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Active shedding of *Neospora caninum* detected in Australian wild canids in a nonexperimental context

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Abstract

Infection with *Neospora caninum* parasites is a leading cause of reproduction losses in cattle worldwide. In Australia, this loss is estimated to total AU$110 million every year. However, despite this considerable economic impact, the transmission cycle and the host(s) responsible for the sylvatic transmission of the parasite remain to be defined. Dingoes (*Canis familiaris*) have been suggested to be a wildlife host of *N. caninum* in Australia, but this is yet to be proven in a nonexperimental setting. This study aimed to determine the prevalence of natural *N. caninum* shedding in Australian wild dogs (defined as dingoes, dingo-domestic dog hybrids and feral dogs) by performing molecular analysis of faecal samples collected in wild dog populations in south-east Australia. Molecular analysis allowed host species identification and dingo purity testing, while genetic analysis of *Coccidia* and *Neospora* conserved genes allowed for parasite identification. Among the 115 samples collected and determined to belong to dingoes, dingo-domestic dog hybrids and foxes, *Coccidian* parasites were detected in 41 samples and *N. caninum* was identified in one sample of canine origin from South East Australia (Mansfield). Across all samples collected in Mansfield only 15 individuals were successfully identified by genotype. Thereby our study determined that 6.7% (1/15, 95% confidence intervals 1.2–29.9) of wild dogs were actively shedding *N. caninum* oocysts at this site. Further, only four individuals were identified at a second site (Swift Creek), and none were positive. This study conclusively confirms the role of wild dogs in the horizontal transmission of *N. caninum* parasites in Australia.

KEYWORDS
18S rRNA gene, dingo, intestinal parasites, ITS1 gene, Nc5 gene, *Neospora caninum*, zoonosis

1 | INTRODUCTION

Distributed widely across Australia, the dingo (*Canis familiaris*) is a generalist and opportunistic carnivore, and the country's top order predator. Prior to European settlements, interactions between dingoes and Aboriginal people were common as dingoes provided communities with protection, companionship and hunting assistance (Balme & O'Connor, 2016). In contrast, the relationship between dingoes and humans changed drastically with European settlement and the implementation of agriculture (Rogers & Kaplan, 2003). Clearing of lands

and farming practices led to dingo attacks on the newly introduced livestock, and arguably the start of the friction between pastoralists and dingoes (Coman, 1972; Harriott et al., 2019; Hytten, 2009; Jones, 2009; Sloan et al., 2017).

Following European settlement in Australia, previously untouched land was encroached upon by human populations, increasing the probability of interactions between dingoes and humans. Consequently, interactions between dingoes and domestic dogs also increased resulting in widespread interbreeding, which ultimately led to a reduction in the number of genetically pure dingoes in the wild (Stephens et al., 2015). As a result, most contemporary Australian wild dogs are dingo-domestic dog hybrids (Claridge et al., 2014), although this may depend on the area inhabited (Cairns et al., 2020; Stephens et al., 2015). With increasing urbanisation and land use, the proximity and interactions with such populations of dingoes and their hybrid counterparts (henceforth collectively referred to as wild dogs) becomes more frequent, so too does their potential role in the transmission of pathogens of agricultural significance, and possibly of public health relevance (Smout et al., 2018). Therefore, understanding the transmission pathways of canid pathogens around human and agricultural settings, is crucial to their management and control in wild and domestic canid populations.

Numerous endoparasites have been reported in the literature to infect wild dogs in Australia (Behrendorff et al., 2016; Coman, 1972; Dunsmore & Spratt, 1979; Durie & Riek, 1952; Jenkins et al., 2008; Jenkins et al., 2014; Ng et al., 2011; Smout et al., 2013; Smout et al., 2018), implicating wild dogs as key players in the transmission cycle of endoparasitic diseases. Wild dogs share land and resources with other sympatric species, providing a possible route of transmission for a sylvatic cycle between wild dogs, and wild and domestic herbivores (King et al., 2011; Smout et al., 2018). In Australia, this predator and prey interaction has been suggested to be involved in the transmission of *Neospora caninum*, a *Coccidian* parasite of agricultural significance (King et al., 2011). *N. caninum* infection is the leading cause of spontaneous abortions in cattle worldwide (Reichel, 2000). Estimated to costs the Australian livestock industry an estimated AU$110 million each year, neosporosis has a substantial economic and social impact on the beef and dairy industries (Dubey, 2003; King et al., 2011; Reichel, 2000).

In North America, the *N. caninum* lifecycle involves a sylvatic and a domestic component of transmission. The sylvatic cycle is maintained by coyotes and wolves consuming infected herbivores such as deer and cattle, while the domestic cycle in maintained through the consumption of infected tissue from hunted wild or domesticated animals (Rosypal & Lindsay, 2005). In Australia, it has been postulated that a parallel lifecycle occurs between wild dogs and macropods (King et al., 2011); however, there is a significant lack of information regarding this specific route of transmission of *N. caninum*. Neosporosis infection in the intermediate host (livestock) can occur through vertical or horizontal transmission. Vertical transmission is thought to account for the majority of infections in Australia, however, recent studies have postulated that vertical transmission alone is insufficient to maintain the observed infection levels in cattle (Donahoe et al., 2015; Reichel, 2000). This therefore implies that horizontal transmission must play a role in the infection cycle of *N. caninum* in Australia.

To address this knowledge gap, recent studies sought evidence of *Neospora* infection in Australian wild dogs using serological and/or molecular diagnostics to document exposure to the pathogen (Barber et al., 1997; King et al., 2012). However, these studies were limited by their ability to demonstrate the source of infection. Indeed, with no evidence of active infection they only indicated exposure to the parasite, thus unable to confirm the prevalence of viable natural infections in wild dog populations (Donahoe et al., 2015). Additionally, Sloan et al. (2017), showed a sixfold increase in seropositive domestic dogs in Victoria, Australia, since a previous study conducted in 1997 (Barber et al., 1997). The reason(s) for this significant increase in domestic dog infections remains to be elucidated, and to do so requires exploring a correlation to a rise in parasitism of wild dogs.

Prior to 2010, domestic dogs were the only confirmed definitive host of *N. caninum* in Australia (King et al., 2010). Even so, evidence implicating domestic dogs' involvement in transmission of *N. caninum* was inconsistent, mostly due to the lack of evidence for the temporal association in the seroconversion of domestic dogs with abortion storms in cattle. In fact, serologically negative dogs on infected cattle farms have been a common occurrence (Barber et al., 1997; King et al., 2010; Reichel, 2000). Nevertheless, it is important to note that the results of serological studies do not discriminate between past and current infections of *N. caninum*, and the sensitivity and specificity of some of the assays used are contentious (Donahoe et al., 2015).

In 2010, King et al. experimentally demonstrated that dingoes are capable of shedding infective *N. caninum* oocysts after ingesting infective bovine tissue. This study suggested that wild dogs could be the missing link between the infection of livestock and wild intermediate hosts. However, there is a shortage of evidence supporting the natural routes of transmission in wild dogs and wild intermediate hosts in Australia. To address this important outstanding question, the present study aimed to determine the prevalence of *N. caninum* shedding in Australian wild dogs by performing molecular analysis of faecal samples.

2 | METHODS

2.1 | Sample collection

Faecal samples from presumed wild dogs were collected in November 2018 using scat detection dogs, targeting rural areas in Victoria, Australia. Collection was focussed on sites where wild dog livestock attacks were reported to be high in 2016–2018 (Pacioni, unpublished), under the assumption that wild dog density in these areas would still be high. In total, 82 samples were collected from the Mansfield region and 33 samples collected around Swifts Creek (Figure 1). Samples were frozen 24–48 hours after collection and stored at −20 °C until required.

FIGURE 1 Geographic location of scats sample collection. A total of 115 faecal samples of presumed wild dogs were collected in rural Victoria, Australia in November 2018. 82 samples were collected in Mansfield and 33 samples in Swifts Creek

2.2 | Genomic DNA extraction

Genomic DNA extractions were performed on all samples using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, 0.25 g of faecal material was mixed in a 2 mL PowerBead Tube and vortexed at maximum speed for 10 minutes to allow mechanical and chemical cell lysis. Total genomic DNA was captured on a silica membrane using a spin column, eluted in 100 μL of elution buffer and stored at −20 °C.

2.3 | Host DNA identification and dingo purity analysis

Host molecular identification was carried out using a separate aliquot from each sample using the QIAamp Fast DNA Stool mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Briefly, epithelial cells were removed by washing the samples with the InhibitEX buffer and, following genomic DNA extraction, all samples were eluted with 60 μL of elution buffer.

Quantitative PCR was performed with primers specific for red fox (*Vulpes vulpes*), cat (*Felis catus*), and dog (*Canis familiaris*) DNA (Supplementary Table S1), and melt curve analysis was carried out as described by Berry and Sarre (2007). We conservatively used a cycle threshold (CT) of less than 25 (Supplementary Table S2), to select samples confirmed to be dogs as candidate for attempting genotyping using 23 microsatellite markers as described in Stephens et al. (2015). This was done because it was demonstrated that success rate in scat samples is dramatically reduced when the CT > 20 (Stephens, 2011; von Thaden et al., 2017).

PCR analysis was conducted in triplicate for each sample, and each allele was confirmed when observed at least twice for heterozygotes and three times for homozygotes. If more than two alleles were observed at one locus across the three PCR replicates, then the locus was coded as missing data.

Genotypes with 14 or more loci successfully amplified were tested for the percentage of dingo ancestry present (purity analysis). Each locus was compared to dingo and dog reference populations using Structure v2.3.3 (Pritchard et al., 2000), as described in Stephens et al. (2015). Dingo ancestry tests were run 10 times for 350,000 iterations with 50,000 burn-in runs using the admixture and correlated allele frequency models (Falush et al., 2003). Structure was run setting the population prior (USEPOPINFO = 1) for the reference specimens only and initialising and updating allele frequencies from these references. Cluster number was set to $k = 2$ in order to assign the test specimens to either the 'dingo' or 'domestic dog' population. The results of 10 structure analyses were then averaged using CLUMPP (Jakobsson & Rosenberg, 2007) to obtain the approximate percentage of dingo DNA present in each sample.

2.4 | PCR amplification of *Coccidia spp* and *Neospora caninum* DNA

Neospora parasites belong to the genus *Coccidia* for which a variety of generic primers targeting the conserved region of 18S ribosomal RNA (18S rRNA) gene have been previously validated and published. To narrow down the identification of *Neospora* parasites among our 115 scat samples we decided to screen for the presence of *Coccidia* DNA by PCR using the 18S rRNA conserved primers cocc18SF/cocc18SR (Martin et al., 2016) or coc1/coc2 (Ho et al., 1997), resulting in a ~400 bp or ~300 bp amplicon, respectively (Figure 3a). Subsequently, a *Neospora* specific screening was conducted with primers targeting a 350 bp fragment of the Nc5 gene with Np21+/Np6+ (Yamage et al., 1996), or a 250 bp fragment of the *N. caninum* ITS1 gene with NN1/NN2 and NP1/NP2 primers (Buxton et al., 1998) (Figure 3a). Genomic DNA of *N. caninum*, *Toxoplasma gondii* and *Sarcocystis gigantea* extracted from parasitic in vitro cultures were used as the positive control for the PCR reactions. The nontemplate control consisted of the PCR mix with nuclease-free water added instead of genomic DNA.

All PCR reactions were performed in a final volume of 25 μL containing 1× Green GoTaq Flexi buffer, 2 mM MgCl2, 10 mM dNTPs, 0.2 μM of both forward and reverse primers, 0.625 units of GoTaq G2 DNA polymerase (Promega, Madison, WI, USA) and 1 μL of total genomic DNA template. DNA amplification with the generic *Coccidia* primers comprised one step of initial denaturation (95 °C for 2 minutes), followed by 35 cycles of denaturation (95 °C for 45 seconds), primer annealing (57 °C for 45 seconds) and extension (72 °C for 45 seconds). The final extension was performed with one step at 72 °C for 5 minutes. The PCR program with the *N. caninum* specific primers consisted of an initial denaturation step (95 °C for 2 minutes), followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds with a final extension step of 5 minutes at 72 °C. DNA amplification was carried out in a thermocycler T100 thermal

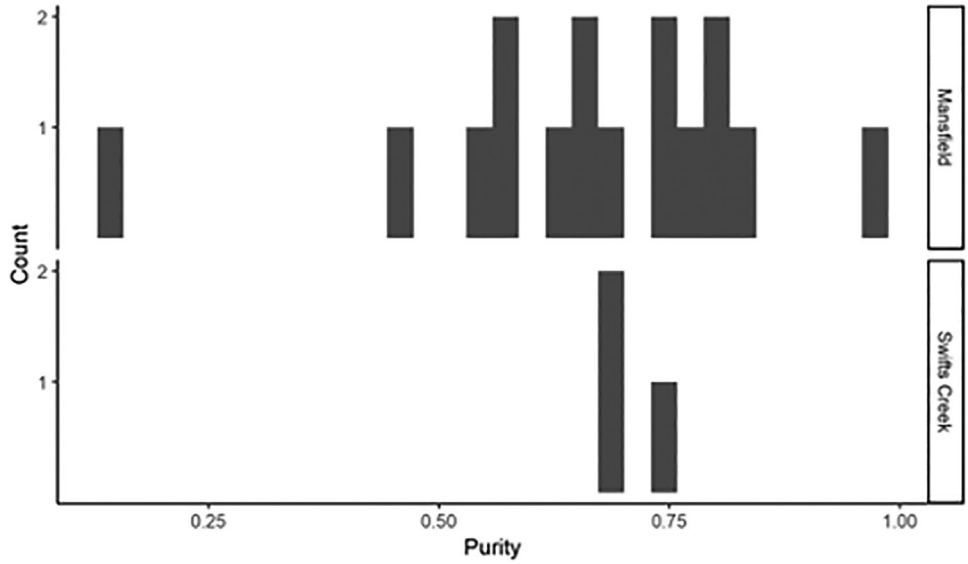


FIGURE 2 Dingo purity analysis. Host species identification was carried out by qPCR analysis on total genomic DNA extracted from each faecal sample. A total of 60 samples were identified as dog and 28 of those were subject to genotype analysis. The dingo ancestry proportion (*x*-axis) for 19 scat samples and the count for each proportion (*y*-axis) is indicated for the two locations of sample collection, Mansfield and Swift creek. 18 samples were identified as dingo-dog hybrids and one sample was classified as pure dingo (> 90% purity)

cycler (BioRad, Hercules, CA, USA). PCR products were visualised by gel electrophoresis on a 2% agarose gel using the high-resolution imaging system ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

Amplicons of the expected size were sequenced by Sanger sequencing at the Australian Genome Research Facility, Melbourne, Australia. DNA sequences were analysed using Geneious software 11.1.4 and compared with reference sequences available in GenBank using BLASTn.

The 95% confidence interval (CI) for the overall prevalence values were calculated using Wilson score interval with the R package binom or Epitools (www.epitools.ausvet.com.au).

2.5 | Phylogenetic analysis

Multiple sequence alignments were performed using Clustal X (Thompson et al., 1997). Pairwise comparisons and phylogenetic tree construction were performed using MEGA 7.0 software (Kumar et al., 2016). Trees were constructed using the neighbour-joining method and distances calculated using Kimura 2-parameter model with bootstrap analysis using 1000 replicates.

Bayesian phylogenetic analyses were carried out in BEAST 2 (Bouckaert et al., 2014) implementing the Yule speciation process as tree prior, assuming a strict clock and conducting 10 million MCMC iterations. bModelTest package (Bouckaert & Drummond, 2017) was used to carry out substitution model average. Tracer (Rambaut et al., 2018) was used to confirm adequate length of the MCMC, enough effective sampling size (ESS) and to verify that 10% 'burn-in' was enough.

3 | RESULTS

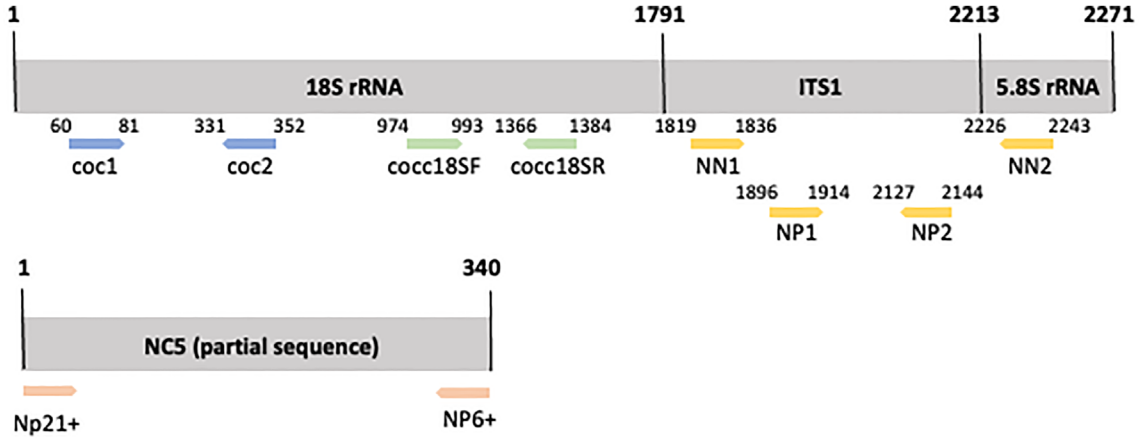
3.1 | Host identification and dingo purity analysis

A total of 115 faecal samples of presumed wild dogs were collected in two different locations in rural Victoria (Figure 1). Following qPCR analysis, we successfully identified the host species in 96% of the samples: a total of 110 scat samples were identified as either fox ($n = 50$) or dog ($n = 60$). None of the samples that generated a product for dogs generated a product for foxes nor vice versa. Interestingly, six samples identified as dog, also produced an amplicon with cat primers. Among the 60 dog samples, genotype analysis was conducted for 28 samples (i.e. those with a CT value > 25 as detailed in the Methods section) corresponding to 19 different individuals. Dingo purity analysis for 19 samples (that included 14 of the 19 identified dogs) was carried out and only one sample was classified as pure dingo (> 90% purity). The other 18 samples were identified as dingo-dog hybrids (Figure 2).

3.2 | Detection of *Coccidia* positive samples by PCR

Generic 18S rRNA primers (cocc18F/cocc18SR) were used to screen for the presence of *Coccidian* parasites. From the 115 scats tested, 41 samples resulted PCR positive (35.7%, 95% CI 27.49–44.74). Among the *Coccidia* positive samples, 51.2% ($n = 21$) were identified as originating from dogs, 43.9% ($n = 18$) from foxes, and in 4.9% ($n = 2$) of the samples the host species was not successfully identified. In addition, 30 out the 41 *Coccidia* positive samples (73.2%) were collected in Mansfield and the remaining 11 samples (26.8%) were collected in Swifts Creek.

a.



b.

Sample ID	Sample origin	Host Species	Primers [target gene]: cocc18SF/cocc18SR [*Coccidia* 18S rRNA]	Np21/Np6 [*N. caninum* NC5]	NN1/NN2 then NP1/NP2 (Nested PCR) [*N. caninum* ITS1]	coc1/coc2 [*Coccidia* 18S rRNA]
WDJ25	Mansfield	Dog (hybrid)	✓	✓	-	-
WDN12	Mansfield	Dog (hybrid)	✓	✓	-	-
WDJ34	Mansfield	Dog	✓	✓	✓	✓
Positive Samples			41 (n=115)	3 (n=41)	1 (n=3)	1 (n=3)

FIGURE 3 PCR amplification of *Coccidia* and *Neospora* DNA. (a) Schematic representation of the 18S rRNA, ITS1, 5.8S rRNA and Nc5 locus with relative location of oligonucleotide primers used in this study. Approximate primer position is provided based on *Neospora caninum* Liverpool strain genome annotation (GenBank accession number U16159) and partial Nc5 (GenBank accession number KP715563). (b) Summary of the PCR screens performed with *Coccidian* and *N. caninum* primers

3.3 | Detection of *Neospora* positive samples by PCR

Following the identification of *Coccidia* DNA by PCR, a *N. caninum* targeted screen was performed on the 41 *Coccidia* positive samples by amplification of the Nc5 gene with primers Np21+/Np6+. Three samples (WDJ25, WDN12 and WDJ34) provided a PCR product, two of which (WDJ25 and WDN12) could not be reliably sequenced (Figure 3b). To further investigate the presence of *N. caninum* in these three samples, a nested PCR was conducted to amplify the *N. caninum* ITS1 gene using primers NN1/NN2 and NP1/NP2, resulting in a positive outcome for sample WDJ34 (Figure 3b). Further, samples WDJ25, WDN12 and WDJ34 were also analysed by PCR with primers coc1/coc2 and only WDJ34 resulted positive (Figure 3b).

For each of the four independent PCR screens performed, amplicons obtained were sequenced using the respective primers. BLAST analysis identified Nc5, ITS1 and 18S rRNA (obtained with coc1/coc2 primers) sequences of sample WDJ34 as belonging to *N. caninum* with nucleotide identities ranging between 97.9% and 100%. Moreover, 18S rRNA sequences obtained with primers cocc18SF/cocc18SR was identified as *Sarcocystis spp*, with nucleotide identities of 99.75% and 100%, respectively. Sequencing analysis of amplicons from samples WDJ25 and WDN12 produced either a DNA sequence of poor quality or a sequence unrelated to *N. caninum*. Therefore, samples WDJ25 and WDN12 were considered negative for *N. caninum* and excluded from further analysis. Importantly, sample WDJ34 consistently tested positive in the four PCR runs performed in this study, and Sanger sequence analysis further corroborated it as *N. caninum*. The WDJ34 sample belonged to a wild dog which was collected from the Mansfield region. Since 15 different wild dog individuals were identified among the samples collected in Mansfield, the prevalence of *N. caninum* at this site was 6.7%. When considering all uniquely identified wild dog individuals ($n = 19$) at both field locations of Mansfield and Swifts Creek, the prevalence was 5.3% (Table 1).

3.4 | Phylogenetic tree analysis confirms WDJ34 sequences cluster with *Neospora caninum*

The amplicon sequence obtained for the 18S rRNA gene (228 bp) and for the ITS1 gene (196 bp) of sample WDJ34, along with representative coccidian parasite sequences (Supplementary Table S3), were used to generate a neighbour-joining tree and a Bayesian phylogenetic tree (Figures 4 and 5) to ensure consistency of the results. Regardless of the

TABLE 1 Prevalence of Neospora caninum in scat samples analysed in this study. Data analysis based on total number of samples and individual wild dogs

	Samples			Individuals[†]		
	Positive/total	%	Confidence intervals[‡]	Positive/total	%	Confidence intervals[‡]
Wild dog						
Mansfield	1/45	2.2	0.4–11.6	1/15	6.7	1.2–29.9
Swift creek	0/15	0	0–20.4	0/4	0	0–49
Total	1/60	1.7	0.3–8.9	1/19	5.3	0.93–24.6
Fox						
Mansfield	0/34	0	0–10.2	N/A		
Swift creek	0/16	0	0–19.4	N/A		

[†]Identification of individual dogs are based on 14–23 locus microsatellite genotypes.
[‡]Confidence intervals are calculated with the R package binom using the Wilson method.

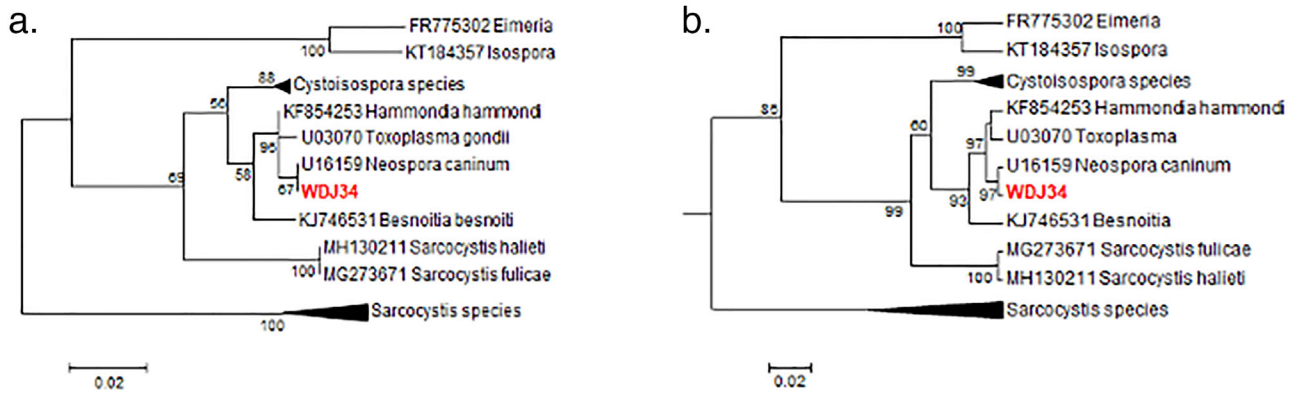


FIGURE 4 Evolutionary relationships of *Neospora* sequence inferred by 18S rRNA gene sequences. WDJ34 sequence amplified with coc1/coc2 primers. Trees were constructed using neighbour-joining (a) and Bayesian (b) analyses. Bootstrap values or posterior probabilities below 50% are not shown. The sample from this study (WDJ34) is highlighted in red

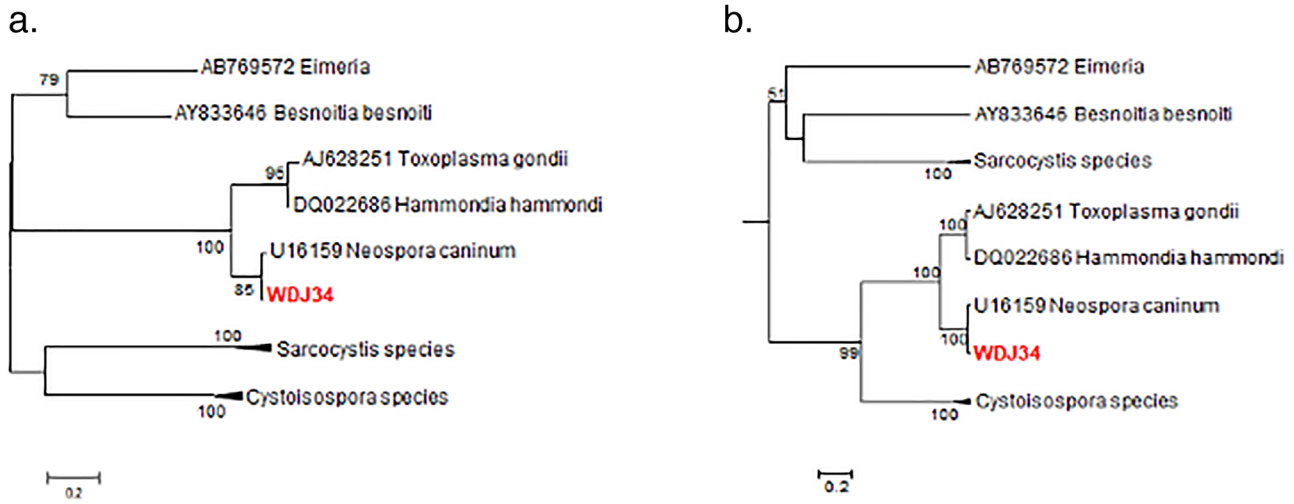


FIGURE 5 Evolutionary relationships of Neospora sequence inferred by using ITS1 gene sequences. WDJ34 sequence amplified with [NN1/NN2 and NP1/NP2] primers. Trees were constructed using neighbour-joining (a) and Bayesian (b) analyses. Bootstrap values or posterior probabilities below 50% are not shown. Sample from this study (WDJ34) is highlighted in red

gene sequence, both analyses confirmed that the WDJ34 sequences cluster with *N. caninum* prototype strain, accession number U16159, and this was supported by bootstrap values of 67% and 82% in the neighbour-joining trees, and by high posterior probability (97% and 100%) in the Bayesian trees providing high confidence in these results.

4 | DISCUSSION

The gastrointestinal parasite *Neospora caninum* negatively affects the health of livestock worldwide, and has a significant impact on the economics of the Australian cattle industry (Reichel, 2000). While vertical transmission is likely the main route for maintaining persistent infections (Anderson et al., 1997; Pare et al., 1996; Reichel, 2000), horizontal transmission may be pertinent to the costly localised outbreaks resulting in abortion storms (French et al., 1999; Reichel, 2000).

In Australia, there is little evidence of the natural routes of horizontal transmission, particularly in wild animals. While the sample size undertaken in this study was relatively small, hence the wide confidence intervals around the prevalence estimates (Table 1), from the individual wild dogs identified, we confirmed a 6.7% prevalence of oocyst shedding in the Mansfield population and an overall prevalence of 5.3% (Table 1). This study is the first to show evidence of *N. caninum* shedding in wild dogs in a nonexperimental context, conclusively confirming previous suggestions associating wild dogs in the horizontal transmission of *N. caninum* (King et al., 2011).

It is important to note that the prevalence of *N. caninum* infection is likely to be greater than that reported in this study as the probability of collecting a faecal sample while the host is shedding infective oocysts is low. While the rate of shedding infective oocysts in dingoes has yet to be determined, in domestic dogs, the shedding rate varies from a few days to several weeks (Cavalcante et al., 2011; Dubey et al., 2007), and is estimated to be similar in other canids (Gondim, 2006; Gondim et al., 2004; King et al., 2010). Moreover, previous studies found differences in the number of oocysts excreted in dogs and coyotes after experimental infection with 10,000 and 250 oocysts excreted per day, respectively (Gondim et al., 2002, 2004).

The failure to find high positivity in faecal samples may be influenced by infrequent oocyst excretion, considering that we collected a limited number of samples over a short time period (Wapenaar et al., 2006). It has been reported that dogs shed oocysts for 5 days or more after ingesting tissues of experimentally or naturally infected animals (Dubey et al., 2007). However, factors affecting oocyst shedding are largely unknown and difficult to investigate due to the low numbers and the erratic nature of oocyst shedding, as well as the high costs involved in housing dogs in a secure facility to allow such studies. Further, the source of infection and the dog's age are thought to influence oocyst shedding. Indeed, there is some evidence that dogs shed more oocysts after ingesting bovine tissues than when fed murine tissues, and pups shed more oocysts than adult dogs (Gondim et al., 2002).

In our study, sample WDJ34 tested positive for both *Neospora* and *Sarcocystis* DNA when analysed with *Coccidia* consensus primers. While coinfection with multiple *Coccidian* parasites is not uncommon (Moré et al., 2008, 2008; Thomasson et al., 2011), when DNA amplification via PCR with generic primers is used as the detection method, it poses the challenge of one parasite masking the presence of another within the same sample. This should be kept in mind especially when a high number of positive samples are detected with consensus primers. In such cases, a follow up with species-specific primers, as performed in this study with *N. caninum* specific primers, can improve sensitivity and specificity of parasite detection.

We recognise that studies on wild animals comprise many intrinsic limitations, including sampling regime, quality of the samples and animal diet. Scat samples analysed in this study were collected from the ground in rural areas of Victoria, Australia. Therefore, the environment and length of exposure to the elements is likely to vary between samples, and it was not possible to determine the history of each sample. Further, the ability of *Neospora* oocysts to survive in the environment is unknown. Although the stability of *Neospora* oocysts in soil is believed to be similar to that of the related *Toxoplasma* parasite (Dubey, 2004; Dubey et al., 2007; Gondim, 2006), which can remain viable in ideal conditions for up to 18 months (Shapiro et al., 2019), this is yet to be empirically determined for *N. caninum* oocysts.

We acknowledge a degree of uncertainty in the species identification undertaken in this study, as this was performed on DNA extracted from scat samples. Wild dogs are known scavengers, therefore the fox DNA identified in the study could originate from dietary sources, rather than host species (and vice versa). Although, traces of hair and bones were detected in some of the samples, we argue that dietary origin of DNA is highly unlikely for all the 50 fox samples identified. Furthermore, the number of wild dogs identified through scat analysis is consistent with those detected by camera traps in the same area, in the same period (CP unpublished data). While the red fox can be seropositive for *N. caninum* and oocysts have been isolated from the central nervous system, no study to date has been able to implicate the fox as a definitive host of this parasite (Almeria et al., 2002; Schares et al., 2002). In the present study, the total number of foxes identified was similar to that of wild dogs ($n = 50$, 43% of the total samples collected). Given the relatively small sample size, and despite the fact that no fox sample tested positive for *N. caninum*, our study does not allow for a definitive conclusion on the role of foxes in the transmission cycle of *N. caninum*.

Considering the limitations discussed above, we conclude that the prevalence of *N. caninum* infection found in this study is likely to be an underestimation of the true shedding rates of *Neospora* parasites. Importantly, our study represents the first categorical demonstration of *N. caninum* parasite shedding in wild dogs in a natural context. Further research is currently ongoing to determine the role of wild dogs in the potential transmission of *N. caninum* to livestock, and the correlation between parasitism in domestic and wild dogs. Research aimed at demonstrating the epidemiological link, or lack thereof, will be instrumental in mitigating the devastating impact that *Neospora* has on the cattle industries in Australia. As the direct route of *Neospora* transmission from dingoes to livestock is currently based around assumptions, with no conclusive study yet performed, a critical next step in understanding the *N. caninum* transmission cycle is to identify the source of

new infections in livestock, and the potential role of dingoes' preys, such as macropods.

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted in the journal's author guidelines page, have been adhered to. No ethical approval was required as this study did not involve the use of animals.

CONFLICT OF INTEREST

The authors confirm that they have no monetary, personal or other conflict of interest in this study.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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