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RESEARCH ARTICLE

Geographic hot spots of dingo genetic ancestry in southeastern Australia despite hybridisation with domestic dogs

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Abstract

Hybridisation resulting from human-driven shifts in species ranges is a global conservation concern. In Australia, hybridisation between dingoes (*Canis dingo*) and domestic dogs (*Canis familiaris*) has been identified as an extinction threat to the dingo, and is thought to be particularly widespread in south-eastern Australia. Here, we investigated the extent of hybridisation between dingoes and dogs in a sample of 783 wild-caught canids from eastern New South Wales, using an established 23-microsatellite test. We then mapped the distribution of these samples and identified three areas that are geographic hotspots of high dingo genetic ancestry using geospatial analysis. Between 9 and 23% of the wild canids that we sampled were classified as only having or likely to have only dingo ancestry. Only 0.6% of the wild canids we sampled were classified as having no dingo ancestry. Introgression from domestic dogs into the southeastern dingo gene pool has been extensive, with 76–88% of sampled dingoes carrying some dog ancestry. Spatial analyses revealed several geographic hotspots of high dingo genetic ancestry within north-eastern New South Wales (NSW) where there was a higher than expected prevalence of dingoes with no domestic dog ancestry. A key finding of our study is the observation of several regions where dingoes were largely free of admixture from dogs. There is an ongoing need for evidence-based strategies to reduce human-driven hybridisation by identifying and maintaining natural barriers to reproduction or limiting opportunities for wild-domesticate hybridisation. Globally, legislators and land managers may need to consider less restrictive species definitions to conserve endangered or ecologically significant taxa.

Keywords Introgression · *Canis familiaris* · *Canis dingo* · Admixture · Microsatellites · Spatial analysis

Introduction

Hybridisation resulting from anthropogenic shifts in species ranges is a global conservation issue affecting many species (Rhymer and Simberloff 1996). Taxa experiencing recent hybridisation events include North American bison (*Bison bison*) (Halbert and Derr 2007), wild cats (Hertwig et al. 2009), bears (Cahill et al. 2015), canids (Bohling and Waits 2011; Hindrikson et al. 2012; vonHoldt et al. 2016; Murphy et al. 2018), salmonoid fish (Muhlfeld et al. 2014) and insects (Sánchez-Guillén et al. 2013). Although hybridisation has been traditionally viewed as a process threatening wildlife populations (Rhymer and Simberloff 1996) it has also been identified as a key mechanism by which species adapt to changing environments both historically and at present (Hoffmann and Sgro 2011; Cahill et al. 2015; Schweizer et al. 2018; vonHoldt et al. 2018).

Anthropogenic hybridisation, where hybridisation is affected by the direct activities of humans i.e. alteration

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of the distribution of species, removal of natural barriers or through uncontrolled diffusion with domesticates, is of major concern to conservationists (Allendorf et al. 2001; Stronen and Paquet 2013; Fitzpatrick et al. 2015; Bohling 2016; van Wyk et al. 2017). The question of how to conserve a species or population subjected to hybridisation is controversial, with some authors arguing that we need to reconsider our use of strict species concepts and insistence on genomic purity (Murray et al. 2015; Morell 2016; vonHoldt et al. 2016). Nevertheless, hybridisation is likely to become an increasingly common concern as humans continue to modify natural environments and climate change leads to shifts in species distributions (Hoffmann and Sgro 2011; Muhlfeld et al. 2014; Canestrelli et al. 2017).

Canids (*Canis* spp.) are particularly vulnerable to hybridisation, due to deteriorating, broken or absent biological barriers to inter-species reproduction and because many canid hybrids are fertile. Biological barriers in canids may be heavily influenced by social collapse, habitat loss and urbanisation. Although hybridisation between canids is a natural process where species ranges overlap, it is an issue of global conservation concern due to the widespread distribution of domestic dogs and resulting introgression of dog genes into native canid populations (Gottelli et al. 1994; Vilà and Wayne 1999; Adams et al. 2003). Hybridisation between wild canid species is also of concern in some regions due to recent range expansions of some canid species. For example, there is evidence of admixture and introgression in grey wolves (Vilà and Wayne 1999; Klütsch et al. 2011; Hindrikson et al. 2012; vonHoldt et al. 2013), coyotes (Wayne and Jenks 1991; vonHoldt et al. 2016) and jackals (Galov et al. 2015). Interestingly, historical introgression of melanism from domestic dogs into North American wolves may have conferred some selective advantage (Anderson et al. 2009; Schweizer et al. 2018) and recent admixture between North American wolves and coyotes may also have been evolutionarily advantageous (vonHoldt et al. 2016).

Dingoes are important for maintaining the health and biodiversity of ecosystems—by suppressing the impacts of introduced mesopredators and both native and introduced herbivore populations (Letnic and Crowther 2012; Letnic et al. 2013; Morris and Letnic 2017). Dingoes (*Canis dingo*) are an early lineage of canine (vonHoldt et al. 2010) that are distinct from modern domestic dogs and wolves (Cairns et al. 2018; Zhang et al. 2018; Smith et al. 2019). Dingoes are closely related to other early dog lineages from Asia (Oskarsson et al. 2011; Sacks et al. 2013; Cairns et al. 2017, 2018) and they have been present in Australia for at least 5000 years, possibly longer (Oskarsson et al. 2011; Cairns and Wilton 2016; Zhang et al. 2018). Europeans introduced modern domestic dog breeds to Australia in 1788, and ever since then there have been concerns about hybridisation between dogs and dingoes and its effect on the dingo's identity and ecological role (Jones 1921; Newsome and Corbett 1985). These concerns are magnified by the fact that it is difficult to discriminate between dingoes (individuals with only dingo ancestry) and admixed dingoes (individuals which have both dingo and dog ancestry) in the field and the laboratory (Crowther et al. 2014; Parr et al. 2016).

Genetic testing of dingoes is used by end users to monitor the prevalence of dog ancestry in wild dingo populations, inform local management decisions and provide ancestry information to captive breeding programs. Information about the presence of dog ancestry in a dingo population can have significant consequences, informing widespread management programs and being used to alter protective legislation. Legislatively, a dingo is of conservation value only if it has no dog ancestry. Previous studies highlighting the high prevalence of dog ancestry in New South Wales (NSW) dingo populations have led to changes in the way that dingoes are managed and conserved (Stephens et al. 2015). The NSW Threatened Species Scientific Committee acknowledges that securing relatively pure dingo populations that include some hybrids may represent the best opportunity for conserving or recovering the Dingo genotype and its ecosystem function in NSW (OEH 2009). Care needs to be taken that management decisions are based on rigorous and accurate information, with local surveys filling in knowledge gaps.

Morphological and genetic studies have been attempting to discriminate dingoes from putative hybrids with little success since the early twentieth century (Jones 1921; Newsome and Corbett 1985; Elledge et al. 2008; Jones 2009; Parr et al. 2016), a situation which has arisen because there are few pre-European dingo specimens available to researchers (Crowther et al. 2014) and the extensive morphological variation in domestic dogs (Drake and Klingenberg 2010). Early methods used to estimate admixture in dingoes relied upon skull morphology (Corbett 2001b), but a drawback of this method is that it requires destructive sampling and may not be useful for differentiating dingoes with domestic dog ancestry (Parr et al. 2016). In the 1990s a microsatellite based DNA test for dingo ancestry was developed (Wilton et al. 1999; Wilton 2001). This method, commonly known as the average 3Q method uses 23 markers, including 21 microsatellites and 2 indel markers (Elledge et al. 2008; Cairns et al. 2011). Stephens et al. (2015) used this same set of 23 markers and incorporated Bayesian clustering as an alternative method of dingo ancestry estimation. Since these DNA tests were developed over 6000 canid samples have been genotyped and are widely used across Australia by government agencies, local management groups, conservation groups, zoos and private dingo owners.

Recent analyses of the mitochondrial DNA genome and nuclear genes indicate that there are two geographically distinct dingo clades, a southeastern clade and northwestern clade (Cairns and Wilton 2016; Cairns et al. 2017). It

is unclear if there is phenotypic subdivision between the genetic dingo clades, although there is some evidence of morphological differentiation across geographical locales (Colman 2015). An implication of there being two geographically isolated dingo clades is that previous attempts to discriminate dingoes from hybrids using skull morphology and genetics may have been confounded by using animals from the southeastern clade (or vice versa) as controls representative of dingoes free from dog admixture (Cairns and Wilton 2016; Cairns et al. 2017).

Criticisms of current dingo DNA testing include reliance on pre-defined geographically restricted reference populations and limited genomic coverage (type and number of markers) (Elledge et al. 2008; Stephens et al. 2015). Stephens et al. (2015) observed a low number of pure dingoes in NSW (1.1%), using an arbitrary threshold of $q \geq 0.9$ to define pure dingoes. Their study included 95 dingo specimens from NSW out of a total of 3637 samples, however their reference population contained mostly dingoes from northern and western Australia. It is possible that ancestry estimates reported for NSW dingoes are biased due to comparison of samples to a different geographical reference population. Furthermore, Stephens et al. (2015) used a smoothing method (kriging) to map ancestry across Australia which may have obscured hotspots of high dingo genetic ancestry in NSW leading to widespread belief that dingoes are virtually extinct in NSW (Corbett 2001a, 2008; Stephens et al. 2015; Allen et al. 2017).

Here, we investigate the ancestry of 783 wild canids from south-eastern Australia using an existing set of 23 markers chosen by Wilton et al. (1999) for diagnosing domestic dog ancestry in dingoes. We compare estimates of dingo ancestry using thresholds of $q \geq 0.9$ and $q \geq 0.8$, and consider the relevance of arbitrary thresholds to ancestry estimation in dingoes under a conservation framework. We utilise two pre-defined reference populations: a dog reference population (66 mixed breed dogs) and a largely south-eastern Australian dingo reference population (the original 37 dingoes from Wilton 2001 or this original reference population and a further 13 dingoes from north-western Australia from Stephens et al. 2015). Our aims were: 1) to determine the extent of dog introgression in our sample of wild caught canids from eastern NSW using the average 3Q method of Wilton (2001) and Bayesian clustering (Stephens et al. 2015); 2) compare the results of our survey to Stephens et al. (2015); and 3) identify areas which are geographic hotspots of high dingo genetic ancestry using geospatial (Gi*) hotspot analysis. We use this dataset to generate hypotheses concerning the factors contributing to wild dingo ancestry in NSW.

Methods and materials

Dingo samples

The NSW National Parks and Wildlife Service and other government land management agencies during the period 1996–2012 (Fig. 1) collected samples during wild canid trapping operations in north-eastern NSW. Trapping occurred on National Parks, Nature Reserves, State Forests and private lands (Online Resource 1). Trapping activities were predominantly aimed at minimising the impact of canid predation on neighbouring livestock operators, but some of the samples collected were from targeted trapping within selected National Parks to assess the conservation status of dingoes in these areas. All samples collected were tested regardless of an individual's morphological characteristics indicating higher or lower dingo/dog ancestry. Samples were collected under Animal Ethics Committee approval No. 010212/01 Dingo/Wild Dog survey of north east NSW. GPS coordinates were recorded for all samples collected (Online Resource 2). A total of 978 samples were collected, those samples with less than 14 microsatellites genotyped or no GPS coordinate recorded were excluded, the remaining 783 samples or 80% were used in this study.

Study area

North-eastern New South Wales has a varied climate, subtropical in the northern coastlands and temperate in the

Fig. 1 The distribution of dingo samples (triangles) collected in New South Wales, Australia

south and at higher elevations (elevation range from 0 to 1500 m). Grazing, forestry and nature conservation are the prevalent land uses in the region with sheep grazing more common on the tablelands, beef cattle and dairying in the lowlands and escarpment forests. There are significant areas of private native forest, but the largest forest areas are in National Parks and State Forests with the majority located in the escarpment ranges and along the coast.

Dingoes historically occupied woodland and grassland areas throughout NSW. Targeted dingo control programs, land clearing and intensive agriculture has reduced their numbers and the available habitat. In NSW, dingoes and their hybrids are largely restricted to public and private lands along the Great Dividing Range and coastal hinterlands.

Management of dingoes, across the study area, and throughout the study period, regardless of tenure, has been driven by the legal requirement that dingoes, hybrids and feral domestic dogs are to be continuously suppressed and destroyed to the extent necessary to minimise their impact on livestock operations. In practice this has involved regular landscape wide strategic control of all wild canids on many sheep and cattle properties, and the adjoining National Parks and State Forests. Additional reactive dingo control occurred on all tenures as required.

Reference Populations

This study uses a set of two reference populations collected by Alan Wilton in the 1990s and used in developing a dingo DNA test (Wilton et al. 1999; Wilton 2001). The dog reference population consists of 66 dogs of mixed breeds sourced from a pound in Sydney (NSW, Australia). The set of 37 reference dingoes was chosen as likely to only have dingo ancestry based on pedigree (captive breeding history), phenotype and/or skull morphology. All of the original reference dingoes were sourced from zoo or captive breeding populations. These dingoes are predominately from "alpine" or southeastern Australia lineages. Skull morphology discrimination was based on the work of Newsome et al. (1980), and Newsome and Corbett (1982, 1985). These two reference populations form the basis of the dingo DNA test which has been used for the last two decades by dingo conservation groups, land managers and government bodies to assess dingo ancestry in Australia. A set of 13 additional reference dingoes were incorporated from Stephens et al. (2015) to provide geographical diversity, these were all wild origin.

Genotyping

Tissue or blood samples were collected and sent to the Wilton laboratory at the University of New South Wales (UNSW) for genetic testing between 1996 and 2012. DNA was extracted from samples using Qiagen DNeasy kits (Qiagen Sciences, Germantown, USA). Samples were then genotyped for a set of 23 microsatellites that are widely used for estimating the degree of admixture in dingoes (Wilton et al. 1999; Wilton 2001; Elledge et al. 2008; Stephens et al. 2015). In 1999, the dingo DNA test developed by Alan Wilton contained a panel of 14 markers which increased to 23 markers over the timeframe that this research was undertaken (Wilton et al. 1999). The 23 diagnostic loci were amplified via PCR in six multiplex panels (Online Resource 3). Negative controls were included in all genotyping multiplexes and batches. Fragment analysis was carried out using an ABI 3730 Sequencer at the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia). Raw genotypes were analysed using GeneMapper v3.7 or v5.0 (Applied Biosystems, Forster City, USA). The PCR multiplex conditions and fragment analysis equipment used in this study are the same as those used to collect and analyse the original reference population data of Wilton et al. (1999).

To maintain consistency across the timespan of this research project, genotype calling was performed or verified by only two trained individuals (Dr Alan Wilton and author KMC), both individuals used the same scoring rules and binning system designed in Genemapper v3.7 and v5.0 (Applied Biosystems). GenAIEx v6.5 (Smouse and Peakall 2012) was used to calculate basic population genetics (Na and Ne), heterozygosity (He, Ho and F) and population differentiation (F_{ST} and G_{ST}) metrics in the dingo and dog reference populations.

Ancestry analysis

Two main methods for estimating ancestry were used; (1) the average 3Q method of Wilton (2001) which uses log-likelihood scores and (2) Bayesian clustering following Stephens et al. (2015). Microsatellite analysis has also been used to estimate canid species ancestries in red wolves and coyotes in North America (Bohling and Waits 2011; Murphy et al. 2018). Stephens et al. (2015) raise several limitations to the average 3Q method including reliance on an a priori dingo reference population that may not be free from domestic dog ancestry and potential under estimation of domestic dog ancestry. We perform both types of analyses because a wide variety of land managers, conservation groups and government agencies are familiar with and currently utilise the average 3Q method and to allow comparison between the two most widely used methods for diagnosing dog ancestry in dingoes.

Average 3Q method

The average 3Q method relies upon log-likelihood (Lod) scores to evaluate the probability that a sample is a dingo

versus a 3/4 dingo hybrid. Lod scores are calculated for each microsatellite locus using the formula log ($P_{dingo}/P_{3/4dingo}$) and then averaged across all the available markers for an individual, giving an average 3Q score (Wilton et al. 1999; Wilton 2001; Elledge et al. 2008). Essentially the average 3Q method uses allele frequencies from the dog and dingo reference populations to estimate likelihood that a specimen is a dingo vs dingo hybrid (Wilton et al. 1999; Wilton 2001; Elledge et al. 2008). The number and proportion of doglike alleles was also calculated; these are alleles that are 10 times more commonly observed in the dog reference population than in the dingo reference population. The proportion of doglike alleles versus average 3Q scores was visualised to compare NSW dingoes to the dingo and dog reference populations. Samples were scored one to seven based on the average 3Q score and number of doglike alleles to assign to ancestry categories (Table 1).

Bayesian clustering method

To estimate the extent of dog introgression in dingoes using Bayesian clustering, simulations were run in STRUCTURE v2.3.4 (Pritchard et al. 2000; Falush et al. 2003) using the 783 wild canids and a set of pre-defined reference populations. Two different reference population sets were used; (1) included the original Wilton reference populations of 37 dingoes and 66 dogs and (2) included the original Wilton reference population plus an additional 13 (score 1 or 2, Table 1) dingoes from North-western Australia to address issues with geographical bias. Similar to Stephens et al. (2015), analyses were run in STRUCTURE (Pritchard et al. 2000; Falush et al. 2003) with the admixture and correlated allele frequency models. Defaults settings for alpha were used. Simulations for K = 1 to K = 10 were run for 200,000 iterations with a 20,000 iteration burn-in period, and 10 replicates were performed.

Analyses including the 783 wild NSW dingoes were run twice, first with the USEPOPINFO flag both on and a second time with the USEPOPINFO flag off. The USEPOPINFO flag allows population allele frequencies to be updated only from the above a priori defined reference population individuals. Individual q-values were averaged across the 10 replicates using CLUMPAK (Kopelman et al. 2015). Individuals were assigned to one of seven categories based on their average q-value representing dingo ancestry (Table 1).

We used the DeltaK method (Evanno et al. 2005) to determine the most appropriate K value. There has been some criticism that the DeltaK method may erroneously support K = 2 (see Janes et al. 2017), this can be overcome if default alpha settings as used in STRUCTURE and if the number of markers is larger than 20. Janes et al. (2017) report that under the above conditions DeltaK performs well with accuracy of 94.6%. To corroborate identification of the most appropriate K value and account for possible bias from uneven sampling (i.e. 116 reference population samples versus 783 wild canid samples) across the dataset we also ran STRUCTURE analyses including only the defined reference populations. Analyses including only the reference populations were run with the USEPOPINFO flag off.

Spatial patterns of ancestry in wild canids

All spatial analyses were implemented using ArcGIS 10.6 (ESRI 2018). The 783 geo-referenced samples were projected into an Albers equal area coordinate system (EPSG:3577) for analysis. The ancestry scores of individuals, assigned based on Table 1 and STRUCTURE q-values, were transformed into two sets. For this purpose, we used the average q-value from analyses run with the USEPOPINFO flag on, K = 2 and with the expanded reference population. The first transform subtracted the scores from 8 to ensure records with higher dingo ancestry had higher positive values. The second was an indicator transform where records with ancestry scores 1–2 (only had dingo ancestry or likely

Table 1 Dingo purity categories and cut offs for average 3Q and STRUCTURE methods

Score	Category	Average 3Q[a] value and doglike allele cut offs	STRUCTURE[b] average q-value cut offs
1	Dingo 1 (dingo with no dog ancestry)	3Q > 0.1 and no doglike alleles	1.0–0.90
2	Dingo 2 (likely dingo with no dog ancestry)	0.05 < 3Q < 0.1 and ≤ 1 doglike alleles	0.89–0.80
3	Dingo with dog ancestry 1 (> 75% dingo)	0 < 3Q < 0.05	0.79–0.70
4	Dingo with dog ancestry 2 (65–75% dingo)	−0.1 < 3Q < 0	0.69–0.60
5	Dingo with dog ancestry 3 (50–64% dingo)	−0.25 < 3Q < −0.1	0.59–0.50
6	Feral dog with dingo ancestry (< 50% dingo)	−0.5 < 3Q < −0.25	0.49–0.25
7	Feral dog	3Q < −0.5	0.24–0.0

[a]According to Wilton 2001 and Wilton et al. 1999

[b]According to Stephens et al. 2015

to only have dingo ancestry) were assigned a value of 1, and all other records were assigned 0. Both sets of data were analysed using kernel density analysis (KDE) and a geospatial hotspot statistic (the Getis-Ord Gi* statistic; Getis and Ord 1992).

The KDE analysis was used to identify regions with concentrations of records with higher likelihoods of dingo ancestry. For this analysis, windows defined using 50 km radius bandwidths were calculated across a 10 km grid, and each record was weighted by its transformed ancestry value.

The Gi* statistic was used to identify areas that contained records with significantly more or less likely dingo ancestry than expected, relative to a random sample of an equal number of records from the data set. A 50 km radius neighbourhood was defined for each sample, with records falling inside a sample's neighbourhood receiving equal spatial weights. The Gi* statistic follows a z-distribution, so values exceeding 1.96 are treated as significantly high at $\alpha = 0.05$ for a two tailed test (Stephenson et al. 2015). We also modelled sample uncertainty using a randomisation analysis. Following Bruce et al. (2014), we randomly removed 10% of the records and recalculated the Gi* scores. This process was repeated 999 times, recording the frequency that each record identified as significantly high at $\alpha = 0.05$ for the full data set was also significantly high for the subsample across the 999 iterations.

Results

Reference populations

As observed by Wilton (2001), there is strong genetic differentiation between the dingo and dog reference populations based on the 23 markers with $F_{ST} = 0.188$ (95% confidence interval 0.126–0.270) and $G_{ST} = 0.183$ (95% confidence interval 0.128–0.261). General population genetics parameters indicate divergence between the dingo and dog reference populations, they also identify that the reference dingo population has higher homozygosity than the dog reference population (Table 2). The dingo reference population had 50% lower observed and expected heterozygosity than the dog reference population (Ho[dog] = 0.700, se = 0.045; He[dog] = 0.741, SE = 0.044; Ho[dingo] = 0.408, SE = 0.051 and He[dingo] = 0.492, SE = 0.060). Patterns of allele frequency differ between the dingo and dog reference populations (Online Resource 4), with some alleles being observed 5–10 times more frequently in dogs than in dingoes; i.e. doglike alleles.

We observed departure from Hardy–Weinberg Equilibrium across several markers and between populations (Table 3). It is not unusual for real populations with population structure to deviate from Hardy–Weinberg Equilibrium and STRUCTURE uses this signal to assist in clustering populations (Pritchard et al. 2000, 2010). Additional STRUCTURE modelling was carried out after removing the three loci (2293, 2257 and AHT125) which deviated from Hardy–Weinberg Equilibrium (Online Resource 5). Individual q-values were consistent between STRUCTURE analyses with and without these three loci, so we utilised the complete marker set in line with Stephens et al. (2015), Elledge et al. (2008) and Wilton (2001).

Ancestry analysis

Average 3Q method

According to the 3Q method, 12.5% of samples were classified as having only dingo ancestry (score 1) or likely to have only dingo ancestry (score 2) (Fig. 2 and Online Resource 2). A total of 77.4% of the samples were admixed dingoes, with both dingo and dog ancestry (score 3–5) and 9.4% of the samples were classified as feral dogs (score 6–7). Only 5 samples (0.6%) were classified as feral dogs with no detectable dingo ancestry (score 7). The proportion of doglike alleles increases as the average 3Q score increases and a majority of the NSW dingoes cluster between the dog and dingo reference populations (Fig. 3). This corroborates the observation that there are few feral dogs observed in this study. Some of the NSW dingoes cluster with the dingo reference population indicating a high level of dingo ancestry.

Table 2 Population genetics parameters for the dingo (n = 50) and dog (n = 66) reference populations

	N	Na	Ne	Ho	Ho	F	F_{ST}	G_{ST}
Dog reference population	66	10.304 (SE = 1.249)	5.698 (SE = 0.694)	0.700 (SE = 0.045)	0.741 (SE = 0.044)	0.059 (SE = 0.014)	0.188 (95% CI 0.126–0.270)	0.183 (95% CI 0.128–0.261)
Dingo reference population	50	6.565 (SE = 0.822)	3.091 (SE = 0.491)	0.408 (SE = 0.051)	0.492 (SE = 0.060)	0.161 (SE = 0.033)		

Na number of alleles, *Ne* number of effective alleles, *F* fixation index

Table 3 Hardy Wienberg Equilibrium divergence across loci in dingo (n = 50) and dog (n = 66) reference populations

Marker	Dog reference population				Dingo reference population			
	df	ChiSq	Prob	Significance	df	ChiSq	Prob	Significance
2293	153	188.782	0.026	*	105	154.838	0.001	**
406	55	51.918	0.593		6	6.405	0.379	
2199	325	355.665	0.116		66	54.304	0.848	
410	78	90.176	0.163		6	56.338	0.000	***
2138	171	193.417	0.115		91	148.629	0.000	***
2175	55	57.843	0.371		28	27.569	0.487	
402	36	40.852	0.266		3	0.021	0.999	
460	28	18.598	0.910		15	18.939	0.216	
2313	153	150.625	0.539		21	25.048	0.245	
434	15	10.648	0.777		6	19.794	0.003	**
2257	45	106.811	0.000	***	21	34.979	0.028	*
2247	153	157.904	0.376		105	242.078	0.000	***
2079	15	17.910	0.267		6	7.757	0.256	
30	55	85.837	0.005	**	21	20.765	0.473	
LEI008	21	14.250	0.859		21	2.321	1.000	
AHT125	45	104.094	0.000	***	15	25.556	0.043	*
AHT103	21	30.820	0.077		21	22.657	0.363	
PEZ1	21	25.089	0.243		6	12.085	0.060	
AHT109	28	17.981	0.927		15	20.422	0.156	
CPH2	15	49.010	0.000	***	1	0.025	0.874	
CXX109	6	15.439	0.017	*	10	6.425	0.778	
M13TT	Monomorphic				1	0.271	0.603	
M13C19	3	0.299	0.960		1	8.943	0.003	**

Key: *P < 0.05, **P < 0.01, ***P < 0.001

Bayesian structuring method

Across all STRUCTURE analyses DeltaK identified K = 2 as being the most appropriate K value for our datasets (Online Resource 6). When STRUCTURE was run with only the dingo and dog reference populations it was able to cleanly separate the reference dog and dingo populations, with limited evidence of dog ancestry in the dingo reference population (Fig. 4).

STRUCTURE average q-value plots depict the ancestry proportion of individual dingoes and the difference between analyses that had the USEPOPINFO flag on or off (Fig. 5, Online Resource 7). According to the STRCTURE simulations using the original Wilton dingo and dog reference populations, 9.3% of samples were classified as having only dingo ancestry or were likely to only have dingo ancestry (score 1–2), 87.4% of samples were admixed dingoes with both dingo and dog ancestry (score 3–5) and 3.3% of samples were feral dogs with scores of 6 or 7 (Fig. 2 and Online Resource 2). Only 5 samples (0.6%) were classified as feral dogs with no detectable dingo ancestry (score 7).

When STRUCTURE analyses were repeated with the expanded reference population a larger proportion of samples (23.1%) were identified as having only dingo ancestry (score 1–2). The ancestry proportion of individual dingoes and the difference between analyses that had the USEPOPINFO flag on or off is depicted in STRUCTURE average q-value plots (Fig. 6, Online Resource 7). A total of 75.4% of samples were classified as admixed dingoes having both dingo and dog ancestry (score 3–5) and only 1.5% of samples were classified as feral dogs (score 6–7). Again only 5 samples (0.6%) were classified as feral dogs with no detectable dingo ancestry (score 7).

Across all STRUCTURE analyses including the 783 wild canids there is a reduction in the overall dingo average ancestry coefficients (q-values) of reference population dingo samples and the wild canid samples when the USEPOPINFO flag is used (Figs. 5, 6).

Spatial patterns of ancestry in dingoes

The Gi* hotspot analyses of 783 dingoes in NSW identified three regions of higher than expected dingo ancestry (Fig. 7). These regions were near Washpool National Park (hotspot A), north of Port Macquarie (hotspot B) and the Myall Lakes region (hotspot C). A region of higher densities is identified by the KDE analysis between hotspots A and B, but this is not identified as a hotspot in the Gi* analysis.

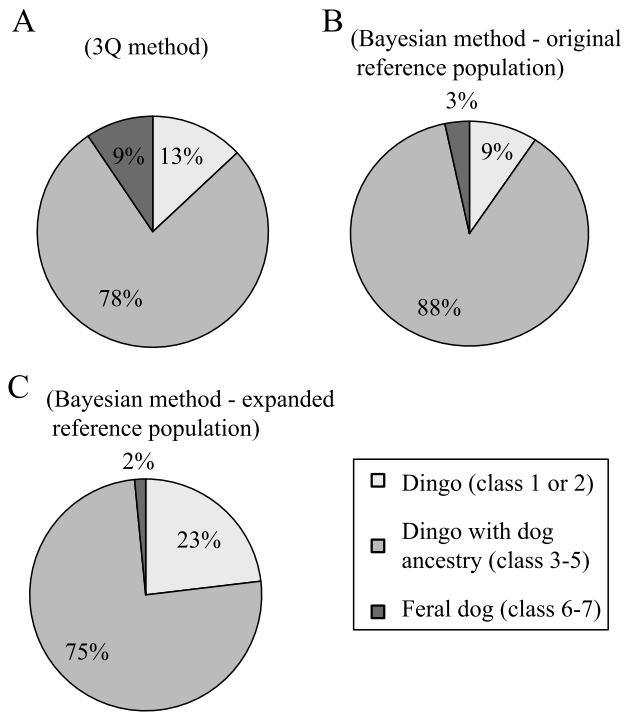

Fig. 2 Pie charts depicting the ancestry of dingoes using three different ancestry estimation methods: **a** 'average 3Q' score; **b** bayesian clustering with the original reference population; **c** bayesian clustering with an expanded reference population

The subsampling analysis identified 43 ordinally weighted records and 34 thresholded records that were significant hotspots for the full data set, but that were significant for fewer than 95% of the random subsamples. All of these are on the fringes of the identified hotspots (Online Resource 8).

Discussion

Our spatial analyses revealed several geographic hotspots in north-eastern NSW where there was a higher than expected prevalence of dingoes with no domestic dog ancestry (Fig. 7). The observation of geographic hotspots of high dingo ancestry in NSW contrasts to the findings of Stephens et al. (2015) where mapping across Australia indicated that NSW had ubiquitous low dingo ancestry with no evidence of regions with high dingo ancestry. Our data challenges the widely posited view that dingoes are virtually extinct in southeastern Australia (NSW, Victoria and the Australian Capital Territory).

Stephens et al. (2015) was the first Australia-wide survey of dingo ancestry, whereas our study represents a more focused survey of the genetic identity of dingoes in eastern NSW. We included 783 specimens from eastern NSW compared to Stephens et al. (2015) who examined 95 specimens from across NSW and ACT. Between 9 and 23% of the wild canids we sampled from eastern NSW were classified as only having dingo ancestry ($q \geq 0.9$) or likely to only have dingo ancestry ($q \geq 0.8$) across the sampled regions of NSW (Figs. 2, 3, 4, 5, and 6). Specifically, we observed 1.9% of wild canids in NSW to be dingoes with no domestic dog ancestry ($q \geq 0.9$), similar to Stephens et al. (2015), who observed 1.1% of wild canids across NSW to be dingoes. We observed that 21.1% of the

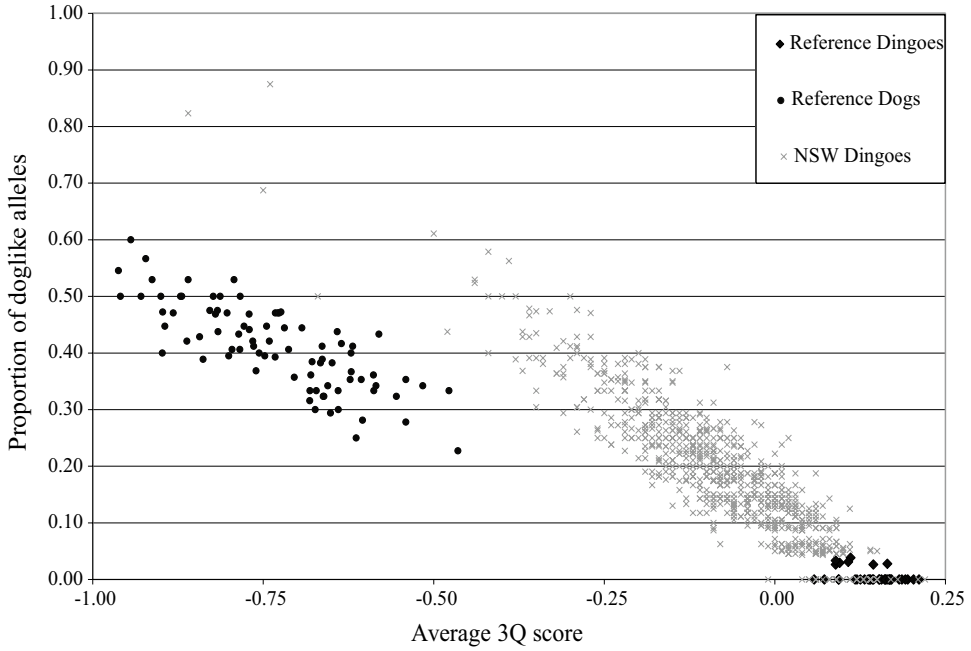

Fig. 3 Proportion of doglike alleles versus calculated average 3Q scores for the NSW dingoes compared to reference dingoes and reference dogs. Average 3Q scores range between 0.25 and −1.0, for interpretation of average 3Q scores see Table 1

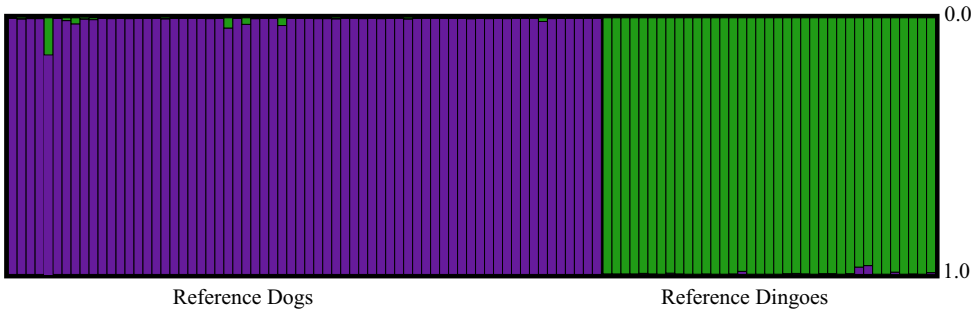

Fig. 4 STRUCTURE estimated ancestry coefficient (q-value) plots depicting K = 2 simulations with the 66 reference dogs and 37 reference dingoes and USEPOPFLAG off

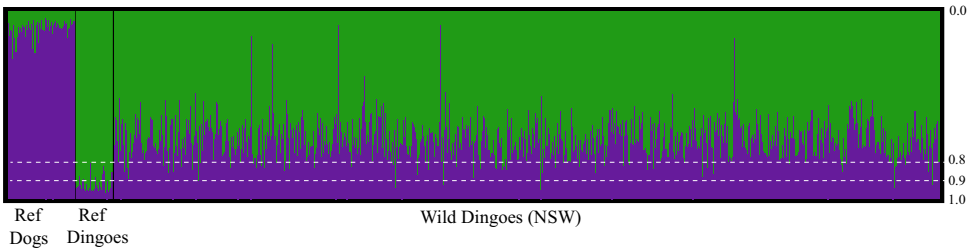

Fig. 5 STRUCTURE estimated ancestry coefficient (q-value) plots depicting 783 NSW dingoes with the original Wilton Reference populations of 66 dogs and 37 dingoes with the USEPOPFLAG on. The q-value thresholds of 0.8 and 0.9 are depicted by the dashed white lines

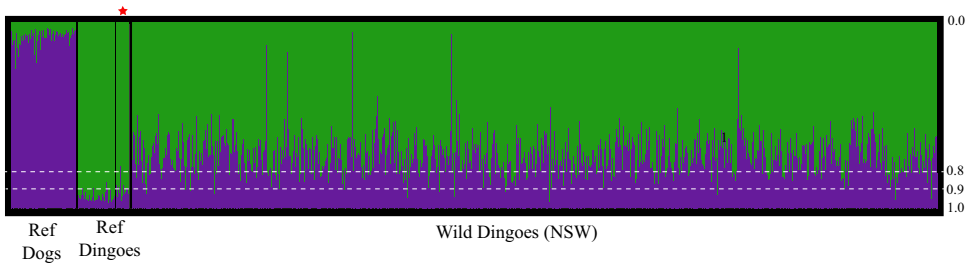

Fig. 6 STRUCTURE estimated ancestry coefficient (q-value) plots depicting 783 NSW dingoes with the expanded Reference populations incorporating 66 dogs and 50 dingoes with the USEPOPFLAG on. Additional reference samples are indicated by the star. The q-value thresholds of 0.8 and 0.9 are depicted by the dashed white lines

eastern NSW wild canids were likely dingoes ($0.9 \geq q \geq 0.8$). Stephens et al. (2015) didn't discuss the distribution of likely dingoes ($0.9 \geq q \geq 0.8$) in NSW, however, based on their supplementary data, likely dingoes ($0.9 \geq q \geq 0.8$) were 23.3% of the wild canid population in NSW. These data highlight the presence of high ancestry dingoes persisting in eastern NSW and challenges the widespread perception that all or most dingoes in NSW are hybrids.

It is important to note that Stephen et al. (2015) used $q \geq 0.9$ as an arbitrary threshold for defining dingoes. Across the population genetics literature, the thresholds used to define pure vs hybrid individuals vary considerably (i.e. $q \geq 0.95$, $q \geq 0.8$, $q \geq 0.725$ etc.) and may depend upon the application of the ancestry assessments (Vaha & Primmer 2006; Rosel et al. 2017; Heppenheimer et al. 2018; Murphy et al. 2018; Hinton et al. 2019; Hulsegge et al. 2019). In a conservation framework it is important to balance the need for accurate ancestry assessments against misclassification of "pure" individuals as hybrids (Vaha and Primmer 2006). In the context of conserving and monitoring wild dingo populations we argue that a threshold of $q \geq 0.8$ balances the need for preserving dingoes with high genetic ancestry against misclassifying pure dingoes as hybrids. Importantly ancestry estimates should consider the presence of at least two distinct genetic dingo clades in Australia and how archetypal the reference populations are. If the reference population is not representative of the genetic identity of dingoes (either from a region or across Australia), then ancestry estimates and strict q thresholds may misclassify pure dingoes as hybrids because they might carry alleles not observed in the reference population.

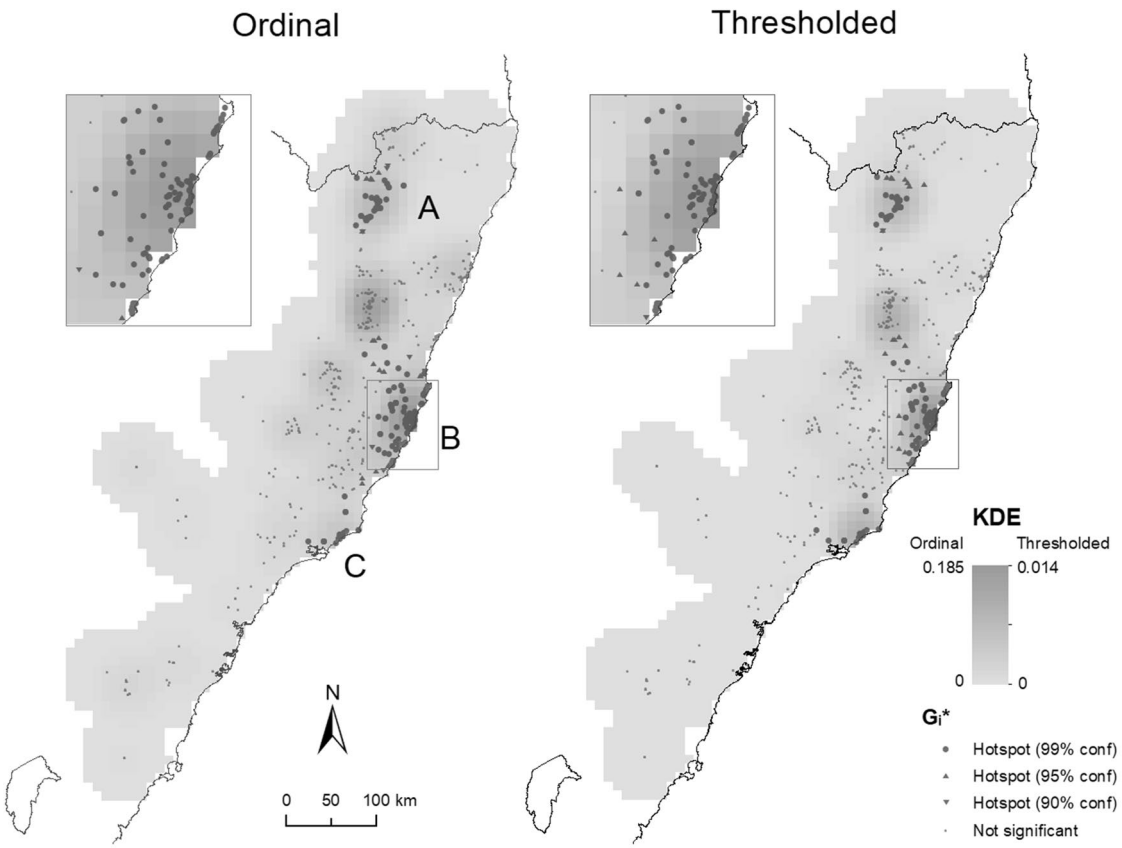

Fig. 7 Kernel density estimator (KDE) surfaces and G_i* hot spots calculated using 783 dingoes collected in NSW. Geographic hotspots are **A** near Washpool National Park, **B** north of Port Macquarie and **C** Myall lakes region

In Stephens et al. (2015), the majority of reference population samples were dingoes from western and northern regions of the continent whereas in this study the majority of the reference population samples were from south-eastern Australia. It is possible that dingoes in some regions may have been misclassified as having dog-ancestry due to allele differences between southeastern and northwestern dingoes.

Our results suggest that feral dogs may be less widespread and dingoes less affected by hybridisation in eastern NSW than previous studies have suggested (Newsome and Corbett 1985; Stephens et al. 2015). Indeed, only 1.5–2.1% of the wild canids we sampled were classified as either feral dogs, or feral dogs with no dingo ancestry. The persistence of hot spots of higher than expected dingo ancestry could be the result of either intensive dingo control occurring on surrounding lands limiting opportunities for hybridisation with domestic and feral dogs, or an area of public or private native forest where the adjoining land use was not affected by the impacts of dingo predation and so little or no control was carried out in the area. Widespread use of "wild dog" terminology in NSW does not accurately represent the genetic identity of wild canids in the region, instead the terms dingoes and dingo hybrids should be adopted.

The existing set of 23 markers can distinguish between dingo and dog reference populations as the populations are divergent (Fig. 4; Table 2). Allele frequencies differ between dingoes and dogs, with some alleles being more commonly observed in dingoes versus dogs or vice versa (Online Resource 4). However, recent genetic studies have observed the presence of at least two divergent populations of dingo on mainland Australia (Cairns and Wilton 2016; Cairns et al. 2017, Cairns et al. 2018). Differences between genetic simulation methods along with the finding of geographic genetic subdivision in dingoes strongly demonstrate the need to incorporate a broad geographic reference population into existing (or future) DNA testing methods. It is important to point out that use of the USEPOPINFO flag in STRUCTURE modelling appears to decrease estimates of dingo ancestry, we hypothesise that running STRUCTURE without the USEPOPINFO flag confounds the analysis because of the high prevalence of domestic dog ancestry in the wild canid sample set and might inflate the dingo ancestry of samples (Figs. 5, 6 and Online Resource 7). We strongly suggest STRUCTURE analyses should be run with the USEPOPINFO flag on (Figs. 5, 6 and Online Resource 7). We observed departures from Hardy–Weinberg Equilibrium at three microsatellite

loci used in the dingo DNA test (Table 3), this violates an assumption of the STRUCTURE software (Pritchard et al. 2000; Falush et al. 2003). However, additional STRUCTURE analyses run with and without the loci deviating from Hardy–Weinberg Equilibrium produce similar ancestry estimates suggesting this has a limited impact on dingo ancestry estimates (Online Resource 5). Ancestry estimates reported by the average 3Q method and USEPOPFLAG STRUCTURE modelling provide a conservative estimate of the dingo ancestry of wild NSW canids.

Our data highlight some important limitations of the current dingo DNA testing methodology: (1) it relies upon a pre-defined reference population; (2) it is based on a limited number of genetic markers and; (3) estimates should be carefully assessed by end users. It is important for end-users of the dingo DNA testing to understand that the methods provide broad estimates such as only having dingo ancestry, likely to only have dingo ancestry, likely to have both dingo and dog ancestry or only dog ancestry (Wilton 2001; Cairns et al. 2011; Stephens et al. 2015). Estimates of dingo ancestry might be biased by missing data, so care should be taken in interpreting results without a full complement (23) of markers. We caution that dingo DNA testing results are estimates of introgression by domestic dogs and the reliability of individual test results should be assessed by considering the reference population used and number of markers tested. The 3Q method provides information about the proportion of alleles that are "doglike" or rarely observed in the reference populations which might be helpful for interpreting results. Land managers and end users should aim to maintain consistent methodology (i.e. 3Q or Bayesian method) across regional areas until an improved method is available and ensure that a geographically appropriate reference population is used.

Improvements in analytical algorithms now allow estimation of introgression history without pre-defining a reference population, even if there are no 100% pure specimens (Maples et al. 2013). However, the type (microsatellite) and limited sample size (23) of markers used in the current dingo DNA test (Wilton et al. 1999; Wilton 2001) inhibits the application of these new algorithms to the current dingo ancestry estimation methods. The limited number and type of genetic markers also limits the sensitivity and accuracy of the testing, specifically estimates based on 23 microsatellites may not reflect genome-wide ancestry, as these 23 markers do not provide coverage across the entire genome. Furthermore, current dingo DNA testing methods are unlikely to identify hybridisation events more than 4 generations ago (Cairns et al. 2011). It is imperative that new DNA testing methods be developed to address these limitations, we suggest harnessing high-throughput SNP genotyping or sequencing methods to capture high-throughput genome wide markers in a cost-effective manner. For example, SNP genotyping has been adopted to more reliably identify hybridisation in European wildcats, SNP data also provides utility to study timing of admixture events, backcrossing and inheritance of domestic vs wild alleles in hybrids (Oliveira et al. 2015; Steyer et al. 2018; Mattucci et al. 2019). Future research should also focus upon identifying geographic conservation hotspots and understanding the process of hybridisation; which may help inform strategies to limit future hybridisation.

Although our results are optimistic in the sense that they show that individuals which only had dingo ancestry persist in eastern NSW and the frequency of domestic dogs was low, it is notable that between 76 and 88% of sampled wild canids had some level of domestic dog ancestry i.e. scores 3–5 (Figs. 2, 3, 4, 5, and 6). This finding confirms those of previous studies that suggest that the south-eastern dingo gene pool has experienced widespread introgression from domestic dogs (Corbett 2001b; Stephens et al. 2015). The geospatial analyses identified geographic hotspots of high dingo genetic integrity within our dataset (Fig. 7). In these regions, there was a higher than expected occurrence of dingoes which only had dingo ancestry or were likely to only have dingo ancestry (scores 1–2). We contend that dingo populations in these regions have conservation value because of their high genetic integrity and this justifies special consideration when developing management plans for wild canids in these areas. In canids, hybridisation driven by human actions is a particularly pressing issue given the genetic compatibility between wild canid species, ongoing lethal control and the wide distribution of domestic dogs across the globe (Adams et al. 2003; Hindrikson et al. 2012; vonHoldt et al. 2013; Bohling and Waits 2015; Galov et al. 2015; Stephens et al. 2015; vonHoldt et al. 2016; Hinton et al. 2018). Our findings highlight a strong need for management strategies to restrict human-driven hybridisation by limiting opportunities for wild-domesticate hybridisation and identifying and maintaining natural barriers to reproduction. Ongoing monitoring of changes in dingo ancestry patterns would be an important component of future dingo management in these areas.

The low prevalence of feral dogs, existence of geographic hotspots of animals that only had dingo ancestry and the generally high rate of introgression raises questions about the history and mechanisms driving genetic introgression between domestic dogs and dingoes. These patterns could be due to three broad processes: (1) ongoing interbreeding between feral domestic dogs and dingoes, (2) occasional mating between domestic dogs and dingoes and/or (3) an artefact of historical hybridisation followed by bottlenecking of dingo populations. These patterns could reflect existing lethal control management programs in south-eastern Australia that might exacerbate hybridisation due to breakdown of social structure and lower abundance of preferred mates (Wallach et al. 2009) and

a long history of sympatry with domestic dogs (Corbett 2001b). Indeed, in North America hybridisation between wild canids (wolves, red wolves and coyotes) is facilitated by human caused mortality (Benson and Patterson 2013; Bohling and Waits 2015; Hinton et al. 2018). We recommend that future research investigate the factors driving spatial variation in dog introgression in dingo populations. We hypothesise that human density and agricultural land use (livestock versus cropping) across time are the factors contributing to patterns of high dingo genetic integrity in regions of NSW. Such information is essential to prevent further dog introgression and assist the conservation of the dingo in NSW.

Traditionally hybridisation is viewed as a process threatening wildlife populations (Rhymer and Simberloff 1996), however, it also facilitates species adaptation to changing environments (Hoffmann and Sgro 2011; Cahill et al. 2015; Schweizer et al. 2018; vonHoldt et al. 2018; Chan et al. 2019). It is possible that there are DNA regions undergoing natural selection in dingoes and dingo hybrids, and that some genomic regions introgressed from domestic dogs are selectively advantageous in dingoes, as in North American wolves (Schweizer et al. 2018). Future research into the genetic identity of dingo hybrids develop our understanding of species adaption, plausibly introgressed DNA from domestic dogs may assist dingoes in persisting in urban or disturbed environments.

Moving forward, conservation and management plans need to encompass evidence-based strategies to reduce human driven hybridisation by identifying and maintaining natural barriers to reproduction or limiting opportunities for wild-domesticate hybridisation. The full conservation and evolutionary implications of hybridisation are poorly understood. We would caution that legislation and policies based on strict genetic definitions of speciation may be counter-productive to the persistence of endangered or ecologically significant taxa (Stronen and Paquet 2013; Fitzatrick et al. 2015; Bohling 2016; van Wyk et al. 2017; vonHoldt et al. 2018; Chan et al. 2019).

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Compliance with ethical standards

Conflict of interest KMC is a scientific advisor to the Australian Dingo Foundation, New Guinea Highland Wild Dog Foundation and New Guinea Signing Dog Conservation Society. No other interests declared.

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