

Genetic perspectives on the historical introduction of the European rabbit (*Oryctolagus cuniculus*) to Australia

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Abstract The introduced European rabbit (*Oryctolagus cuniculus*) is one of Australia's most damaging invasive alien species, both in terms of ecological and economic impact. Biological control of rabbits using the myxoma and rabbit haemorrhagic disease viruses has been undertaken in Australia since the mid-1950s, and locally varying genetic resistance to these biocontrol viruses has been reported. The efficacy of biocontrol agents may be influenced, among several factors, by the genetic background of rabbit populations. Therefore, understanding the invasion process of rabbits in Australia, and their resultant population structure, remains crucial for enhancing future rabbit management strategies. Using reduced-representation sequencing techniques we genotyped 18 Australian

rabbit populations at 7617 SNP loci and show that Australia's invasive rabbits form three broad geographic clusters representing different ancestral lineages, along with a number of highly localised, strongly differentiated lineages. This molecular data supports a history of multiple independent rabbit introductions across the continent followed by regional dispersal, and the resulting patchwork genetic structure may contribute to variation across the country in rabbit resistance to the viral biocontrols. Our study highlights the importance of using genome-wide molecular information to better understand the historical establishment process of invasive species as this may ultimately influence genetic variability, disease resistance and the efficacy of biocontrol agents.

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Introduction

The successful establishment of the European rabbit (*Oryctolagus cuniculus*) in Australia, and subsequent population boom after introduction by European settlers in the mid-1800s, is unsurprising given the species' generalist herbivorous diet and famously high fecundity (Tablado et al. 2009). However, the rapid

spread of the rabbit throughout the southern half of Australia—described as the world’s fastest mammal invasion (Caughley 1977)—contrasts starkly with modern understanding of rabbit ecology. Rabbits have small home ranges and are generally dependent on burrows for shelter against both predators and an inhospitable climate, making them poor natural dispersers. Studies indicate that dispersing sub-adult rabbits rarely travel further than 500 m to a neighbouring pre-established warren, although rare dispersals over 1.5 km have been recorded (Parer 1982; Richardson et al. 2002).

What then can explain the rapid colonization of the rabbit in Australia? Although a successful introduction of wild English rabbits by Thomas Austin of Barwon Park, Victoria in 1859 is commonly credited as the primary source of the original rabbit plague, evidence gathered from contemporary newspapers indicates that rabbit introductions were commonplace in that period, with several additional documented successes (Peacock and Abbott 2013). This provides an alternative hypothesis: rather than dispersing continent-wide from a few primary introductions, rabbits were actively spread throughout the country by vagile human colonisers, dispersing under their own power at a more localised scale.

The historical dispersal pathways of the European rabbit in Australia are of modern consequence through their impact on the current genetic composition of rabbits, which in turn influences the effectiveness of pest management practices. Overgrazing by rabbits in Australia is responsible for an estimated AU\$200 million in agricultural losses per annum (Cooke et al. 2013), as well as widespread damage to terrestrial ecosystems by preventing regeneration of palatable native plants (Bird et al. 2012; Cooke 2012; Mutze et al. 2016) and supporting large populations of introduced predators (Holden and Mutze 2002; Pedler et al. 2016). Landscape scale management of rabbits has primarily been achieved through two introduced viral bio-controls: myxomatosis and rabbit haemorrhagic disease virus (RHDV). Geographic differences in biocontrol efficacy have been observed. By challenging rabbits from populations across the country with low doses of RHDV, Elsworth et al. (2012) found evidence of varying resistance to the virus across populations. This may be due to varying selection pressures acting on the locally available genotypes of individual rabbits (Schwensow et al. 2017a, b).

To investigate the genetic differentiation among invasive Australian rabbit populations, we used double-digest genotyping-by-sequencing [ddGBS, (Poland et al. 2012)] to detect genome-wide SNPs in $N = 413$ rabbits sampled continent-wide across 18 populations. As well as the inherent improved insights into the colonisation of Australia by the rabbit, understanding the genetic structure of Australian invasive rabbits will have two demonstrable benefits for pest management strategies. Firstly, the extent of genetic variation between rabbit populations may reflect varied potential for developing resistance to RHDV, myxomatosis, or any future new biocontrols. Documenting this variance will thus guide understanding of the variation that can be expected in efficacy of present and future nationwide biocontrol initiatives. Secondly, the degree of connectivity between rabbit populations will influence the size of effective rabbit management units such that re-immigration from neighbouring areas following control activities is minimised, as has previously been implemented for rats on South Georgia (Robertson and Gemmell 2004) and feral pigs in Australian rangelands (Cowled et al. 2008).

Methods

Sample collection

Ear tissue was scavenged from wild rabbits shot throughout 2014; during regular activities of readers of the Australian Shooter magazine. Tissue was immediately stored in DESS (20% dimethyl sulphide, 0.25 M disodium EDTA, saturated with NaCl), and frozen at $-20\text{ }^{\circ}\text{C}$ upon receipt. The GPS location of each sample was recorded, with 18–49 rabbits sampled from within a maximum 65 km radius at each of 11 sites throughout Western Australia (WA), South Australia (SA), Victoria (Vic), Australian Capital Territory (ACT) and New South Wales (NSW). Genomic DNA was also obtained from rabbits trapped in 2009 at eight additional sites, including Southern Queensland (QLD) during a previous challenge study experiment by Elsworth et al. (2012) and from a site at Turretfield in SA collected in 2014 as part of an ongoing capture–mark–recapture study. All sample locations are summarised in Table 1; see “Results” section for map.

Table 1 Rabbit sample locations used in this study, grouped by state

Site	State	Population cluster	Mean longitude	Mean latitude	N	Collection year
WA1	WA	Perth	114.2034	− 27.8897	17	2014
WA2	WA	Perth	115.9746	− 29.9885	18	2014
WA3	WA	Perth	117.8554	− 34.7799	47	2014
SA1	SA	Perth	135.0620	− 33.5638	15	2014
SA2	SA	Central	137.9865	− 27.8148	17	2014
SA3	SA	Central	138.6361	− 31.4547	18	2009
SA4	SA	Adelaide	138.8222	− 34.5604	15, 20	2009, 2014
QLD	QLD	Central	142.5956	− 25.9008	10	2009
NSW1	NSW	Central	144.9076	− 35.5975	34	2014
NSW2	NSW	Central	149.4667	− 33.3600	12	2009
NSW3	NSW	Sydney	150.7676	− 34.0721	19	2014
NSW4	NSW	Central	151.8194	− 29.3097	17	2014
NSW5	NSW	Brisbane	152.0294	− 29.0286	20	2014
ACT	ACT	Central	149.3934	− 35.0893	37	2014
VIC1	VIC	Central	142.4164	− 34.6342	10	2009
VIC2	VIC	Melbourne	142.0439	− 38.3308	11	2009
VIC3	VIC	Melbourne	144.1424	− 37.5883	22, 38	2009, 2014
VIC4	VIC	Melbourne	144.3736	− 37.0175	16	2009

Rabbit sample locations used in this study, grouped by state. N reflects final sample numbers after sequence filtering (total N = 413). Population cluster as determined by majority ancestry in fastSTRUCTURE

State abbreviations: WA Western Australia, SA South Australia, QLD Queensland, NSW New South Wales, ACT Australian Capital Territory, VIC Victoria

DNA extraction and sequencing

DNA was extracted from roughly 6 mm² of each rabbit ear tissue sample using the Gentra Puregene tissue extraction protocol after desalting in 1 ml TE buffer for at least 1 h, and eluted in 100 µl TLE buffer. Approximately 200 ng of whole genome DNA extract was used to prepare a double digest GBS library following the protocol of Poland et al. (2012), using New England BioLabs PstI-HF as enzyme 1 and New England BioLabs MspI-HF as enzyme 2. GBS library was purified using the Qiagen QIAquick PCR Purification Kit, and fragments < 200 bp in length removed using 1.1× ratio of Agencourt AMPure XP beads. Ninety-six samples including a negative control were pooled per run for 75 bp single-end sequencing by the Australian Genome Research Facility on an Illumina NextSeq 500.

Genotyping and SNP filtering

Raw sequence reads were filtered for quality (sliding window phred score limit of 10) and adapter presence, trimmed to 40 bp and demultiplexed using the process_radtags program from the software Stacks v1.34 (Catchen et al. 2013). Reads were then mapped to the rabbit genome assembly OryCun2.0 (available from the National Center for Biotechnology Information (NCBI) at www.ncbi.nlm.nih.gov/genome/316?genome_assembly_id=203429) using the bwa aln/samse functions from the software Burrows–Wheeler Aligner (Li and Durbin 2009) with default values. The Stacks v1.34 pipeline ref_map was then used to call SNPs with a minimum stack depth of 10, minimum minor allele frequency of 0.01 and minimum of 80% of samples represented. Files were checked for quality at each stage using FastQC. Based on the Stacks sumstats output, loci that mapped to the X-chromosome (NC_013690.1) were removed from the dataset along with one member of any pair of loci that mapped within 20,000 bp of each other to

minimise linkage between loci. Rabbits with > 40% missing data were removed from the dataset.

Loci under selection can potentially bias analysis of population structure (Beaumont and Nichols 1996), therefore loci with outlying F_{ST} values were identified for removal using the software package Bayescan 2.1 (Foll and Gaggiotti 2008). Bayescan 2.1 was run with prior odds of 100 for the neutral model, sample size of 5000, a thinning interval of 10, twenty pilot runs of length 5000 and a burn-in of length 50,000. Samples were grouped into ‘populations’ based on sample site. Any locus detected as an F_{ST} -outlier at a false discovery rate of 0.05 was removed from the dataset.

Analysis of population structure

Population structuring and sample ancestry estimation was investigated using three approaches: (1) multivariate analysis through discriminant analysis of principal components (DAPC) with the R package adegenet 2.0.1 (Jombart et al. 2010); (2) population genetic model-based Bayesian clustering with the program fastSTRUCTURE (Raj et al. 2014); and (3) a spatially explicit least-squares optimisation approach with the package Tess3R (Caye et al. 2016) run through R 3.2.5 with Rstudio 1.0.136. Tess3R differs from fastSTRUCTURE in its inclusion of geographic proximity information and a model-free algorithm, whereas fastSTRUCTURE is based on population genetic models of Hardy–Weinberg equilibrium.

Genetic clusters for DAPC were inferred through K-means clustering with the find.clusters function. The optimal value for K was chosen at the minimum Bayesian information criterion (BIC) score using 20 replicates of 1×10^7 iterations for $K = 1$ –25. DAPC was then used for each sample to assign membership probabilities to these clusters. To avoid overfitting, the number of principal components retained during DAPC was selected using the cross-validation function. fastSTRUCTURE was run for $K = 1$:12 with a simple prior. Tess3R was run with ten replicates for each of $K = 1$:20 under default parameters. Admixture proportions for the four most supported values of K for fastSTRUCTURE and Tess3R were visualised through the barplot.tess3q function of Tess3R. Where several sample sites were supported to form an ancestral cluster those site clusters were re-run separately in fastSTRUCTURE, using both the simple and

logistic prior, in order to examine substructure within the cluster.

GenAlEx v6.503 (Peakall and Smouse 2012) was used to find overall F_{ST} value through Analysis of Molecular Variance (AMOVA) with 9999 permutations and to perform Mantel tests for isolation-by-distance through correlation between linear genetic distance and both linear and log-linear geographic distance with 9999 permutations. Expected and observed heterozygosity, average number of alleles per locus and number of private alleles were also calculated for fastSTRUCTURE clusters normalised to 19 individuals each (equivalent to the total sample size of the smallest cluster). Arlequin 3.5.2.2 (Excoffier and Lischer 2010) was used to generate a pairwise F_{ST} matrix, F_{ST} significance was assessed with 10,100 permutations using $\alpha = 0.05$ with Bonferroni correction.

Results

Sequencing recovered 1675 million reads yielding 39,753 SNP loci with over 10x coverage, of which 7821 remained after quality control and filtering. At a false discovery rate of 0.05 Bayescan identified 204 outlier loci, yielding a set of 7617 selectively neutral loci for population structure analysis, typed across 413 rabbits from 18 sampling locations.

k-Means clustering supported an optimum of five clusters to best describe the rabbit population structure of our continent-wide rabbit samples, with the Bayesian Information Criterion forming a distinct minimum ‘elbow’ at this point for 9 of 20 replicates and a further 5 replicates ambiguous between $k = 4$ and $k = 6$. Cross-validation supported retention of 50 principal components during DAPC. Three of these clusters (dubbed Central, Melbourne and Perth) represent contiguous geographic regions, while the remaining clusters represent individual sample sites SA4 and NSW3. DAPC based on the five clusters detected minimal overlap between clusters (Fig. 1). The first discriminant function clearly separated clusters Adelaide and Perth from the remaining groups, while the second isolated Sydney, and a third discriminant function clearly distinguished all clusters.

The chooseK component of FastSTRUCTURE analysis supported six population clusters at the

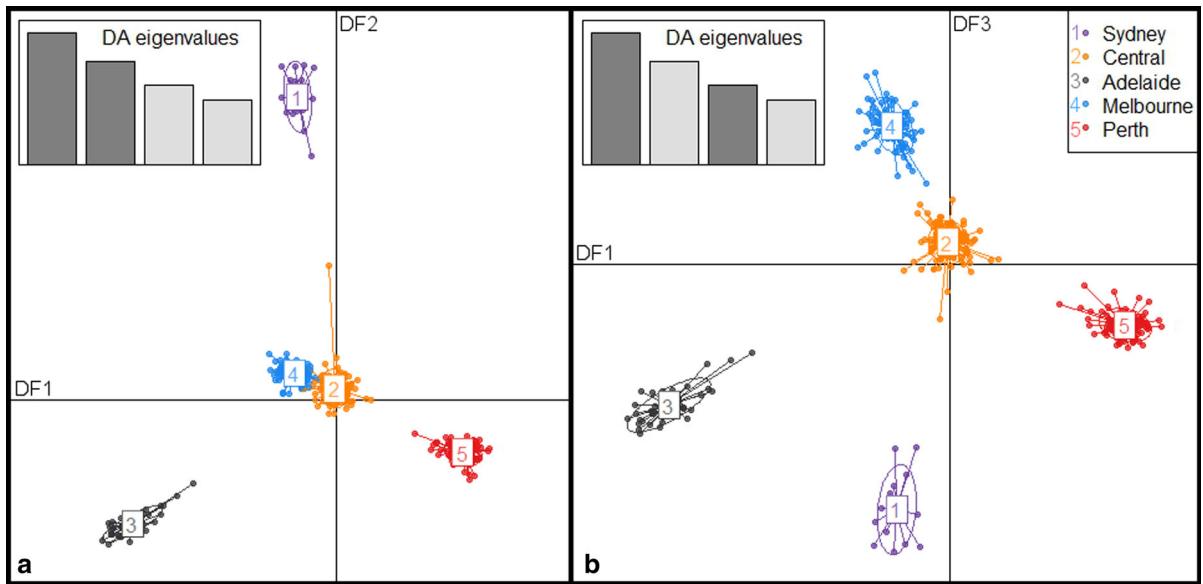


Fig. 1 Discriminant analysis of principal components (DAPC) scatter plot of five Australian rabbit clusters, produced using the adegenet package of R. Insets show relative eigenvalues for the four discriminant functions (plotted functions in darker grey).

a X-axis represents discriminant function 1, y-axis represents discriminant function 2. **b** X-axis represents discriminant function 1, y-axis represents discriminant function 3

maximum marginal likelihood value; while Tess3R did not support a distinctive best value for K , suggesting that further sub-structuring is present within the six clusters. Independent population clustering by both fastSTRUCTURE and Tess3R (Fig. 2) showed groupings largely congruent with those supported by k -means clustering in adegenet. All sites from Western Australia along with SA1 in South Australia formed a consistent group (predominantly red in Fig. 2, cluster hereafter called Perth), while sites in southern Victoria formed a second consistent grouping (predominantly blue in Fig. 2, cluster hereafter called Melbourne). Sites SA4, NSW3 and NSW5 each represented distinct genetic lineages, hereafter named for their nearby state capital cities Adelaide, Sydney and Brisbane (grey, purple and green respectively in Fig. 2), while the remaining central and eastern sites formed a large cluster with varying levels of admixture from neighbouring clusters (predominantly yellow sites in Fig. 2, which we will hereafter refer to as the Central cluster). Of the 413 samples, 379 were assigned to the same groups by both fastSTRUCTURE and Tess3R, although Tess3R generally supported a greater level of admixture compared to fastSTRUCTURE (Fig. 2b). Of the 34 samples which differed between the two programs 17 were from Site

NSW4 which was assigned to the central cluster by fastSTRUCTURE but grouped with the neighbouring Brisbane cluster by Tess3R. When examined separately in both fastSTRUCTURE and Tess3R each of the three broader geographic clusters showed evidence of further substructuring aligning strongly with sample site location.

Pairwise F_{ST} values between sampling locations ranged from 0.007 to 0.247 and only one of 190 site pairs was not significantly different (see Fig. 3). In line with population clustering results F_{ST} values were generally larger for comparisons involving SA4, NSW3 or NSW5. SA1 also had high pairwise F_{ST} values with all pairs outside of Western Australia (mean $F_{ST} = 0.151$, $\sigma = 0.039$), particularly when paired with neighbouring SA4 ($F_{ST} = 0.247$). Pairwise F_{ST} values between years at SA4 and at VIC3 were low (0.055 and 0.021 respectively) but still significant.

AMOVA testing found significant differentiation in the sample sites ($F_{ST} = 0.108$, $P < 0.001$) with 7% of molecular variance apportioned to differences among sites. A further 3% of molecular variance was attributed to differences among clusters as identified by fastSTRUCTURE, and 23% to differences among individuals. We did not find evidence of correlation between genetic and geographic

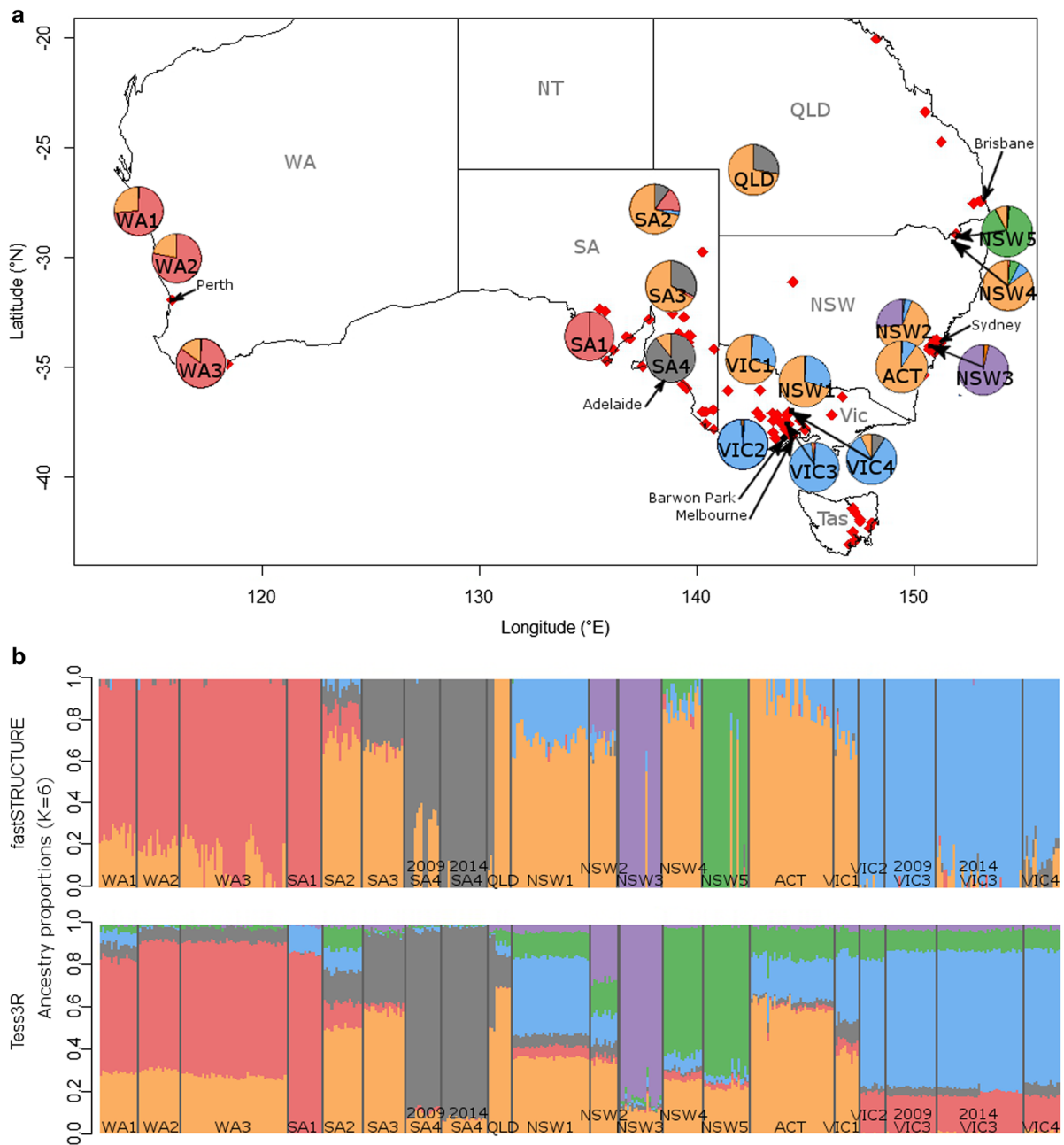


Fig. 2 **a** Map of rabbit sample sites. Pie chart colours represent proportions of ancestry for rabbit sample sites used in this study as estimated by fastSTRUCTURE with $K = 6$. Sample sites numbered from west to east in each state, as in Table 1. Historical rabbit introduction records of successful or unknown outcome reported by Peacock and Abbott (2013) are represented as red diamonds, noting some may be hidden by pie charts. The

Barwon Park release site in Victoria and state capital cities are specifically indicated. **b** Bar charts of individual rabbit ancestry proportions as estimated with $K = 6$ by fastSTRUCTURE (above) and Tess3R (below). Individuals grouped by sample site, numbered as in a). Figure produced using GNU Image Manipulation Program and the R packages Tess3R, maps, mapplots and oz

	WA1	WA2	WA3	SA1	SA2	SA3	SA4 (2009)	SA4 (2014)	QLD	NSW1	NSW2	NSW3	NSW4	NSW5	ACT	VIC1	VIC2	VIC3 (2009)	VIC3 (2014)	VIC4
WA1	0																			
WA2	0.017	0																		
WA3	0.018	0.029	0																	
SA1	0.069	0.080	0.075	0																
SA2	0.029	0.048	0.046	0.114	0															
SA3	0.060	0.078	0.072	0.159	0.021	0														
SA4 (2009)	0.102	0.114	0.105	0.194	0.065	0.076	0													
SA4 (2014)	0.161	0.173	0.164	0.247	0.126	0.115	0.055	0												
QLD	0.073	0.088	0.081	0.167	0.035	0.083	0.100	0.143	0											
NSW1	0.050	0.066	0.060	0.122	0.027	0.052	0.087	0.150	0.054	0										
NSW2	0.059	0.076	0.067	0.143	0.032	0.089	0.131	0.169	0.120	0.038	0									
NSW3	0.141	0.152	0.139	0.195	0.112	0.137	0.166	0.221	0.142	0.115	0.086	0								
NSW4	0.059	0.071	0.063	0.137	0.026	0.066	0.105	0.163	0.068	0.038	0.045	0.129	0							
NSW5	0.109	0.126	0.116	0.191	0.085	0.117	0.156	0.212	0.115	0.090	0.095	0.175	0.079	0						
ACT	0.047	0.067	0.062	0.126	0.028	0.055	0.091	0.151	0.055	0.031	0.038	0.122	0.029	0.084	0					
VIC1	0.069	0.088	0.077	0.153	0.040	0.089	0.137	0.178	0.099	0.045	0.086	0.140	0.062	0.114	0.047	0				
VIC2	0.054	0.071	0.057	0.117	0.037	0.117	0.156	0.187	0.145	0.026	0.123	0.115	0.055	0.104	0.035	0.094	0			
VIC3 (2009)	0.054	0.075	0.063	0.120	0.045	0.111	0.146	0.187	0.126	0.040	0.105	0.123	0.062	0.112	0.048	0.092	0.088	0		
VIC3 (2014)	0.049	0.068	0.064	0.102	0.046	0.077	0.110	0.171	0.074	0.038	0.046	0.110	0.051	0.106	0.049	0.051	0.007	0.021	0	
VIC4	0.065	0.085	0.080	0.136	0.054	0.085	0.115	0.171	0.099	0.050	0.069	0.128	0.068	0.121	0.060	0.072	0.057	0.062	0.050	0

Fig. 3 Pairwise site F_{ST} matrix, as determined by Arlequin 3.5.2.2 (Excoffier and Lischer 2010). Colour gradient with green = lower differentiation between populations, red =

higher differentiation between populations. All values significant at $\alpha = 0.05$ with Bonferroni correction except SA2/NSW2

Table 2 Genetic diversity statistics by population cluster

	Central	Perth	Melbourne	Adelaide	Sydney	Brisbane
Mean allelic richness	1.905	1.846	1.894	1.732	1.803	1.731
Private alleles	53	35	89	22	84	10
Mean expected heterozygosity	0.283	0.268	0.286	0.230	0.263	0.241
Mean observed heterozygosity	0.271	0.246	0.272	0.225	0.251	0.254

Genetic diversity statistics by population cluster. N = 19 randomly chosen individuals per cluster

distance (mantel test simulated $P = 0.293$ and 0.482 for geographic distance and $\log(1 + \text{geographic distance})$ respectively).

The total number of private alleles for each cluster (prior to normalisation) were: Perth = 4, Central = 20, Melbourne = 11, Adelaide = 2, Sydney = 15 and Brisbane = 2. Private alleles in the larger clusters were, on average, present at lower frequency than those in clusters comprising a single sampling site (mean private allele frequency 7.5% vs 20.9%, $P < 0.0001$). Clusters that comprised multiple sampling sites had higher genetic diversity than clusters that comprised a single sampling location, shown by greater allelic richness (mean number of alleles per locus) (1.846–1.905 vs 1.731–1.803) and greater expected heterozygosity (26.8–28.6% vs 23.0–26.3%). Cluster genetic diversity is detailed in Table 2.

Discussion

A history of multiple introductions

Caughly's (1977) observation of Australia's colonisation by rabbits as being the fastest mammal colonisation in history is oft-cited and fits well with the social narrative of Australia's overwhelming rabbit plagues. While the rapid initial spread of rabbits across this landscape is undisputed, the popular notion that rabbits achieved this feat through natural dispersal in a single wave from Barwon Park has been challenged by historical evidence of multiple introductions by Stodart and Parer (1988) and Peacock and Abbott (2013). The molecular evidence presented here supports the historical records, in suggesting that while natural dispersal has likely played a large role in regional range expansions, continent-wide movement

was also facilitated by repeated human-mediated introductions.

Analyses of Australia's rabbit population structure through three independent approaches based on principal components (DAPC), bayesian clustering (fastSTRUCTURE) and spatially explicit least-squares optimisation (Tess3R) all yield a concordant notion of six genetic lineages. Of these, three are geographically broad lineages roughly representing Western Australia (Perth), Southern Victoria (Melbourne) and Central Australia/New South Wales (Central), with evidence of moderate admixture across their boundaries (as in Fig. 2). The remaining three comprise individual sample sites—SA4 (Adelaide), NSW3 (Sydney) and NSW5 (Brisbane)—and are notable for their much greater genetic differentiation at a very localised geographic scale.

Remarkably, fastSTRUCTURE suggested very little common ancestry between rabbits from NSW4 and NSW5, despite the two sites being separated by less than 40 km and with no readily apparent geographic or biological barrier. Tess3R's grouping of NSW4 and NSW5 is the most substantial departure from the fastSTRUCTURE results, and is likely a result of the spatial constraints in the Tess3 algorithm which ensure that geographically close populations are more likely to share ancestry than those that are far apart (Caye et al. 2016). It appears that NSW4 and NSW5 lie at the boundary of the Central and Brisbane ancestral groups, and represent admixed populations. Substantial admixture is indicated both in the uncertain ancestry of NSW4 (Fig. 2) and the unexpectedly high heterozygosity present in NSW5 when compared to other clusters (Table 2).

The substantial differentiation of rabbits in the Greater Western Sydney area (NSW3) is further supported by moderately high pairwise F_{ST} values with all other sites (0.086–0.221) and the presence of 84 private alleles (many at high frequencies), confirming the results of Phillips et al. (2002) who tentatively found differentiation between rabbits in Sydney and surrounding areas using allozyme data.

The strong structuring with admixture along cluster boundaries observed in Australia's rabbit population is consistent with two possible scenarios, genetic drift or multiple introductions, both of which are not mutually exclusive. A single introduced population experiencing a dramatic decrease in migration following nationwide dispersal could have formed pockets of

differentiation as a result of genetic drift. Rabbits in Australia have undergone repeated population bottlenecks driven by control activities. At introduction in 1950 myxomatosis caused up to 99% rabbit mortality (Fenner et al. 1953), and initial RHDV mortality was recorded as high as 95% in 1995 (Mutze et al. 1998). Bottlenecks such as these compound the effect of genetic drift in isolated populations because rare alleles present in surviving individuals become disproportionately common among their descendants, while lost alleles are not quickly replaced by mutation or immigration. In this manner more isolated populations such as those at sites SA4, NSW3 and NSW5 may have become differentiated from surrounding sites. There is some limited evidence for a change in allele frequencies over time at our SA4 site where the pairwise F_{ST} between samples taken in 2009 and 2014 was low (0.055) but still significant, suggestive of ongoing genetic drift.

The observed population structure may also result from multiple independent introductions to different regions which are experiencing admixture at the boundaries of dispersal. These independent historical introductions can account for the genetic differentiation of population clusters through either differing rabbit source populations or founder effects caused by strong population bottlenecks in isolated populations at introduction. A combination of genetic drift and multiple introductions is also possible. It may be that some ancestral clusters result from isolated populations experiencing strong genetic drift, while others represent unique introduction events. Without comparative genetic information from source populations it is difficult to distinguish between these alternative histories, however, two factors lead us to suggest that multiple independent introductions are likely to have contributed to the differentiation between our genetic clusters. Isolation-by-distance was not observed at a continent-wide scale, which would have been expected in a range expansion from a single successful introduction such as from Barwon Park. Although genetic drift could reduce the correlation between genetic and geographic distance over time, the broad scale of clusters and extent of admixture observed at cluster boundaries suggests that the clusters may not be sufficiently isolated for drift to cause such strong differentiation. Further, the presence of numerous private alleles in each population cluster can more parsimoniously be explained by multiple introductions

than by mutation in just 150 years. The particularly high number of private alleles in the Melbourne and Sydney clusters in particular suggest that several introductions may have contributed to the gene pools in these locations. Multiple introductions is supported by contemporary accounts (Peacock and Abbott 2013).

Our support for multiple historic rabbit introductions in Australia challenges the conclusions of Zenger et al. (2003) who investigated the genotypic variation of Australian rabbits by comparing seven microsatellite markers in five Australian rabbit populations to those from European sourced rabbits. Zenger et al. (2003) interpreted their microsatellite diversity within the context of isolation-by-distance, assuming that all rabbit populations in Australia stemmed from the Barwon Park plague via a series of range expansions. Under this assumption they expected allelic diversity to decrease towards the edge of the species range as a result of allelic drop-out from sequential founder effects. With this scenario in mind they concluded that the surprising abundance of unique and rare alleles that they observed in rabbits from Wellstead in WA, at the furthest edge of this range, were a result of rapid population expansion offsetting the colonisation founder effect. Our results suggest that the Western Australian rabbit population is the result of a separate historical introduction, forming a genetic cluster distinct from the Southern Victorian rabbits around the Barwon Park release site. Our strong clustering of Western Australian rabbits with rabbits from SA1 on the Eyre Peninsula of South Australia is concordant with historical accounts from Abbott (2008) that place South Australia as a major source of rabbits travelling overland to WA. We therefore postulate that, rather than being a distant migration from Barwon Park, Zenger et al.'s unexpectedly diverse Wellstead rabbits are in fact the product of migrants stemming from the Eyre Peninsula (where successful introductions were reported at Point Lowly (1860–1864), Middlecamp (pre-1873) and Franklin Harbour (pre-1878)—see Peacock and Abbott 2013), perhaps augmented by local introductions such as that recorded at Cheyne Beach in 1886 (Peacock and Abbott 2013).

Our findings assign each of Australia's state capitals its own ancestral rabbit genotype, a pattern that bears a striking resemblance to that found by Andrew et al. (2017) for Australia's introduced house sparrows. Like sparrows, rabbits are typically very

sedentary but capable of moderate dispersal under appropriate conditions (Parer 1982; Richardson et al. 2002); it is therefore unsurprising that the two species would follow a similar genetic pattern arising from early (perhaps repeated) introductions to major settlements followed by regional dispersal. Peacock and Abbott (2013) document 223 rabbit introductions to Australia during the mid-1800s, of which at least 32 were reported successful in historic articles. These span much of the country, as indicated in Fig. 2a, including plausible sources for independent introductions associated with each of the ancestral clusters identified here, and the well-known release at Barwon Park in 1859 which could be a primary source of all populations within the Melbourne ancestral cluster.

The patterns of ancestry shown in Fig. 2 suggest that while some populations have remained largely localised (Sites SA4, NSW3 and NSW5) others appear to have dispersed widely, resulting in the Perth, Central and Melbourne clusters. An interesting avenue of further research will be identifying the causes of this variation in dispersal, which may include local climate, landscape barriers, or factors affecting propagule pressure such as early control efforts and predator impact (as per Peacock and Abbott (2013)).

The rabbit introduction records assembled by Peacock and Abbott (2013) include both intentional and accidental releases, and undoubtedly represent only a fraction of total releases occurring at that time period. One account from c. 1820 states “probably a 100 or more distinct efforts were made by as many of the first settlers to breed rabbits in New South Wales” (*The Australasian* 20 April 1918 page 723). It is apparent that the importance of rabbits as a source of portable protein for early European settlers resulted in substantial human-mediated propagule pressure across the continent. That this pressure resulted in numerous independent population establishments is evidenced by the six ancestral clusters identified in our analyses. While the rapid colonisation of Australia by rabbits remains remarkable, it appears that natural dispersal was given a substantial head-start by association with early European colonisation.

Implications for pest management

Outside the agricultural areas, where physical control of rabbits is cost effective, management of rabbit populations in Australia currently relies on viral

biocontrols to suppress numbers across a vast and sparsely populated landscape. Although both myxomatosis and RHDV were highly effective on naïve rabbit populations, mortality rates have decreased over time. Several studies (summarised by Kerr 2012) have found evidence of adaptive resistance to myxomatosis in wild rabbits, as well as attenuation of the virus to maximise rabbit–rabbit transmission. Similarly, challenge studies by Elsworth et al. (2012) found reduced infection rates for RHDV in some wild rabbit populations.

Understanding rabbit population structure is a key step in understanding documented variability in biocontrol resistance. In this study we have identified three primary genetic lineages of Australian rabbits, as well as the presence of highly localised lineages which may represent independent local rabbit introductions. The lineages identified here bear no apparent relationship to the distribution of RHDV resistance identified by Elsworth et al. (2012). Elsworth et al. (2012) found no significant resistance in the rabbits from VIC2 or QLD (Melbourne and Central clusters respectively), and high levels of RHDV resistance in rabbits from VIC1, VIC3, and SA4 (Central, Melbourne and Adelaide clusters respectively). This suggests that resistance to RHDV may have evolved several times independently at a more localised scale, however further research will be required to determine the specific genes involved and whether they differ among rabbit lineages. Where lineages exhibit strain-specific viral resistance this will aid prediction of the impact of biocontrol efforts in different regions and create opportunities for regional customisation of future biocontrol releases.

The genetic structure of invasive populations has at times been used to inform pest management strategies through the identification of ‘management units’—regions between which there is minimal gene flow, due to geographic or other barriers—in which the species of interest can be suppressed or eradicated with minimal chance of reinvasion. One example of this is the identification of appropriate management units for feral pigs (*Sus scrofa*) in Australian rangelands by Cowled et al. (2008) who suggest that the failure of previous pig management attempts was caused by a disparity between the area of management and the geographic range of the sub-population. There has been some concern that, given the speed and scale of historical rabbit invasions, the scale of appropriate

management units for this species may simply be too large for viability. Previous studies of rabbit variation in more localised areas have produced varied results. Richardson (1980) found variation in allozyme frequencies between NSW subpopulations as little as 1 km apart. In contrast, Fuller et al. (1996) found no evidence of differentiation between nine populations of arid Queensland rabbits within a 25 km radius using five allozyme loci. Phillips et al. (2002) found differentiation in mtDNA haplotype frequencies between rabbits from Sydney, inland NSW and Victoria which could represent our Sydney, Central and Melbourne ancestral clusters. Although our results indicate that the bulk of Australian rabbits do segregate into three geographically broad clusters, the presence of highly localised populations at Sites SA4, NSW3 and NSW5, as well as distinct cluster substructuring to the scale of sample site, suggest that the treatment of local populations as management units may indeed be viable. The conflicting results of Richardson (1980) and Fuller et al. (1996) indicate that the scale of appropriate management units may vary. Further studies using genome-wide sequencing techniques at regional scales will be required to identify the factors that influence the scale of localised gene flows (and thus the appropriate scale of management units).

Conclusion

Using reduced-representation sequencing techniques we have shown that Australia’s introduced rabbits form three broad geographic clusters representing different ancestral lineages, along with a number of highly localised, differentiated lineages. Rather than the oft-cited single plague of rabbits dispersing rapidly from an introduction at Barwon Park in Victoria, this molecular data supports a history of multiple independent rabbit introductions across the landscape, highlighting the importance of explicitly testing popular assumptions of species invasion history. This new insight into the population structure of Australia’s rabbits provides a foundation for further research to examine the impact of rabbit lineages on biocontrol resistance and optimal management units to enhance management strategies for this challenging pest species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal ethics All animal samples used were either archival material from previous studies or scavenged from regular public pest control activities.

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