Species-level identification of trypanosomes infecting Australian wildlife by High-Resolution Melting - Real Time Quantitative Polymerase Chain Reaction (HRM-qPCR)

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ABSTRACT

Conventional nested PCR and Sanger sequencing methods are currently the gold standards for detecting trypanosomes in wildlife. However, these techniques are time-consuming and can often overlook mixed infections. True trypanosome prevalence can thus be underrepresented. Here, we designed an 18S rDNA-based real-time quantitative PCR (qPCR) assay coupled with High-Resolution Melting Analysis (HRMA) to detect and discriminate three *Trypanosoma* species (*T. copemani, T. noyesi,* and *T. vegrandis*) commonly infecting Australian marsupials. A total of 68 genetically characterised samples from blood and tissue were used to validate the High-Resolution Melting - Real Time Quantitative Polymerase Chain Reaction (HRM-qPCR) assay. A further 87 marsupial samples consisting of blood, tissue and *in vitro* cultures derived from wildlife blood samples, were screened for the first time using this assay, and species identity confirmed using conventional PCR and Sanger sequencing. All three *Trypanosoma* species were successfully detected in pure cultures using the HRM-qPCR assay, and in samples containing mixed trypanosome infections. Of the 87 marsupial samples screened using the HRM-qPCR assay, 93.1% were positive for trypanosomes, and 8.0% contained more than one trypanosome species. In addition to the three targeted *Trypanosoma* species, this assay was also able to detect and identify other native and exotic trypanosomes. The turnaround time for this assay, from sample preparation to obtaining results, was less than 2 h, with a detection limit of 10 copies of the amplicon in a reaction for each of the targeted trypanosome species. This more rapid and sensitive diagnostic tool provides a high throughput platform for the detection, identification and quantification of trypanosome infections. It will also improve understanding of host diversity and parasite relationships and facilitate conservation management decisions.

1. Introduction

Trypanosomes are flagellate protozoan parasites responsible for trypanosomiasis, a potentially fatal disease of humans and animals worldwide (Hoare, 1972). These unicellular haemoparasites inhabit the blood, and in some cases, tissues of vertebrate hosts and are usually transmitted through arthropod or leech vectors (Hoare, 1972; Stevens et al., 2001; d'Avila-Levy et al., 2015; Maslov et al., 2019). Trypanosome infections in wildlife were often underreported due to opportunistic sampling or subclinical infections. However, increased surveillance and improved sensitivity of detection methods have overcome this problem, revealing a high prevalence and diversity of trypanosomes in Australian wildlife (Averis et al., 2006; Botero et al., 2013; Northover et al., 2019).

The first report of native trypanosome species in Australian wildlife was published in the 1950s (Mackerras, 1959). Decades later, ten species of native trypanosomes infecting Australian wildlife have been taxonomically described (Thompson et al., 2014; Botero et al., 2016).

The unravelling of evolutionary host-parasite relationships using molecular platforms has inadvertently revealed the complexities of Australian trypanosome diversity. The number of formally described indigenous trypanosomes has now been superseded by classifications based on genomic sequences. There is still considerable genetic diversity yet to be explored, but these will need to be supported by morphological and biological data (Votypka et al., 2015; Cooper et al., 2018). Native trypanosome parasites of Australian wildlife have a high level of intra-specific variation with many species comprising more than one genotype

([Noyes et al., 1999](); [Paparini et al., 2011](); [Botero et al., 2013](), [2018]()). It is also common for multiple trypanosome species to infect a host concurrently (polyparasitism or mixed infections) ([Paparini et al., 2011](); [Thompson et al., 2013](); [Botero et al., 2013](); [Cooper et al., 2018]()). Although indigenous trypanosomes have been considered non-pathogenic in Australian wildlife, growing evidence suggests that these parasites may adversely affect host health ([Pickering and Norris, 1996](); [MacPhee and Greenwood, 2013](); [Botero et al., 2013](); [Austen et al., 2015]()). For example, recent research has demonstrated that some species of marsupial trypanosomes are potentially more pathogenic than others and that mixed infections may exacerbate the impact on the health of hosts ([Botero et al., 2013](); [Godfrey et al., 2018]()).

Nucleic acid-based amplification techniques such as nested PCR and Sanger sequencing remain the most widely used tools for screening for trypanosomes present in blood and tissue of wildlife ([d'Avila-Levy et al., 2015]()). Generic amplification of trypanosome DNA using nested PCR is a widely used tool; however, it requires Sanger sequencing to resolve species identity. Species-specific primers are capable of distinguishing between species when insertions and deletions (INDELS) of genomic segments are targeted, but they may not always discriminate between genotypes of the same species without the aid of Sanger sequencing ([Botero et al., 2013](); [Votypka et al., 2015](); [Cooper et al., 2018](); [Maslov et al., 2019]()). Such targeted approaches may also fail to identify *Trypanosoma* species outside of the primer range. Furthermore, low stringency PCRs can overlook samples with mixed infections leading to a misrepresentation of one species and thus misinterpretation of true infections. A very efficient assay for detecting these parasites should not only target and discriminate more species, but should also significantly reduce handling thereby reducing the chances of laboratory contamination, and should require less processing times by eliminating some post-PCR procedures such as gel electrophoresis and Sanger sequencing.

Real-time quantitative Polymerase Chain Reaction paired with High-Resolution Melting Analysis (HRM-qPCR) is a highly sensitive tool for DNA detection, quantification and genotyping. High-Resolution Melting Analysis employs a saturated dye (e.g. SYTO 9) which allows for detection of specific amplicons and the generation of a melt curve with data collected over a period with small increments in temperature. A single base-pair change can shift the melting temperature (Tm). The Tm is defined as the point at which 50% of the DNA is double-stranded, and 50% is single-stranded (Applied Biosystems, California, USA, 2009). Methylation analysis, mutation scanning, and genotyping can all be resolved using this method ([Garritano et al., 2009]()). The technique can be an invaluable resource in the parasitology field and has been successfully used to discriminate between different species of parasites based on the GC contents and sequence lengths or strand complementary ([Limor et al., 2002](); [Kirkpatrick et al., 2009](); [Lalonde and Gajadhar, 2011](); [Rahimi et al., 2019]()). However, no such method is currently available for the efficient detection of trypanosomes in Australian wildlife.

The aim of this study was, therefore, to develop a HRM-qPCR assay capable of detecting, identifying and quantifying several species of *Trypanosoma*. The assay was validated with three *Trypanosoma* species (*T. noyesi*, *T. vegrandis*, and *T. copemani*) commonly infecting several species of Australian marsupials.

2. Materials and methods

2.1. Samples

Blood samples were collected from woylies, brushtail possums and chuditch between 2013 and 2016 at various locations within Southwestern Australia (Upper Warren Region and Dryandra), in collaboration with the Western Australian Department of Biodiversity, Conservation and Attractions (DBCA) under Murdoch University Permit Numbers WC2172-08, WC2350-10 and RW2659/14 and DBCA permit numbers DECAEC/52/2009 and NS1182-06. The animals were captured

in Sheffield cages (Sheffield Wire Products, Western Australia, Australia) with DBCA approved bait, and were restrained by experienced handlers. A maximum of 1 ml of blood was drawn from the lateral caudal vein (conscious) by approved Murdoch University personnel and stored in EDTA tubes (Greiner Bio-One, Germany) at −20 °C until further processing. The animals were then released at the point of capture no later than 3 h after sunrise. Tissues were also harvested from five frozen woylie carcasses (euthanased animals or roadkill) donated by DBCA and stored in 70% ethanol until DNA extraction. The blood of fourteen woylies was collected and cultured *in vitro* to isolate trypanosome species. The cultured trypanosomes were incubated at 37 °C with no CO_2 and in two culture media: RPMI with Fetal Calf Serum (FCS) and RPMI without FCS (Thermo Fisher Scientific, Victoria, Australia). Live cultures of *Trypanosoma cruzi*, *Trypanosoma rangeli* and *Trypanosoma microti* were purchased from the American Type Culture Collection and were used to assess if the HRM-qPCR assay could detect and identify exotic trypanosomes.

Genetically characterised DNA from blood and tissues of sixty-eight marsupials were used as templates to validate the HRM-qPCR assay. Once validated, the assay was used to screen for the presence of different species/genotypes of trypanosomes in a further 87 woylie samples consisting of blood, tissues, and *in vitro* cultures of Australian trypanosomes.

2.2. DNA extraction

In accordance with the manufacturer's protocol, DNA was extracted from blood and *in vitro* cultures (200 μl) of Australian trypanosomes and live cultures from the American Type Culture Collection, and tissues (25 mg) using the QIAamp 96 DNA Blood and Tissue Kit (Qiagen, Hilden, Germany), and eluted in 60 μl of the supplied AE buffer. Previously screened wildlife samples known to be free of trypanosome infection were used as negative controls in the DNA extraction process and the PCR assays to rule out cross-contamination and non-specific amplification.

2.3. Primer design

2.3.1. A pair of HRM-qPCR primers (TrypF: AGCCTGAGAAATAGCTACCAC and

TrypR: CGAACCCTTTAACAGCAACA) were designed in this study to amplify a region of 246 bp from the 18S rDNA gene of trypanosomes. The primers were designed to bind to conserved sites but flanked regions with genetic variability among the different trypanosome species. To identify conserved binding sites for the primers, an alignment was generated using published sequences from eight *Trypanosoma* species (using the MUSCLE alignment plugin in Geneious ([Edgar, 2004]()) and the expected sequences of the amplicons re-aligned using Bioedit ([Hall, 1999]()) to indicate nucleotide diversity in DNA of the species ([Fig. 1]()). All sequences were retrieved from the databases of the National Centre for Biotechnology Information (NCBI). Australian trypanosome sequences used in the design included, but were not limited to, genotypes belonging to *T. copemani*, *T. noyesi* and *T. vegrandis*.

2.4. HRM-qPCR conditions

All HRM-qPCR assays were carried out in a RotorGene Q machine (Qiagen, Hilden, Germany) in a 10 μl reaction volume consisting of 1 μl of a specific concentration of DNA template, 0.2 μM of each primer, 5 μl MeltDoctor™ HRM master mix containing SYTO 9 dye (Applied Biosystems, California, USA) and 3.6 μl sterile, deionized water. The thermal profile for each qPCR consisted of a pre-PCR step of 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 15 s and 30 s at the annealing temperature of 60 °C. Immediately following qPCR, HRM was carried out to identify the species using the melt curve analysis program. The HRM conditions were as follows: an increase in temperature from
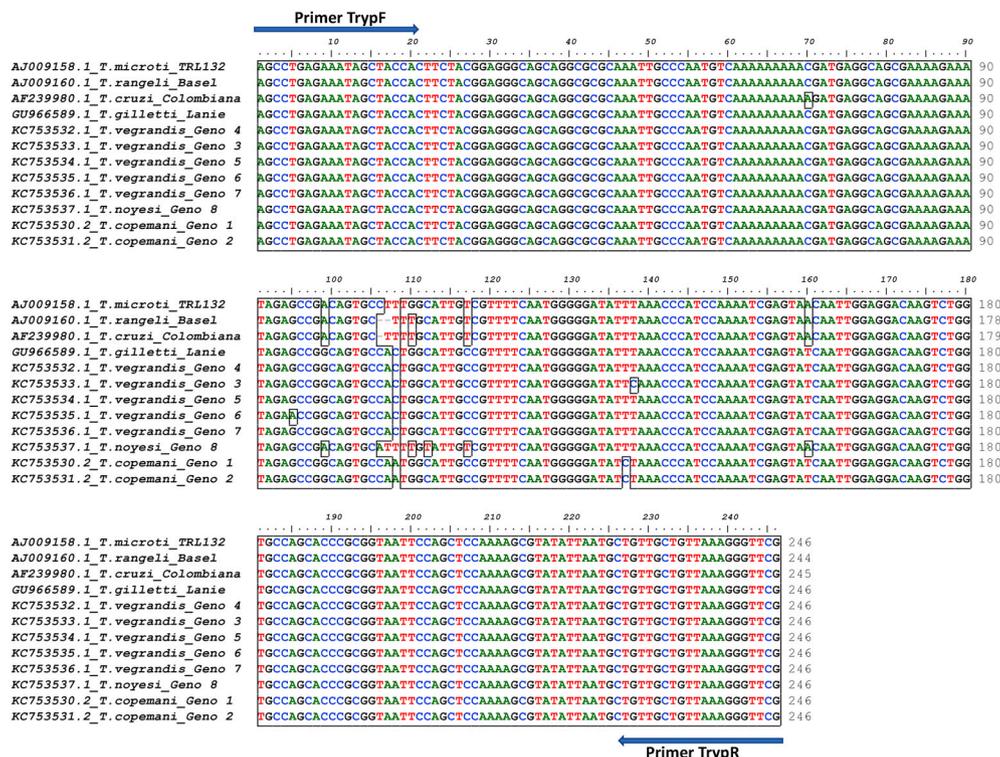
Fig. 1. Multiple sequence alignment of a portion of the 18S rDNA of seven Trypanosome species and subsequent genotypes used to design the HRM-qPCR assays.

60°C to 95 °C by 0.2 °C increments with a 2-s hold after each step. The average melting temperature (T_m) for each parasite species was determined using the High-Resolution Melt Software v 3.01 (Applied Biosystems, California, USA).

2.5. Cloning DNA of Australian trypanosomes

Genomic DNA of several trypanosome species including *T. noyesi* genotype 8 (G8), *T. vegrandis* genotype 6 (G6), *T. vegrandis* genotype 7 (G7), *T. copemani* genotype 1 (G1), *T. copemani* genotype 2 (G2), and *T. cruzi* were amplified using the HRM-qPCR primers and the amplicons cloned so the plasmid DNA could be used as internal controls in the HRM-qPCR. The amplicons were isolated from 1% agarose gels and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA) and quantified using the Nanodrop ND-100 Spectrophotometer (Thermo Fisher Scientific, Victoria, Australia). They were then cloned using the pGEM®-T Easy Vector system II (Promega, Wisconsin, USA) according to the manufacturer's instructions (Promega, Wisconsin, USA). Successful cloning was confirmed using PCR. *EcoRI* restriction digestion (New England Biolabs, Victoria, Australia) and Sanger sequencing using the Big Dye Terminator Sequencing Chemistry (Applied Biosystems, California, USA). DNA quantities for restriction digestions and sequencing were determined using the Nanodrop ND-100 Spectrophotometer (Thermo Fisher Scientific, Victoria, Australia). The plasmid DNAs were stored at −20 °C.

2.6. Detection limit of the HRM-qPCR assay

To determine the limit of detection for the HRM-qPCR assay, a standard curve was created by plotting the threshold cycle (Ct) against a series of six 10-fold dilutions of a set initial amount for each purified plasmid from each of the following trypanosome species used for validation: *T. copemani* G2, *T. noyesi* G8 and *T. vegrandis* G7. To achieve a reliable curve for amplification of the DNA of each target species, the plasmid DNA was amplified in duplicates for each standard dilution point over the selected dilution range of 10^5- 10^0.

2.7. Validation of HRM-qPCR specificity and detection of mixed infections

The HRM-qPCR primer pair were designed as generic primers for all known trypanosome species infecting all the wildlife samples used in the study. DNA from *Trypanosoma microti*, *T. cruzi* and *Trypanosoma rangeli* were subjected to HRM-qPCR to assess assay suitability for detecting trypanosomes exotic to Australia. The melting profiles for each species was determined, and the identity of the species confirmed using the nested PCR and Sanger sequencing methods described by Botero et al. (2013).

The following mock infections were created by mixing equal amounts of DNAs cloned from the species to assess the specificity of the assay in detecting mixed infections: *T. copemani* G2/ *T. noyesi* G8; *T. copemani* G2/ *T. vegrandis* G7; *T. vegrandis* G7/ *T. noyesi* G8; and *T. copemani* G2/ *T. vegrandis* G7/ *T. noyesi* G8. The specificity of the assay was further assessed by using DNA templates known to contain other parasite species commonly infecting woylies, brushtail possums and chuditch. These parasites included Piroplasm species (*Theileria* spp. and *Babesia* sp.) occurring in the blood and the tissue cyst Coccidian parasites (*Toxoplasma gondii*).

2.8. Reliability of HRM-qPCR in detecting trypanosomes in wildlife samples

A total of 155 wildlife samples were screened with the established HRM-qPCR assay: of these, 68 had been previously screened and were used to validate the assay whereas the remaining 87 were screened for the first time. Amplicons were visualised on a 1.5% agarose gel stained with SYBR Safe (Invitrogen, Victoria, Australia) and viewed using an LED light transilluminator. Successful PCRs was confirmed by the presence of the expected band size. The amplicons were purified using an in-house filter tip method and sequenced (Tautz and Renz, 1983; Yang et al., 2013). The presence of DNA belonging to more than one Trypanosome species in the samples (represented by double or triple peaks in the HRM analysis) was confirmed using specific nested PCRs

and Sanger sequencing as previously described by Botero et al. (2013). The PCR controls for all reactions included pre-PCR and post-PCR negative controls; a DNA extraction blank; and a known PCR positive control.

Sequencing was carried out at the Western Australian State Agricultural Biotechnology Centre (SABC) at Murdoch University, using an ABI Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystems, California, USA) and an ABI 3730 96 capillary machine. Both strands of DNA for each amplicon were sequenced and were edited and analysed using Geneious (Edgar, 2004). Edited sequences were initially compared with published sequences in the NCBI sequence database using BLAST, and the sequence identities confirmed by comparing with a local sequence database created with sequences of trypanosome species commonly infecting Australian wildlife using MUSCLE (Edgar, 2004).

2.9. Phylogenetic analysis of amplified 18S rDNA of trypanosomes

Molecular phylogenetic analysis of the amplified section of 18S rDNA gene of 12 Trypanosomes species used in this study was conducted using MEGA7 (Kumar et al., 2016). The relationship was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei (1993). The tree was constructed using 1000 bootstraps with the highest log likelihood shown (Felsenstein 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value.

3. Results

3.1. The HRM-qPCR assay specificity

Genomic DNA of *T. noyesi* G8, *T. vegrandis* G7, *T. copemani* G1 and *T. copemani* G2 were amplified by the primers developed for the assay (Fig. 1). These amplicons were cloned and the plasmid DNA used to first validate the assay, and also as internal controls for the HRM-qPCR assays. The oligonucleotide primers successfully amplified the cloned DNA of *T. copemani* G1 and G2, *T. noyesi* G8 and *T. vegrandis* G7. The amplification plots showed distinct melting temperature curves for each of the trypanosome species indicating the assay could concisely discriminate between the species (Fig. 2A and B; Supplementary Figure 1; and Table 1). However, the melting temperature curves for *T. copemani* G1 and G2 were the same; they are therefore referred to as *T. copemani* from here on.

Genomic DNA purified from the cultures of *T. microti*, *T. cruzi* and *T. rangeli* was tested in this assay to confirm the range of detection within the *Trypanosoma* clade. As shown inFig. 2A and B and Table 1, all six species were successfully detected using this method, producing unambiguous melt temperatures that allow precise identification of the species. Sanger sequencing of amplicons obtained from nested PCRs confirmed the identity of these species. The diversity in the sequences that allowed clear discrimination of the species was confirmed by the

Table 1
Primer Tm range (°C) for plasmid DNA and genomic DNA of six trypanosome species.

| Trypanosome species	Tm range °C
Australian trypanosomes	Cloned DNA
T. copemani G1/G2	82.10–82.30
T. noyesi G8	80.34–80.60
T. vegrandis G7	80.90–81.10
Exotic Trypanosomes	**Genomic DNA**
T. microti	81.64–81.66
T. rangeli	81.44–81.46
T. cruzi	81.14–81.20
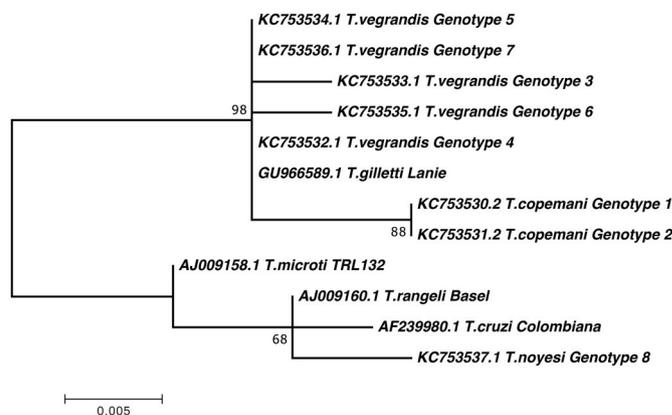
Fig. 3. Phylogenetic tree of seven Trypanosome species and subsequent genotypes constructed with sequences of the amplicons generated by the HRM-qPCR primers.

phylogenetic tree generated using sequences of the amplicons (Fig. 3). The close relationship between the *T. copemani* genotypes G1 and G2, revealed in the phylogenetic tree also confirmed the similarity in the melting temperature curves generated for the genotypes (Fig. 3).

The assay was specific and reliable and did not amplify any of the parasitic species which were also present in the wildlife samples. These parasites included the blood parasite Piroplasms and the tissue cyst coccidian parasites *Toxoplasma gondii*.

3.2. Assay detection limit

Dilutions of the cloned DNA of *T. copemani, T. noyesi* G8 and *T. vegrandis* G7 were each used to generate standard curves from which the detection limits of the assays were derived. The series of 10-fold dilutions, performed in duplicates, ranged from 100,000 to 1 copy number (s)/μl for each trypanosome species. The lowest detectable limit of the assay for all three target sequences was 10 copies of the amplicon in a reaction (Fig. 4). Dilutions lower than 10 copies of the amplicon in a reaction produced inconsistent curves. The amplification efficiency of
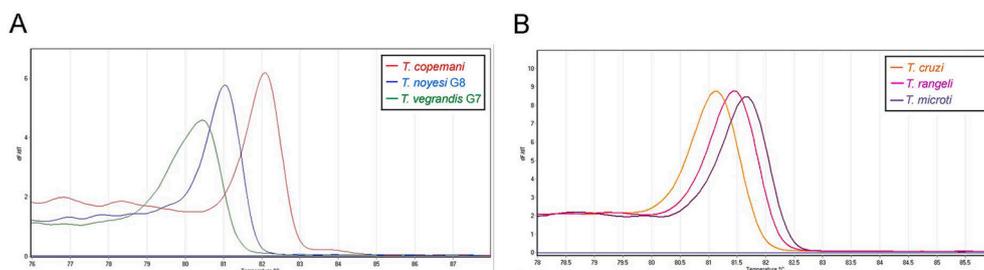
Fig. 2. A-B: (A) Derivative melt curves of *T. copemani, T. noyesi* G8*, T. vegrandis* G7; (B) Derivative melt curves of *T. microti, T. cruzi* and *T. rangeli*.
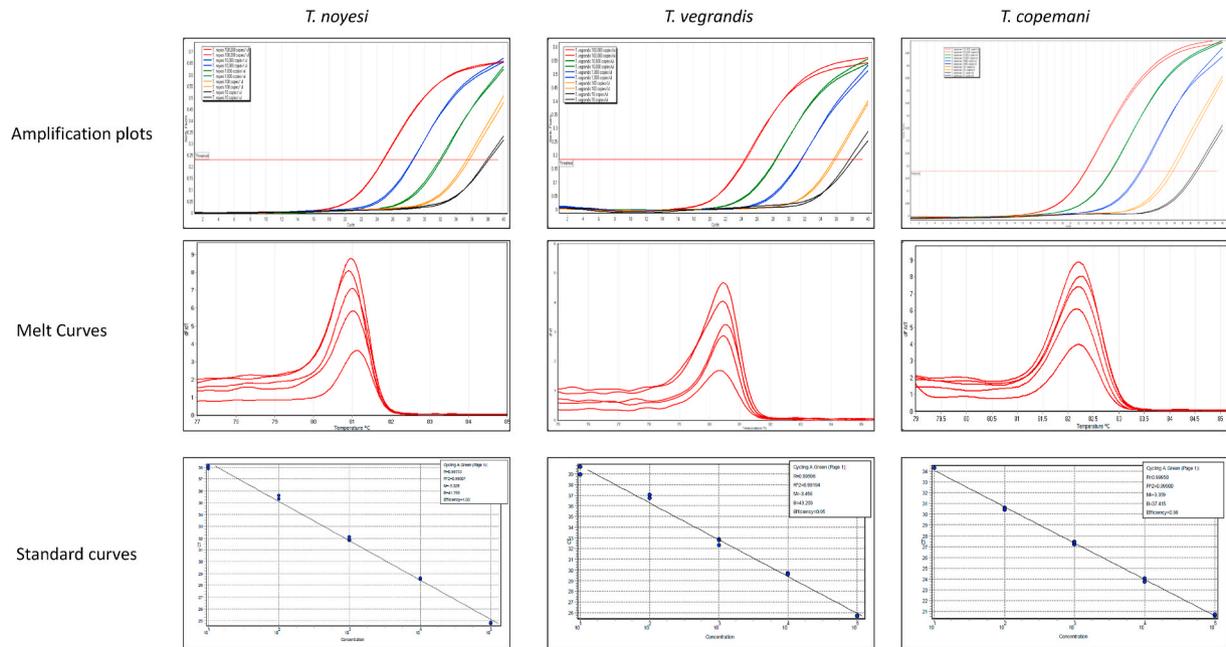
Fig. 4. Amplification plots, melt curves and standard curves of *T. copemani*, *T. vegrandis* G7 and *T. noyesi* G8 prepared from a plasmid containing trypanosome species.

DNA from all three species were within acceptable range as indicated by the gradients, R^2 and efficiencies of the amplifications. Notably, the amplification efficiencies for *T. noyesi* G8 was 100%, with those of *T. copemani* and *T. vegrandis* G7 being 98% and 95% respectively (Fig. 4).

3.3. Ability of the developed HRM-qPCR assay to detect mixed infections

A 1:1 mixture of different combinations of the cloned DNA from *T. noyesi* G8, *T. copemani* and *T. vegrandis* G7 were used as templates to determine if the HRM-qPCR assay could detect individual species in mixed infections. DNA of the species in two of the three mock double mixed infections, the combinations of *T. copemani*/ *T. vegrandis* G7 and *T. copemani*/ *T. noyesi* G8, were accurately distinguished by the assay (Fig. 5A–C). For these, the melting curve peaks contributed by the two component species could be clearly seen on the derivative melting curve plots. However, in some combinations the peaks generated were wider

compared to the peaks in the assays where individual cloned DNA were analysed, indicating the respective melting temperatures slightly increased or decreased when DNA of multiple species was present (Fig. 5A and B). The HRM-qPCR analysis of DNA involved in the mocked mixed infection of *T. vegrandis* G7/ *T. noyesi* G8 and *T. vegrandis* G7/ *T. noyesi* G8/ *T. copemani* (Fig. 5D) inconsistently produced defined peaks and sometimes generated a single intermediate peak with a slight shoulder possibly due to the closeness of the melting temperature peaks for DNA of single Trypanosome species (*T. vegrandis* G7: Tm = 80.36–80.54; *T. noyesi* G8: Tm = 80.90–81.10). Also, the HRM-qPCR assay did not consistently discriminate between the DNA of the three different species from a mixture; for these either three wider peaks were produced or there were two peaks with one of them presenting as a slight shoulder indicating the presence of a second DNA as seen in the *T. vegrandis* G7/ *T. noyesi* G8 mocked infection (Fig. 5D).
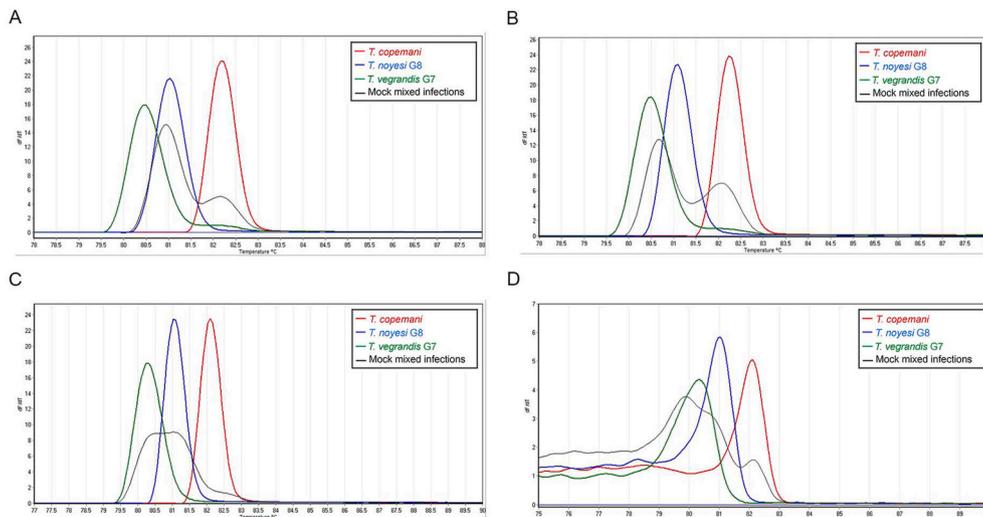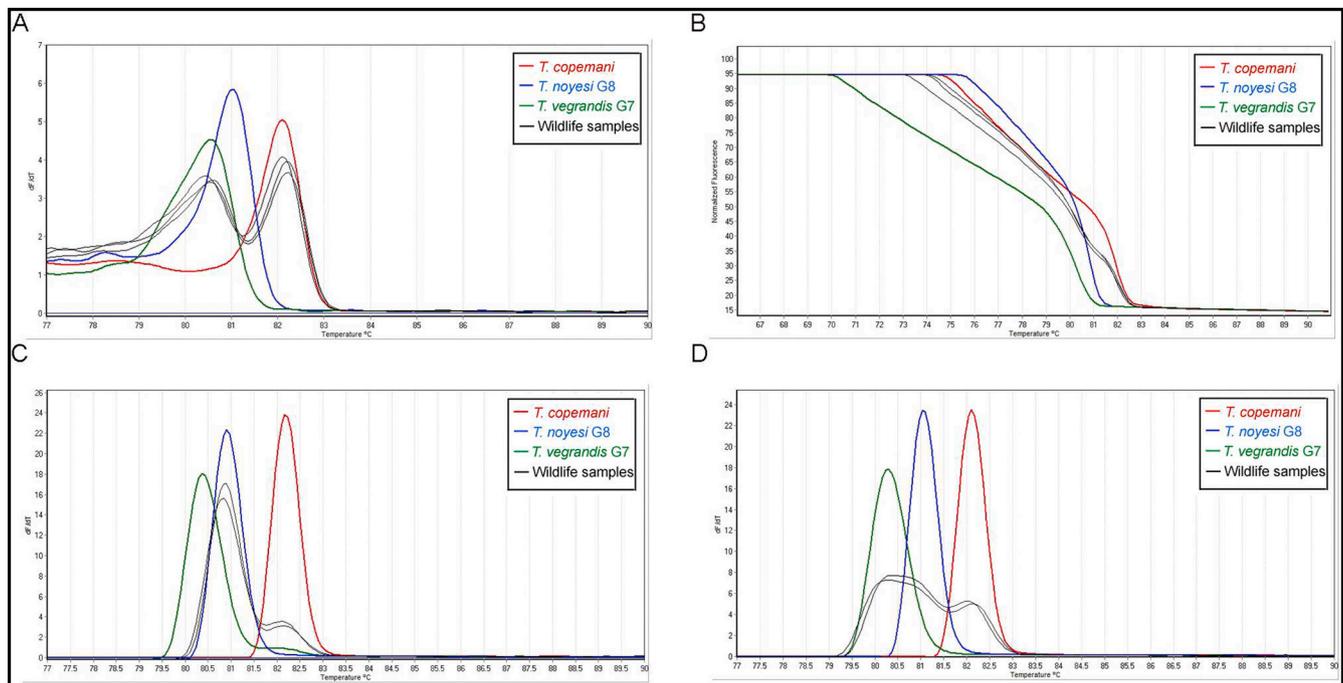

Fig. 5. A-D: Derivative melt curves showing mock mixed infections generated from plasmid clones containing the following DNA: (A) *T. noyesi* G8 and *T. copemani*; (B) *T. vegrandis* G7 and *T. copemani*; (C) *T. vegrandis* G7 and *T. noyesi* G8; (D) *T. vegrandis* G7, *T. noyesi* G8 and *T. copemani*.

Fig. 6. A-D: (A) Derivative melt curves of wildlife samples containing *T. copemani* and *T. vegrandis* G7 infections from woylie tissue and the (B) the respective normalised melt domains; (C) Derivative melt curves of wildlife samples containing mixed infections with *T. copemani* and *T. noyesi* G8 in woylie blood; (D) Derivative melt curves showing wildlife samples containing mixed infections of *T. copemani*, *T. noyesi* G8 and *T. vegrandis* G7 in woylie tissue.

3.4. Screening wildlife samples using the developed HRM-qPCR

Of the 155 wildlife samples screened using the HRM-qPCR assay, 68 samples had been previously genetically characterised using nested PCR and Sanger sequencing (Botero et al., 2013). These samples were used to validate the assay. All three *Trypanosoma* species (*T. copemani*, *T. noyesi* G8 and *T. vegrandis* G7) were successfully detected in the samples as shown in Fig. 6A–D, and the HRM-qPCR results correlated well with our previous screening results using nested PCR and Sanger sequencing. The HRM-qPCR assay detected single and multiple infections: *T. noyesi* G8 (n = 1), *T. vegrandis* G7 (n = 3), *T. copemani/ T. noyesi* G8 (n = 1), *T. copemani/ T. vegrandis* G7 (n = 1) and *T. copemani/ T. vegrandis* G7/ *T. noyesi* G8 (n = 1), in samples that nested PCR and Sanger sequencing failed to detect previously. In addition, three samples were misdiagnosed as having just one infection (*T. copemani*) using the traditional PCR and sequencing however the HRM-qPCR detected co-infections with *T. noyesi* G8 (n = 2) and *T. vegrandis* G7 (n = 1). From the known samples, the HRM-qPCR detected single infections of trypanosome species and co-infections: *T. copemani* (n = 35), *T. vegrandis* G7 (n = 3), *T. noyesi* G8 (n = 1), *T. copemani/ T. noyesi* G8 (n = 5), *T. copemani/ T. vegrandis* G7 (n = 9) and *T. copemani/ T. vegrandis* G7/ *T. noyesi* G8 (n = 3). No amplification was detected in five samples known to be negative for trypanosomes infection.

A total of 87 wildlife samples consisting of *in vitro* cultures, blood and tissue samples, were screened for the first time using the HRM-qPCR method developed. Positive results were later confirmed using nested PCR and/ or Sanger sequencing. Trypanosome species were detected in 93% of the samples and mixed infections were detected in 8%. The HRM-qPCR detected the following trypanosome species and combinations in the wildlife samples: *T. copemani* (n = 50), *T. vegrandis* G7 (n = 8), *T. noyesi* G8 (n = 10), *T. copemani/ T. noyesi* G8 (n = 3), *T. copemani/ T. vegrandis* G7 (n = 4). The melting temperature range for all samples containing more than one trypanosome species slightly increased compared to those obtained from samples containing only one species as shown in Fig. 6A–D. Table 2 shows the melting temperature range (single and mixed infections) for each trypanosome species.

When screening wildlife samples, a defined peak was detected outside of the range obtained when the cloned DNA was used (81.9–82 °C). Sequencing revealed this product to be *T. vegrandis* G6. Using the same approach, a second genotype was detected (*Trypanosoma* sp. ANU2) within the same range as *T. copemani* (82.1–82.3 °C). Both of these genotypes are known to infect woylies. *T. vegrandis* G6 and *Trypanosoma* sp. ANU2 were both detected in six woylie tissue samples. Mixed infections containing *T. noyesi* G8/ *T. vegrandis* G7 were not detected in any of the wildlife samples.

4. Discussion

This is the first study to use HRM technologies to screen for Australian *Trypanosoma* species. Unlike current gold standard tools, this two-step approach (qPCR and HRMA) is completed within a single closed tube, requiring a single pair of universal trypanosome oligonucleotides and a new generation saturating dye, SYTO9, to detect multiple species of Australian trypanosomes including *T. copemani*, *T. noyesi*, *T. vegrandis*, and other native and exotic trypanosomes such as *T. cruzi*, *T. rangeli* and *T. microti*. The primers designed in this study did not only have the capacity to discriminate between multiple trypanosome species, but it also detected intraspecific variation. Here, we describe a screening technique that allows for high-throughput and sensitive detection of *Trypanosoma* species in single and mixed infections without the need to carry out species-specific PCRs.

Our knowledge on *Trypanosome* diversity and polyparasitism, across

Table 2
High resolution melting temperature range for each trypanosome species.

Species	Tm range (°C) for samples with one or more infections.	
	Clone DNA mixture (mimicry of single infections)	Wildlife blood sample range (mixed infections)
T. copemani	82.04–82.24	82.00–82.70
T. noyesi G8	80.80–81.20	80.66–81.40
T. vegrandis G7	80.20–80.60	79.86–80.66

multiple hosts, has surged over the last two decades coinciding with the emergence of diagnostic platforms such as nested PCR and sequencing (Grybchuk-Ieremenko et al., 2014; Kozminsky et al., 2015; Spodareva et al., 2018). In Australia, recent studies using Next Generation Sequencing, an advanced diagnostic tool, have shown that a marsupial host can be infected with up to five trypanosome species (Barbosa et al., 2017; Cooper et al., 2018). It is likely that trypanosome prevalence has been underrepresented in previous studies due to the sensitivity and capabilities of the methods selected for detection of infections at the time. Here, we have demonstrated this by using HRM-qPCR to screen wildlife samples that were previously screened using conventional trypanosome generic PCRs followed by Sanger sequencing or PCRs using trypanosome species-specific primers. Our assay detected about 10% more infections than when traditional PCRs and sequencing were used. Furthermore, the new assay detected mixed infections in three samples that were misdiagnosed as having just one infection using traditional PCR and sequencing. Our HRM-qPCR successfully detected three common trypanosome species, *T. copemani*, *T. noyesi* and *T. vegrandis*, infecting Australian wildlife with assay sensitivity as low as 10 copies of the amplicon in a reaction.

Establishing positive controls to determine the melting temperature profile for each trypanosome species is fundamental when designing an HRM-qPCR assay. Previous studies have shown that analyses of DNA of some species and genotypes of parasite can produce misleading melt curves because they generate bimodal melt curves. The initial perception is of mixed infections however an amplicon containing an uneven distribution of stable GC rich regions can produce this profile. In this study, no bimodal melting pattern was detected from cloned DNA of *T. copemani* G1/G2, *T. noyesi* G8 or *T. vegrandis* G7 and each species produced an unambiguous melt curve.

The assay's ability to detect mixed infections was assessed by generating different mock mixed infections containing cloned DNA of *T. copemani*, *T. noyesi* G8 and *T. vegrandis* G7. The derivative melt curves generated from the cloned DNA of *T. noyesi* G8 and *T. vegrandis* G7 typically produced a wider peak with a slight shoulder indicating the presence of the two species. Conversely, *T. copemani* consistently produced a defined derivative melt curve when mixed with either *T. vegrandis* G7 or *T. noyesi* G8. When the DNA of all three species were mixed, the typical melt profile produced a defined curve for *T. copemani*, followed by the typical *T. noyesi* G8/ *T. vegrandis* G7 curve, and one wide curve with a slight shoulder. Our results using wildlife samples were consistent with the derivative melt curves obtained in mock mixed infections with the cloned DNA of the three species. However, the Tm range for each of the targeted trypanosome species slightly increased in combination with other trypanosome species (species confirmation was resolved by species-specific nested PCR or Sanger sequencing). This could be due to small sequence variants such as single nucleotide differences resulting in a melt curve shift. Deciphering such melt curves can prove to be problematic when the melting range differs from the melting temperature of the cloned DNA controls. Further confirmation such as targeted species-specific PCR or the use of generic trypanosome PCRs followed by Sanger sequencing may be required to determine infections outside of the Tm range. The use of positive controls in experiments will help override this problem, by determining their melt profile.

The HRM-qPCR assay successfully detected the following trypanosome co-infections in wildlife samples: *T. copemani/ T. vegrandis* G7; *T. copemani/ T. noyesi* G8 and *T. copemani/ T. vegrandis* G7/ *T. noyesi*. Interestingly, mixed infections containing *T. vegrandis* G7 and *T. noyesi* G8 were not detected in wildlife samples screened in this assay. One possible reason is that such mixed infection may not exist in the samples. This may be due to interspecific competition whereby one species may moderate the sequential establishment of another. The competition may mask the infection of the controlled species or the DNA of the controlled species may be beyond detectable limit. This phenomenon has also been reported in mixed infections of *T. copemani* and *T. vegrandis* in wildlife samples from the UWR (Thompson et al., 2014).

This study selected a 246 nucleotide-long segment within the variable region of the 18S rDNA gene for trypanosome species. Although detection of trypanosome species and discrimination was observed using the HRM-qPCR, the observation of intraspecific variation (*T. vegrandis* G7 and *T. vegrandis* G6) and overlap of melt curves between species (*T.* sp. ANU2 and *T. copemani*) added to the complexity of the assay. Studies have shown that the genomes of trypanosome parasites of Australian wildlife have a high level of heterogeneity within species (Botero et al., 2013; Cooper et al., 2018). This diversity may be a reason for unique melting temperatures obtained in this study. The thermal melt profiles identified in this assay are most likely due to the proximity of the melting temperature for each species and genotype. Consequently, species such as *T. vegrandis*, with high GC content and genotypic variation, will produce unique melt curves for each genotype. Unfortunately, despite the *Trypanosoma* sp. ANU2 and *T. copemani* probably being different species based on phylogenetic analysis (Cooper et al., 2018), the 18S rDNA sequence region selected for this assay may be very similar for both species. Re-evaluating primer design using more diverse regions of the 18S rDNA to enable amplification and discrimination of more trypanosome species should be visited in order to develop a quick and a more accurate diagnostic screening tool, able to distinguish not just species but between genotypes.

Our HRM-qPCR additionally amplified DNA belonging to *T. microti*, *T. cruzi* and *T. rangeli*, and all species could be identified by a different melting temperature curve. The ability of this technique to detect a broad range of trypanosome species is important in terms of wildlife health as knowledge of host-parasite relationships is limited. A recent study using Next Generation Sequencing found that trypanosomes endemic to Australia demonstrated low host specificity, high intraspecific diversity and low geographic boundaries (Cooper et al., 2018). Furthermore, how these trypanosomes are transmitted or their potential to cause disease is yet to be determined with no knowledge of their vectors.

It is becoming increasingly important to understand the impact and transmission of parasites when considering fauna conservation and management. Thus HRM-qPCR provides that fundamental platform capable of screening multiple trypanosome species and genotypic variation within species, providing an indication of prevalence without the need for more laborious and time-consuming detecting methods such as nested PCRs and Sanger sequencing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2020.11.003.

Permits

All fieldwork was carried out under DBCA permit numbers DECAEC/52/2009 and NS1182-06, and Murdoch University ethics approval numbers WC2172-08, WC2350-10 and RW2659/14.

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