Author/s:
Jabbar, A;Cotter, J;Lyon, J;Koehler, AV;Gasser, RB;Besier, B

Title:
Unexpected occurrence of Haemonchus placei in cattle in southern Western Australia

Date:
2014-01-01

Citation:
Jabbar, A., Cotter, J., Lyon, J., Koehler, A. V., Gasser, R. B. & Besier, B. (2014). Unexpected occurrence of Haemonchus placei in cattle in southern Western Australia. Infection Genetics and Evolution, 21, pp.252-258. https://doi.org/10.1016/j.meegid.2013.10.025.

Persistent Link:
https://hdl.handle.net/11343/44069

Unexpected occurrence of *Haemonchus placei* in cattle in southern Western Australia

Abdul Jabbar, Jenny Cotter, Jill Lyon, Anson V. Koehler, Robin B. Gasser, Brown Besier

PII: S1567-1348(13)00397-3
DOI: http://dx.doi.org/10.1016/j.meegid.2013.10.025
Reference: MEEGID 1787

To appear in: *Infection, Genetics and Evolution*

Received Date: 10 July 2013
Revised Date: 4 October 2013
Accepted Date: 27 October 2013

Please cite this article as: Jabbar, A., Cotter, J., Lyon, J., Koehler, A.V., Gasser, R.B., Besier, B., Unexpected occurrence of *Haemonchus placei* in cattle in southern Western Australia, *Infection, Genetics and Evolution* (2013), doi: http://dx.doi.org/10.1016/j.meegid.2013.10.025

Infection, Genetics and Evolution
MEEGID-D-13-00371-R3

Unexpected occurrence of *Haemonchus placei* in cattle in southern Western Australia

Abdul Jabbar[a,1], Jenny Cotter[b,1], Jill Lyon[b], Anson V. Koehler[a], Robin B. Gasser[a], Brown Besier[b]

[a] *Faculty of Veterinary Science, The University of Melbourne, Werribee, Victoria 3030, Australia*
[b] *Department of Agriculture and Food, Albany, Western Australia 6330, Australia*

_____

* Corresponding author. Address: Faculty of Veterinary Science, The University of Melbourne, Werribee, 3030, Australia .Tel.: +61 3 9731 2022; fax: +61 3 9731 2366.
E-mail addresses: jabbara@unimelb.edu.au (A Jabbar).

[1]Equal contribution

A B S T R A C T

Haemonchus placei is an abomasal parasite of cattle, primarily in tropical and subtropical areas of the world. In Australia, this nematode can be extremely pathogenic in summer rainfall areas, particularly in the hot, sub-tropical Kimberley region, in the far north of the state of Western Australia (WA). Although cattle are occasionally transferred to southern parts of WA, it was believed that *H. placei* did not occur in southern regions of WA, as it is less cold-adapted than *H. contortus,* and the free-living stages would not develop during the cold winter and dry summer periods. Here, we show that, although *H. contortus* is found in cattle in the temperate southern region of WA, it appears that *H. placei* also occurs in southern WA. While investigating the prevalence of anthelmintic resistance in nematodes of cattle in WA, the existence of *H. placei* was suspected on a range of participating farms, following the morphological examination of third-stage larvae cultured from faeces, and of adult worms recovered from sheep experimentally infected with these larvae. Genomic DNAs from individual worms as well as eggs from pooled faecal samples from seven farms in southern WA were subjected to PCR-based mutation scanning and sequence analyses of the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA. The results showed that both *H. contortus* and *H. placei* were harboured by cattle. This first record of *H. placei* in cattle in southern WA raises questions as to the prevalence and distribution of this parasite in other temperate and cool climatic regions of Australia. Although clinical disease due to *H. placei* has not yet been seen in southern WA, global, climatic trends might suggest an increased importance of this parasite in the longer term.

Keywords:
Haemonchus placei
Nematode
Ecology
Cattle
Geographical distribution

1. Introduction

Members of the genus *Haemonchus* Cobb, 1898 (Nematoda: Trichostrongyloidea) are important abomasal nematodes of domestic ruminants (Anderson, 2000) and are responsible for significant economic losses in sheep and goats in tropical and subtropical regions of the world (O'Connor et al., 2006). These nematodes are transmitted orally from contaminated pasture to the host through a complex life cycle (cf. Veglia, 1916; Anderson, 2000): eggs are excreted in host faeces; the first-stage larva (L1) develops inside the egg to then hatch and moult to the second- (L2) and third-stage (L3) larvae. The host becomes infected when infective L3s are ingested, which then exsheath and, after a histotrophic phase, develop via fourth-stage larvae (L4s) to dioecious adults. *Haemonchus* spp. feed on blood from capillaries in the abomasal mucosa, and cause haemorrhagic gastritis, anaemia, oedema and associated complications, often leading to death in severely affected animals, particularly sheep and goats (Anderson, 2000).

Important species are *H. contortus* (Rudolphi 1803) Cobb, 1898, which principally infects sheep and goats, but can also be found in cattle and some species of deer (Eve and Kellogg, 1977; Anderson, 2000), and *H. placei* (Place 1893) Ransom 1911, which is primarily a parasite of cattle (Anderson, 2000). However, it is also known that both species can simultaneously infect cattle and small ruminants, particularly on communal pastures (Achi et al., 2003; Amarante et al., 1997; Jacquiet et al., 1998).

In Australia, *H. placei* in cattle and *H. contortus* in sheep can be extremely pathogenic in summer rainfall areas (O'Connor et al., 2006). In Western Australia (WA), *H. placei* has been recognised as a common nematode of young beef cattle in the Kimberley district (Fig. 1), an area in the north of WA (summer rainfall zone), although disease outbreaks are rare (B. Besier unpublished findings) (Fig. 1). By contrast, the agricultural region in southern WA is in a Mediterranean climatic zone (Fig. 1), characterised by hot dry summers and receiving

predominantly winter rainfall. In high rainfall and coastal zones within this region, *H. contortus* regularly parasitizes sheep, with an occasional 'spillover' into cattle in situations of communal grazing.

While investigating the prevalence of anthelmintic resistance in young cattle in southern WA (J. Cotter, unpublished), the existence of *H. placei* was suspected on a range of participating farms, following the morphological examination of L3s cultured from cattle faeces, and of adult worms recovered from sheep following experimental infection with these larvae. The present study was conducted to confirm that, although *H. contortus* is occasionally found in cattle in southern WA, *H. placei* also occurs in this region. We used morphological and molecular methods to characterize *H. placei* and *H. contortus* present here in young cattle.

2. Materials and methods

2.1. Study area and farms

The study area was in the south-west of WA (Fig. 1). In contrast to much of WA, the coastal rim maintains some green pasture throughout most of the year, including Kikuyu grass, annual ryegrass and clovers. The coastal city of Albany in the Great Southern Region of WA (latitude 35.03 °S, longitude 117.88 °E, elevation 3 m) has a temperate climate (temperature: winter (July) 8-15 °C; summer (January) 15.5-23 °C) and receives an annual rainfall of 600-800 mm (Australian Bureau of Meteorology; www.bom.gov.au). Beef cattle farms in the region represent mostly self-replacing herds, with an average of approximately 200 breeding cows, with some larger herds of up to 2,000 cattle, mostly of British breeds. The farms (n = 7) involved in the present study were within 40 km of the coast, in the vicinity of Albany (Table 1).

103

104 *2.2. Coprological methods used for preliminary investigations*

105 In 2010 and 2011, faecal samples were collected from young cattle (8-10 months of age)
106 during the course of a series of anthelmintic resistance trials (manuscript in preparation). For
107 each treatment and control group, individual faecal egg counts (FEC) were performed on 4 g
108 of faeces from individual cattle (n = 1425) on participating farms (n = 19) using the modified
109 McMaster technique (Whitlock, 1948), and the remaining pooled faeces from each farm were
110 subjected to larval culture for seven days at 25 ºC (MAFF, 1986). Cultured L3s were
111 identified as previously described (Dikmans and Andrews, 1933; Keith, 1953).

112

113 *2.3. Experimental infection of sheep with* Haemonchus *larvae from cattle*

114 Based on size differentiation of cultured L3s derived from cattle faeces (section 2.2.), *H.*
115 *contortus* and *H. placei* were suspected. To confirm the presence of *H. placei*, a sheep was
116 infected with L3s to produce adult worms. Specifically, an adult, helminth-free Merino sheep
117 was treated with PYRIMIDE 3-Way Combination Drench for Sheep® (abamectin 0.8 g/L,
118 albendazole 20.0 g/L, levamisole 25.5 g/L, Novartis Animal Health, Australia). After three
119 weeks, the sheep was orally infected with 5000 L3s, containing 27% *Haemonchus* larvae in a
120 mixture with other strongylid nematode larvae, and housed for 40 days before euthanasia, to
121 collect adult worms. Abomasal contents were passed through a 1 mm sieve and resuspended
122 in a tray, from which adult *Haemonchus* were isolated. Spicule length and vulval flap
123 morphology of these individual adults were recorded according to previously published
124 articles (Roberts et al., 1954; Bremner, 1956). The worms were stored in a mixture of alcohol
125 (70%) and glycerol (5%) until molecular characterisation (September 2012).

126

127 *2.4. Collection of eggs to confirm the presence of* Haemonchus placei *in cattle*

In February 2013, in order to verify that *H. placei* was still cycling on the farms where it was detected one year before, pooled faecal samples (from 20 weaner cattle, 6 to 9- months old, from each of seven farms) were collected. Faecal egg counts were performed to establish the presence of strongyles, and a lectin binding assay (Palmer and McCombe, 1996; Colditz et al., 2002) was used to confirm the presence of *Haemonchus*. Strongylid eggs were isolated from faeces as described previously (Bott et al., 2009).

2.5. Molecular methods

2.5.1. Isolation of genomic DNA

Prior to DNA isolation, ethanol was removed from individual worms by rehydration. Then, individual adults of *Haemonchus* (n = 9) were incubated in ~ 200 µl of 20 mM Tris-HCl (pH 8.0), 100 mM EDTA, 1% sodium dodecyl-sulphate containing 10 mg/ml proteinase K (Amresco Inc., USA) at 37 °C for 18 h. Genomic DNA was isolated from the homogenised suspension using mini-columns (Wizard® DNA Clean-Up Kit, Promega, USA). Genomic DNA from strongylid eggs from faeces was isolated using PowerSoil® DNA Isolation Kit (MO BIO Labs, Inc., USA) according to the manufacturer's protocol.

2.5.2. PCR amplification, single-strand conformation polymorphism (SSCP) and sequencing

The second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA (including flanking sequence) was amplified by PCR from the genomic DNA (10-20 ng template) from individual worms using primers NC1 (5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3'). PCRs were conducted in 50 µl volumes containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl (Promega), 3.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dNTP), 50 pmol of each primer and 1 U of Go*Taq* polymerase (Promega) using the following cycling conditions: 94°C for 5 min, then 35 cycles of 94°C for

30 s, 55°C for 30 s and 72°C for 30 s, followed by 72°C for 10 min. Negative (no-DNA) and known positive (*H. contortus*) controls were included in each set of PCRs. Amplicons were subjected to agarose (1.5%) gel electrophoresis, and photographed upon transillumination using a GelDoc system (BioRad, Hercules, USA). *Haemonchus* DNA was detected in genomic DNA from strongylid eggs from faeces using a real-time PCR method (Bott et al., 2009).

ITS-2 amplicons from 92 samples of *Haemonchus* spp. were subjected to SSCP analysis using protocol B of Gasser et al. (2006) to screen for sequence variation within and among individual worms. One to five amplicons representing each unique SSCP profile were treated with shrimp alkaline phosphatase and exonuclease I (Fermentas Inc., USA), and sequenced (BigDye® Terminator v.3.1 chemistry, Applied Biosystems, USA) using primers NC1 and NC2 in separate reactions. The quality of individual sequences was assessed visually using the program Geneious Pro 5.6.5 (Biomatters Ltd., New Zealand).

2.5.3. Sequence comparisons and phylogenetic analyses

Prior to phylogenetic analyses, ITS-2 sequences were subjected to BLASTn analysis (http://blast.ncbi.nlm.nih.gov) to identify the best matches to all nucleotide sequences available in current databases. Sequence differences were calculated by pairwise comparison. Subsequently, all distinct ITS-2 sequences determined in the present study were aligned with a selected subset of closely related reference sequences using the program Clustal X (Larkin et al., 2007), and alignments were adjusted manually. Phylogenetic analyses of the sequence data (ITS-2) was conducted by Bayesian inference (BI), employing the Monte Carlo Markov Chain (MCMC) method in MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and distance-based Neighbour Joining (NJ) methods. For BI, the likelihood parameters were set based on the Akaike Information Criteria (AIC) test in Modeltest v.3.7 (Posada and Crandall, 1998). The general time-reversible model of evolution,

180 with gamma-distribution and a proportion of invariable sites (GTR + Γ + I), was utilised for
181 the analysis of the sequence data. Sequence data were also analyzed using the Neighbour-
182 Joining (NJ) method employing PAUP (PAUP 4.0b10) where molecular distances were
183 estimated by the general time-reversible model of evolution and the nodes were tested for
184 robustness by 100,000 bootstrap replicates. Phylogenetic trees constructed using the BI and
185 NJ methods were examined for concordance in topology.
186
187 **3. Results**
188
189 *3.1. Morphological identification of L3 and adult stages of Haemonchus*
190 Anthelmintic resistance testing of cattle in 2010 and 2011 on 19 farms throughout Western
191 Australia had identified gastrointestinal nematodes of the genera *Cooperia onchophora,*
192 *Haemonchus* spp., *Oesophagostomum* spp., *Ostertagia ostertagi* and/or *Trichostrongylus* spp.
193 (J. Cotter, unpublished). *Haemonchus* was found on 17 of the 19 farms, and results from
194 larval culture and differentiation had indicated the existence of *H. placei* in cattle on 17 of
195 these farms. FECs for *Haemonchus* in cattle (n = 1425) from these farms ranged from a mean
196 of 0 to 100 eggs per gram.
197 In faecal samples from cattle on 7 farms in 2013, lectin binding assays on eggs
198 isolated from the samples after worm egg counts indicated that *Haemonchus* was present on
199 all farms, with mean FEC from 2 to 90 eggs per gram (this technique does not allow
200 differentiation to the species level).
201 On the basis of morphological measurements (spicules, and vulval morphology), both *H.*
202 *placei* (80%)and *H. contortus* (20%) were identified from adult worms recovered from the
203 abomasa from the sheep experimentally infected with L3s originally derived from pooled
204 faeces from cattle from one farm.

205

206

207 *3.2. Molecular identification*

208 In order to verify the existence of *H. placei* in the study area, adults of *Haemonchus* from the
209 experimentally infected sheep and strongylid nematode eggs from cattle faeces were
210 subjected to molecular investigation.

211 ITS-2 amplicons of the expected size (~310 bp) were produced from the genomic DNAs
212 from individual adults of *Haemonchus* (n = 92) collected from the experimentally infected
213 sheep. Ten distinct ITS-2 profiles (designated A–J) were detected by SSCP analysis (Table
214 2). Selected ITS-2 amplicons (n = 1-5) representing each of these SSCP profiles were
215 sequenced. The lengths of individual ITS-2 sequence types, mean nucleotide frequencies,
216 polymorphic positions (if any), G+C content and respective accession numbers are listed in
217 Table 2. Sequencing revealed ten different sequence types (231 bp; GenBank accession nos.
218 KF364623-KF364632).

219 These ten sequence types were aligned over 243 positions with ITS-2 reference sequences
220 for *H. contortus* (n = 13) and *H. placei* (n = 5) (Stevenson et al., 1995; Cerutti et al., 2010;
221 Brasil et al., 2012; Gharamah et al., 2012) and for *Bunostomum phlebotomum*, *Nematodirus*
222 *rupicaprae*, *Oesophagostomum columbianum* and *Trichostrongylus axei* ('outgroups') (n = 4)
223 (Hoste et al., 1995; Newton et al., 1998; Gasser et al., 1999; Jex et al., 2009) and subjected to
224 phylogenetic analyses. The analyses unequivocally identified *H. placei* and *H. contortus* with
225 strong nodal support (posterior probability values 0.8-1.00; bootstrap values 80-98%) (Fig.
226 2). The topologies of the trees constructed using the two different algorithms were the same
227 with only minor variation in nodal/bootstrap support values (see Fig. 2). This analysis
228 revealed 78 and 14 adults of *H. placei* and *H. contortus*, respectively. Five distinct genotypes
229 were characterized within each species, which differed in sequence by 0.5-3.5% (accession

nos. KF364623-KF364627) and 0.5-0.9% (accession nos. KF364628-KF364632), respectively, upon pairwise comparison.

SSCP analysis and sequencing of ITS-2 ampicons from the strongylid eggs from cattle faeces, recovered in 2013 (section 2.4), revealed *H. placei* (accession no. KF364623) on farms 1-6 and *H. contortus* (accession no. KF364628) on farm 7 (Table 1) based on a perfect match to the sequence of *H. placei* (genotype A; accession no. KF364623) or *H. contortus* (genotype F; accession no. KF364628).

4. Discussion

Morphological examination of L3s cultured from faeces and of adult worms from sheep with experimental infections with such larvae revealed the presence of *H. placei* in young cattle in the temperate southern region of WA, which was confirmed by molecular study. This was a new and unexpected finding. While *H. placei* is endemic in tropical and subtropical zones in Australia, including in the Kimberley region in the far north of WA, it had not been found previously in more temperate, southern regions. *H. placei* might have been occasionally introduced to southern regions via cattle transported from the northern, endemic zone, but environmental constraints that limit the distribution of *H. contortus* were expected to apply to an even greater degree to the less cold-tolerant *H. placei*. In southern WA, studies with *H. contortus* in the relatively temperate south-coastal environment of the Albany region (Besier and Dunsmore, 1993a,b) indicated that the development of the free-living stages on local pastures was limited to short periods in autumn and spring, and ceased for several months in the hot, dry summer periods and during the relatively cold winters. As the environmental extremes of both summer and winter are greater in inland and more northern areas, the ecological requirements explain the geographical distribution of *H.*

255 *contortus*, which is found largely in the milder coastal and high-rainfall areas of the state.
256 Rarely, *H. contortus* is found in laboratory submissions from sheep in arid inland parts of
257 southern WA, but FECs are invariably negligible (B. Besier, unpublished findings).

258 In the present study, we also confirmed that that *H. placei* was still cycling on the farms
259 even after one year where it was detected first time. The incapability of *H. placei* to tolerate
260 lower temperature was expected to severely restrict its establishment within the agricultural
261 region of south-western WA (Fig. 1). For example, minimum temperatures of ≤ 13 °C have
262 been shown to prevent the hatching of *H. placei* eggs under laboratory conditions, while *H.*
263 *contortus* eggs are reported to hatch at 10-11 °C (Le Jambre 1981, Besier, 1992). Within the
264 southern agricultural region, mean monthly minimum temperatures of more than 13°C are
265 recorded only for the months of December to March (south of Perth), and November to April
266 (north of Perth) (Australian Bureau of Meteorology; www.bom.gov.au). Under these
267 conditions, in the months sufficiently warm enough to allow *H. placei* egg development,
268 conditions in most of southern WA are too dry (generally less than 25 mm rainfall per month)
269 and hot (mean monthly temperatures of more than 25°C) for the successful development of
270 *H. contortus* eggs, particularly on pastures composed of annual plant species (Besier and
271 Dunsmore, 1993a; O'Connor et al., 2006). The only location where summer temperatures (for
272 at most a 2-month period) are sufficiently mild enough to allow *H. contortus* development is
273 along the south coast (mainly between Albany and Esperance), although conditions are also
274 dry at this time. This ecological model indicates little opportunity for the development of L3s
275 of *H. placei* on pasture, and supports the lack of previous reports from southern WA.

276 Detection of *H. placei* in areas previously considered unfavourable raises questions about
277 the applicability of environmental data from studies with *H. contortus* in sheep for these
278 predictions. However, the taxonomic similarity of *H. contortus* and *H. placei* (see Gibbons,
279 1979) suggests that the ecological behavior of both species will also be similar, and studies of

Ostertagia (now *Teladorsagia*) *circumcincta* from sheep and *O. ostertagi* from cattle (Young, 1983) showed little difference in over-summer L3 survival rates in relation to the nature of host faecal deposits. A more likely explanation of the survival of *H. placei* in southern WA is that micro-environmental conditions allow L3 development in situations not indicated by general ecological models. Considerably better survival of L3s of *H. contortus* occurred for eggs from sheep faeces when deposited on to pastures of perennial (summer-green) grass species, compared with those deposited on to dry pastures (Besier and Dunsmore, 1993b). Importantly, perennial pastures (particularly Kikuyu grass, *Pennisetum clandestinum*) were present on all farms studied here and on which *H. placei* was found. In addition, all farms on which *H. placei* was located in the relatively temperate south coast region, where summer temperatures are mostly mild and summer rainfall is common. Although *Haemonchus* was found in cattle outside of this area, the species in these samples has not yet been investigated. Further study should assist in determining whether *H. placei* is restricted to more temperate parts of the southern agricultural region, or whether the ecological determinants for this species should be revised to include survival and development in more extreme environments of WA. A potential role for hypobiosis (seasonally arrested development) cannot be excluded, but is not considered likely. Although well-recognized in *H. contortus* in extremely dry or cold climates (Gibbs 1986), hypobiosis has not yet been reported in either *Haemonchus* species in southern parts of Australia.

The presence of both *Haemonchus* species in cattle on one farm, and of *H. contortus* only on another, is not unexpected. *Haemonchus* spp. are occasionally detected in faecal samples submitted to local laboratories from young cattle in this region, though never in association with overt parasitism (B. Besier, unpublished), and, until recently, were considered to be *H. contortus*, as cross-infection with this species among ruminant hosts is well-recognized (Riggs, 2001; Akkari et al., 2013). Local patterns of occurrence are likely to be determined

by the opportunity for infection with each species. For example, *H. contortus* is common on sheep farms in the more temperate parts of the southern agricultural region of WA, and young cattle on these properties are likely to acquire short-term infection with *H. contortus*, although bovine haemonchosis has not been detected in this region. In the present study, it could not be established whether *H. placei* was recently introduced to southern WA by the introduction of cattle from the Kimberly region and/or other parts of the country, or whether this species of nematode was always present in the region and was found incidentally. Most of the farms studied here had closed herds and did not introduce cattle from other regions of WA and or elsewhere. However, some farms (nos. 2, 4 and 5, Table 1) introduced cattle from the Kimberley region about 18 years ago, and it is possible that *H. placei* was brought to southern WA with these cattle. Based on the low *Haemonchus* egg counts found in cattle, clinical disease would not be expected, and it is possible that inapparent *H. placei* infection had been present for some time.

It has been suggested that climate change will alter the risk of infectious disease outbreaks, including for ruminant nematodes, by extending the seasonal window for parasite growth and by increasing the rate of transmission (Kenyon et al., 2008, Morgan and van Dijk, 2012). Rainfall in the South West agricultural region has declined significantly over the past 30 years compared with early 20th century (Carmody, 2010), with a greater variability in seasonal patterns and a greater proportion of rainfall in summer. In addition, it has been proposed that by 2030 the average annual temperatures are likely to rise 0-2 °C in southern WA (Carmody, 2010). Therefore, it is possible that changes in environmental conditions in recent decades might explain the presence of *H. placei* in southern WA, and increased summer rainfall may increase its importance in the region. However, further investigations of the ecology of *H. placei* are necessary to provide environmental data for modelling predictions of the likely response to changes in climate.

Based on the PCR–coupled mutation scanning analysis of the ITS-2 sequences determined herein, we defined five genotypes each for *H. contortus* and *H. placei*. Two genotypes of *H. placei* were identical to previously reported ITS-2 sequences (GenBank accession nos. JN128896 and JQ342249) from Brazil (Brasil et al., 2012), whereas the other three matched with those (JQ342248, X78812, JN128895) reported from Australia and Brazil (Stevenson et al., 1995; Brasil et al., 2012) (see Fig. 2). Similarly, *H. contortus* sequences determined herein matched previously published sequences of the ITS-2 from Australia, Brazil, Italy, Malaysia and Yemen (see Fig. 2) (Stevenson et al., 1995; Cerutti et al., 2010; Brasil et al., 2012; Gharamah et al., 2012).

In conclusion, this report demonstrates the advantage of using a combined morphological/molecular approach for the differential diagnosis of nematode infections, particularly where species identification is essential for the interpretation of new epidemiological information. The approach is applicable irrespective of the developmental stage of the parasite involved, thus providing a reliable and powerful tool for understanding the ecology of the free living stages of parasites. Although there is an extensive literature available on the development and behavior of the free-living stages of *H. contortus* in sheep, only a few studies have been undertaken to understand the epidemiology of *H. placei* in cattle. Further studies are required to elucidate the ecology of free living stages of *H. placei* in this region, and to indicate the prevalence and potential impact of *H. placei* on young cattle in the southern parts of Australia.

Acknowledgements

354 The infection experiment was approved by the Ethics Committee of the Department of
355 Agriculture and Food, WA (AEC 2-10-10). The able assistance of Heide Gutelich, Jo Hislop
356 and Esther Spence from the Albany Animal Health Laboratories is gratefully acknowledged.
357 The molecular work in the present study was supported by the Australian Research Council
358 (RBG) and Collaborative Research Grant from The University of Melbourne (AJ).

359

360 **References**

361 Achi, Y.L., Zinsstag, J., Yao, K., Yeo, N., Dorchies, P., Jacquiet, P., 2003. Host specificity of
362 *Haemonchus* spp. for domestic ruminants in the savanna in northern Ivory Coast. Vet.
363 Parasitol. 116, 151–158.
364 Akkari, H., Jebali, J., Gharbi, M., Mhadhbi, M., Awadi, S., Darghouth, M.A., 2013.
365 Epidemiological study of sympatric *Haemonchus* species and genetic characterization
366 of *Haemonchus contortus* in domestic ruminants in Tunisia. Vet. Parasitol. 193, 118–
367 125.
368 Amarante, A.F., Bagnola Junior, J., Amarante, M.R., Barbosa, M.A., 1997. Host specificity
369 of sheep and cattle nematodes in Sao Paulo state, Brazil. Vet. Parasitol. 73, 89–104.
370 Anderson, R.C., 2000. Nematodes parasites of vertebrates: their development and
371 transmission, second ed. CAB International, Wallingford, UK, pp. 105–109.
372 Besier, R.B., 1992. Intraspecifc variation in *Haemonchus contortus*: ecological studies with
373 an isolate from Western Australia. PhD Thesis, Murdoch University, Australia.
374 Besier, R.B., Dunsmore, J.D., 1993a. The ecology of *Haemonchus contortus* in a winter
375 rainfall region of Australia. 1. The development of eggs to infective larvae. Vet
376 Parasitol. 45, 275–292.
377 Besier, R.B., Dunsmore, J.D., 1993b. The ecology of *Haemonchus contortus* in a winter
378 rainfall region of Australia. 2. The survival of infective larvae on pasture. Vet.
379 Parasitol. 45, 293–306.
380 Bott, N.J., Campbell, B.E., Beveridge, I., Chilton, N.B., Rees, D. Hunt, P.W., Gasser, R.B.,
381 2009. A combined microscopic-molecular method for the diagnosis of strongylid
382 infections in sheep. Int. J. Parasitol. 39, 1277–1287.

383 Brasil, B.S., Nunes, R.L., Bastianetto, E., Drummond, M.G., Carvalho, D.C., Leite, R.C.,
384 Molento, M.B., Oliveira, D.A., 2012. Genetic diversity patterns of *Haemonchus*
385 *placei* and *Haemonchus contortus* populations isolated from domestic ruminants in
386 Brazil. Int. J. Parasitol. 42, 469–479.
387 Bremner, K.C., 1956. The parasitic life-cycle of *Haemonchus placei* (Place) Ransom
388 (Nematoda: Trichostrongylidae). Aust. J. Zool. 4, 146–151.
389 Carmody, P., 2010. Climate adaptation for South West Agricultural region. Farmnote 413.
390 Department of Agriculture and Food, Government of Western Australia.
391 Cerutti, M.C., Citterio, C.V., Bazzocchi, C., Epis, S., D'Amelio, S., Ferrari, N., Lanfranchi,
392 P., 2010. Genetic variability of *Haemonchus contortus* (Nematoda:
393 Trichostrongyloidea) in alpine ruminant host species. J. Helminthol. 84, 276–283.
394 Chilton, N.B., Newton, L.A., Beveridge, I., Gasser, R.B., 2001. Evolutionary relationships of
395 trichostrongylo- nematodes (Strongylida) inferred from ribosomal DNA sequence
396 data. Mol. Phylogenet. Evol. 19, 367–386.
397 Colditz, I.G., Le Jambre L.F., Hosse,R., 2002. Use of lectin binding characteristics to
398 identify gastrointestinal parasite eggs in faeces. Vet Parasitol. 105, 219–227.
399 Dikmans, G., Andrews, J.S., 1933. A comparative morphological study of the infective larvae
400 of common nematodes parasitic in the alimentary tract of sheep. Trans. Am. Microsc.
401 Soc. 52, 1–25.
402 Eve, J.H., Kellogg, F.E., 1977. Management implications of abomasal parasites in
403 southeastern white-tailed deer. J. Wildl. Manage. 41, 169–177.
404 Gasser, R.B., Rossi, L., Zhu, X., 1999. Identification of *Nematodirus* species (Nematoda:
405 Molineidae) from wild ruminants in Italy using ribosomal DNA markers Int. J.
406 Parasitol. 29, 1809–1817.

Gasser, R.B., Hu, M., Chilton, N.B., Campbell, B.E., Jex, A.J., Otranto, D., Cafarchia, C., Beveridge, I., Zhu, X., 2007. Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation**.** Nature Protoc**.** 1, 3121**–**3128**.**

Gharamah, A.A., Azizah, M.N., Rahman, W.A., 2012. Genetic variation of *Haemonchus contortus* (Trichostrongylidae) in sheep and goats from Malaysia and Yemen. Vet. Parasitol. 188, 268–276.

Gibbs, H.C., 1986. Hypobiosis in parasitic nematodes – an update. Adv. Parasitol. 25, 129–174.

Gibbons, L.M., 1979. Revision of the genus Haemonchus Cobb, 1898 (Nematoda: Trichostrongylidae). Syst. Parasitol. 1, 3–24.

Hoste, H., Chilton, N.B., Gasser, R.B. and Beveridge, I. 1995. Differences in the second internal transcribed spacer (ribosomal DNA) between five species of *Trichostrongylus* (Nematoda: Trichostrongylidae) Int. J. Parasitol. 25, 75-80.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Keith, R.K., 1953. The differentiation of the infective larvae of some common nematode parasites of cattle. Aust. J. Zool. 1, 223–235.

Kenyon, F., Sargison, N.D., Skuce, P.J., Jackson, F., 2008. Sheep helminth parasitic disease in south eastern Scotland arising as a possible consequence of climate change. Vet. Parasitol. 163, 293–297.

Jacquiet, P., Cabaret, J., Thiam, E., Cheikh, D., 1998. Host range and the maintenance of *Haemonchus* spp. in an adverse arid climate. Int. J. Parasitol. 28, 253–261.

Jex, A.R., Waeschenbach, A., Hu, M., van Wyk, J.A., Beveridge, I., Littlewood, D.T. and Gasser, R.B., 2009. The mitochondrial genomes of *Ancylostoma caninum* and

Bunostomum phlebotomum--two hookworms of animal health and zoonotic importance. BMC Genomics 10, 79

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

Le Jambre, L.F., 1981. Hybridisation of Australian *Haemonchus placei* (Place, 1893), *Haemonchus contortus cauygensis* (Das and Whitlock, 1960) and *Haemonchus contortus* (Rudolphi, 1803) from Louisiana. Int. J. Parasitol. 11, 323–330.

Morgan, E.R, van Dijk, J. 2012. Climate and the epidemiology of gastrointestinal nematode infections of sheep in Europe. Vet. Parasitol. 189, 8–14.

Newton, L.A., Chilton, N.B., Beveridge, I. and Gasser, R.B., 1998. Systematic relationships of some members of the genera *Oesophagostomum* and *Chabertia* (Nematoda: Chaberti-ae) based on ribosomal DNA sequence data Int. J. Parasitol. 28, 1781–1789.

O'Connor, L.J., Walkden-Brown, S.W., Kahn, L.P., 2006. Ecology of the free-living stages of major trichostrongylid parasites of sheep. Vet. Parasitol. 142, 1–15.

Palmer, D.G., McCombe, I.L., 1996. Lectin staining of trichostrongylid nematode eggs of sheep: Tapid identification of *Haemonchus contortus* eggs with peanut agglutinin. Int. J. Parasitol. 26, 447–450.

Posada, G., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Riggs, N.L., 2001. Experimental cross-infections of *Haemonchus placei* (Place, 1893) in sheep and cattle. Vet. Parasitol. 94, 191–197.

454 Roberts, FHS., Newton-Turner, H., McKevett, M. 1954. On the specific distinctness of the
455 ovine and bovine 'Strains' of *Haemonchus contortus* (Rudolphi) Cobb (Nematoda:
456 Trichostronglidae). Aust. J. Zool. 2, 275–295.
457 Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under
458 mixed models. Bioinformatics 19, 1572–1574.
459 Stevenson, L.A., Chilton, N.B., Gasser, R.B., 1995. Differentiation of *Haemonchus placei*
460 from *H. contortus* (Nematoda: Trichostrongylidae) by the ribosomal DNA second
461 internal transcribed spacer. Int. J. Parasitol. 25, 483–488.
462 Veglia, F. 1916. The anatomy and life history of the *Haemonchus contortus* (Rud.). The third
463 and Fourth Reports of the Director of veterinary Research , Department of
464 Agriculture, Union of South Africa, pp. 349-500.
465 Whitlock, H.V., 1948. Some modifications of the McMaster helminth egg counting technique
466 and apparatus. J J. Counc. *Sci.* Ind. Res. (Australia) 21, 177–180.
467 Young, R.R., 1983. Populations of free-living stages of *Ostertagia ostertagi* and *O.*
468 *circumcincta* in a winter rainfall region. Aust. J. Agric. Res. 34, 569–581.

469

470 **Figure legends**

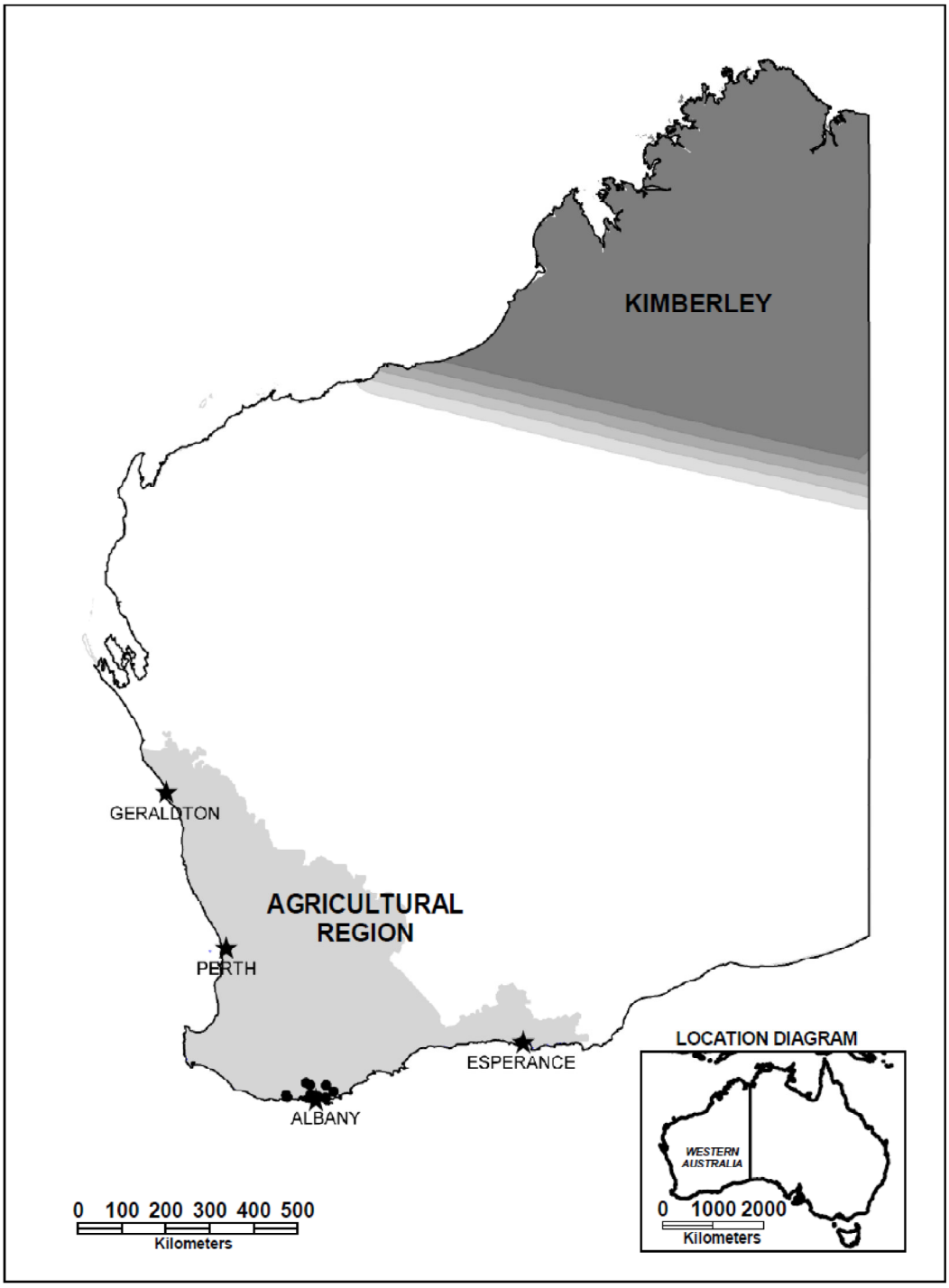
471

472 **Fig. 1.** Map showing geographical location of the study area in the agricultural region in
473 Western Australia. Black dots in this area near Albany show the location of farms included in
474 the study. An endemic area, i.e., Kimberly region, in the state endemic for *Haemonchus*
475 *placei* is also shown. Inset shows an Australian map.

476

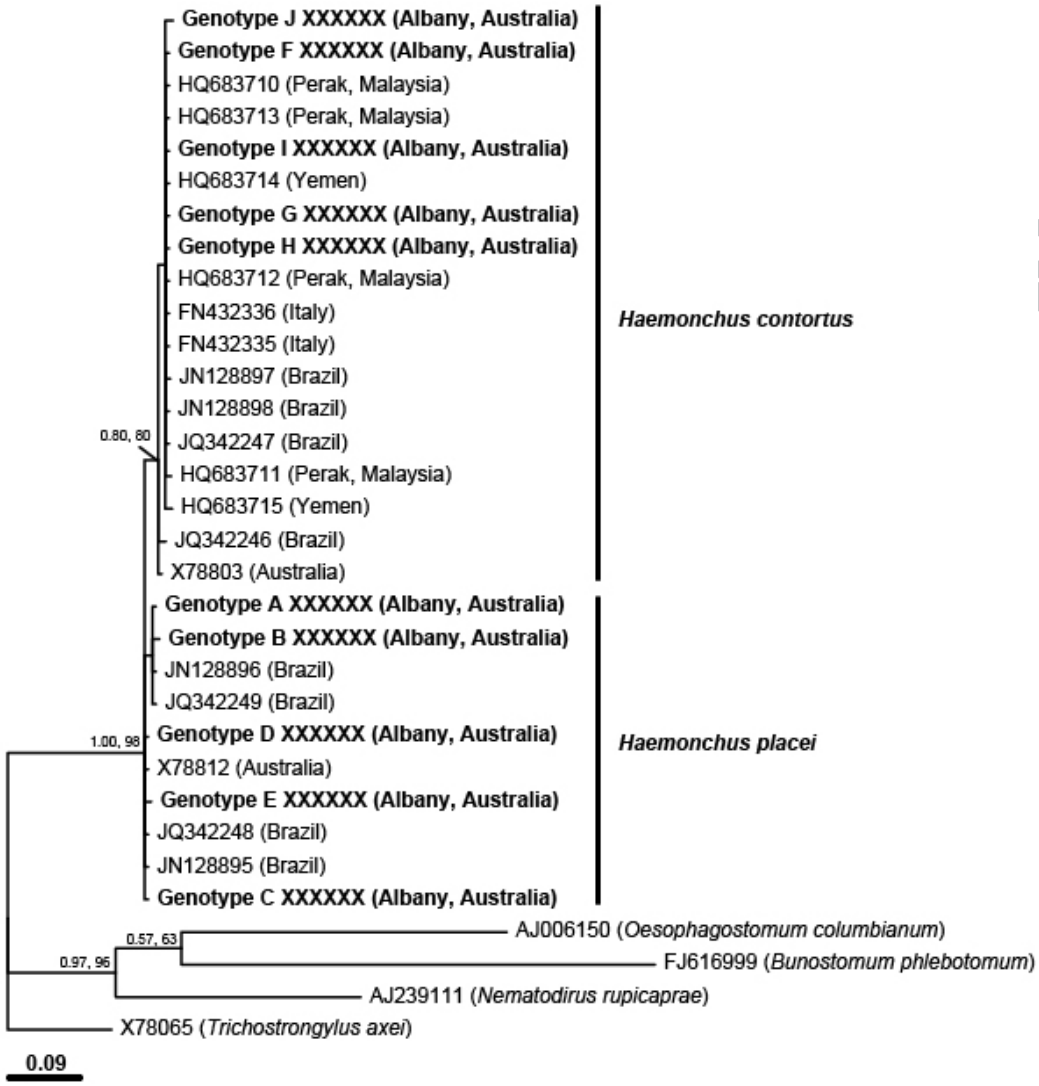
477 **Fig. 2.** Phylogenetic analysis of the ITS-2 sequence data representing *Haemonchus* spp. from
478 the southern Western Australia (present study: bold-type) and sequence data for previously
479 published sequences. *Bunostomum phlebotomum*, *Nematodirus rupicaprae*,
480 *Oesophagostomum columbianum* and *Trichostrongylus axei* represent outgroups. Bayesian
481 inference (BI) and neighbour-joining (NJ) methods were used to infer phylogenetic
482 relationships. Nodal support is given as a posterior probability (pp) for BI (top) and bootstrap
483 value for NJ (bottom). The scale bar indicates distance.

484



485
486
487 **Fig. 1.**

488



489

490

491 **Fig. 2.**

493 **Table 1**

494 Characteristics of beef cattle farms near Albany and Denmark sires, Western Australia

Farm No. Local location	Breed of cattle (age in months)	Introduction of cattle in the last five years and/or earlier	Sheep on and/ or adjoining farms	Communal grazing[a]
1 Marbellup	Red Angus/Sussex/ South Devon weaners (~8 -10 months)	Original cows from within agricultural (Ag) region. Bulls sourced from within Ag region.	Sheep were run concurrently until 10 years ago	None
2 Narrikup	Red Angus weaners (~ 8-10 months)	Kimberley cattle brought to Ag region (1994), transferred to current property12 months later. Other cattle sourced from within Ag region. Semen imported	Sheep were run concurrently until 1995	None - occasional ovine or bovine stray
3 Denbarker	Murray Grey weaners (~8-10 months)	Original cows from within Ag region. Bulls sourced within Ag region.	Sheep were run concurrently until 5 years ago	None
4 Kentdale	Angus and Murray Grey weaners (~8-10 months)	Kimberley cattle brought on in 1993. Bulls sourced from within the Ag region.	No sheep and no neighbors with sheep	None
5 Narrikup	Angus weaners (~8-10 months)	Original cows from within Ag region, 1995.	Small sheep flock run concurrently and goats run on adjoining farm	Yes, periodically with goats on adjoining farm
6 Young's Siding	Murray grey/Angus /Simmental weaners (~8-10 months)	Original cows from within Ag region. Bulls sourced from within the Ag region.	Sheep were run concurrently until 5 years ago	none
7 Kalgan	Hereford weaners (~8-10 months)	Original cows from within Ag region. Bulls sources from within the Ag region.	Sheep are run concurrently	None

495 [a] Grazing with other animals, including sheep, goats, alpacas and horses; .

Table 2

The classification of adult *Haemonchus* spp. specimens based on single-strand conformation polymorphism (SSCP) profiles for the ITS-2 used in the present study. The sequence linked to each unique SSCP profile is represented by its GenBank accession number, its length, polymorphism and G+C content. Mean nucleotide frequencies for the main sequence types are also provided.

Genotype			**ITS-1**						
	SSCP profile (no. of samples with this profile)	Accession no.	Length (bp)	Polymorphism[a] (alignment position)	GC content (%)	Mean nucleotide frequencies			
						A	C	G	T
A	Hp-1 (28)	KF364623	231	---	32.90	0.30303	0.16017	0.17749	0.35931
B	Hp-2 (47)	KF364624	-do-	C/G (21)	32.90	---	---	---	---
C	Hp-3 (1)	KF364625	-do-	R (24), T/A (65), Y (103), R (219)	32.90	---	---	---	---
D	Hp-4 (1)	KF364626	-do-	T/A (65)	32.90	---	---	---	---
E	Hp-5 (1)	KF364627	-do-	C/G (21), T/A (65)	33.33	---	---	---	---
F	Hc-1 (7)	KF364628	231	---	33.77	0.30736	0.15152	0.17749	0.36364
G	Hc-2 (1)	KF364629	-do-	W (196)	33.77	---	---	---	---
H	Hc-3 (1)	KF364630	-do-	G/C (21)	32.47	---	---	---	---
I	Hc-4 (2)	KF364631	-do-	T/A (196)	33.77	---	---	---	---
J	Hc-5 (3)	KF364632	-do-	T/C (22)	33.77	---	---	---	---
Total (nematodes)	**92**								

[a]Polymorphism for each sequence type was assessed by aligning these sequences with the reference sequences (Stevenson et al., 1995). R = A/G; K = G/T; S = C/G; Y = C/T

505 **Highlights**

506 • *Haemonchus placei* is an abomasal parasite of cattle
507 • This parasite primarily occurs in summer rainfall areas of the world
508 • Here we show that *H. placei* also occurs in a winter rainfall area of Australia

509