetically diverse than their native counterparts especially when there are very few founding events (Le Cam *et al.* 2020; Puillandre *et al.* 2008; Rollins *et al.* 2013), leading to additional hurdles in effectively monitoring the distribution and abundance of invasive taxa with microsatellites.

As next-generation sequencing technologies have developed and become more accessible, the use of multiplexed amplicon panels to sequence single nucleotide polymorphism (SNP) markers have become a viable alternative to microsatellite markers for genotyping non-invasive samples (Erwin *et al.* 2021; Harmoinen *et al.* 2021; Hayward *et al.* 2022). SNP markers can comprise shorter fragment lengths and incorporate fewer genotyping errors than microsatellites, and results are more easily reproduced across laboratories (Flanagan and Jones 2019; Gettings *et al.* 2015). Amplicon-based SNP markers are also directly sequenced whereas microsatellites are characterised via changes in allele fragment sizes; the sequencing of SNPs can therefore reduce bias in the interpretation of outputs by automating the SNP calling process (Eriksson *et al.* 2020). While the costs of processing SNP panels are lower than microsatellite panels (Eriksson *et al.* 2020; von Thaden *et al.* 2017), there are currently far fewer SNP panels available than microsatellite panels, and SNP panels generated for population genetic and individuals' identification are generally not applicable to a broad range of taxa like some microsatellite panels. This means that the characterisation of SNPs through reduced representation sequencing is often required prior to developing amplicon panels, thereby increasing time and costs of developing and implementing SNP panels.

As genomic studies have become more commonplace for population genetic analysis of wildlife, many large genomic datasets are easily accessible online. These genomic datasets have the potential to be utilised to develop for amplicon SNP sequencing in non-invasive samples, thereby circumventing initial time and costs associated with generating this data for taxa of interest. Here, we utilise a reduced representation sequencing dataset generated by (Hu *et al.* 2019) for several species of deer belonging to the family Cervidae, to create multiple SNP amplicon panels to assist in the management of invasive deer species introduced to Australia. Six deer species were introduced to Australia in the 19th century and have established wild, self-sustaining populations: sambar deer (*Cervus unicolor*), Javan rusa deer (*Cervus timorensis*), fallow deer (*Dama dama*), hog deer (*Axis porcinus*), red deer (*Cervus elaphus*), and chital (*Axis axis*). Population genetic studies can provide critical information for improving management (Davies *et al.* 2021; Hill *et al.* 2022), however, most deer are elusive and difficult to sample, making non-invasive sampling using more easily collected faecal samples an attractive source of DNA material. Complicating this is that a number of species share overlapping distributions across Australia, and issues exist in identifying species based on scat morphology (Bowkett *et al.* 2013; Costa *et al.* 2017), so genetic analysis is often necessary for species identification. Here, we develop an interspecies SNP amplicon panel capable of distinguishing between species, with an intraspecies polymorphic amplicon panel specific, which will provide important genetic information about multiple deer species present within a site, and more-in depth estimates of abundance and genetic structure for individual deer species.

The aims of this study are to demonstrate the utility of publicly available reduced representation sequencing datasets to develop SNP amplicon panels for the monitoring of wildlife species, by using sambar deer present in Australia as a case study. Sambar deer are considered one of the most

successful deer introductions to Australia, where both abundance and distribution of the species continues to increase across south-eastern Australia (Bentley 1978; Crittle and Millyn 2020; Forsyth *et al.* 2015; Moloney *et al.* 2022). Overabundant populations of deer are known to cause a variety of negative impacts in Australia (Bennett and Coulson 2010; Cripps *et al.* 2019; Davis *et al.* 2010; Hampton and Davis 2020; McDowell 2007), with local management via culling undertaken to reduce these impacts; however, at present it is difficult to measure the success of ongoing culling at reducing local abundances of deer. Sambar deer are elusive and difficult to monitor and have been shown to avoid areas where human disturbance has recently occurred (Semiadi *et al.* 1994), with monitoring of this species therefore reliant on non-invasive techniques in order to measure changes in abundance and dispersal in managed areas. Sambar deer are also known to co-exist with a number of deer species, and, of particular importance, hybridise with the closely related rusa deer (Hill *et al.* In Review; Martins *et al.* 2018)

We develop an interspecies amplicon panel capable of distinguishing between four deer species introduced to Australia through fixed SNP differences, and an intraspecies amplicon panel developed for sambar deer. Cross amplification of the intraspecies amplicon panel to rusa deer is explored, and amplification success across invasive and non-invasive sample types is also considered for both panels. Finally, a comparison of the intraspecies SNP amplicon panel to an existing microsatellite panel is undertaken to demonstrate the effectiveness of the SNP amplicon panel in distinguishing individuals and estimating abundance.

METHODS

AMPLICON MARKER DEVELOPMENT AND TESTING

Reduced representation sequencing data for sambar deer were downloaded from GenBank under the BioProject PRJNA355630; this dataset includes samples from Cervus elaphus, C. nippon, C. albirostris, C. eldii, C. unicolor, and Rangifer tarandus, with multiple samples per species (Hu et al. 2019). A two-step process was undertaken using Geneious 9.1.8 (Kearse et al. 2012) to identify SNPs for the development of amplicons. Firstly, a de novo assembly using C. unicolor (BioSample: SAMN06093039) sequence reads was undertaken using the Geneious assembler with high sensitivity settings. However, as there are several C. unicolor subspecies, to increase the likelihood that amplicons would be suitable across all sambar species and other closely related deer, sequence reads from Cervus elaphus, C. nippon, C. albirostris, C. eldii, C. unicolor, and Rangifer tarandus from this BioProject were then mapped back against the consensus contigs generated from the de novo assembly, and contigs that consisted of multiple species were used for SNP development. Assemblies were screened for SNP variation and primers were designed for variant contigs to amplify fragment lengths between 100-150 base pairs (bp). Primer pairs for 157 amplicons were successfully created using this method.

Amplicons were tested in Australian samples of sambar deer and rusa deer to confirm SNP polymorphism, and cross species utility in samples of sambar deer, fallow deer, hog deer, and rusa deer. Initial testing was completed on tissue, blood, and scat samples in order to evaluate any possible issues in amplification that may arise due to sample quality (Table 1). Tissue and blood samples were provided by either recreational hunters or from control operations, and scat samples were collected in the field by swabbing the outside of a single scat with sterile cotton swabs and storing the swabs in a 1.5 mL Eppendorf tube containing Longmire buffer (Davies et al. 2019). All samples were frozen at -20°C prior to DNA extractions. Tissue samples were extracted using a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. Blood samples were extracted using a QIAamp DNA Blood Mini Kit using 125 uL of starting material, following the manufacturer's instructions. Swabs were extracted using a modified DNeasy Blood and Tissue Kit (Qiagen) protocol, as outlined in (Davies et al. 2019).

Table 1 Genetic samples obtained from four species of deer to test amplicon polymorphism

	Sambar deer	Rusa deer	Fallow deer	Hog deer	TOTAL
Tissue	14	4	5	7	30
Blood	9	15	-	-	24
Scat	10	-	-	-	10
TOTAL	33	19	5	7	64

Amplicons were multiplexed and sequenced using a dual-indexing process, with an initial PCR to amplify the product of interest, and a subsequent PCR to attach indexes and Illumina adapters. Amplicon primer pairs were divided into six multiplexes of 23-28 primer pairs in each multiplex. For each multiplex, primers were added in equal proportions to a stock concentration of 250 nM per primer. Multiplex PCR amplification were carried out in 10 uL reactions, comprising 6 uL of Qiagen Multiplex PCR Master Mix, 2 uL of pooled primers, and 2 uL of template DNA. PCR reaction conditions were 95°C for 15 mins, 20 cycles of 94°C for 30 sec, 57°C for 1 min 30 sec, 72°C for 1 min 30 sec, and a final extension of 72°C for 10 min. Illumina adapters and indexes were then attached to the PCR products from the first amplification to ensure identification of individual samples at the demultiplexing stage. Reactions for this PCR are carried out in 15 uL, using 7.25 uL of MyTaq RedMix (Bioline), 0.75 uL of index primer pairs (10 uM), 4 uL of H_2O, and 3 uL of PCR product. Cycling conditions were 95°C for 3 min, 10 cycles of 95°C for 20 sec, 60°C for 15 sec, 72°C for 30 sec, and a final extension of 72°C for 3 min. PCR products from this reaction were then cleaned to remove primer dimer using an AmpureXP protocol with a SPRI magnetic bead mix. Samples were quantified using a NanoDrop Lite Spectrophotometer (Thermo Fisher) and normalised for pooling. A final library of all samples was prepared as per Illumina MiSeq protocol, and sequencing was performed using a MiSeq Reagent v2 (2x250bp) sequencing kit.

IDENTIFICATION OF AMPLICON PANELS

Demultiplexed fastq files generated from the Illumina MiSeq run were imported into *CLC Genomics Workbench 7* (CLC bio, Inc.), where fastq files were filtered and aligned to the reference amplicons from BioProject PRJNA355630. *Geneious 9.1.8* was then used to generate alignments for each amplicon comprising rusa, sambar, fallow, and hog deer samples, and each alignment was inspected by eye to identify SNPs and confirm polymorphisms. Two amplicon panels were generated from the dataset, one showing fixed SNP differences that can be used for delineating the four deer species, and the second showing within species polymorphism for sambar deer/rusa deer. Data was therefore separated at this stage to analyse an interspecies amplicon panel, and an intraspecies amplicon panel.

INTERSPECIES AMPLICON PANEL

Eleven amplicons were identified that showed fixed SNP differences between each of the four deer species (Table 2). The number of SNPs present on each of these amplicons ranged from 3-8, with 56 SNPs in total present across the 11 amplicons.

Table 2 Amplicons designed for species differentiation between four species of deer

Amplicon	Forward	Reverse	Size (bp)	No. of SNPs
14521	AGCTAAAGTGAAAGGCAAAATATGA	AACAAGTACTAAATGAAACCTATCCTG	108	5
43032	CAGTTTGCCCTTCAAGTTTTCA	AGGTGTCAAGGTGATGAAGGAC	108	5
32213	TGCTGGAACAATTAGATGTCCA	AGAGTTTTGTTGTTTTGTGCTCT	139	5
36842	AGGAAAAGGGCGTCTTGAGA	CCAACCAATCTACCAAAAACCCC	131	4
34972	AGTTGCATTTGTCAGCAACC	CAGGCTTTGAAGGTAGAGTAACA	99	5
45313	TGCTGGGAAACAAACACTCG	GTCAGACAAGTTGTAGTGGAAGC	127	5
25874	TCCAAGCTCCAATCCCTGAA	TCCTTGTCTGTGTAGCAGTTGG	100	3
25456	TGGAGGATCAGTGTGTTGGT	TTGTTCGGTTGGGCAAAGAC	137	7
11241	GCATCTCCACACACAAAGATGT	TCTCAGTAAGAAAAAGCTGAGTA	130	8
5220	TCCAGTCTTATCTAAACAAGCCTC	TCTCTGTGCCTTCAAGAAGTCTT	100	4
11845	GGGCAGAGATAAGAGGAACCAG	ATCTATAGCAGGTCCTTGGTAAA	100	5

The program *GTscore 1.3* was used to extract the target SNPs in the fastq files and provide read counts for each amplicon, using the AmpliconReadCounter command (McKinney *et al.* 2020) available at https://github.com/gjmckinney/GTscore. Average read depth and genotype rate for each SNP was then calculated for each species using the *GTscore R* pipeline; rusa blood samples were not included in this analysis to provide a consistent comparison of the performance of tissue samples across each species. To observe potential changes to read depth and genotype rate based on sample type, sambar deer blood and scat samples were also analysed. All samples and amplicon data were then combined, and the package *poppr 2.9.3* (Kamvar *et al.* 2014) was used to filter out individuals with >10% missing data, and loci with >10% missing data, removing 33 samples and 0 loci. A UPGMA dendrogram was then generated in *poppr* using 1000 bootstrap replicates to demonstrate the separation of species using this amplicon dataset. From this panel and corresponding mitochondrial data (not shown), a hybrid individual was detected, comprising a morphological and mitochondrial identification to sambar deer, however comprising nuclear genotypes consistent with rusa deer. This individual was also identified as a hybrid individual via DArT sequencing analysis (Hill *et al.* In Review).

INTRASPECIES AMPLICON PANEL

Amplicons that were identified as either polymorphic within sambar or rusa deer samples, or showed species differences between sambar and rusa, were included in the intraspecies amplicon panel (Table 3). *GTscore* and the corresponding *R* pipeline was used as described above to extract target SNPs, provide read counts for each amplicon, and calculate average read depth and genotype rate for each SNP. This was calculated for each species and sample type separately. Observed and expected heterozygosity (H_O and H_E) was calculated for each SNP/species using *GenAlEx 6.51*, with the hybrid sample identified using the interspecies amplicon removed from the sambar population for this analysis (Table S1) (Peakall and Smouse 2006). All samples were then combined to demonstrate the discrimination power of the marker panel between rusa and sambar deer samples. Loci and samples were filtered using *poppr*, with an emphasis on retaining as many loci as possible. Initial filtering removed samples and loci that comprised missing values above 75%, removing 6 rusa deer blood samples and 24 loci. The same filtering steps were then repeated with a more stringent cut-off, with samples containing more than 25% missing data removed, and loci with greater than 10% missing data removed, leaving 23 individuals and 141 loci. A principal component analysis (PCA) was plotted using the *R* package *adegenet 2.1.5*, retaining two axes (Jombart 2008).

Table 3 Amplicons designed for population genetic analysis in sambar deer and rusa deer. Contigs with an asterisk (*) are also present in the interspecies amplicon panel, and contigs indicating 'species differences' show fixed differences between rusa deer and sambar deer at the same base pair position. Multiplex number is indicative of the final multiplexes used to analyse the sambar deer King Lake population

Contig	Forward	Reverse	Size (bp)	SNPs	Polymorphic	Multiplex
40	GGGGGAAAAGATAGTGAAAAAGGG	CTCAGCTCTCCATATCAGGAAGT	101	1	Rusa	3
111	GAGCCTGGAAGTTCATATAGCCA	CTGTCTGGGGGTCCTGTGTC	93	1	Rusa	3
587	CTTTTTATTGCGCCGCTTGG	AACAGCAAATCTAAACCTCTCCT	124	1	Both	3
612	CAGCAGTGAGTGTCTGTGGA	ACACATCAGTTATTTGCTGTACT	133	3	Sambar	1
623	CAGGATGAAGAAAGGTGGGCT	GCTGAACTTTGAGCTGCCTG	116	4	Sambar	2
642	CCAACTGTCACCTGCGAAAC	TCTTTGAAGGAAAATAGCGTGA	132	4	Both	2
871	GAAACTGCAACCAAAGGGGC	TTAGAGCCCCATCCAACAGC	91	2	Both	3
2123	CAACTGAACTACCAGGGAAACA	CCTTAGGTCCTGGAGAGGGA	110	2	Both	3
2590	CGGTTATCCCCCAACACAAA	GGATTTCGAAATGTGGACACGT	107	3	Species differences	2
3621	TGGACAGCACAATAAAAATAGAAA	GCCTCCTCTGTCAAAGATAAGGT	105	4	Rusa	3
4124	ATTTCTTTTTGTGGAAACAAGAGAGG	TCTGGAGTCAGAGGTCCCAG	100	3	Rusa	2
4502	TTTCTGGCACACCCTCCTAAC	AGTCAGATCTTATTCAAGAAACTGG	101	1	Sambar	2
5935	CCCTAGGTCCCATTTTCCCAG	AACCCTCAGCCACTCACCT	105	1	Sambar	2
6629	CCTAATGGCTGCCTCAAAAGC	AGAGTAGCAGTTGATCTTTCTAAA	105	5	Both	1
6908	TGGGGGTGATGAAAATGTTCT	CACATACCATACATTTCACTCTTT	106	5	Sambar	3
8968	TTGCTGGGTTTTTAGTTCCCAC	GCAACAACACGGATGAACCC	100	1	Rusa	2
10030	AACTATGTGGGCAAATGTTCAGTG	CCCCATAAGCAACCCAGGAA	100	3	Rusa	2
10357	GCAAGAGAATTTGAGAAGATGACT	ACTCAAAGCTTCCAATGTAGT	150	7	Both	3
10381	GGACATAGGTGGTAAGAGCTGG	TTGGGTTCCCTTCTCCTCAC	104	1	Both	1
11768	AACAGGTAGTAAAGCATACAGAT	TCCAAGTGCCTAAACTCTGCA	123	1	Sambar	3
12780	TGTCTCAGACAGAAAGAAAGATT	ACTATACGGTCTACAAAGCCAAAA	100	2	Both	3
13087	TGGCTCTCATTCTTTGCCCA	GTGTACTGGCTGCTAGAGAGT	100	3	Sambar	1
13200	CTCTCCCCAGGTACCCCC	GGCACCCTGTCCTTGGACT	125	2	Both	3
13479	GGGTTCATATGCCCTGTTG	CCTGTTTTCCTTGCTCTTATCT	102	3	Sambar, species differences	1
13829	TTCCATCCTATTGAGTGGATTTT	TTGATCTGAGTAACTTAGACCAAA	107	2	Sambar	2
14594	GCTATGTGGAGGGTAGAAGGG	TCAGACTTCAAGGGTCATTGCT	103	1	Sambar	2
14646	ATTTACCTTGCTTCATTCTCAGAAT	TGAGCTATGTGACTTTGGGTT	102	1	Sambar	1
14860	TCAATGGAGCCCAGAACCTG	AGAAAGACTGGTCAAAGAGTTGT	108	1	Sambar	2
14897	GCCACAGAACTCTTGTATATCTCA	TCTACTTCTGAACATCTGGCTGT	103	1	Rusa	1
15190	ACAGACTCAGCTGAGGTCTGA	TCACTCGTCACATATCTGTCACA	124	1	Both	2
15236	AGCTGTTGGGATGATGCAGA	CTCATTCCCACCTCCAGCAC	113	2	Rusa	1
16424	AAAAAGCACCACCAAGTGTG	TGCAGTTTCTCACCCCTGTT	129	2	Both	1
17035	AAGGGATGGAGTCTGGATTGG	ACCTGCTGCAAAGTCATCTCT	108	3	Both	3
17329	TTTGACTGGTGAGTGGTCCC	GGACTGCATAGTGACCTGCT	122	3	Both	3
17922	AGGAATTGCAGGGTGAAGAAT	AGAGTGACAGTTCTCACAGCT	119	1	Both	3
18505	GCCAGGAACGTCCCCCTC	CCCGGGTGTGAATAGGGTAT	128	1	Sambar	3
19107	GCTGAGGAGCACAAGAAGAAG	TCTCAGACCTGGGCTTGG	137	2	Both	1
19173	CCTAATCAGCTTCCCATCCCC	TGCTCACTCTCAACTTCTGCA	110	1	Sambar	1
19597	AGCAGGGAATGGGAAGCTTG	CTCCTTTGGTTGGACTCCGA	115	1	Species differences	2
19603	TAACTGCAGCAGTAAGTCAAG	TATTCAGCCTGCCCCCAAGT	84	2	Both	1
19664	TGACTGACTGAATTTCCCCAGG	GTTTAGTGTATCCTCAACCCTTATCA	106	2	Rusa	3
19774	AACAGTCTGTGCTCTTGGGG	ACAGCTCAACACCTTGGAGC	89	3	Both	1
19782	TCATAGCCTAGTTGAAGCCACA	GCTGGCTGAGAAGAGCAGAA	121	3	Both	1
20321	ACGCTCTATGCCTAACACTGG	TGAATTTACCCTAATGAGCACTCA	129	1	Sambar	3
20869	ATGTCCTTCCCATATCTTCTCACT	ACCAGAACAGGACTGATGGG	103	3	Sambar	3
21214	TTTTGCTAGGTGCTTCTCTACT	AGAGCATCTGTTCTCTTCTATAGG	125	3	Both	3
21978	AAGCACAGTGTGGATATTCTCA	GTCTCCCAAGTCCATTTTCCG	101	1	Both	1
22720	GCTCAAGAGGTTCCCAGACC	TCCCAACATAGATAAGCTGCTGC	107	4	Both	1
22814	AGGAGGAGGAAGCAGAGGG	ACTCTGTGCTGCTTTCTCCTC	106	4	Both, species differences	2
23634	ACTTGCAAGCTTTACACAGAGT	TCTGTTACAATCTAAAGTTTCCTGTGC	115	3	Both	2
23911	TCCCAGCCCTTATGTCCCTC	AGATAATGCCTGTGCGGGAG	125	6	Both	2
24072	GCTGGTTTATCTCCATCATAATGAACT	GGAAAGTGGTGGATCTCCTG	100	2	Sambar, species differences	3
24346	ACTAATTACAATTTTCCCCAAAAAGAC	CTTGGATGCAGTAGTAAGTGGGA	125	1	Sambar	1
25231	AAAGGCTTGTCTGTTTTAGGACA	ATGCTAGGCAGTGCTTTTCA	100	1	Sambar	2
25325	AGGAAATCACCTGCCAAGCA	ATGAATTTAGTTTTCTGGACCCTAAG	118	2	Sambar, species differences	1
25785	TGGACCAGTGACCTATGTCT	ACTCTGAACTCTTGTACTTTTCTCTGT	136	8	Sambar, species differences	1
25814	TCCAGACAAACAGGACATCAGG	GAGGTGCCACGCTGATACC	103	3	Rusa, species differences	3
27139	GAGCCTGTGCCTCTGGACTA	CCTGGGGGAGGGAAATACC	116	5	Both	3
27617	ACACAAATAGAAGAGACGTAAAGTG	CCCCTTCCTTTCTTGCTTTGA	107	1	Sambar	1

27654	AAACTTACAGAAAAGTCGCAAG	ATGCCTTTGGGATCCTCTGC	85	2	Sambar	3
27912	AGGAGTTGGGGAGACTTACCT	AGAGAATATCTGGTCCAGACTTCTC	127	4	Sambar, species differences	3
28161	CCTGCCTCCAAGATAGAGACAG	CTTCCCTTTGGTGGTGGTGT	121	1	Sambar	2
28878	TAAAGACGCAAAGGCAGTGG	CATCCGGCACAGTGAGGTG	114	3	Both, species differences	1
30970	GGGTCCAGCATCTAATTTGCA	TGTTGGAGTGATCCTGAGAGA	105	1	Sambar	3
31650	CTGGGCAATAATGTAGGGAGGG	CACCTACCTCAGCCGGAATG	113	4	Both	2
32119	AGAGGTTTACAGAAGATGAATAACAAA	CAATTCAGTTTACCTTTCAGTAAGACT	133	2	Both	2
32124	GAAAGAACTGCCAAGGCTGC	GATGGAACTAAGAGGGGCTCA	120	3	Species differences	1
32213*	TGCTGGAACAATTAGATGTCCA	AGAGTTTTGTTGTTTTGTGCTCT	141	2	Species differences	2
32401	GGTGTCTGCTCTCAGCTCAG	GGGGGAAGCTAGCTGATGAA	131	5	Both	1
33321	TCCCTCAAAGTCCTCCCAGT	TGCAACTAATACCTCAAGGACA	108	1	Rusa	3
33913	TGCTCTGGCATCTAGTGGGT	CGGGGAGGCTCTTGTGAATT	71	1	Sambar	2
35101	CGGACACCACCAGGGAAG	TAAGCAAACCCTCTTGGCG	120	3	Rusa	1
35495	GCTGCTCACTGGGTCTGATT	AGTTTGAGCTCTGCTTCCAGT	110	4	Both	2
36470	CTGTCTCTAAAGCTCCTGGTT	ATGTTTGTGACCACCTGTGAA	136	1	Rusa	3
36792	ACATTTCCTCTCTCATCTTTCCACT	AGCTCTCTGTACACATAGTGGG	116	1	Sambar	1
38487	AATGGTTACTGAGCCTTCGT	CTCCCTACCCAGGAACACCT	91	3	Species differences	3
40125	CTTTAGAAAGAAGGGCTGTGCC	CAGTTTCACTGCCGCTGTAG	119	3	Both	2
40254	CTGAATGTAATCTTACCTGGGGG	GCACACGGCTGAAGTACAAAG	129	1	Sambar	2
41405	TTCCTTGGTCACCTCTGCCT	CTTGAAAGGGGTGGGGAAGG	109	2	Rusa, species differences	2
42004	AGCTTCGTTATTGGCATTGAC	CTGGATGAAAGCTCATTCCACC	106	2	Sambar, species differences	2
43032*	CAGTTTGCCCTTCAAGTTTTCA	AGGTGTCAAGGTGATGAAGGAC	108	1	Rusa	2
44774	ACCCAGTACTTTTCCACACCTC	GGGCTCCCCTGACAAAAG	101	2	Rusa, species differences	1
45313*	TGCTGGGAAACAAACACTCG	GTCAGACAAGTTGTAGTGGAAGC	127	2	Sambar, species differences	1

AMPLICON COMPARISON TO STRS

SAMPLE COLLECTION AND DNA EXTRACTION

In order to test the performance of the amplicon panel developed here, the panel was applied to 92 sambar deer scat samples collected from Kinglake National Park in Autumn 2018 as part of an ongoing study. Scat samples were collected along transect lines, and the outside of a single scat was swabbed with a sterile cotton swab and stored in a 1.5 mL Eppendorf tube containing Longmire buffer. Individual scats were also collected and stored in 5 mL containers to provide an additional sample to accompany the swabs. All samples were frozen at -20°C prior to DNA extractions, and swabs were extracted using a modified DNeasy Blood and Tissue Kit (Qiagen) protocol, as outlined in (Davies *et al.* 2019). A subset of samples were also extracted from the scats collected, using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturers' instructions, using scrapings from the outside of a single scat as the starting material.

AMPLICON SEQUENCING

Primer pair interactions among the amplicons described in the intraspecies panel above were assessed with the program *AutoDimer* (Vallone and Butler 2004) and three new multiplexes developed (Table 3) (Vallone and Butler 2004). Library preparation for Illumina MiSeq sequencing of the amplicon panel was the same as described above, however, to account for lower DNA quality typical of scat samples, the number of PCR cycles in the initial amplification stage were increased from 20 cycles to 30 cycles. SNPs were identified, and average read depth for each locus and genotyping rate of each sample calculated using *GTscore*.

STR GENOTYPING

Fourteen STR loci were chosen for amplification in the sambar scat samples, based on previous successful amplification of these loci in Australian populations of sambar deer (BL42, IDVGA55, INRA121, TGLA53, TGLA57, Ca18, Ca43, CelJP38, RT7, OarFCB5, BM757, BMC1009, Apo4, ApoV135) (Davies *et al.* 2019; Davies *et al.* 2021; Hill 2021). PCRs were carried out in 12.5 uL singleplex reactions, containing 6.25 uL of MyTaq RedMix (Bioline), 0.4 uL each of forward and

reverse primers (10 uM), 1.95 uL of H_2O, 1.5 uL of MgCl, and 2 uL of template DNA. PCR cycling conditions comprised an initial denaturation at 95°C for 5 minutes, 40 cycles of 95°C for 30 seconds, annealing step for 30 seconds, 72°C for 1 minute, and a final extension of 72°C for 10 minutes. Annealing temperature varied between individual STRs. Samples that did not initially amplify were run using a temperature gradient, with the same mastermix and PCR protocol (range of 50°C-60°C annealing step) until a fragment could be successfully amplified for each STR. Dilutions of DNA samples were also attempted to facilitate successful amplification. Markers OarFCB5, BM757, and BMC1009 could not be consistently amplified, therefore leaving 11 STR loci for genotyping. PCR products were visualised on a 2% agarose gel and samples with clear bands present were sent to the Australian Genome Research Facility (AGRF) for genotyping. Genotypes were then visualised and scored using *Geneious 9.1.8*.

DATA FILTERING AND ANALYSIS

The SNP amplicon and STR datasets were imported into *R*, and SNPs with a read depth below 5 were removed from the dataset. The package *poppr* was then used to filter both datasets for missing data, monomorphic loci, and minor allele frequencies. Of the 92 scat samples processed with both amplicon and STR markers, a total of 87 samples amplified at least one amplicon in the SNP dataset, and 82 samples amplified at least one marker in the STR dataset. Six loci were removed from the SNP dataset as they did not amplify in any sample (contigs 36214, 21214, and one locus each on contigs 10357 and 13479). Samples comprising over 75% missing data were removed, filtering 25 samples from the amplicon SNP dataset, and 12 samples from the STR dataset. Loci that failed to amplify in more than 50% of samples were then filtered, removing 39 loci from the SNP amplicon panel, and one locus from the STR dataset. A second stage of filtering samples and loci was then completed with more stringent cut-offs. Samples that failed to amplify more than 25% of loci were removed, filtering 22 samples from the SNP panel, and 25 samples from the STR panel. Loci that comprised missing data greater than 5% in the remaining samples were then filtered, excluding a further 69 loci from the SNP dataset, and 4 loci from the STR dataset. Removal of markers that were monomorphic or comprised a minor allele frequency (MAF) of 0.01 were then filtered, removing 36 uninformative loci from the SNP panel. No loci were removed from the STR panel at this stage. Final panels comprised 40 samples and 48 SNP markers for the amplicon SNP dataset, and 45 samples and 6 STRs for the STR dataset. Samples that were successfully amplified in both datasets were retained for further analysis, leaving 36 samples. Loci were then checked again in both datasets to ensure no monomorphic markers were present. Average read depth of the SNP markers retained in the final dataset ranged from 13.52-166.99. Number of alleles ranged from 2-4 in the final STR panel. The SNP dataset was then analysed using two different methods; the first involved retaining only one SNP from each amplicon to ensure unlinked loci were not present in downstream analyses, and the second involved recoding amplicons with multiple SNPs into single, multiallelic loci to reflect haplotype diversities within these amplicons. A total of 9 amplicons were affected by these methods (contigs 13087, 13479, 31650, 612, 623, 6629, 23911, 35495, and 32401). The number of multi-locus genotypes (MLGs) was calculated using *Colony 2.0.6.4* by identifying the number of clones present in the SNP and STR datasets (Jones and Wang 2010). The pairwise full likelihood combined method for analysis was chosen with high precision, and a 0.05 allele dropout rate and false allele rate per locus were also assumed. *Colony* was then run five times to ensure convergence of results. One sample from each unique multi-locus genotype identified by *Colony* was retained for analyses of observed and expected heterozygosity and probability of identity in both the SNP and STR datasets. These statistics were calculated for each dataset using *GenAlEx 6.51*. In order to determine the number of STR loci needed to achieve similar probability of identity values observed using the SNP amplicon panel, the average probability of identity of all STR markers was calculated and then cumulatively added until probability of identity values were similar between the two panels.

RESULTS

Using the reduced representation sequencing data available from BioProject PRJNA355630 uploaded to GenBank (Hu et al. 2019), primer pairs for a total of 157 amplicons were developed and tested across six multiplexes for amplification success and analysed to detect species differences across all four species sampled (sambar, rusa, hog, and fallow), and identify polymorphisms within rusa and

sambar deer. Only four amplicons failed to amplify sufficient samples for analysis, and five amplicons were monomorphic across all species tested. An additional five amplicons showed random insertions/deletions across sequences that were not specific to species or indicative of intraspecies polymorphisms and were therefore deemed uninformative for further analysis. A majority of the amplicons showed species level differences, with 101 amplicons showing species specific SNPs between at least two species. A total of 68 amplicons showed polymorphisms within the sambar deer samples, while 49 amplicons were polymorphic within rusa deer.

INTERSPECIES AMPLICON PANEL

Average read depth was consistent across the 11 amplicons in the sambar deer tissue samples, ranging between 19.76-80.57 (Fig.1). Conversely, wide ranges of average read depth were observed in fallow, hog, and rusa deer tissue samples. Each of these species recorded the highest average read depth at amplicon 43032, with depths of 168.1 in rusa deer, 203.11 in hog deer, and 211.48 in fallow deer. Lowest average read depth for these three species were all below 10 but differed at amplicons. Genotyping rate was above 0.8 for all amplicons except two; these were amplicon 25874 in hog deer with a genotyping rate of 0.57, and amplicon 14521 in rusa deer, with a genotyping rate of 0.75 (Fig.1). Across sambar deer sample types, read depth and genotyping rate decreased along with decreasing quality of genetic samples (Fig.1). The scat samples comprised the lowest genotyping rates and average read depths, with seven amplicons showing an average read depths below 10, and five amplicons comprising a genotyping rate below 0.4. Blood samples showed a similar pattern, with amplicons with an average read depth of 10 or lower also comprising low genotype rates below 0.44.

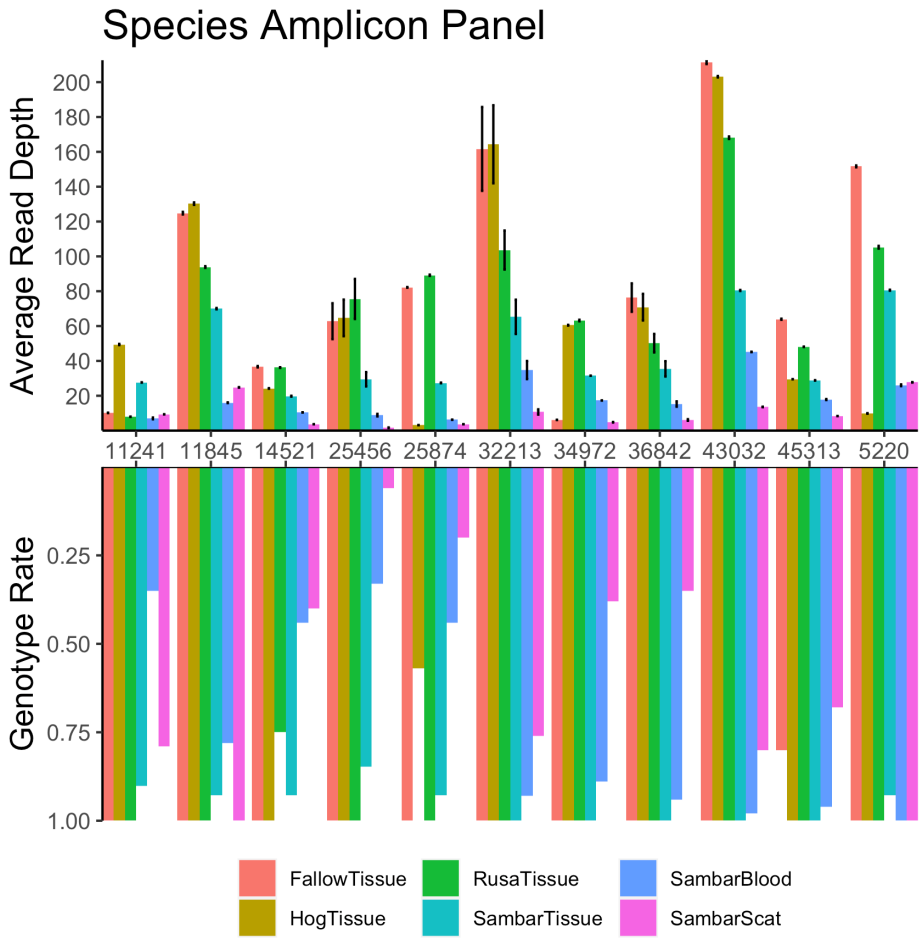

Fig.1 Average read depth and genotyping rate of interspecies amplicon panel across all four species of deer after filtering. Amplicon names are indicated between the two plots. Sample sizes are fallow deer n = 5, hog deer n = 7, rusa deer n = 19, sambar tissue n = 14, sambar blood n = 9, sambar scat n = 10. Error bars represent standard deviation

After filtering samples with missing data, a UPGMA phylogenetic tree comprising 31 samples and all interspecies amplicon loci (56 loci) was generated. Four distinct clades were observed in the tree, corresponding to each of the four deer species (Fig.2). A single sambar sample was grouped into the rusa deer clade; this deer was morphologically identified as a sambar deer at the time of sample collection and contains a mitochondrial haplotype consistent with sambar deer in Australia (data not shown). This sample was taken from Port Macquarie in New South Wales where sambar and rusa deer are both present, and has additionally been confirmed as a hybrid in previous research (Hill *et al.* In Review).

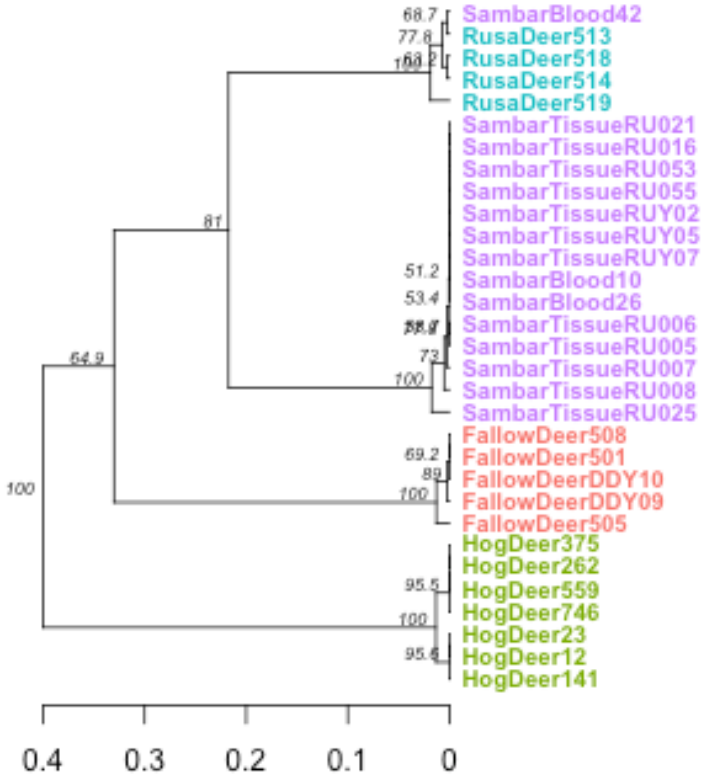

Fig.2 Phylogenetic tree based on 56 loci across 11 amplicons designed for species differentiation. Bootstrap values above 50 are indicated above each node

INTRASPECIES AMPLICON PANEL

A total of 198 SNPs across 83 amplicons were identified in the rusa (n=19) and sambar (n=33) samples that were one of 1) polymorphic in either sambar or rusa samples, 2) polymorphic in both species, 3) showed species level differences between rusa and sambar samples (Table 3). Average read depth and genotype rate varied across amplicons and sample types, however in general a low read depth for a given amplicon also resulted in lower genotyping rates (Table 4). Read depth was generally lowest in the sambar scat samples, however an average read depth of 555.3 was observed at amplicon 587; this amplicon showed high amplification across all sample types in both species. A number of amplicons failed to amplify in different sample types/species; this included one amplicon in the sambar scat samples, 12 amplicons in the sambar blood samples, and 13 amplicons in the rusa tissue samples. All amplicons amplified in both species for at least one sample type.

In the rusa tissue samples, average read depth ranged from 7.5-469.25 (amplicons 2590 and 587 respectively) and genotype rate ranged from 0.5-1, with 48 amplicons amplifying across all samples.

In the rusa blood samples, the lowest average read depths were observed at amplicons 28161, 23634, 24346, and 14594 with a read depth of 1, and the highest at amplicon 587 (171.67). Genotype rate ranged from 0.07 in four amplicons (40254, 27654, 28161, and 14594, to 1 in three amplicons (587, 33321, and 30970). In the sambar tissue samples, average read depth ranged from 3.33-819.33 (amplicons 21214 and 13087 respectively), and genotyping rate ranged from 0.14-1, with the lowest genotype rate attributed the amplicon 21214. A total of 52 amplicons comprised a genotype rate of 1 across all sambar deer tissue samples. Average read depth in the sambar blood samples ranged from 3.25 at amplicon 2590, to 426.25 at amplicon 587. Genotyping rate in the sambar blood samples ranged from 0.33-1, with amplicon 28161 comprising the lowest rate, and 31 amplicons amplifying all sambar blood samples, showing a genotype rate of 1. Only one amplicon failed to amplify in the sambar scat samples, however, average read depth was low, with three amplicons comprising a read depth of 1 (25231, 24072, and 23634). Again, amplicon 587 comprised the highest average read depth of 555.3. Genotype rate for these samples ranged from 0.2-1.

Table 4 Average read depth and genotype rate of the intraspecies amplicon panel across different sample types from rusa deer and sambar deer. Values in brackets indicate standard deviation

	Rusa Tissue		Rusa Blood		Sambar Tissue		Sambar Blood		Sambar Scat	
Amplicon	**Read Depth**	**Genotype Rate**	**Read Depth**	**Genotype Rate**	**Read Depth**	**Genotype Rate**	**Read Depth**	**Genotype Rate**	**Read Depth**	**Genotype Rate**
40	78.5 (27.01)	1	14.75 (14.45)	0.80	42.93 (30.87)	1	28.63 (11.53)	0.89	34.3 (21.52)	1
111	27 (9.83)	1	7.29 (5.12)	0.93	13.79 (11.74)	1	13.38 (6.46)	0.89	13 (6.46)	1
587	469.25 (349.37)	1	171.67 (183.99)	1	495.14 (134.15)	1	426.25 (169.84)	0.89	555.3 (323.71)	1
612	44.08 (7.39)	1	7.06 (7.12)	0.73	24.79 (19.85)	1	13.88 (7.53)	0.89	18.93 (10.69)	1
623	47.75 (10.77)	1	4.96 (4.14)	0.80	19.27 (11.57)	1	13.56 (8.84)	0.89	11.65 (7.48)	1
642	19.06 (4.95)	1	3.66 (2.62)	0.53	10.98 (6.50)	1	6.66 (3.29)	0.89	9.44 (6.42)	0.8
871	102 (20.3)	1	17.93 (16.11)	0.93	49.86 (35.02)	1	45 (23.60)	0.89	27.65 (22.99)	1
2123	20.88 (6.98)	1	4.39 (2.99)	0.60	10.57 (7.33)	1	7.19 (4.00)	0.89	7.15 (3.38)	1
2590	7.50 (4.12)	1	2 (0.98)	0.67	4.52 (1.53)	1	3.25 (2.09)	0.89	3.56 (1.87)	0.9
3621	25.36 (11.67)	0.88	4.71 (3.93)	0.52	10.64 (8.95)	0.95	9.14 (7.35)	0.81	9.56 (6.27)	0.8
4124	54.67 (16.08)	1	9.81 (8.78)	0.93	31.64 (18.76)	1	15.92 (8.00)	0.89	20 (13.18)	1
4502	63 (25.01)	1	12 (11.79)	0.93	38.14 (30.71)	1	28.13 (12.70)	0.89	21.5 (12.87)	1
5935	87.75 (21.27)	1	3.89 (2.37)	0.60	139.5 (122.08)	1	15.44 (12.71)	1	32.4 (29.06)	1
6629	161.95 (20.18)	1	7.6 (8.41)	0.60	117.73 (87.71)	1	38.04 (40.64)	1	55.24 (50.40)	1
6908	53.70 (14.87)	1	4.44 (3.29)	0.33	60.11 (45.18)	1	18.55 (18.37)	0.89	21.54 (17.73)	1
8968	126.50 (21.98)	1	5.73 (6.28)	0.73	104.64 (80.68)	1	25.5 (24.12)	0.89	35.3 (30.31)	1
10030	44.75 (7.69)	1	7.44 (7.51)	0.91	22.67 (14.78)	1	16.63 (9.53)	0.89	17.77 (10.27)	1
10357	65.92 (11.97)	0.86	5.62 (6.64)	0.54	44.11 (34.47)	0.98	10.52 (12.53)	0.97	13.18 (11.15)	0.96
10381	214.75 (42.18)	1	14.45 (14.54)	0.73	165.79 (132.69)	1	75.67 (68.01)	1	86.8 (100.69)	1
11768	55.25 (13.65)	1	2.22 (1.56)	0.60	49.14 (38.00)	1	10.56 (11.00)	1	17.3 (16.17)	1
12780	-	-	3.86 (2.67)	0.23	177 (29.79)	0.21	-	-	2.56 (1.86)	0.8
13087	-	-	4.5 (4.35)	0.22	819.33 (166.25)	0.21	-	-	6.62 (8.46)	0.7

13200	-	-	6.5 (1.73)	0.13	730.67 (120.70)	0.21	-	-	4.88 (5.99)	0.8
13479	-	-	11.17 (0.41)	0.13	675.22 (103.43)	0.21	-	-	6.94 (6.92)	0.6
13829	-	-	4 (3.46)	0.13	301.33 (66.24)	0.21	-	-	3.38 (3.18)	0.8
14594	-	-	1 (0)	0.07	28.5 (20.89)	1	10.5 (14.10)	0.89	3.57 (3.21)	0.7
14646	34 (7.0)	0.75	4 (3.79)	0.47	18.57 (13.74)	1	10.13 (9.89)	0.89	3.75 (2.55)	0.8
14860	45.67 (9.02)	0.75	3.38 (3.25)	0.53	25 (17.61)	0.93	13.33 (12.92)	1	3.38 (3.25)	0.8
14897	53 (3.46)	0.75	6.56 (7.30)	0.60	25.31 (18.68)	0.93	12.56 (14.13)	1	2.83 (2.64)	0.6
15190	21 (2.65)	0.75	4 (2.16)	0.27	8.64 (7.09)	1	6.71 (5.91)	0.78	2.17 (2.04)	0.6
15236	82.50 (10.13)	0.75	3.41 (3.30)	0.73	36.89 (26.79)	1	14.44 (11.87)	0.89	3.55 (2.61)	1
16424	126.50 (9.89)	0.75	9.89 (11.86)	0.60	93.11 (70.56)	1	30.28 (31.28)	1	9.89 (8.69)	0.9
17035	275 (35.32)	0.75	21.95 (28.42)	0.84	119.38 (94.61)	1	83.44 (81.76)	1	13.93 (10.80)	0.97
17329	97.22 (4.84)	0.75	5.03 (5.39)	0.73	56.67 (43.38)	1	20.81 (20.66)	1	4.83 (3.10)	0.78
17922	152.67 (8.08)	0.75	8.78 (15.62)	0.60	68.86 (57.33)	1	44 (49.13)	1	8.4 (5.48)	1
18505	55.67 (0.58)	0.75	3 (3.5)	0.40	35 (25.91)	1	12.33 (13.60)	1	3.44 (2.79)	0.9
19107	53.50 (5.92)	0.75	3.38 (5.34)	0.53	17.86 (16.35)	1	10 (9.36)	0.78	2 (2.15)	0.7
19173	70.33 (25.01)	0.75	6.1 (7.64)	0.67	46.64 (35.99)	1	23.25 (18.96)	0.89	4.13 (3.76)	0.8
19597	52 (3.46)	0.75	5 (6.28)	0.33	26.29 (21.85)	1	11.75 (12.45)	0.89	4.33 (2.34)	0.6
19603	-	-	20.25 (17.04)	0.13	454.67 (69.64)	0.21	-	-	4.36 (4.33)	0.7
19664	73.50 (17.54)	0.75	3.5 (2.48)	0.60	46.23 (32.70)	0.93	16.22 (12.43)	1	5.44 (4.08)	0.8
19774	100.17 (11.27)	0.50	7.31 (8.67)	0.58	53.52 (42.17)	1	26.38 (24.97)	0.96	6.15 (5.76)	0.87
19782	110.9 (45.76)	0.83	11.21 (14.61)	0.76	88.14 (66.73)	1	40.74 (36.59)	1	10.46 (8.80)	0.93
20321	53.67 (10.26)	0.75	2.33 (1.21)	0.40	28.46 (19.35)	0.93	9.75 (10.17)	0.89	2.63 (2.50)	0.8
20869	79.22 (21.61)	0.75	6.22 (7.15)	0.60	40.74 (30.94)	1	15.93 (14.16)	1	5.5 (4.21)	0.8
21214	433.67 (52.19)	0.75	34.94 (37.07)	0.78	3.33 (2.58)	0.14	61.67 (90.30)	0.67	-	-
21978	221.50 (65.36)	1	3.17 (2.04)	0.40	72.31 (79.58)	0.93	15.38 (17.13)	0.89	2.57 (2.07)	0.7
22720	103.38 (27.82)	1	2.79 (2.04)	0.40	40.5 (40.57)	0.93	10.81 (12.28)	0.89	1.6 (0.50)	0.5
22814	111.31 (18.38)	1	4.07 (6.09)	0.47	51.14 (61.80)	1	10.6 (11.40)	0.83	1.83 (1.03)	0.58
23634	55.92 (18.23)	1	1 (0)	0.16	18.60 (23.44)	0.93	6.47 (4.19)	0.63	1 (0)	0.27
23911	239.50 (90.99)	1	3.05 (2.68)	0.47	125.75 (139.52)	1	18.65 (21.27)	0.89	4.10 (3.09)	0.7
24072	54.63 (15.19)	1	3.5 (0.58)	0.13	15.43 (18.43)	1	5.14 (5.93)	0.78	1 (0)	0.3
24346	97.75 (30.02)	1	1 (0)	0.20	23.92 (25.22)	0.93	5.38 (5.15)	0.89	1.5 (1)	0.4
25231	30.25 (11.93)	1	1.5 (0.71)	0.13	19.69 (22.06)	0.93	4.86 (4.14)	0.78	1 (0)	0.3
25325	28.33 (9.18)	1	2 (0.67)	0.33	38.12 (35.61)	0.93	8.29 (6.64)	0.78	2.25 (1.54)	0.6
25785	52.03 (27.32)	1	1.25 (0.44)	0.20	9.32 (8.71)	0.85	6.38 (6.43)	0.51	1.29 (0.46)	0.3
25814	99.42 (27.96)	1	2.87 (2.47)	0.33	39.82 (40.90)	0.93	7.15 (5.65)	1	2 (0.74)	0.4
27139	107.75 (30.86)	1	1.27 (0.55)	0.29	44.6 (45.89)	0.93	11.46 (10.08)	0.58	2.45 (2.14)	0.4

27617	-	-	8.33 (10.12)	0.20	461.5 (325.94)	0.29	-	-	8.14 (8.49)	0.7
27654	-	-	13.5 (0.71)	0.07	439.83 (84.17)	0.21	-	-	3.17 (3.07)	0.9
27912	65.19 (14.58)	1	1.58 (0.51)	0.20	22.15 (22.57)	0.93	4.93 (4.16)	0.75	1.27 (0.46)	0.38
28161	22.67 (9.87)	0.75	1 (0)	0.07	13.08 (12.27)	0.93	4.67 (3.21)	0.33	1.5 (0.71)	0.2
28878	117.17 (28.45)	1	1.71 (0.92)	0.38	41.48 (46.56)	1	7.52 (8.14)	1	1.3 (0.48)	0.33
30970	207.25 (38.91)	1	12.8 (18.33)	1	105.57 (69.73)	1	55.89 (52.59)	1	17.89 (20.67)	0.9
31650	131.81 (52.71)	1	16.79 (22.96)	0.87	154.59 (103.58)	1	96.78 (100.84)	1	34.33 (29.85)	0.9
32119	15.50 (6.65)	1	3 (3.09)	0.33	6.74 (5.71)	0.82	4.77 (4.97)	0.72	4.67 (3.26)	0.6
32124	196.08 (52.70)	1	15.97 (25.67)	0.87	127.86 (82.49)	1	59.59 (54.96)	1	15.47 (15.92)	1
32213	97.13 (28.0)	1	8.69 (8.08)	0.53	59.11 (37.08)	1	31.33 (31.51)	1	9.95 (9.22)	0.95
32401	114.10 (32.06)	1	9.78 (12.06)	0.69	99.9 (74.53)	1	51.73 (49.18)	1	15.78 (12.06)	1
33321	195.75 (33.14)	1	15.33 (22.31)	1	124.5 (85.20)	1	60.89 (63.83)	1	21.22 (20.80)	0.9
33913	-	-	22 (1.41)	0.13	599 (97.81)	0.21	-	-	5.43 (5.94)	0.7
35101	16.42 (7.75)	1	3.33 (4.76)	0.80	9.71 (6.37)	1	9.15 (7.94)	1	4.39 (1.20)	0.6
35495	205.63 (44.10)	1	19.14 (25.80)	0.93	198.91 (128.82)	1	99.28 (84.82)	1	38.08 (39.70)	1
36470	185.5 (50.22)	1	11.5 (15.19)	0.80	111.07 (81.20)	1	51.11 (47.89)	1	22.7 (25.97)	1
36792	-	-	5.25 (8.5)	0.27	683.67 (134.08)	0.21	-	-	3.75 (3.73)	0.8
38487	-	-	16.5 (4.93)	0.13	666.78 (141.66)	0.21	-	-	6.25 (8.77)	0.8
40125	56.58 (6.32)	1	5.58 (8.81)	0.73	15.29 (10.96)	1	11.67 (14.27)	1	3 (1.68)	0.83
40254	-	-	5 (0)	0.07	231.33 (28.31)	0.21	-	-	3.25 (3.86)	0.4
41405	243 (54.91)	1	18.5 (27.70)	0.93	194.64 (109.21)	1	97.78 (93.05)	1	29.2 (35.57)	1
42004	211.88 (15.09)	1	19.79 (28.44)	0.80	116.14 (74.16)	1	63.89 (59.75)	1	19.8 (18.72)	1
43032	168.25 (21.70)	1	13.17 (15.39)	0.80	80.57 (58.39)	1	45.56 (45.64)	1	13.6 (11.16)	1
44774	187 (20.35)	1	15.43 (20.26)	0.93	120.96 (81.99)	1	61 (56.01)	1	16.75 (17.27)	1
45313	48 (12.13)	1	5.75 (7.52)	0.67	28.89 (21.10)	1	17.83 (16.29)	1	8.39 (7.81)	0.9

PCA analysis identified two distinct clusters, corresponding to a sambar deer group and a rusa deer group (Fig.3). Again, a single sambar deer sample was assigned to the rusa deer group; this is the same sample identified with the species amplicon panel as a rusa x sambar hybrid. Planes 1 and 2 explained 67.5% and 4.9% of the variance observed respectively in the PCA plot.

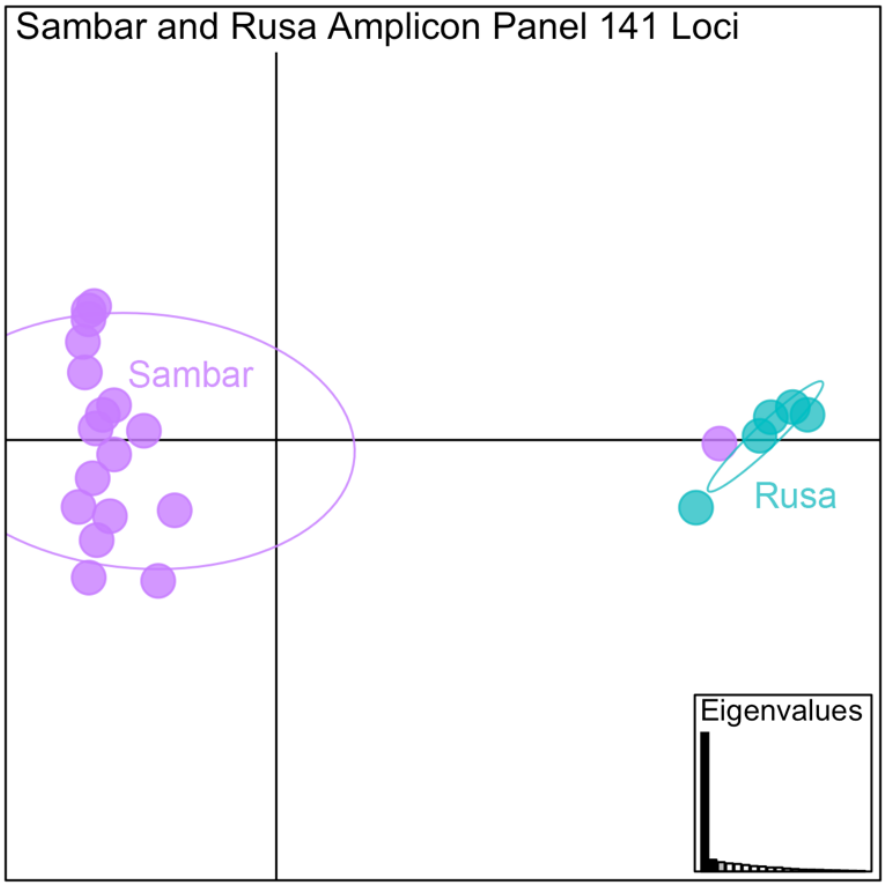

Fig.3 Principal component analysis plot using the sambar deer and rusa deer amplicon panel, retaining 141 loci

AMPLICON SNPS AND STRS COMPARISON

A total of 46 samples were successfully amplified for 56 SNP loci, and 45 samples were amplified using 6 microsatellite loci. To ensure direct comparison between both marker panels, only 38 samples amplified by both marker sets were retained for further analysis.

Identification of clones in *Colony* revealed a total of 18 unique multi-locus genotypes (MLGs) in the SNP amplicon panel, and 19 unique MLGs in the microsatellite dataset (Fig.4). Probabilities of MLGs were higher in the SNP amplicon dataset compared to the STR dataset, with 10 MLGs comprising a probability of 0.99 or higher in the SNP amplicon dataset, while only 5 MLGs showing at a probability of 0.99 or higher in the STR dataset. This also corresponds to the number of samples assigned to an MLG with a high probability; 21 samples in total were assigned with probabilities >0.9 in the SNP dataset, compared to only five samples in the STR dataset. Probabilities <0.5 were observed for three MLGs comprising three samples for the SNP amplicon dataset, compared to six MLGs comprising 21 samples in the STR dataset. Four MLGs were consistent across both datasets, albeit at varying probabilities.

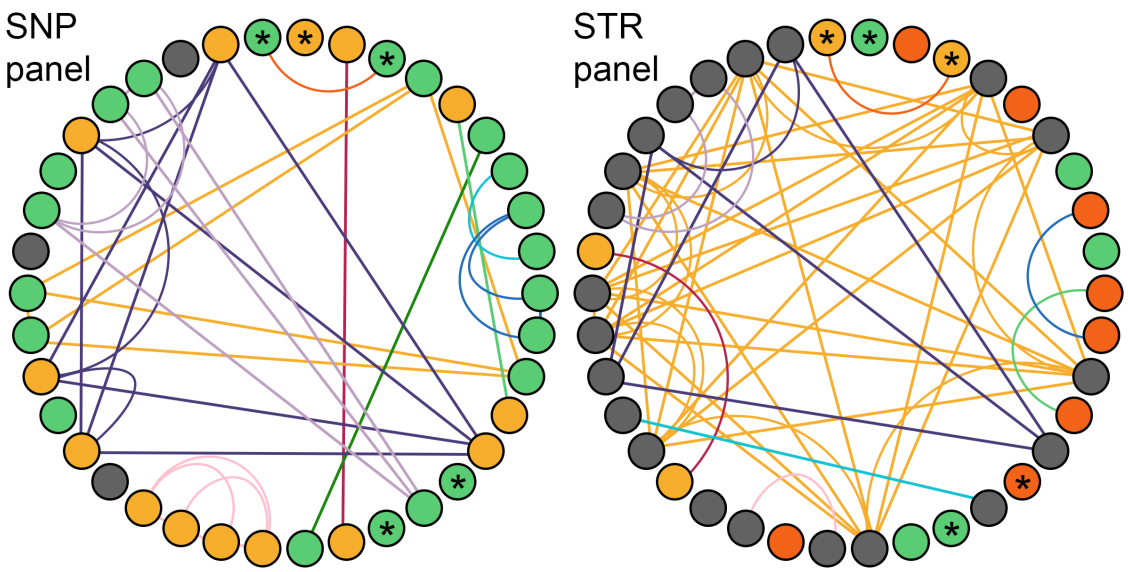

Fig.4 Network graphs depicting individual samples identified as clones in the SNP amplicon and STR datasets. Circles represent each sample, with samples shown in the same order in both networks. Circle colours represent multi-locus genotype (MLG) probabilities from Colony, with green circles comprising a probability >0.9, orange circles a probability between 0.7-0.89, yellow circles between 0.5-0.69, and grey circles comprising a probability <0.5. Coloured lines show samples belonging to the same MLG. Samples with an asterisk (*) were identified as the same MLG in both SNP amplicon and STR datasets

One sample from each unique MLG was retained for analyses of observed and expected heterozygosity and probability of identity for both marker panels, regardless of probability values for each MLG identified in *Colony*, leaving 18 samples in the SNP amplicon dataset and 19 samples in the STR dataset. Average number of alleles observed in the STR dataset was 2.833. While expected heterozygosity was similar between the SNP amplicon and STR panels (0.378 and 0.322 respectively), observed heterozygosity was much greater in the SNP amplicon panel with a value of 0.574, compared to 0.202 observed in the STR dataset.

The final cumulative probability of identity value of 1.1×10^{-2} and 4.7×10^{-19} was observed for the STR and SNP amplicon datasets respectively (Fig.5). The most informative locus in the STR panel was IDVGA55 with an individual probability of identity of 1.7×10^{-1}, while the most informative locus in the SNP amplicon panel comprised an individual probability of identity value of 3.8×10^{-1}. A total of 28 SNP markers shared this value. In order to create an STR panel with the same power to discriminate individuals as the SNP amplicon panel, a total of 54 STR markers would be needed, based on the average probability of identity per marker for the STRs genotyped in this study.

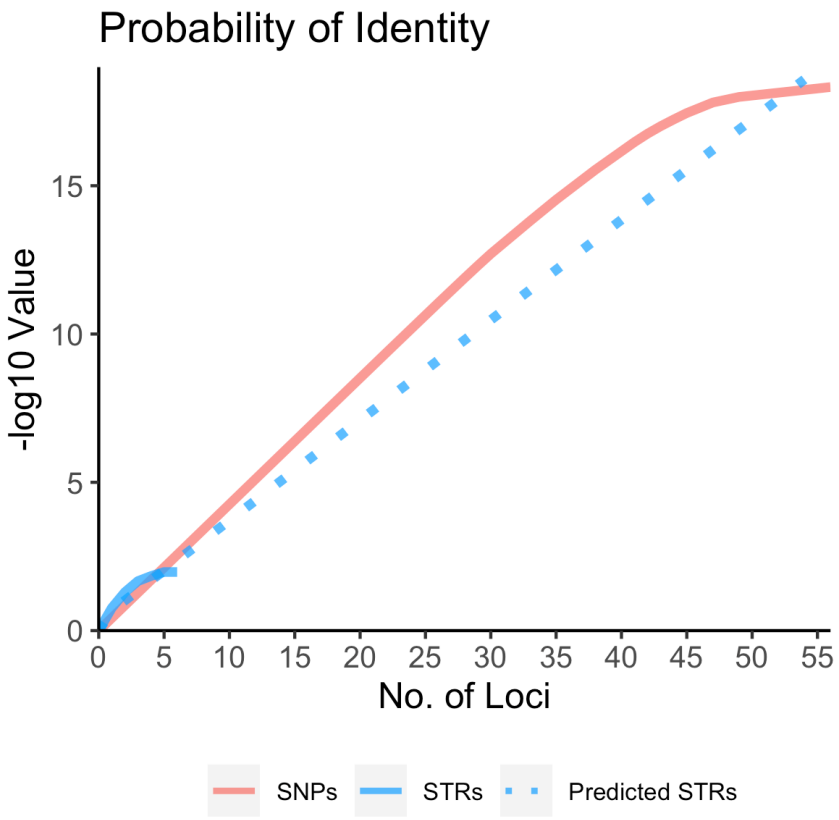

Fig.5 Cumulative probability of identity values for the final STR panel (6 STRs) and final SNP amplicon panel (56 SNPs) using one sample from each unique multi-locus genotype identified in each dataset (n=19 in STR panel, n=18 in SNP panel). The predicted number of STR markers necessary to achieve the same probability of identity value as the SNP amplicon panel is shown by the dotted line. Values have been -log10 transformed, so larger values represent a lower total probability of identity

DISCUSSION

When developing marker panels for the monitoring of species via non-invasive samples, much of the time and cost is dedicated to generating genomic datasets with enough resolution to allow SNP identification and primer design. However, as large genomic datasets from previous research become more readily available online in databases such as GenBank, these initial steps can be eliminated, saving time and money that can be better utilised for species monitoring. Here, we show how it is possible to create multiple SNP amplicon panels from pre-existing genomic data available online with applications in species identification and individualisation of single species. We also demonstrate how SNP amplicon panels can outperform STR panels when identifying individuals, especially in a species where genetic bottlenecks or founder events are likely to have occurred and, hence, STR variability is limited.

AMPLICON PANELS

The amplicons chosen for the interspecies amplicon panel were able to successfully distinguish between the four deer species sampled in this study, using 56 SNPs present in 11 amplicons. A hybrid sample was additionally detected using this panel, however showed a consistent profile to rusa deer rather than intermediate genotypes between sambar and rusa deer and would not have been detected as a hybrid if morphological and mitochondrial data did not exist for this sample. This sample was identified as a backcross to rusa deer in previous work (Hill *et al.* In Review), and it is therefore unsurprising that this individual comprised a profile consistent to rusa in the interspecies amplicon panel. An additional individual morphologically identified as a sambar was shown to be a backcross to sambar in (Hill *et al.* In Review) (sample SambarBlood10), and this hybrid class was not detected in any analyses in the present study. F_1 hybrids and backcrosses can be detected using SNP assays

that employ fixed SNP differences (Lee *et al.* 2014; Nussberger *et al.* 2013), and it is possible that the interspecies amplicon panel described here is also capable of identifying hybrids, however further testing of the panel on samples of known hybrid classification is necessary to confirm this. While the rusa backcrossed individual included in the initial analysis would not have been detected as a hybrid if additional morphological and genetic data was unknown, the addition of a mitochondrial marker to the amplicon panel is likely to be of benefit for hybrid identification, particularly for F_2 and backcrossed hybrid classes.

While fixed differences were observed between all four deer species sampled (sambar, rusa, fallow, and hog deer) using the interspecies amplicon panel, an additional two deer species established in Australia, red deer and chital, were not sampled in this study. Further validation of this panel to include samples of red deer and chital would be beneficial to ensure that fixed differences are also present in these species, and that the panel is robust in delineating all free-ranging deer species present in Australia.

While the intention of the intraspecies marker panel was for use in monitoring sambar deer populations across Australia, the inclusion of amplicons showing fixed differences between sambar and rusa, and SNPs only polymorphic in rusa, was logical considering the close relationship between the two species (Martins *et al.* 2018). We have demonstrated that our intraspecific panel is suitable for identifying unique individuals in sambar deer for future mark recapture studies, however, further validation of the power of this panel to identify individual rusa deer samples is necessary.

SAMPLE TYPES AND AMPLICON PERFORMANCE

Unsurprisingly, amplification of both amplicon panels was affected by sample type, with scat samples showing the lowest average read depths and genotype rates of all sample types tested in sambar deer. Exposure to sunlight, moisture, temperature, and time between sample deposition and collection for genetic analysis have been shown to affect genotyping success (Hájková *et al.* 2006; Stetz *et al.* 2015); these factors are unlikely to have affected SNP amplification in the tissue and blood samples as these were collected from fresh material during deer culls, however are likely to be significant issues in the scat samples collected in this study. Roughly half of the scat samples collected and genotyped for both the SNP amplicon and STR panels failed to amplify any fragments, suggesting that amplification failure is unlikely to be an issue with panel performance and more likely to be due to sample degradation. No quality control processes were implemented during laboratory preparation of scat samples in this study however these will be necessary in future to further reduce costs associated with non-invasive monitoring of sambar deer. Utilising qPCR methods to test likely amplification success has been used as a quality control step prior to downstream lab processes and sequencing (Hayward *et al.* 2022; Hayward *et al.* 2020), and may be beneficial for further non-invasive monitoring of sambar deer.

COMPARISON OF AMPLICON SNPS TO STRS

Overall, the SNP amplicon panel described in this study for sambar deer individualisation appears to outperform STRs when identifying unique individuals. Unique MLGs were detected with higher confidence when using the SNP panel, and a lower cumulative probability of identity was observed using the SNP panel. The predicted number of STRs required to achieve similar performance to the SNP amplicon panel was 54 STRs, only slightly lower than the number of SNPs retained in the final probability of identity analysis. This is likely due to the low number of alleles identified in the STRs used in this study (average 2.833 across all STR loci) and highlights the importance of considering the biological history of populations of interest prior to genetic analysis. Populations that have undergone recent declines through population bottlenecks or founder events are likely to experience a reduction in genetic diversity, which can affect the performance of marker panels. Introduced populations typically show a reduction in genetic diversity compared to their native counterparts (Puillandre *et al.* 2008), and marker panels developed on native populations of a species may be less effective for population genetic analysis in introduced populations.

Therefore, there is a need for the validation or development of panels specifically for use in introduced populations. The creation of SNP amplicon panels allows many more markers to be amplified for

analysis, circumventing the issue of low allelic diversity in introduced populations and increasing confidence in statistical analyses, and avoids known issues with STR genotyping in non-invasive samples such as genotyping error rates (Pompanon *et al.* 2005).

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APPENDIX 4. HYBRIDISATION RATES, POPULATION STRUCTURE AND DISPERSAL OF SAMBAR DEER (*CERVUS UNICOLOR*) AND RUSA DEER (*CERVUS TIMORENSIS*) IN SOUTH-EASTERN AUSTRALIA

HYBRIDISATION RATES, POPULATION STRUCTURE, AND DISPERSAL OF SAMBAR DEER (*CERVUS UNICOLOR*) AND RUSA DEER (*CERVUS TIMORENSIS*) IN SOUTH-EASTERN AUSTRALIA

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SUMMARY

Understanding connectivity and dispersal capability is important for management of invasive deer species in Australia. Genetic analysis of sambar deer (*Cervus unicolor*) and rusa deer (*Cervus timorensis*) was undertaken to assess broad-scale population structure across south-eastern Australia, and additionally characterise hybridisation rates between the species. Multiple hybrid classes were detected in the dataset, and population structure was evident across sambar deer populations. Broad-scale population structure was less apparent in rusa deer, however analyses of dispersal capability suggest that movement is limited in both species, allowing the management of distinct genetic units.

Image credit, Arthur Rylah Institute

ABSTRACT

Context: Introduced populations of sambar deer (*Cervus unicolor*) and rusa deer (*Cervus timorensis*) are present across south-eastern Australia and are subject to local population control to alleviate their negative impacts. For management to be effective, identification of dispersal capability and management units are necessary. These species also readily hybridise, so additional investigation of hybridisation rates across their distributions is necessary to understand the interactions between the two species.

Aims: Measure the hybridisation rate of sambar and rusa deer, assess broad-scale population structure present within both species and identify distinct management units for future population control, and measure the likely dispersal capability of both species.

Methods: A total of 198 sambar deer, 189 rusa deer, and 3 suspected hybrid samples were collected across Victoria and New South Wales (NSW) and sequenced using the Diversity Arrays Technology DArT sequencing platform. After filtering, 14,099 polymorphic single nucleotide polymorphism (SNP) markers were retained for analysis. Hybridisation rates were assessed before the data was split by species to identify population structure, diversity indices, and dispersal distances.

Key results: Across the entire dataset, 17 hybrids were detected. Broad-scale population structure was evident in sambar deer, but not between the sites where rusa deer were sampled. Analysis of dispersal ability showed that a majority of deer movement occurred within 20 km in both species, suggesting limited dispersal.

Conclusions: Distinct management units of sambar deer can be identified from the dataset, allowing independent population control. While broad-scale population structure was not evident in the rusa deer populations, dispersal limits identified suggest that rusa sites sampled in this study could be managed separately. Sambar x rusa hybrids are present in both Victoria and NSW and can be difficult to detect based on morphology alone.

Implications: Genetic analysis can identify broad-scale management units necessary for population control, and considerations of dispersal capability can assist in delineating management units where broad-scale population structure may not be apparent. The negative impacts associated with hybridisation require further investigation to determine if removal of hybrids should be considered a priority management aim.

Keywords: invasive species, pest species, hybridisation, wildlife management, connectivity, dispersal, management units, non-native deer

Running head: Sambar and rusa deer population genetics

INTRODUCTION

The global desirability of deer as a hunting resource has prompted both historical introductions outside of their native range and contemporary anthropogenic movements of deer across landscapes (Dolman and Wäber 2008). As the abundance and distribution of non-native deer populations increase, their undesirable environmental, economic, and social impacts also increase (Cripps *et al.* 2019; Davis *et al.* 2016; Hampton and Davis 2020; Honda *et al.* 2018). In Australia, deer were first released in the 19th century, predominantly by Acclimatisation Societies, with some of these introductions establishing self-sustaining wild populations (Bentley 1998; Moriarty 2004b). Today, six deer species are present in Australia: sambar deer (*Cervus unicolor*), fallow deer (*Dama dama*), red deer (*Cervus elaphus*), Javan rusa deer (*Cervus timorensis*), chital (*Axis axis*), and hog deer (*Axis porcinus*). Deer populations have not been considered high priorities for control in Australia until the last decade (Bomford and Hart 2002; Davis *et al.* 2016). Now, most States and Territories list deer as a pest species (Davis *et al.* 2016), with localised management undertaken for all six deer species to reduce their abundances and limit range expansions. Despite these efforts, the abundance and

distribution of many of these species and their undesirable impacts continue to increase (Bennett and Coulson 2010; Davis *et al.* 2010; Forsyth *et al.* 2015; Forsyth *et al.* 2016; Roberts *et al.* 2015).

Sambar deer are one of the most successful deer introductions to Australia (Bentley 1978; Long 2003). Introduced by the Victorian Acclimatisation Society to several sites in Victoria during the 1860s and 1870s, the population has expanded to occupy ~29% of Victoria's land area (Forsyth *et al.* 2015), with the population continuing to expand further north into New South Wales (NSW) (Crittle and Millyn 2020). Isolated populations of sambar deer are also present in western Victoria and the Northern Territory (Davies *et al.* 2021; Moriarty 2004b). The closely related rusa deer was also introduced to Australia by Acclimatisation Societies in the 19th century but has a much smaller range than sambar deer. Rusa deer were released in Victoria, NSW, and Western Australia (WA), however the WA population is thought to have failed to establish, and the population in Victoria was extirpated in the 1940s (Long 2003). Self-sustaining populations of rusa deer have also established in Queensland, likely from deer farm escapees and releases (Bengsen *et al.* 2022b; Dryden 2000). The current distribution of rusa deer in NSW appears to be patchy, however large populations are present in Wollongong, Forster, and Port Macquarie (Fig. 1). The expansion of distributions for both sambar and rusa deer appear to have been additionally aided by translocations of animals to new sites, and releases/escapes from deer farms during the 1990s (Moriarty 2004b). Both species are farmed in Australia, however sambar and rusa deer farms comprise only a small proportion of all deer farming in the country (Shapiro 2010).

The abundance and distribution of sambar deer and rusa deer are increasing across south-eastern Australia. In Victoria, annual recreational harvesting of sambar deer has escalated over the last decade, with an estimated 131,258 sambar harvested in 2019 alone (Moloney *et al.* 2022; Moloney and Hampton 2020). Population growth of the species in Victoria is currently estimated at 15% annually (Watter *et al.* 2020). An annual population growth of 10% was estimated for rusa deer in Royal Park between 1999-2000 (Moriarty 2004a), however, no recent estimates of population growth are available for this species. Nonetheless, given that rusa deer distribution continues to increase in NSW (Crittle and Millyn 2020) it is likely that their abundance is also increasing. As the abundance and distribution of both species rises, the negative impacts associated with deer over-abundance become apparent, including destruction of native flora through bark stripping and trampling, soil erosion, competition with native herbivores for food sources, and wallowing (Bennett and Coulson 2010; Davis *et al.* 2016; Davis *et al.* 2008; Keith and Pellow 2005; McDowell 2007). Deer in Australia have also been shown to carry diseases that could impact livestock (Huaman *et al.* 2020) and can additionally pose risk to humans through deer-vehicle collisions which are likely to become more commonplace as deer encroach peri-urban environments (Burgin *et al.* 2015; Davies *et al.* 2019). Rusa and sambar deer are also known to hybridise (Martins *et al.* 2018), with suspected hybrids thought to be present in the wild in Australia (Forsyth *et al.* 2015). These hybrids may pose additional negative impacts as some genetic fitness may be associated with hybrids (heterosis), which can promote changes to life history traits or phenotypes that may be even more detrimental to native environments than either parent species (Iacolina *et al.* 2019). Sambar and rusa hybrids grow rapidly and changes in body mass can promote further damage to native environments or increase the severity of deer-vehicle collisions where larger animals are involved (Pacioni *et al.* 2021; Tuckwell 1998). Currently, the extent of hybridisation within sambar and rusa deer populations in Australia is unknown.

To mitigate the negative impacts of sambar and rusa deer across south-eastern Australia, population control through ground- and helicopter-based shooting is often undertaken. Such control can reduce deer densities locally (Bengsen *et al.* 2022a), but understanding larger-scale population structure, connectivity and dispersal capability, is important for identifying larger-scale and longer-term priorities as well as estimate the time needed for reinvasion. (Comte *et al.* 2022; Leslie Jr 2011; Semiadi *et al.* 1994). While monitoring of sambar and rusa deer can be difficult using traditional techniques due to their elusive nature, genetic analysis of populations of interest can assist in determining movement and population structure within each species and is critical to identify distinct management units where targeted control can take place. These methods have been widely implemented for a range of invasive species globally to identify management units for targeted population control or eradication (Cowled *et al.* 2008; Fraser *et al.* 2013; Mora *et al.* 2018; Sjodin *et al.* 2020) and present a viable

approach to inferring population structure and potential management units across south-eastern Australia. While previous genetic studies have examined sambar deer connectivity in Victoria (Davies *et al.* 2021), and fine-scale structure of rusa deer in Royal National Park, and Wollongong in NSW (Li-Williams *et al.* in review - this issue; Webley *et al.* 2004), the present study aims to expand the sampling range of both species to include additional sites across Victoria and NSW to assess broad scale population structure in both species across south-eastern Australia, and use 1000s of highly resolving single nucleotide polymorphism (SNP) markers that will additionally aid in the identification of potential hybrids. Specifically, we aim to 1) determine the interactions between sambar and rusa deer where the two species co-occur through hybrid analysis, 2) assess any broad-scale population structure present within sambar deer and rusa deer and identify any distinct management units for each species, and 3) measure the likely dispersal capability of each species.

METHODS

SAMPLE COLLECTION

Samples of free-ranging sambar deer and rusa deer were collected between 2013-2021, predominantly during targeted culling operations carried out by local government agencies in New South Wales and Victoria (Fig.1, Table 1). These samples primarily consisted of tissue collected from ear tips using sterile surgical scissors, scalpels and tweezers and stored in 5 mL vials containing 100% ethanol, or via biopsy punches using Allflex Tissue Sampling Applicators and Tissue Sampling Units. In NSW samples were collected from three known sambar populations; Albury/Murray River in South NSW (possible natural range expansion); Werakata National Park in the Hunter Valley (likely originated from a failed sambar and rusa farm) and Harrington (unknown source). Additional incidental sambar samples were collected from a combination of Pest Control operations and animals culled by commercial harvesters or landholders. Blood samples of sambar and rusa were also collected from a cull conducted at Port Macquarie in North NSW in 2018. The sex of each animal, an indication of whether each animal was an adult or juvenile, and the sampling location were recorded. Deer that showed intermediate characteristics that may indicate the animal was a hybrid were also noted, including atypical body size and ear morphology.

DNA ISOLATION AND SEQUENCING

Blood and a subset of the tissue samples were extracted using a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturers' instructions. Approximately 150 uL of blood was used as starting material for blood extractions, and all samples were eluted using 100 uL of deionised H_2O. DNA was quantified using a Qubit 2.0 Fluorometer (Invitrogen), and samples were then either diluted with deionised H_2O or concentrated using an RVC 2-18 Rotational Vacuum Concentrator (John Morris Scientific) to obtain final concentrations between 5-15 ng/uL.

DNA extracts and the remaining tissue samples were sent to Diversity Arrays Technology (DArT) in Canberra, Australia, for DNA extraction and genotyping by sequencing. Sequencing was conducted using an Illumnina HiSeq 2500, using 1.2 million reads per sample, and reads were aligned to a red deer (Cervus elaphus) genome available on GenBank (BioProject PRJNA324173). Rusa deer samples sequenced by Li-Williams et al. (in review – this issue) were additionally combined with the dataset. A total of 5 samples did not provide sufficient data following sequencing, leaving 404 samples for further analysis.

SNP FILTERING

DArT sequencing returned 53,946 single nucleotide polymorphisms (SNPs), of which 41,492 were successfully aligned to the red deer genome. Of these SNPs, 1373 were aligned to either the X or Y red deer chromosome, and to avoid any potential sex-linked markers appearing in the dataset, these SNPs were removed from further analysis. Genotypes were analysed with *dartR 2.0* (Mijangos *et al.* 2022) except when otherwise specified. Loci were filtered with a minimum call rate of 0.95, a minimum read depth of five for each allele (i.e., the reference and alternative allele), and a minimum reproducibility of 0.99. Individuals were removed if they had less than 80% of loci genotyped. Only one SNP was kept where multiple SNPs within the same read were present, with the SNP comprising the highest repeatability and information content chosen. Monomorphic loci and loci with the minor

allele observed only once (i.e., singleton) or twice in the whole dataset were discarded. At the end of these steps, the dataset comprised 390 individuals and 14,333 loci.

This dataset was initially inspected using a Pearson Principal Component Analysis and analysed with the program *fastStructure 1.0* (Raj *et al.* 2014), with output files processed using *StructureSelector* (Li and Liu 2018). We considered the profile of the marginal likelihood and the minimum number of the model components needed to explain the structure in the data to select the optimal number of clusters (K). Both priors, beta and logistic distributions, were used for the allele frequencies. With the beta prior we tested a wide range of K values (1 to 9), however with the logistic prior we limited the analysis to between 1 and 8 as guided by the previous results. Based on the results of the analyses described above, up to five possible genetic clusters were identified. Partitioning the data based on these five clusters, we evaluated possible departure from Hardy-Weinberg equilibrium (HWE) for each locus following recommendations from (Waples 2015) and (De Meeûs 2018). Briefly, the exact tests (Wigginton *et al.* 2005) for departure from HWE were carried out within each population for each locus with a = 0.05. The results were compared with the null expectations of the possible number of significant tests if these were obtained by chance alone (both overall and across multiple populations for the same locus). The Fisher's global test (Fisher 1970) was also assessed to evaluate whether there is at least one test that is truly significant in the series of tests conducted (De Meeûs *et al.* 2009). Furthermore, we assessed the correlation between F_{IS} and F_{ST} (Nei 1978) to investigate potential causes of departure from HWE to determine if loci out of HWE should be removed (e.g. because of null alleles or selection), or retained (e.g. small population size) because the latter can provide important biological insights. Following these filtering steps, a total of 14,099 polymorphic SNP loci and 390 samples were retained for the final analysis, comprising 198 sambar deer samples, 189 rusa deer samples, and 3 suspected hybrids based on morphological identification.

DATA ANALYSIS

From this final dataset, we conducted several analyses, some of which required subsets of samples as summarised in the flowchart (Fig.2) and described in more detail below.

HYBRIDISATION

A Pearson principal component analysis was undertaken for the final dataset using the gl.pcoa function in *dartR* and plotted using *ggplot2 3.3.6* (Wickham 2016). Analyses of fixed loci differences between the rusa and sambar deer were calculated using the gl.fixed.diff and gl.collapse functions in *dartR*, with all suspected hybrid samples removed for this analysis. Private alleles between rusa and sambar deer were calculated using the gl.report.pa function in *dartR*.

FastStructure was used for the final dataset using the methods described above and limiting the number of possible clusters between 1 and 6. We additionally tested for structure using the R package *tess3r 1.1.0* (Caye *et al.* 2016), which provides a spatially explicit analysis of population structure. For the entire dataset, Ks 1-9 were tested with 20 repetitions per K, 100,000 iterations, and a tolerance level of 1e-7 using the ‘projected.ls’ method. Best fit for K was determined via evaluation of cross-validation criterion for each K. The program *NewHybrids 1.1* (Anderson and Thompson 2002), implemented in the R package *parallelnewhybrid 0.0.0.9002* (Wringe *et al.* 2017) was used to assess hybrid classes within the dataset. NewHybrids assigns samples to categories of either parental species, F_1 and F_2 hybrids, or backcrosses to either of the parental species. As this software has a 200 loci limit, loci with missing data were removed using the gl.filter.callrate function in *dartR* using a threshold of 1, and the dataset converted to *NewHybrids* format using the gl.nhybrids function, retaining the first 200 loci ranked on information content using the ‘AvgPIC’ method. Thirty samples of sambar and 15 samples of rusa were randomly assigned as parental species, based on samples identified in *fastStructure* with a Q value above 0.9999 when analysed with K = 2. A burn-in of 100,000 and 500,000 sweeps were used, with 5 replicate runs completed.

INTRASPECIES POPULATION STRUCTURE AND DIVERSITY

Following the hybridisation and species differentiation analysis, the dataset was further subset by species and geographic region to detect weaker structure possibly present in the data. When subsets of data were analysed, loci that were monomorphic within each dataset were also removed (Fig. 2).

Analyses for *fastStructure*, *Tess3*, Pearsons principal components analysis, and the fixed differences analysis were repeated for each species separately (i.e., rusa and sambar deer), using the same methods as described above. For the PCA and fixed differences analysis, hybrid samples were removed.

Pairwise F_{ST}s, diversity indices, inbreeding coefficient F_{IS}, and effective population size (N_e) were all calculated for the distinct geographic regions identified in both the rusa and sambar deer datasets. Pairwise F_{ST} was calculated using gl.fst.pop, while observed and expected heterozygosity (H_O and H_E), and F_{IS} were calculated using the gl.report.heterozygosity function in *dartR*. Effective population size (N_e) was calculated using *NeEstimator V2.1* implemented through the gl.LDNe function in *dartR* (Do *et al.* 2014), using the linkage disequilibrium method. We used a 0.05 allele frequency cut-off and reported the 95% confidence intervals calculated via jack-knifing of samples. Analyses of H_O, H_E, F_{IS}, and N_e were also run with hybrid samples removed.

DISPERSAL DISTANCE

We investigated whether the genetic data would provide any indication of the dispersal distances in sambar and rusa deer using a spatial autocorrelation analysis (Double *et al.* 2005; Peakall *et al.* 2003; Smouse and Peakall 1999). This analysis aims to evaluate whether samples collected within a distance class are genetically more similar (i.e., related) than would be expected at random. A pairwise genetic distance was constructed as 1 – number of mismatches between pairs of samples while a geographical distance matrix included the linear distance between sampling points (in km) computed using the R package *raster* (Hijmans 2022). Custom distance classes were used, with endpoints: 5, 10, 15, 20, 25, 50, 81, 243, and 729 km. Following (Double *et al.* 2005; Peakall *et al.* 2003; Smouse and Peakall 1999), the 95% confidence intervals around the null hypothesis of no spatial autocorrelation were computed conducting 1,000 permutations and these data were used for the one-tail test, while 1,000 bootstraps were conducted to estimate the 95% confidence intervals around the spatial correlation coefficients. The general code used for computing these statistics (gl.spatial.autoCorr) was made publicly available in the development version of the R package *dartR* (https://github.com/green-striped-gecko/dartR/tree/dev). These analyses were conducted after removing all juveniles and individuals of unknown age, separately for the rusa deer samples, and the south NSW/Gippsland and Melbourne, and north NSW sambar deer clusters. The samples from Werakata NP were not included in this analysis due to small sample sizes. For each dataset, these analyses were also repeated separating males and females.

Analysis of kinship was undertaken using *Colony 2.0.6.8* to assess the dispersal of kin throughout the landscape and infer contemporary dispersal (Jones and Wang 2010). *Colony* was run using the same datasets as described above for the spatial autocorrelation analysis, with hybrid samples removed, and assuming polygamy in both males and females, inbreeding, and no sibship prior or candidate parents. The pairwise full likelihood combined method for analysis was chosen with high precision and updating allele frequencies, and a 0.05 allele dropout rate and false allele rate per locus were also assumed. *Colony* was run three times to ensure convergence of results.

RESULTS

HYBRIDISATION

PCA analyses identified three distinct genetic clusters within the entire dataset, corresponding to one rusa deer group, and two groups of sambar deer broadly split into a northern NSW and southern NSW/VIC group (Fig.3). A number of samples from NSW fall between the rusa and sambar clusters, in addition to two samples from VIC that were flagged as potential hybrids based on morphological identification. A third sample that was identified as a potential hybrid based on morphological I.D. is clustered with the NSW sambar group. After removing suspected hybrid samples and reclassifying two samples that had been misidentified (the morphologically identified hybrid sample clustering in the sambar NSW group, and a sample morphologically identified as rusa but genetically assigned as sambar), fixed difference analysis found 2798 loci were fixed between the rusa and sambar samples (false positive expectation 0.4, $p < 0.0001$), with all sambar clusters identified in the PCA collapsing

into a single sambar population (Supplementary Material 1 Fig.1). A total of 4439 private alleles were present within the rusa cluster, and 9685 private alleles present in the sambar cluster.

FastStructure analysis of the entire dataset indicated that K = 2 clusters were present, corresponding to the two species (Fig.4a). Similar to the PCA, a number of samples were intermediate between the two species clusters, including seven samples from North NSW, eight samples from Wollongong and two samples from South NSW/VIC. Results from *Tess3* were largely congruent with *fastStructure* results, with the optimal K value at K=2 and clusters corresponding to the two species (Fig.4b). However, additional mixing of the two clusters was evident in the *Tess3* results in the North NSW group, while the samples that showed intermediate genotypes between the two clusters in Wollongong from the *fastStructure* analysis were less evident in the *Tess3* plots.

NewHybrids analysis revealed that intermediate samples identified in *fastStructure* and *Tess3* comprised a range of hybrid classes (Fig.4c). In North NSW, F_1 hybrids, as well as hybrids backcrossed to either rusa or sambar deer, were detected in this region. Samples from Port Macquarie comprised two individuals that had backcrossed to rusa, with one sample each having been morphologically identified as a sambar and rusa deer, and two individuals backcrossed to sambar deer, both morphologically identified as sambar deer during sample collection. Samples obtained from Cattai Wetlands comprised one F_1 hybrid that had been morphologically identified as a rusa deer, and one individual backcrossed to sambar deer, identified during sample collection as a sambar deer. A single sample from Harrington was identified as an F_1 hybrid, previously classified as a rusa deer. In Wollongong, all eight samples showing intermediate genotypes from *fastStructure* analysis were assigned as backcrosses to rusa deer. The two suspected hybrids collected in Victoria were confirmed to be hybrid animals, with the Croajingolong sample identified as an F_1 hybrid, and the Cloverlea sample assigned as an F_2 hybrid. The third suspected hybrid sample collected from Cattai wetlands (North NSW) was assigned to the parental sambar class and comprised a Q value of 0.999 for the sambar deer cluster in *fastStructure*. Finally, a second individual from Cattai Wetlands morphologically assigned as a rusa deer was in fact a sambar deer based on *fastStructure* and *NewHybrids* results.

INTRASPECIES POPULATION STRUCTURE AND DIVERSITY

The dataset was subsequently split into a rusa deer group and a sambar deer group to further elucidate any fine scale structure present within each species. Both *fastStructure* and *Tess3* identified K = 2 genetic clusters in the rusa dataset, however these clusters separated the parental rusa from the hybrid samples and were not reflective of population structure detected between sites (Supplementary Material 2 Fig.1). Within the sambar deer, *fastStructure* indicated K = 2 genetic clusters, again separating parental sambar from hybrid samples (Supplementary Material 2 Fig.2), however, *Tess3* identified K = 5 genetic clusters within the sambar samples (Fig.5). While one of these clusters comprised only hybrid samples, the remaining four clusters were associated with geographic region, with a distinct cluster identified in North NSW, a second cluster specific to Werakata NP, and the final two clusters mixed between South NSW/Gippsland and Melbourne. Interestingly, a single sample from North NSW collected from Willow Tree NP appeared to cluster with the South NSW/Gippsland and Melbourne samples.

PCA plots for each species with hybrid samples removed conformed with the sambar results from *Tess3*, with four groups broadly corresponding to North NSW, Werakata NP, South NSW/Gippsland, and Melbourne, and intermixing between South NSW/Gippsland and Melbourne, and the single Willow Tree sample clustering with the Victorian samples (Fig.6). Fixed differences analysis between these sambar populations only showed fixed differences between Werakata NP and North NSW, with only four fixed differences observed. No sambar deer populations were shown to be significantly different, and all collapsed into a single population. When the single Willow Tree sample was removed, and fixed difference analysis rerun, the number of fixed differences between Werakata NP and North NSW rose to five, however all populations were still not significantly different and again collapsed into a single population. Within the rusa samples, some slight clustering can be observed between Port Macquarie and Wollongong which was not detected by *fastStructure* or *Tess3* (Fig.6). No fixed differences were detected between these two sites, with both populations collapsing into a single rusa group.

Pairwise F_{ST}s between species and geographic regions for sambar were all statistically significant, with comparisons between rusa and sambar deer comprising the highest pairwise values between 0.884-0.912 (Table 2). Within the sambar deer geographic regions, pairwise F_{ST} values ranged from 0.035-0.231, with the lowest comparison between Werakata NP and Gippsland, and the highest pairwise value between Melbourne and Werakata NP.

Observed and expected heterozygosity within the major geographic regions identified for rusa and sambar deer showed that the rusa NSW population comprised the lowest values, with a H_O of 0.056 and a H_E of 0.057 with hybrid samples retained, and 0.043 for H_O and H_E with hybrid samples removed (Table 3). Sambar deer present in North NSW comprised the highest observed and expected heterozygosity with hybrid samples included, with a value of 0.153 for each. However, when hybrid samples were removed from this analysis, the sambar population present in Werakata NP comprised the highest H_O and H_E values, followed by South NSW/Gippsland. F_{IS} values were similar when hybrids were present and absent in the dataset and ranged from -0.069 in the sambar Werakata NP population to 0.083/0.068 in the sambar South NSW/Gippsland region. Estimates of effective population size were highest for sambar deer populations present in Victoria and South NSW, with an estimated N_e of 126.9 across South NSW/Gippsland, and 101.1 within the sambar Melbourne samples (Table 3). The rusa populations in NSW comprised an N_e of 63 with hybrids retained and 61 with hybrids removed, and the lowest estimates were observed in the sambar populations in northern NSW, with values of 2.8 in Werakata NP and only 0.7 in North NSW, however this value increased to 11.2 when hybrids were removed (Table 3).

DISPERSAL DISTANCES

The distance class 5-10 km was marginally significant (p=0.047) for the rusa NSW dataset (r=0.0001) (Supplementary Material 3 Fig.1). For the same dataset, the distance classes 20-25 and 25-50 km were also significant (p≤0.028) with negative coefficients (r=-0.0002 and -0.0001 respectively). The last distance class (>243 km) was also significant (p=0.037) and positive (r=0.00015). No distance classes were significant when rusa deer males were analysed alone and only the distance classes > 15 km had a significant (p≤0.04) and negative correlation coefficient (r≤0.0003) when females were analysed alone. Kinship analysis revealed 89 full sibling pairs with probabilities above 0.9 in the Rusa NSW dataset, with no half sibling pairs identified. Of these sibling pairs, 5 were from Port Macquarie, and the remaining 84 from Wollongong, with no siblings identified between the two sites. Within Port Macquarie, four sibling pairs were sampled less than 1 km apart, with one pair separated by 2.6 km (Fig. 7). At Wollongong, 39 sibling pairs were sampled less than 1 km apart, 29 sibling pairs were identified between 1 - 4.9 km, 4 pairs were separated by a distance between 5 – 9.9 km, and 10 pairs sampled greater than 10 km apart, with the largest distance between two siblings recorded at 25.8 km (Fig. 7). Two sibling pairs were identified with a sample where no location information was taken, so kin dispersal could not be measured.

The overall spatial autocorrelation analyses were not significant for the North NSW sambar deer group (Supplementary Material 3 Fig.2) nor were the ones with males only. When females were analysed separately, only the first distance class (0-5 km) returned a significant (p=0.007) correlation coefficient (r=0.0057). Kinship analysis identified 7 full sibling pairs and 17 half sibling pairs between the North NSW sites, with 11 pairs collected from the same sites, and 13 pairs collected from different sites. Pairs sampled from the same site showed dispersal distances <2 km, while pairs sampled from Cattai Wetlands and Harrington showed dispersal distances between 6.6 – 8.7 km, accounting for 5 sibling pairs (Fig. 7). Surprisingly, kin pairs were identified between Cattai Wetlands and Harrington to Port Macquarie, with dispersal distances between 38.1 – 40 km; two individuals from Cattai Wetlands, and one individual from Harrington, are responsible for the kin pairings to Port Macquarie, with the two samples from Cattai Wetlands themselves also relatives (Fig. 7). A total of 6 sibling pairs were identified between Cattai Wetlands and Port Macquarie, and 2 sibling pairs between Harrington and Port Macquarie.

The first three distance classes (<15 km) of the sambar deer from South NSW/Gippsland and Melbourne were significant (p<0.0001) with correlation coefficients between 0.001 and 0.008 (Supplementary Material 3 Fig.3) while the last five (distance classes >25 km) had significant (p≤0.016) negative correlation coefficients (range -0.002 – -0.001) except for the 50-81 km distance

class, which had a positive correlation coefficient (r=0.001). When the two sexes were analysed separately, this pattern was consistent in females with all distance classes being significant except for the distance class 50-81 km. Only the first three distance classes (<15 km) and the last two (>243 km) were significant (p<0.0001) in males, with positive correlation coefficients in the first three and negative in the last two distance classes. When comparing the auto correlograms for the two sexes with this dataset, although the correlation coefficients had the tendency of being higher for females, these were not greatly different, and the 95% confidence intervals were mostly within each other range for the same distance classes (Fig.8). Kinship analysis identified 35 full sibling pairs with values above 0.9. Sixteen of these pairs comprised generic location coordinates for both samples so movement could not be measured, however all pairs were sampled from the same sites. For the other pairs identified, 10 were sampled <1 km apart, 4 sibling pairs were observed between 1 – 4.9 km, one pair was detected between 5 – 9.9 km, and 4 sibling pairs were sampled at a distance >10 km (Fig. 7). Of the sibling pairs identified at a distance greater than 10 km, 2 of these were between the Melbourne and South NSW/Gippsland regions. The largest dispersal distance of 61.2 km was observed between two sambar deer present in Kosciuszko National Park.

DISCUSSION

HYBRIDISATION

A total of 17 hybrids were identified in the dataset, representing multiple hybrid classes in both NSW and Victoria. These samples comprised < 1% missing data except two samples from Port Macquarie that had between 14 and 16% missing data. Of the three samples flagged as hybrids prior to genetic analysis, only two were confirmed to be hybrids, and comprised an F_1 and an F_2 hybrid. On the contrary, 11 individuals identified as rusa deer and four identified as sambar deer were in fact F_1, F_2 hybrid or backcrosses. These results demonstrate the difficulties in distinguishing hybrids based on morphology alone, and although some intermediate characteristics can be observed in sambar x rusa hybrids, particularly in antler growth patterns, ear morphology, and increased body size in the smaller rusa deer (Bentley 1978), these characteristics are likely to become less apparent as F_1 and F_2 hybrids backcross to parental species. Similar patterns have been observed in white-tailed deer (*Odocoileus virginianus*) x mule deer (*Odocoileus hemionus)* hybrids and hog deer (*Axis porcinus*) x chital (*Axis axis*) hybrids, where hybrid individuals phenotypically resemble a parental species rather than intermediate characteristics of both parents (Combe *et al.* 2022; Hill *et al.* 2019), and in sika (*Cervus nippon*) x red deer (*Cervus elaphus*) hybrids where hunters misidentified 21% of deer at sites where hybrids were known to occur (Smith *et al.* 2014). It is therefore not surprising that a majority of the hybrids detected through genetic analysis in this study were not flagged as suspected hybrids prior to analysis and highlights the need for genetic testing of sambar and rusa deer to accurately determine hybridisation rates between the species. Deer hybrids can present as larger in body size than their parental species, and this phenomenon is often encouraged in the deer farming industry to promote larger meat yields (Pearse 1992; Tate *et al.* 1997; Tuckwell 1998). Hybridisation in ungulates has also been shown to increase disease resistance (Barbato *et al.* 2017; Grossen *et al.* 2014), growth rate and body weight (Asher *et al.* 1996; Ismail and Saidi 2009; Senn *et al.* 2010), and is suggested to aid in population growth and dispersal (Goedbloed *et al.* 2013; Iacolina *et al.* 2019; Manunza *et al.* 2016). These characteristics may lead to an increase in negative impacts associated with deer in areas where the sambar, rusa, and their hybrids co-occur, with further research necessary to understand the full scale of the negative impacts likely to be associated with the presence of rusa x sambar hybrids in the Australian landscape.

While many of the hybrids identified in this study were collected from sites where both sambar and rusa deer occur (Port Macquarie, Cattai Wetlands, Harrington, Wollongong), hybrids were additionally detected at two sites in Victoria (Croajingolong and Cloverlea), where rusa are not believed to be established (Bentley 1978; Forsyth *et al.* 2015). Dispersing male rusa deer have been observed in Victoria close to the NSW border (Forsyth *et al.* 2015), and so it is possible that a dispersing rusa individual has encountered the Victorian sambar population which has given rise to the hybrid sample collected from Croajingolong. At Cloverlea, where natural dispersal of rusa is highly unlikely, an escapee from a nearby local farm managed for recreational hunting is the most likely explanation for the hybrid observed at this site, however it is unclear if a hybrid or a pure rusa individual has escaped,

with the parental rusa subsequently interbreeding with the local sambar population. The hybrid detected in Cloverlea also highlights the ongoing issues of deer escapees, and how these escapees can further damage native environments and counteract control efforts.

POPULATION STRUCTURE

Genetic structure was observed between the major geographic areas sampled for sambar deer, particularly between North NSW and South NSW/Gippsland and Melbourne, while populations of rusa deer did not appear to be genetically distinct. Fine-scale genetic analysis of rusa deer in Wollongong does show evidence of population structure (Li-Williams *et al.* in review - this issue), however, the different spatial scales between this work and the present study, coupled with differences in SNP filtering, has likely led to the differences observed between the two studies. Also, it is important to note that Li-Williams *et al.* (in review – this issue) removed 'outliers' from their datasets which were identified as rusa back-crosses in our study and the presence of these individuals in our analysis may mask more subtle population structure within rusa that is present in the data. These two studies highlight the importance of assessing genetic structure at multiple spatial scales to understand the connectivity between multiple populations and how populations may have arisen in the landscape, and additionally investigating genetic structure within more localised areas to identify fine-scale structure that can assist with local management of the species.

The genetic structure evident between Victoria and South NSW to the North NSW sambar populations is likely due to different founding events between the two regions and subsequent genetic drift. Genetic structure is evident in invasive species where multiple introductions have occurred (Johansson *et al.* 2018; Mora *et al.* 2018; Zalewski *et al.* 2010) and can additionally be observed as invasive species continue to expand their distributions (Kajita *et al.* 2012; Short and Petren 2011). The lack of genetic structure observed in sambar deer in Victoria and South NSW suggests a relatively high level of gene flow across Gippsland and South NSW. Sambar were released at several sites in the Gippsland region in Victoria in the 19th century by the Victorian Acclimatisation society, with this population believed to have naturally dispersed as far as the Blue Mountains region near Sydney. North NSW sambar deer are believed to have established relatively recently, with deer in Port Macquarie first being observed in the 1980s, and deer in Werakata NP released/escaped from farms in the 1990s. The analyses of genetic diversity seem to support this, with low estimates of heterozygosity and N_e compared to the southern populations. Deer releases and escapees from farms are estimated to account for 35% of the deer populations present in Australia, second only to translocations as the source of new deer populations (Moriarty 2004b). Genetic structure attributed to multiple founding events and subsequent genetic drift has been observed in sambar deer previously (Davies *et al.* 2021), and combined with the results from the present study, suggests that these factors are the major contributors to genetic structure observed in the species in Australia.

Although genetic structure was consistently identified in analyses between the North NSW and South NSW/Gippsland sambar groups, the extent of population structure was less apparent between South NSW/Gippsland and Melbourne. Intermixing between these two regions is evident in the tess3 and PCA analyses, however pairwise F_{ST} between these two regions was higher than the comparison between South NSW/Gippsland and North NSW (0.16 and 0.097 respectively). The Melbourne population likely represents the range edge of sambar deer from the Gippsland population, and so the pattern observed here may be reflective of range edge effects in the Victorian population. Populations on the edges of a given species' distribution can show reduced genetic diversity and population structure, and higher levels of inbreeding than core range populations, however these findings are not always consistent across species (Arnaud-Haond *et al.* 2006; Assis *et al.* 2013; Eckert *et al.* 2008). Estimates of genetic diversity and inbreeding through F_{IS} in the sambar Melbourne population were consistent with the other sambar regions sampled, potentially due to the proximity of Melbourne to one of the initial release sites of sambar in Victoria in the 19th century (Kinglake) (Forsyth *et al.* 2015). Alternatively, it is possible that landscape and urbanisation may play some role in the genetic structure observed in the Melbourne population. The sambar deer samples collected in Melbourne were predominantly from the peri-urban region of the city, and previous studies of deer show that urban habitats can lead to genetic differentiation within urban sites and between rural areas (Blanchong *et al.* 2013; Fraser *et al.* 2019). Genetic analysis of rusa deer in a peri-urban environment

has demonstrated fine-scale population structure is present across the region (Li-Williams *et al.* in review - this issue) and therefore additional sampling of sambar deer across peri-urban Melbourne and surrounds, and an assessment of landscape features that may facilitate or inhibit movement, is also warranted to further understand the patterns of population structure observed in the present study.

DISPERSAL DISTANCE

Across all datasets, shorter distance classes (<20 km) have generally positive and significant correlation coefficients for both sambar and rusa deer. The correlation coefficients rapidly drop for distances >20 km becoming often significantly negative. While this pattern was strongly pronounced in the southern cluster of sambar deer, it was less so in rusa deer and the northern cluster of sambar deer. The point where positive (and significant) spatial correlation coefficients become not significant and close to zero are usually interpreted as the limit of the dispersal distance of the species (in a specific habitat). On the contrary, consistently negative coefficients at large distance class could be interpreted as being beyond the possible dispersal distance, causing an isolation by distance effect. Conversely, the sample size was much lower in the northern sambar deer dataset, however, given that the two sambar deer datasets have a similar pattern these results suggest that the natural dispersal distance in sambar deer within Australia is likely to be <20 km, while it is unlikely that sambar deer disperse beyond 50 km. Rusa deer seem to have a more homogeneous dispersal pattern within 20 km, but similarly it would appear unlikely that individuals of this species disperse beyond 50 km. These data also suggest that both sexes have similar dispersal capacity although the majority of females may have a slightly reduced dispersal distance.

Both sambar and rusa are considered sedentary species (Long 2003), with home range size estimates of rusa showing home ranges encompassing roughly 4 km^2 with a core range of 1 km^2 in Queensland, Australia, (Amos et al. 2022), consistent with results taken elsewhere (Santosa et al. 2015; Spaggiari and de Garine-Wichatitsky 2006). Estimates of home range in sambar deer appear to vary based on habitat, season, and sex, but most recent analyses in Taiwan suggest average home ranges of 1.43 km^2 in males and 0.7 km^2 in females, which is largely consistent with previous work (Chatterjee et al. 2014; Leslie Jr 2011; Yen et al. 2019). Contemporary dispersal via kinship analysis showed that rusa and sambar movements were largely within these ranges, with over half of all kin pairs moving between 0-5 km for both species, however some dispersal events were above 10 km, with the highest reported distance of 61.2 km between two sambar deer. It is not possible to confirm through these analyses if a single deer has travelled this distance, or if both sibling pairs have equally dispersed at smaller distances; nonetheless, the estimates of contemporary dispersal measured through both spatial autocorrelation and kinship would suggest that movement is largely limited. However, some consideration of the drivers of dispersal and how sambar and rusa have come to disperse across much of Victoria and NSW is needed. Larger home ranges in male sambar deer are attributed to the exploratory nature of males during rut to find females (Chatterjee et al. 2014), and it is common for males and juveniles to be the predominant dispersers in many mammalian species (Dobson 1982; Shaw et al. 2006). Dispersal can also be driven through attempts to avoid inbreeding with relatives during breeding seasons (Biosa et al. 2015; Long et al. 2008), and environmental factors such as habitat cover, landscape features, and bushfires may also be important to understand natural deer movement (Davis et al. 2016; Kelly et al. 2014; Long et al. 2005; Long et al. 2010). However, given the patchy distribution of some of the genetic clusters identified in both the sambar deer and rusa deer datasets, particularly between Willow Tree NP and South NSW/Gippsland in sambar deer (~490 km distance between the nearest sites), and Wollongong and Port Macquarie in rusa deer, anthropogenic dispersal may also provide some explanation. Releases/escapees of deer from farms have already been discussed as a potential cause of some of the genetic structure and hybrids observed in the dataset (see above) and are additionally likely to contribute to increases in deer distributions. Additionally, translocations by hunters keen to expand hunting opportunities to their local areas can lead to increases in range of sambar and rusa deer. Given the large distance between the sample collected from Willow Tree NP and the other samples assigned to this cluster in South NSW/Gippsland, and the high degree of genetic similarity, it is likely this sample represents a translocated animal. Translocated deer and pigs have been detected via genetic methods in Australia previously (Hill et al. 2022; Spencer and Hampton 2005) and elsewhere in the world (Carden et al.

2011; Frantz et al. 2006; McDevitt et al. 2009), with many of the deer populations present in Australia today believed to have arisen through recent translocations (Moriarty 2004b). The suspected translocated animal in the present study was culled within a National Park, where recreational hunting is prohibited, which suggests this individual has potentially dispersed from its initial release site. Additional genetic sampling of this site and surrounds is necessary to understand the extent of translocations at this area.

MANAGEMENT IMPLICATIONS

Measurements of dispersal capabilities of rusa and sambar deer, and the presence of population structure between sambar deer sites sampled, provide some insights for population control moving forward. Although rusa populations in Wollongong and Port Macquarie appear genetically similar, the dispersal capability of this species suggests they cannot naturally disperse between these sites and can be managed separately. However, additional sampling in Port Macquarie can confirm that kin do not appear between these two sites. Assessment of genetic structure within a single population of rusa deer (Li-Williams *et al.* in review - this issue) also suggests that localised management units of rusa deer can additionally be identified for further management intervention and highlights the need for localised studies in both species in the future. In sambar deer, population structure is evident, suggesting multiple management units where population control can be undertaken independently. Currently, Port Macquarie is managed separately to Cattai Wetlands and Harrington, however the results reported here suggest that gene flow exists between these sites, with kinship pairs observed between the three areas. It would therefore be beneficial in future to coordinate population control of these sites to achieve greater reductions in deer density. Further sampling of deer at these sites would also be of value to determine if the connectivity observed extends to additional deer species. Rusa deer occur at these sites as well, in addition to fallow deer (*Dama dama*), red deer, and chital (*Axis axis*) at Port Macquarie (Pacioni *et al.* 2021) and understanding dispersal pathways and connectivity early in the invasion process can greatly assist in successfully managing problematic species (Adams *et al.* 2014; LaRue *et al.* 2011). The Werakata NP population appears isolated from all other sambar deer populations, however, additional monitoring is necessary to understand the range of this population.

The South NSW/Gippsland population of sambar deer will be much more challenging to manage given the scale of gene flow across this region. Management of the species should continue in high priority sites such as National Parks to reduce their impacts, and results of dispersal capability of sambar deer should assist in determining local scales of population control to reduce reinvasion. Further research is necessary across Gippsland and South NSW to determine potential landscape features that may be facilitating or limiting gene flow in sambar deer. Forest cover has already been suggested as important for sambar deer dispersal (Davies *et al.* 2021), so confirming this in addition to identifying potential barriers can assist in using landscape or human-made features to aid control within this region. However, challenges in ensuring any artificial barriers to deer dispersal do not also negatively impact movements of native species are also likely to arise (Jones *et al.* 2021).

Lastly, further research is necessary to understand the impacts rusa x sambar hybrids are likely to have on the Australian landscape, and if management at sites where hybrids occur should be considered a high priority to prevent further spread. Measuring phenotypic, behavioural, and fitness changes in hybrids compared to parental species is warranted to understand the scope of this potential threat. Currently, hybridisation appears mostly isolated to the North NSW region, however, further sampling of the contact zone between the two species at the Victorian/NSW border will elucidate the likelihood of hybrids further spreading throughout this region.

CONFLICTS OF INTEREST

David Forsyth was a guest Associate Editor for this special issue. Despite this relationship, he did not at any stage have editor-level access to this manuscript while in peer review, as is the standard practice when handling manuscripts submitted by an editor to this journal. *Wildlife Research* encourages its editors to publish in the journal, and they are kept separate from their manuscripts' decision-making process. The other authors have no relevant financial or non-financial interests to disclose

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FIGURE CAPTIONS

Fig. 1 Map of final samples of sambar (circles) and rusa deer (squares) genotyped for this study. b) shows all sites sampled, with a) and c) providing further detail of North NSW and South NSW/Gippsland/Melbourne sampling sites respectively. Samples that were either morphologically or genotypically identified as hybrids are shown as purple triangles. Colours of circles/squares indicate major geographic regions identified during population genetic analysis; blue = sambar Melbourne, green = sambar South NSW/Gippsland, orange = Rusa NSW, pink = Werakata NP, red = sambar North NSW. Distributions of both species are shown in yellow (sambar) and green (rusa)

Fig.2 Flowchart summarising the datasets (green) and analyses (purple) undertaken post-filtering

Fig.3 Principal component analysis plot of rusa and sambar deer, based on 390 samples and 14,099 polymorphic SNP loci. Samples identified as hybrids in this plot are based on morphological identification prior to genotyping

Fig.4 a) Best fit K=2 plot generated from fastStructure analysis b) Best fit K = 2 plot generated from Tess3 analysis c) NewHybrids plot showing individual assignment to parental or hybrid classes. Samples appear in the same order in all plots, with broad geographic range highlighted at the top of the figure

Fig.5 Best fit K = 5 plot and map generated from Tess3 analysis for all sambar deer samples where genetic clusters are colour-coded. Broad geographic range is identified at the bottom of the bar chart, along with the Werakata NP site and Willow Tree samples which form part of North NSW

Fig.6 Principal component analysis plot for major geographic regions of a) sambar deer, and b) rusa deer, with all hybrid samples removed

Fig.7 Bar plot showing the frequency of kinship pairs observed at a range of dispersal distances for both sambar deer and rusa deer. All kinship pairs comprised a probability value >0.9

Fig.8 Auto correlogram with distance classes in km on the x-axis and spatial correlation coefficient (r) on the y-axis for males and females from the southern sambar deer cluster. Error bars are estimates of the 95% confidence intervals obtained via bootstrap analysis. Note that the x-axis is broken to improve visibility

TABLES

Table 4 Final sample sizes for sambar deer, rusa deer, and morphologically identified hybrids genotyped for this study. Sites with an asterisk (*) were grouped into the geographic regions 'sambar North NSW' or 'Rusa NSW' for analysis. M/F/U indicates male/female/unknown sex, A/J/U indicates adult/juvenile/unknown age. Samples collected from Wollongong were provided by Li-Williams et al. (in review – this issue)

	Sambar Deer		**Rusa Deer**		**Suspected Hybrids**		
Geographic Region	Sex M/F/U	Age A/J/U	Sex M/F/U	Age A/J/U	Sex M/F/U	Age A/J/U	Total
Melbourne	15/34/4	46/0/7	-	-	-	-	53
Gippsland/South NSW	34/42/33	48/2/59	-	-	2/0/0	0/0/2	111
***Wollongong**	-	-	86/88/1	138/36/1	-	-	175
Werakata National Park	2/2/2	3/0/3	-	-	-	-	6
***Harrington**	0/0/6	0/0/6	0/0/1	0/0/1	-	-	7
***Cattai Wetlands**	7/3/0	10/0/0	1/1/0	2/0/0	1/0/0	1/0/0	13
***Willow Tree National Park**	1/0/0	1/0/0	-	-	-	-	1
***Port Macquarie**	4/9/0	13/0/0	3/8/0	11/0/0	-	-	24
Total	63/90/45	121/2/75	90/97/2	151/36/2	3/0/0	1/0/2	390

Table 2 Pairwise FST between the major geographic regions identified in the rusa and sambar deer samples. All pairwise comparisons were significant with p<0.001

	Rusa NSW	**Sambar North NSW**	**Sambar Werakata**	**Sambar Gippsland**
Sambar North NSW	0.892			
Sambar Werakata	0.906	0.134		
Sambar Gippsland	0.884	0.097	0.035	
Sambar Melbourne	0.912	0.166	0.231	0.160

Table 3 Diversity indices for rusa and sambar deer based on distinct geographic regions observed. n = sample number, HO = observed heterozygosity (SD), HE = expected heterozygosity (SD), FIS = inbreeding coefficient, Ne = effective population size (95% CI)

	Hybrid samples retained					**Hybrid samples removed**				
Species/ Region	n	H_O	H_E	F_{IS}	N_e	n	H_O	H_E	F_{IS}	N_e
Rusa NSW	187	0.056 (0.124)	0.057 (0.117)	0.021	63 (56 - 70.2)	177	0.043 (0.127)	0.043 (0.120)	0.010	61 (53.5 – 69.9)
Sambar North NSW	33	0.153 (0.146)	0.153 (0.138)	0.014	0.7 (0.1 - 2.3)	28	0.090 (0.168)	0.089 (0.161)	0.012	11.2 (6.9 – 18.6)
Sambar Werakata	6	0.143 (0.231)	0.123 (0.177)	-0.069	2.8 (0.9 - infinite)	6	0.143 (0.231)	0.123 (0.177)	-0.069	2.8 (0.9 - infinite)
Sambar South NSW/ Gippsland	111	0.117 (0.162)	0.127 (0.170)	0.083	126.9 (96.1 - 171.3)	109	0.107 (0.168)	0.114 (0.176)	0.068	126.9 (96.4-177.6)
Sambar Melbourne	53	0.101 (0.172)	0.103 (0.171)	0.028	101.1 (92.8 - 204.6)	53	0.101 (0.172)	0.103 (0.171)	0.028	101.1 (92.8 - 204.6)

APPENDIX 5. FALLOW DEER POPULATION GENETICS

FALLOW DEER POPULATION GENETICS PRELIMINARY REPORT JULY 2022

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ABSTRACT

Fallow deer (*Dama dama*) is an invasive species in Australia, which can have negative environmental impacts and pose a biosecurity risk. To improve management, it is first necessary to understand the population size, distribution and connectedness of falllow deer across regions. Here we use genetic data generated from 92 tissue samples to determine the population structure of fallow deer in south-eastern Australia. Our results revealed, firstly, that fallow deer in Tasmania have limited genetic structure, suggesting a highly connected population or shared low genetic diversity from a single founding event. Secondly, we identified one genetically diverse population in peri-urban Melbourne that was diverged from all other deer in the sample, indicating that this population may have been founded recently from a unique source. Finally, we found that, while most mainland sites were diverged from the Tasmanian population, one Victorian population at Yellingbo had ancestry from both Tasmania and the mainland. Yellingbo also appeared to be an isolated population and we thus propose it is a good candidate for eradication. These results suggest that animals may be escaping from farms or otherwise have been deliberately moved between regions. We recommend the establishment of a genetic database of farmed animals and imported deer to determine the contribution of farm escapees to the maintenance or establishment of wild deer populations which would assist feral deer management in south-eastern Australia.

BACKGROUND

European fallow deer were first introduced into Tasmania in the 1830's and were subsequently translocated to the mainland by acclimatization societies (Moriarty, 2004). Since then, wild deer populations across Australia have increased substantially, particularly in the last four decades, and are having increasingly negative impacts on the environment and agriculture (Davis et al., 2016). Fallow deer now have established populations in five of the six Australian states, with the largest in Tasmania (Moriarty, 2004). To mitigate their negative impacts, state run control programs aim to cull deer populations to limit population growth and expansion. However, we currently lack a good understanding of the connectedness of deer populations in Australia, which would allow control programs to more targeted and therefore efficient.

This project aims to use genetic analysis to improve understanding of the relatedness and structure of fallow deer (*Dama dama*) populations in south-east Australia. Insights from project data will help to develop a strategic management plan for deer control by identifying management units, determining the most appropriate scale of control and helping to estimate the likelihood of successful eradications. Here, we report preliminary results from our first batch of sequencing data, generated in June 2022.

SAMPLE COLLECTION AND SEQUENCING

Samples were collected between 2017 and 2021, with a majority collected in 2020 and 2021. State agencies and their contractors were engaged to collect biopsies from deer culled during control programs. Our sample set consisted of tissue samples from 36 individuals from Victoria, 19 from NSW and 39 from Tasmania. Tissue samples (*n*=94) were sent to Diversity Array Technology (DArT) and were processed using their genome-complexity reduction method, with samples from other deer species, to produce a large genetic dataset of single nucleotide polymorphism (SNP) markers.

From DArT we received genotypes of 92 fallow deer individuals. The remaining two samples failed to produce genotypes. The dataset was imported into R and initial filtering performed using the dartR package. Details of the initial filtering steps performed are given in the Appendix. After filtering, our dataset consisted of 2039 SNPs and 91 individuals (Table 1).

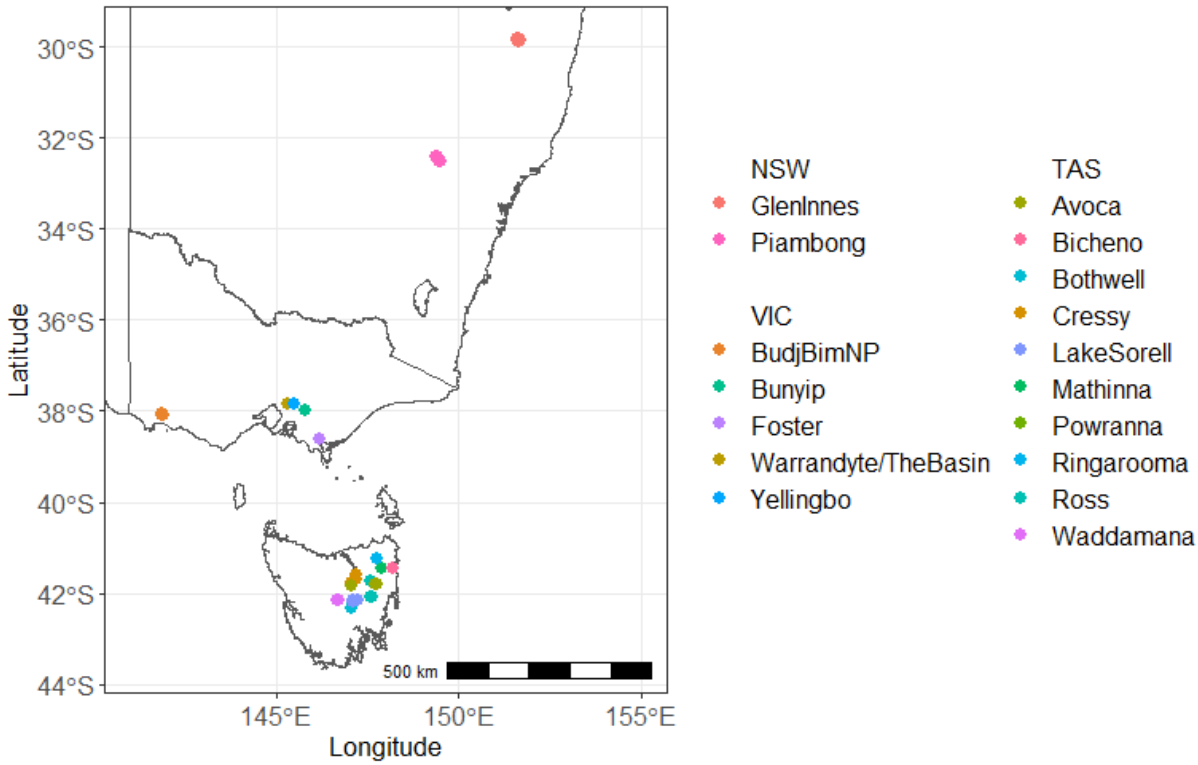

Figure 1. Sampling locations of the 91 fallow deer samples included in analyses after filtering.

POPULATION GENETIC ANALYSES

POPULATION STRUCTURE

We first ran a principal components analysis (PCA) to examine population structure within our sample set. PCA is a statistical technique for exploring datasets with a large number of measurements by reducing those measurements to a few 'principal components' (PCs), which explain the main patterns and can be easily visualized. Plotting the first two PC's of the fallow deer data shows two interesting patterns. The first PC, which explained 33.1% of the variation in the dataset, significantly separates a group of Victorian fallow deer from all other individuals in our sample set (Figure 2). These individuals came from a single peri-urban site in outer north-east Melbourne. This site was labelled either The Basin or Warrandyte by collectors, although the suburb of Warrandyte is further north-west than the geographic coordinates of these samples indicate. We refer to these samples as Warrandyte/The Basin. This divergence of the Warrandyte/The Basin samples is especially unusual as the site is only 20km from another site, Yellingbo (Appendix Figure A1). These data also suggest that Tasmanian fallow deer are more similar to individuals from NSW, than Warrandyte/The Basin individuals are to

other Victorian deer. The second PC separated samples from Tasmania and most of the mainland. Individuals from Yellingbo fell between these two groups, suggesting gene flow between Tasmania and this Victorian population.

We also re-ran this analysis with samples from Warrandyte/The Basin excluded as removing highly diverged groups can sometimes reveal more fine-scale patterns within the less diverged groups. However, in this reanalysis, the same general results were observed as when samples from Warrandyte/The Basin were included (Appendix Figure A2).

Table 1. Number of samples (n) from each state in each age class included in analyses after filtering.

State	Age	*n*
NSW	Adult	9
	Juvenile	10
	Unknown	0
VIC	Adult	22
	Juvenile	11
	Unknown	2
TAS	Adult	1
	Juvenile	24
	Unknown	12

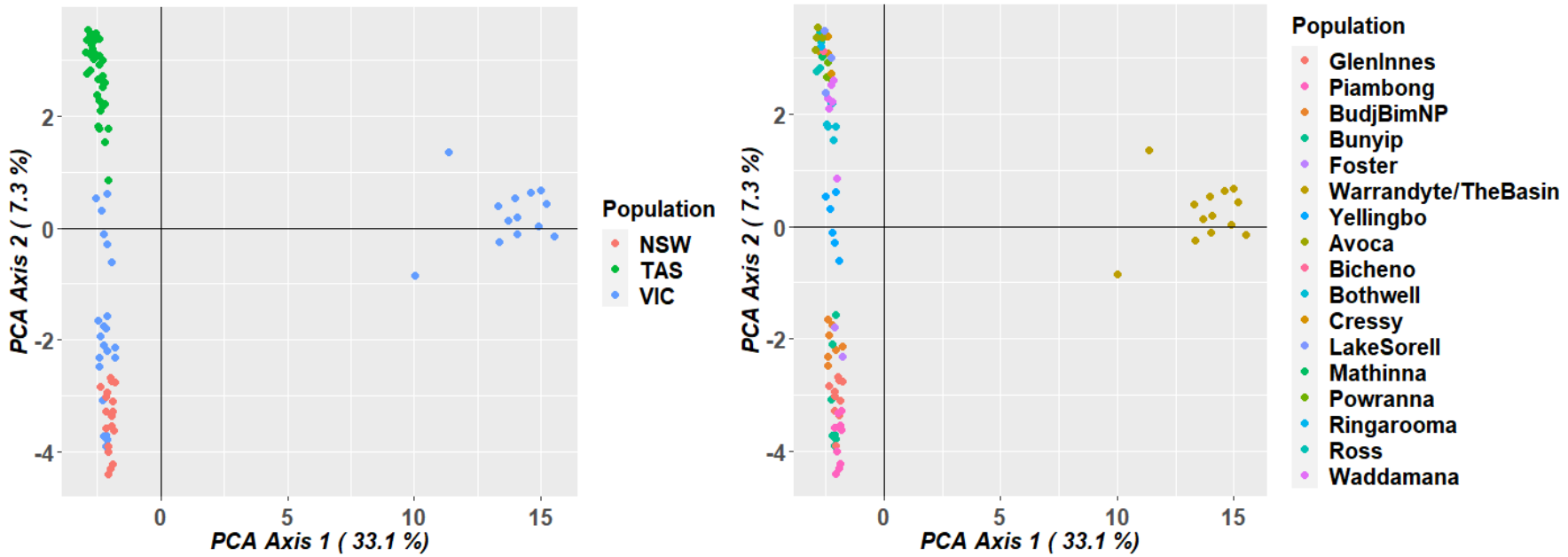

Figure 2. Principal Components Analysis plots of the fallow deer dataset. Each point represents an individual. The first is coloured by state and the second plot is coloured by the specific collection site.

GENETIC CLUSTERING

We next used the program fastSTRUCTURE to define clusters based on genetic similarity and estimate the proportion of each individual's genome that is derived from each of these clusters. Our fastSTRUCTURE results suggest either 2 or 3 clusters are present in the data. These groupings largely reflect the PCA results. With two clusters selected, the Warrandyte/The Basin samples are separated from all other samples (Figure 3). When three clusters are selected, the remaining samples are split into two groups, Tasmania and the rest of the mainland, with Yellingbo representing a mixture of both these groups (Figure 3). Excluding the Warrandyte/The Basin samples gave similar results with Yellingbo representing an admixed group of Tasmanian and mainland individuals (See Appendix Figure A3).

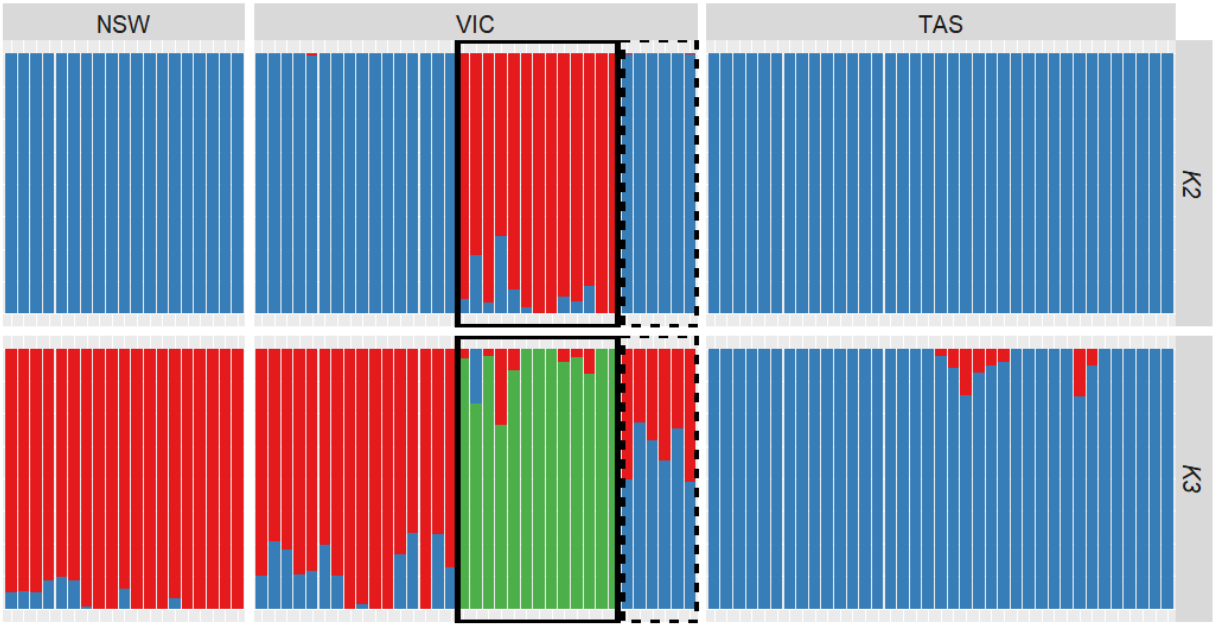

Figure 3. fastSTRUCTURE results. Each bar represents an individual, grouped by the state at which they were sampled. Colours represent the estimated proportional ancestry from each cluster. Results of analyses are shown for which the number of clusters (K) was assumed to be 2 or 3. Samples from Warrandyte and The Basin are highlighted by the solid box, samples from Yellingbo are highlighted by the dotted box.

MULTI-SPECIES ANALYSIS

Given these unusual results, and because DArT processed multiple plates of different deer species for us at the same time, we wanted to check that none of the Warrandyte/The Basin samples had been mis-identified or mis-labelled as an incorrect species. We went back to the entire, multi-species dataset provided to us by DArT and filtered as above, with some small changes to account for the multi-species nature of the dataset. Details of the initial filtering steps performed are given in the Appendix. After filtering, our multi-species dataset consisted of 11873 SNPs and 532 individuals. We then conducted a second PCA using this dataset (Figure 4). This showed convincingly that all our fallow deer samples grouped together to the exclusion of the other species in our dataset (sambar and rusa deer), confirming that species mix-ups had not occurred.

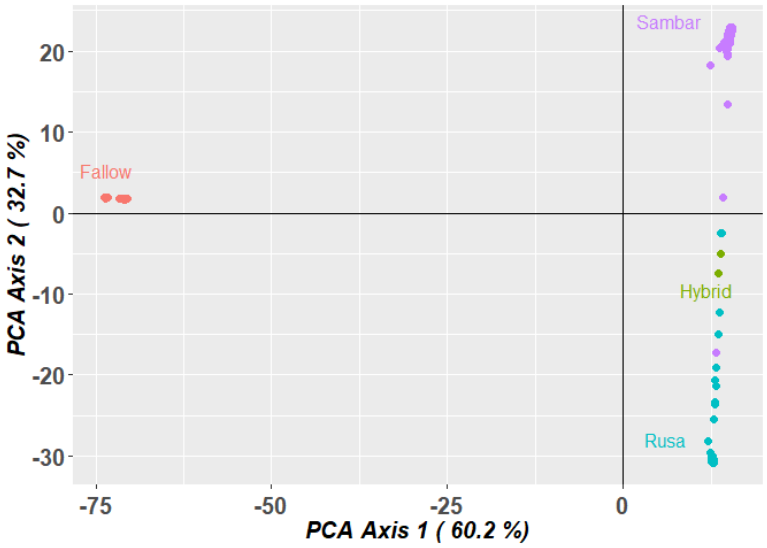

Figure 4. Principal Components Analysis plots of the multi-species deer dataset. Each point represents an individual and colours represent the species. Note there is a continuum between rusa and sambar deer, suggesting hyrbidization between these species. This is the subject of a separate report and is not discussed further here.

REPRESENTATION OF RELATIVES

We next checked whether the strong divergence of Warrandyte/The Basin individuals could be driven by an over-representation of close relatives within samples from that site. Relatives may be over-represented within particular locations because shooters are likely to target entire groups that may be composed of family units. To preliminarily gauge the number of relatives present in our sample set, we calculated genetic dissimilarity (Hamming distance) between all pairs of sampled individuals. We found that pairs of individuals from the same locations were more genetically similar to each other than pairs of individuals at different sites, consistent with sampling of relatives or a pattern of isolation by distance (where genetic distance is correlated with geographic distance). However, Warrandyte/The Basin individuals were far less similar to each other than pairs at other sites (Figure 5), suggesting that overrepresentation of relatives cannot explain the strong divergence pattern.

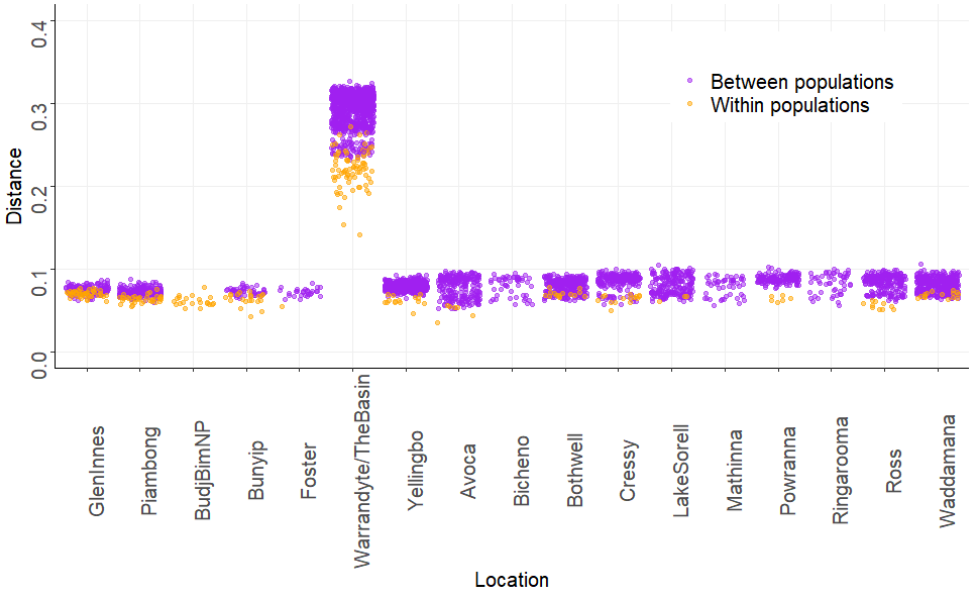

Figure 5. Pairwise genetic dissimilarly (or Hamming distance) between all pairs of individuals in our sample set. Each point represents a pair of individuals. The x-axis represents the location from which the first individual in each pair originated, and points are coloured by whether the second individual in that pair originated from the same location as the first (yellow dots), or a different location (purple dots).

GENETIC DIVERSITY

To further explore what is driving the separation of Warrandyte/The Basin individuals, we next calculated two measures of genetic diversity; rarefied allelic richness and observed heterozygosity. We found that, in both measures, Warrandyte/The Basin stood out as significantly more diverse than all other populations.

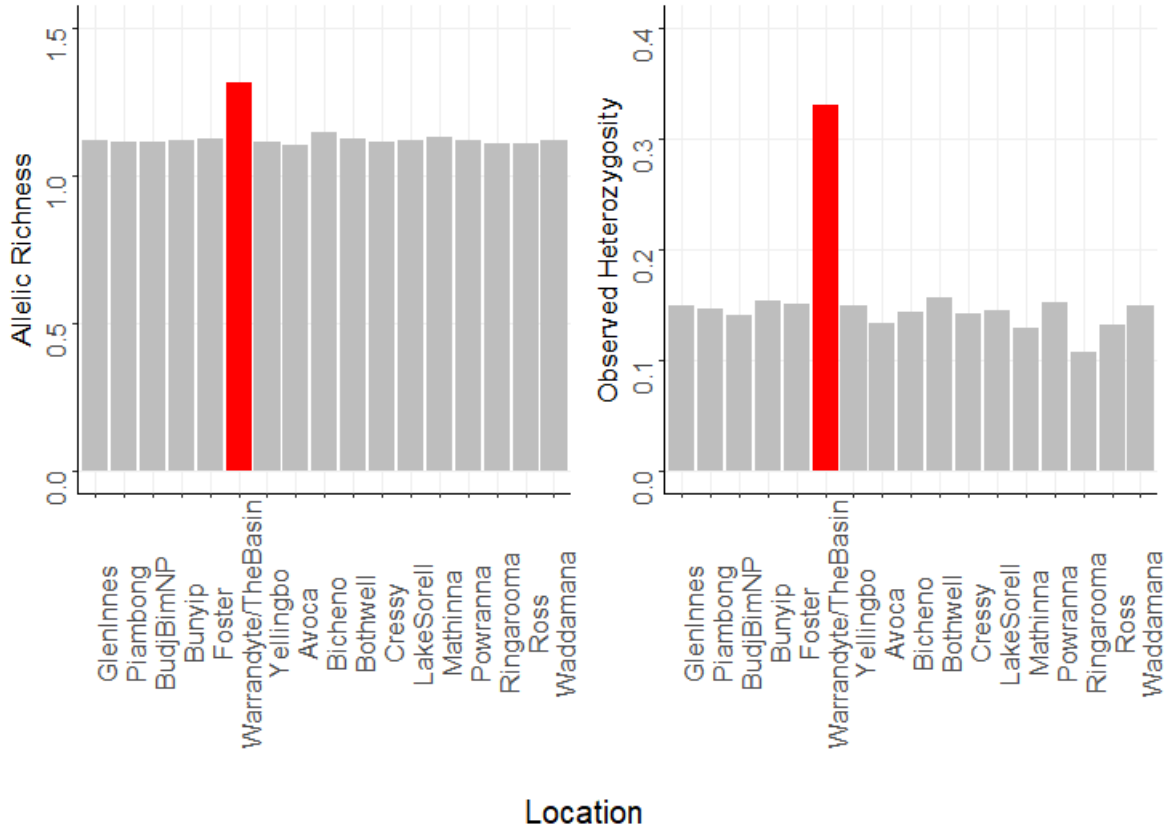

Figure 6. Measures of genetic diversity across sampling locations. Left plot: allelic richness. Right plot: observed heterozygosity. The highly diverged location, Warrandyte/The Basin, is highlighted in red.

DISCUSSION

FALLOW DEER IN THE WARRANDYTE/THE BASIN AREA ARE GENETICALLY DISTINCT

Our results suggest that the fallow deer population in the Warrandyte/The Basin area was founded (or admixed with) a completely different source population to all other samples in our dataset. The localised nature of the unusual genetics suggests that, either, this population is completely isolated from other populations on the mainland, or, the input of genetic material from the different source population occurred recently and has not yet had time to enter into other populations via dispersal.

We are aware of anecdotal evidence of semen being imported into Australia by deer farmers to establish populations with particular traits (e.g. antler shape, body size or coat color). Farm escapees descended from recently imported genetics could explain the uniqueness of Warrandyte/The Basin individuals. However, representative samples from farmed deer are needed to explore this possibility.

TASMANIA AND MAINLAND FALLOW DEER ARE ISOLATED FROM EACH OTHER EXCEPT AT YELLINGBO

Excluding samples from Warrandtye/The Basin, our results show a divergence between Tasmanian and most mainland fallow deer populations. Many populations of fallow deer on the mainland were originally sourced from the already-established Tasmanian population by acclimatization societies and individual settlers in the 1800s (Bentley 1998). The genetic divergence of contemporary Tasmanian

and (most) mainland fallow deer suggests that there has been limited gene flow between the populations since then.

One exception, however, is at the Yellingbo site in Victoria. This population stands out as it appears to be composed of admixed individuals with ancestry from both the mainland and Tasmanian fallow deer populations. This admixture could be the result of deliberate release of Tasmanian deer in the Yellingbo area, or from farm escapees which had bred with Tasmanian stock. The localisation of the admixed individuals and their low genetic diversity (similar to all other sites except Warrandyte/The Basin) suggests that this admixture did not occur recently (which we would expect to lead to higher heterozygosity) and that Yellingbo is an isolated population.

Genetic similarity suggests connected populations or shared ancestry and low diversity

Within the mainland and Tasmania, most sites are only moderately diverged. This finding is consistent with previous work showing that the Tasmanian population was relatively unstructured (Webley et al., 2007). This pattern may be driven by a shared founding history and low genetic diversity, although it could also indicate that individual deer are moving or dispersing between these 'populations'.

Future analyses could exploit the good sampling distribution we have from Tasmania to estimate home range sizes and dispersal distances. That information could also help us understand how fallow deer populations are connected on the mainland. However, most of the samples from Tasmania are either juveniles (less than 24 months old based on the collector's judgement) or are missing age information. It is important for genetic-geographic analyses that only adult individuals are included as juveniles are likely to not yet have dispersed and will their inclusion will downwardly bias estimates of dispersal distance.

RECOMMENDATIONS

Based on our results we have three recommendations for the management of and future research into fallow deer in Victoria and south-eastern Australia more broadly. Firstly, establishing a genetic database of farmed deer should be a priority. A well-curated database would allow genetic tracing of wild-shot deer and allow us to determine the contribution of farm escapees to the maintenance and establishment of wild deer populations. Furthermore, a database of imported semen (including country of origin and location of populations established from imported genetic material) would also help in determining the origins of the unusual genetics around Warrandyte/The Basin. Secondly, Yellingbo may be a good candidate for eradication as it appears isolated from other fallow deer populations in Victoria. Finally, following up age data for those samples missing this information, particularly in Tasmania, should be attempted to allow robust estimates of home-range size and dispersal distances.

ACKNOWLEDGEMENTS

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APPENDIX

FILTERING – FALLOW DEER DATASET

We removed untyped SNPs, SNPs with a reproducibility score of less than 0.99, SNPs that were genotyped in fewer than 80% of individuals and that were called based on fewer than five or more than 300 reads per allele. We then removed individuals that had >20% missing data across all SNPs. We next pruned the dataset by removing one of each pair of SNPs which occur on the same locus. Finally, we removed invariant (monomorphic) and low frequency (singletons and doubletons) SNPs.

Table A1. Details of the initial filtering steps and remaining SNPs and individuals after each step.

Filtering step	SNPs remaining	Individuals remaining
Raw data	88852	92
Remove untyped SNPs	62413	92
Reproducibility score < 0.99	55307	92
SNPs with more than 20% missing data	45188	92
SNPs with <5 or >300 reads per allele	33543	92
Individuals with more than 20% missing data	33543	91
SNPs on the same locus	32735	91
Monomorphic SNPs	5171	91
Singleton and doubleton SNPs	2039	91

FILTERING – FALLOW, SAMBAR AND RUSA DEER DATASET

To filter the dataset that contained multiple species (fallow, sambar and rusa deer), we used a similar strategy as above, but were less stringent when filtering individuals for missing data, and more stringent when filtering loci for missing data. This ensured that we retained only SNPs that were typed across all species and did not unnecessarily remove individuals for comparison.

Table A2. Details of the filtering steps for the multi-species sample set, including remaining SNPs and individuals after each step.

Filtering step	SNPs remaining	Individuals remaining
Raw data	88852	557
Remove untyped SNPs	88852	557
Reproducibility score < 0.99	76276	557
Individuals with more than 40% missing data	76276	532
SNPs with more than 5% missing data	12649	532
SNPs with <5 or >300 reads per allele	12317	532
SNPs on the same locus	12178	532
Monomorphic SNPs	11937	532
Singleton and doubleton SNPs	11873	532

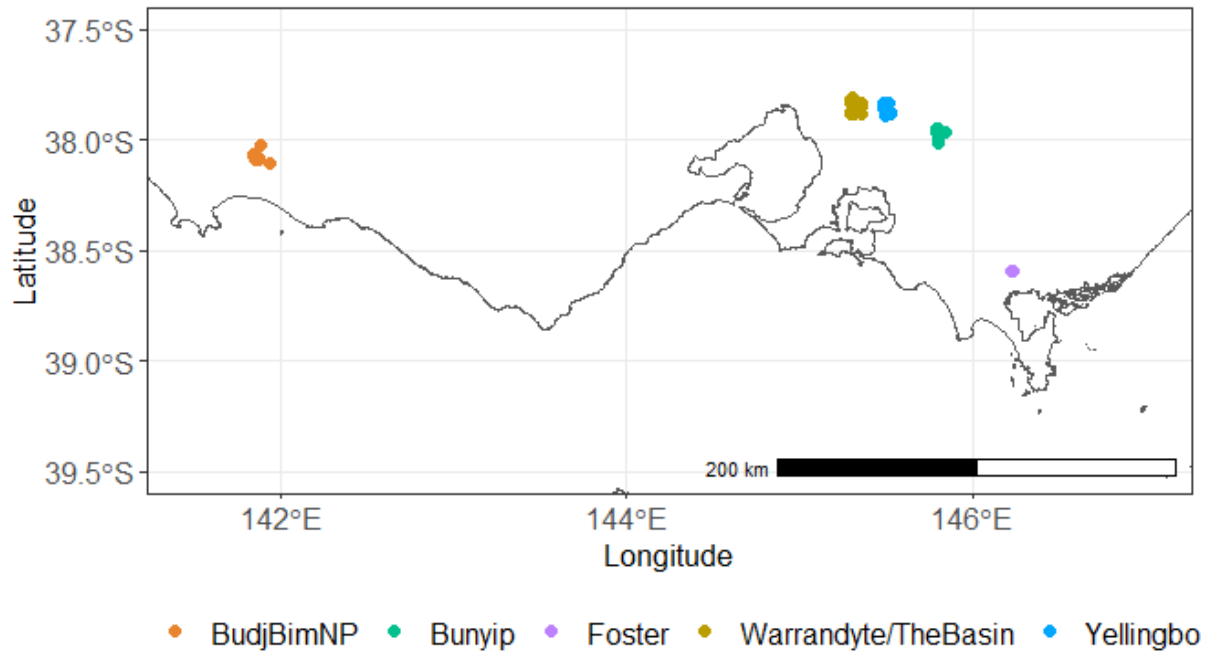

Figure A1. Sampling locations of the 36 Victorian fallow deer samples in our sample set.

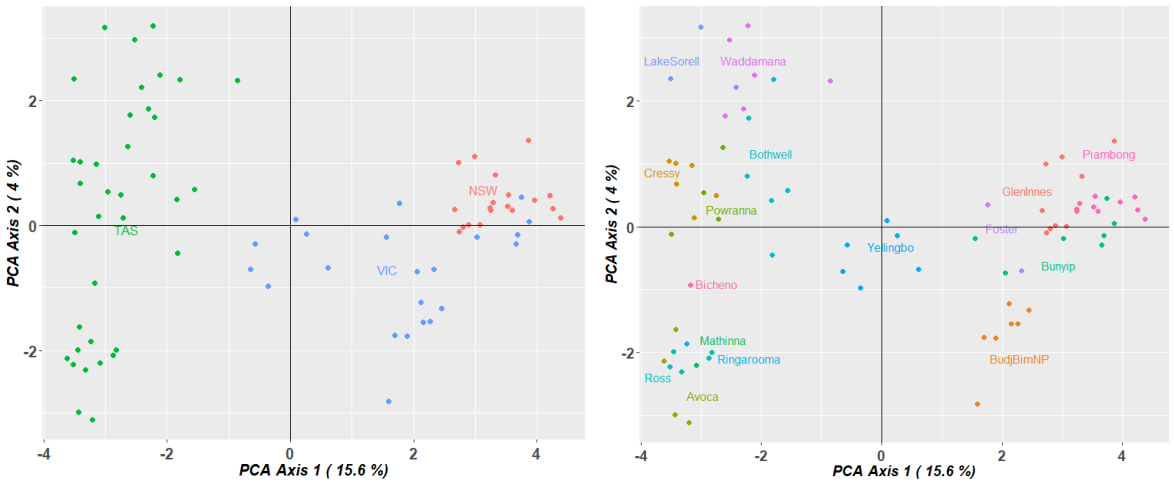

Figure A2. Principal Components Analysis plots of the fallow deer dataset excluding the highly divergent Warrandyte and The Basin individuals. Each point represents one individual. The first plot is coloured by state and the second plot is coloured by the specific collection site.

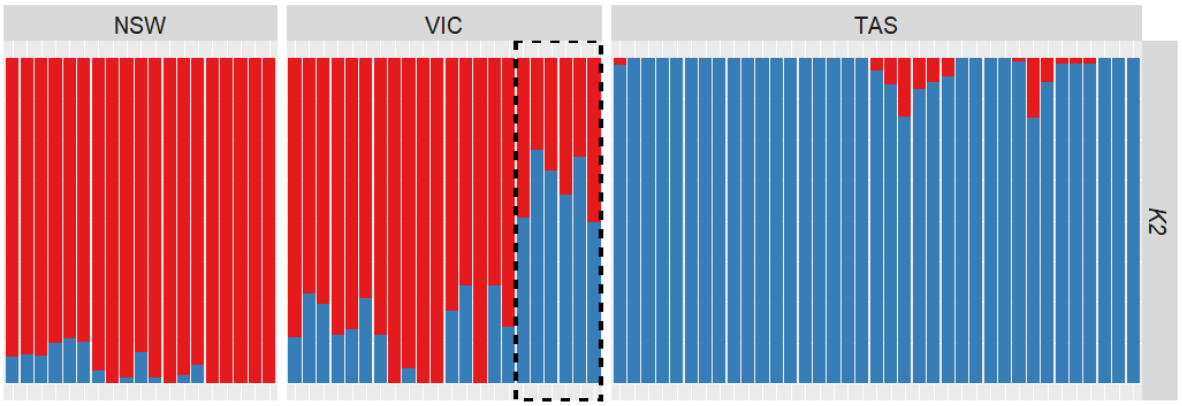

Figure A3. fastSTRUCTURE results excluding the highly divergent Warrandyte and The Basin individuals. Each bar represents an individual, grouped by the state at which they were sampled. Colours represent the estimated proportional ancestry from each cluster. Results of analyses are shown for which the number of clusters (K) was assumed to be 2. Samples from Yellingbo are highlighted by the dotted box.

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