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3 ORIGINAL PAPER

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5 **Description of *Cloacina atthis* sp. nov. from the stomach of the euro**

6 **(*Macropus robustus*) (Marsupialia: Macropodidae) from Western**

7 **Australia based on morphological and molecular criteria**

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9 **Ian Beveridge • Hanh Nguyen • Simon Nyein • Chung Cheng • Anson Koehler •**
10 **Mary E. Shuttleworth • Robin B. Gasser • Abdul Jabbar**

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15 Nucleotide sequence data reported in this paper are available in the GenBank database

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18 Ian Beveridge (✉) • Hanh Nguyen • Simon Nyein • Allan Cheng • Anson Koehler •
19 Mary E. Shuttleworth • Robin B. Gasser • Abdul Jabbar
20 Faculty of Veterinary Science, The University of Melbourne, Victoria, Australia
21 e-mail: ibeve@unimelb.edu.au

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26 **Abstract** A new species of the strongyloid nematode from the genus *Cloacina*
27 (Chabertiidae: Cloacininae) is described from the stomach of the hill kangaroo or euro
28 (*Macropus robustus*) (Marsupialia: Macropodidae) from Western Australia. *Cloacina*
29 *atthis* sp. nov. was found only in euros from the Pilbara region in the northwest of
30 Western Australia, in spite of extensive collecting of the same host species from around
31 the Australian continent. *Cloacina atthis* is most closely related to *C. clymene*, a species
32 found in the same host species but only in the eastern half of the continent; the two
33 species differ in minor morphological features (the shape of the wall of the buccal
34 capsule, spicule lengths, the degree of sclerotization of the gubernaculum and the shape
35 of the vagina) as well as in differences in the internal transcribed spacers of ribosomal
36 DNA. This study highlights the importance of using molecular methods when
37 investigating the apparently disjunct distributions of strongyloid nematodes.

38

Introduction

Strongyloid nematodes occur primarily in the large intestines of vertebrates, often occurring in large numbers and forming species flocks, with multiple congeneric or conspecific species found in a single host (Kennedy and Bush 1992). The most outstanding example and the strongylid parasites of the large intestine of equids, with 19 genera and 64 species belonging to the family Strongylidae currently described (Lichtenfels et al. 2008). Similarly complex associations occur in the large intestines of elephants and rhinoceroses (four genera and 49 species belonging to the strongyloid tribes Quiloninea, Kiluluminea and Murshidiinea) (Chabaud 1957, Round 1968, Beveridge and Jabbar 2013) as well as in tortoises (Lichtenfels and Stewart 1981), although the latter communities have been less well documented. Recent studies have shown the importance of molecular as well as morphological data in characterising species within this group with the increasing recognition of the existence of cryptic species (Hung et al. 1997, 1999; McLean et al, 2012; Beveridge and Jabbar 2013).

Kangaroos and wallabies (family Macropodidae) harbour an extremely diverse fauna of strongyloid nematodes in their sacculated forestomachs (Beveridge and Spratt 1996) all belonging to the Chabertiidae and currently comprising 36 genera and 292 species (Appan et al, 2004, Beveridge, 1999, 2001a,b, Beveridge and Chilton 2001, Beveridge and Speare 1999, Chilton et al, 2002, Huby-Chilton et al, 2002). However, recent studies suggest that molecular methods are likely to reveal larger numbers of morphologically cryptic species within these hosts (Chilton et al, 2009, Tan et al, 2012).

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63 Of the currently recognised genera, the genus *Cloacina* is the most highly speciose with
64 110 species described to date (Beveridge 1998, 1989; Beveridge and Speare 1999;
65 Beveridge 2002), but with additional species awaiting description (Beveridge 1998). Of
66 the various kangaroos and wallabies, the hill kangaroo, variously known as the euro or
67 wallaroo (*Macropus robustus*) is host to at least 19 species of *Cloacina* (Beveridge 1998),
68 again with some additional species awaiting description. *Macropus robustus* is one of the
69 most widely distributed species of kangaroos (Van Dyck and Strachan 2008). Four
70 subspecies are recognised, with *M. robustus robustus* occurring along the eastern coast of
71 Australia, *M.r. woodwardi* restricted to the tropical north and northwest, *M.r. erubescens*
72 occurring across much of the remainder of inland Australia and *M.r. isabellinus* restricted
73 to Barrow Island off the coast of Western Australia (Clancy and Croft 2008). The
74 parasites of *M. robustus* from central and northern Queensland have been investigated by
75 Beveridge et al. (1998), but records of its helminth parasites from other parts of the
76 continent are limited.

77 Recent collections of parasites from *M. robustus* in the Pilbara region (north west) of
78 Western Australia resulted in the recovery of specimens initially attributed to *C. clymene*,
79 but which differed in some relatively minor morphological features. Subsequently, a
80 preliminary examination of DNA sequence data suggested that these specimens
81 represented a distinct species, an hypothesis supported by further morphological
82 examination. Here, we describe a new species, closely related to *C. clymene*, based on
83 both morphological and molecular evidence, and address the question of the possible
84 evolution of such geographically isolated sister species.

Materials and methods

Morphological identification

Nematode specimens were collected either from fresh road kills or from animals shot commercially. Nematodes were washed in saline and were either frozen for molecular studies or were fixed in Berland's fluid (95% glacial acetic and 5% formaldehyde) and stored in 70% for morphological studies. Specimens fixed for morphological studies were subsequently cleared in lactophenol, and illustrations were made using a drawing tube attached to an Olympus BH-2 microscope. Measurements are presented in millimetres as the mean and range for 10 specimens. Specimens of the new species have been deposited in the Western Australian Museum, Perth, Western Australia (WAM), the South Australian Museum, Adelaide, South Australia (SAM) and The Natural History Museum, London (BMNH), UK. In illustrations of the apical views of the cephalic ends of nematodes, the dorsal aspect is oriented towards the top of the page. The format of the description and the use of morphological terminology for the genus follow Beveridge (1998). In addition, following Beveridge (1998), the new species is named after a classical deity.

Molecular characterisation

For molecular studies, frozen specimens of *C. atthis* sp. nov. ($n = 31$) and *C. clymene* ($n = 17$) were dissected and a mid body region (3-4 mm) removed for molecular analysis. The anterior and posterior regions of the body were cleared in lactophenol to confirm the identification of the species, and representatives of these specimens have been deposited in SAM (46963-5). Genomic DNA was isolated from individual nematodes by suspending them in DNA extraction buffer (20 mM Tris-HCl [pH 8.0], 100 mM ethylenediaminetetraacetic acid, and 1% sodium dodecyl-sulphate) containing 10 mg/ml proteinase K (Amresco LLC, Solon, OH, USA) and isolated from the homogenised suspension using a mini-column (Wizard[®]DNA Clean-Up System, Promega, Madison, WI, USA) according to the manufacturer's protocol. Two nuclear ribosomal loci (ITS-1 and ITS-2) were PCR-amplified using the primer pairs NC13-NC16 and NC1-NC2, respectively (Chilton 2004). For each set of PCRs, negative (no-DNA) and known positive controls were included.

Both ITS-1 and ITS-2 amplicons were subjected (separately) to PCR-based single-strand conformation polymorphism (SSCP) analysis (Gasser et al. 2006; protocol B) to display sequence variation within and among amplicons. For each locus, amplicons representing each unique SSCP profile were subjected to bi-directional, automated sequencing using (separately) the same primers employed in PCR. The quality of each sequence was assessed by appraising its electropherogram using the program BioEdit (Hall 1999). Polymorphic sites were designated using International Union of Pure and Applied Chemistry (IUPAC) codes.

Phylogenetic analyses

The ITS-1 and/or ITS-2 sequences of *C. atthis* and *C. clymene* were aligned with reference sequences for closely related species (i.e. *Cloacina caenis*, *Cloacina cloelia*, *Cloacina ernabella*, *Cloacina pearsoni*, and *Cloacina robertsi* as outgroups) using the program Clustal X (Thompson et al. 1997). The alignments were adjusted manually employing the program BioEdit, and phylogenetic analyses were performed on individual (ITS-1 or ITS-2) or concatenated (ITS-1 + ITS-2) sequence datasets. Each concatenated sequence was derived from the same individual nematode. Following initial phylogenetic analysis, only *C. cloelia* was used as an outgroup. Analyses were conducted by Bayesian inference (BI), employing the Markov chain Monte Carlo (MCMC) method in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The likelihood parameters for BI were based on the Akaike Information Criteria (AIC) test in Modeltest v3.7 (Posada and Crandall 1998). For all three datasets, AIC revealed the general time-reversible model of evolution, with gamma-distribution and a proportion of invariable sites (GTR+ Γ + I), as the ‘best’ model. Estimates of the base frequencies, the substitution rate model matrix and the proportion of invariable sites were fixed. Posterior probabilities (pp) were calculated using 2,000,000 generations, employing four simultaneous tree-building chains, with every 100th tree being saved. At this point, the potential scale reduction factor approached one, and the standard deviation of split frequencies was <0.01. A consensus tree (50% majority rule) was constructed based on the final 75% of trees generated by BI. Sequence data were also analysed using the Neighbour-Joining (NJ) method employing PAUP (PAUP 4.0b10) where molecular distances were estimated by the general time-reversible model of evolution, and the nodes were tested for

robustness by 100,000 bootstrap replicates (Felsenstein 1985). Phylogenetic trees constructed using the BI and NJ methods were examined for concordance in topology.

Geographical data

To establish the geographical distributions of both *C. atthis* sp. nov. and *C. clymene*, all records from SAM together with data from Beveridge et al. (1998) as well as unpublished collection data were compiled. In instances where several collection localities were proximate (e.g., station properties in the region of Charters Towers, Queensland) records of occurrence were combined. For the new species, all collections were made either north or south of the Fortescue River Roadhouse in the Pilbara region of Western Australia or south of Roebourne in the same area. For these collections, only two principal sites are indicated (Fig. 13).

Results

Cloacina atthis Beveridge sp. nov. (Figs. 1-12)

Description

Small nematodes; cuticle not inflated in oesophageal region; transverse annulations prominent, 0.025 apart. Submedian cephalic papillae prominent, 0.018 long, projecting anteriorly from peri-oral cuticle; distal segment ovoid, 0.008 long; proximal segment cylindrical, 0.010 long; 2 amphids present. Buccal capsule shallow, symmetrical in lateral views; thick walled, walls spindle shaped and inclined obliquely in dorso-ventral and lateral views, capsule slightly elongated dorso-ventrally in apical views; anterior margin of buccal capsule sinuous. Dorsal oesophageal tooth absent. Leaf crown elements 6 in number with inconspicuous tips; peri-oral cuticle associated with leaf crown elements

greatly inflated into lip-like structures. Oesophagus claviform, without pre-neural swelling; without internal denticles; bosses absent in internal lining. Nerve ring in mid-oesophageal region or slightly posterior to it; deirid immediately posterior to buccal capsule; excretory pore posterior to oesophago-intestinal junction.

Male. Measurements of 10 specimens, types. Length 13.4-16.4 (15.2); maximum width in mid-body 0.50-0.63 (0.59); buccal capsule 0.080-0.100 (0.091) wide, 0.030-0.045 (0.037) deep; oesophagus 0.72-0.81 (0.75) long; nerve ring 0.40-0.50 (0.47), excretory pore 0.86-1.20 (1.08) and deirids 0.10-0.15 (0.12) from anterior extremity. Spicules 4.00-4.63 (4.22) long; gubernaculum 0.03-0.120 (0.060) long. Bursa with virtually no divisions between lobes; dorsal lobe without median indentation; dorsal lobe not elongate but swollen in lateral and apical views; ventro-ventral and ventro-lateral rays apposed, reaching margin of bursa; externo-lateral ray stout, divergent from lateral trunk, not reaching margin of bursa; medio-lateral and postero-lateral rays apposed, reaching margin of bursa, externo-dorsal ray arises from lateral trunk, not reaching margin of bursa; dorsal ray divides in mid-region; secondary subdivision at $\frac{3}{4}$ length; external branchlets short, directed laterally; internal branchlets longer, not reaching margin of bursa. Genital cone with elongate ventral lobe bearing single papilla and paired short dorsal appendages. Gubernaculum weakly developed, and extremely variable in size, almost invisible in dorso-ventral views. Spicules elongate with transversely striated alae; anterior extremity knobbed; tips pointed, with alae diminishing in width gradually towards tips then enlarging to form flange around tip containing 2 minute branches of spicule.

199

200 Female. Measurements of types. Length 21.2- 23.1 (21.9); maximum width in mid-
201 body 0.77-0.90 (0.85); buccal capsule 0.085-0.105 (0.095) wide, 0.035-0.050 (0.043)
202 deep; oesophagus 0.76-0.96 (0.89) long; nerve ring 0.37-0.50 (0.46), excretory pore 0.85-
203 1.15 (1.05) and deirids 0.10-0.18 (0.13) from anterior extremity; tail 0.26-0.35 (0.30)
204 long, with prominent mucro; vulva 0.61-0.85 (0.69) from posterior end; vagina vera 2.20-
205 2.80 (2.44) long, proximal vagina straight, recurved anteriorly, most proximal region
206 invested with musculature; eggs 0.150-0.160 (0.152) long, 0.070-0.080 (0.077) wide.
207 Female tail short, conical; vagina opens anterior to anus; eggs ellipsoidal.

208

209 **Types:** Holotype and allotype from stomach of *Macropus robustus erubescens*, 57 km
210 NE of Fortescue River Roadhouse, Pilbara Region, Western Australia (WA) (21° 17'S
211 116° 8'E, WAM V8240 and V8241; paratypes: same data, 3 males, 3 females, WAM
212 V8242, 5 males, 7 females SAM 45945, 1 male, 1 female, BMNH 2014.4.14.1-2.

213

214 **Additional specimens examined:** from stomach of *Macropus robustus erubescens*: 2
215 males, 8 females, 6 km NE of Fortescue River Roadhouse, Pilbara Region, WA (SAM
216 46018); 14 males, 18 females, 31 km NE of Fortescue River Roadhouse, Pilbara Region,
217 WA (SAM 45940); 7 males, 9 females, 45 km NE of Fortescue River Roadhouse, Pilbara
218 Region, WA (SAM 45965); 1 male, 6 females, 45 km NE of Fortescue River Roadhouse,
219 Pilbara Region, WA (SAM 45937), 3 males, 9 females, 4 km S of Fortescue River
220 Roadhouse, Pilbara Region, WA (SAM 45950); 2 males, 1 female, 17 km SW of

221 Roeburne, WA (20° 46'S 117° 9'E) (SAM 45928); 8 males, 12 females, 5 km S of
222 Roebourne, WA (SAM 45956).

223

224 **Site in host:** sacculated forestomach

225

226 **Etymology:** this species is named after Atthis, the goddess of rugged coastlines, alluding
227 to the region in which the species was collected.

228

229 **Remarks**

230 The species described here is very similar to but is distinguishable from *C. clymene*. The
231 clavate oesophagus, the six bulbous lips and the morphology of the cephalic papillae
232 indicate its affiliation with this species using the key of Beveridge (1998). However, the
233 specimens described here differ in several significant characteristics. In the male, the
234 spicules of *C. atthis* are shorter than those of *C. clymene* (4.00-4.63 in *C. atthis*, 4.86-5.60
235 in *C. clymene*) and while the gubernaculum is prominent and ovoid in dorso-ventral
236 views in *C. clymene*, it is poorly sclerotised and highly variable in shape in *C. atthis*. The
237 spicule tip described here differs slightly from that of *C. clymene* provided by Beveridge
238 (1998). However, a re-examination of the spicule tips of both species has revealed that
239 they are identical and the description of the spicule tip of *C. clymene* presented here
240 replaces that of Beveridge (1998). In the female of *C. clymene*, the ascending or distal
241 vagina vera has a constant twist or convolution (Beveridge 1998, Fig. 172) which is
242 absent in *C. atthis* (Fig.12). In addition, in both sexes, the shape of the buccal capsule
243 differs. In *C. atthis*, the wall of the buccal capsule is spindle shaped and oriented at an

oblique axis in optical section (Figs. 2 and 3), while in *C. clymene* it is ovoid and oriented sagittally (Beveridge 1998, Figs. 161-163). For these reasons, *C. atthis* is considered to be a species closely related to but independent from *C. clymene*.

Molecular data

DNA from 48 individual nematodes were subjected to PCR-based SSCP analysis of ITS-1 (430 bp) and ITS-2 (275 bp) amplicons of *C. atthis* and *C. clymene*. For *C. atthis*, one profile was displayed for both ITS-1 and ITS-2. For *C. clymene*, one and two different SSCP profiles were displayed for ITS-1 and ITS-2, respectively. The ITS-1 and ITS-2 sequences for *C. atthis* and *C. clymene* were characterised from nine and 15 sequenced samples, respectively. The features and accession numbers of sequences are listed in Table 1. Pairwise comparisons revealed sequence differences of 10.3% (ITS-1) and 8.6% (ITS-2) between *C. atthis* and *C. clymene*.

The concatenated ITS-1 + ITS-2 sequences representing *C. atthis* and *C. clymene*, respectively, were aligned over 608 positions with a reference sequence of *C. cloelia* (FM992612; outgroup) and subjected to phylogenetic analyses. Sequences of *C. atthis* and *C. clymene* formed monophyletic clades with strong nodal support (pp = 1.0; bootstrap value = 100%) (Fig. 14). Separate analyses of ITS-1 and ITS-2 sequence datasets produced trees with identical topologies to that generated for the concatenated ITS-1 + ITS-2 dataset (not shown). In addition, the topology of the trees constructed using the two different algorithms (BI and NJ) was the same (see Fig. 14).

Discussion

Until now, *C. clymene* has been found only in *M. robustus* in eastern Australia (eastern Queensland and New South Wales), but has not been found in extensive collections of the species from the Northern Territory, South Australia or the south of Western Australia (Fig. 13). In eastern Australia, it has been found principally in *M. r. robustus*, with a single male collected from *M. r. erubescens* in western New South Wales (SAM 33852). In northern Queensland, the precise boundary between the two host subspecies is unclear (Richardson and Sharman 1976). In New South Wales, the two subspecies differ distinctly in colour, whereas in northern Queensland, they are only differentiable using biochemical methods (Richardson and Sharman 1976). Most samples of *C. clymene* are probably from *M. r. robustus*, but some of the more western collections in Queensland may be from *M. r. erubescens*. Further studies of the hosts in this region will be needed to clarify the situation. The occurrence of a sister species to *C. clymene* as a highly disjunct population in *M. r. erubescens* in the Pilbara region of Western Australia cannot be explained on the basis of the pattern of subspeciation of the hosts, although the genetic structure of populations of *M. robustus* has not yet been examined in detail (Eldridge et al. 2014). The subspecies *M. r. isabellinus* is limited to Barrow Island off the Western Australian coast close to the localities at which the new species was collected. However, the Barrow Island subspecies is morphologically distinct from the mainland forms and has not been examined for parasites. A recent genetic study (Eldridge et al. 2014) suggests that *M. r. isabellinus* is closely related to *M. r. erubescens* on the adjacent mainland but that the differentiation between *M. r. robustus* and *M. r. woodwardi* arose

290 due a Pleistocene barrier. Additional studies are required to identify genetic groupings
291 within *M. r. erubescens* in Western Australia. Other molecular studies of the parasitic
292 nematodes (*Hypodontus macropi* and *Labiosimplex longispicularis*) and cestodes
293 (*Progamotaenia festiva* and *P. macropodis*) of *M. robustus* have revealed genetic
294 differences in these parasites concordant with the pattern of speciation of the host,
295 although difficulties in identifying subspecies in northern Queensland exist in each of
296 these studies (Chilton et al. 2011, 2012; Beveridge et al. 2007; Hu et al. 2005).

297 Smales (2011) described *Labiosimplex camporum* as a new species from the same
298 hosts as *C. atthis*, although in this instance, the nematode was also found in sympatric red
299 kangaroos, *Macropus rufus*. As is the case with *C. atthis*, *L. camporum* has only been
300 found at this single locality, even though its presence would have been noticed at all other
301 sites at which *M. robustus* was examined for parasites (Fig. 13). Similar extensive
302 surveys of the parasites of *M. rufus* across the continent have not revealed this species at
303 other localities (unpublished data). As a consequence, no obvious explanation for the
304 existence of these distinctive species in the Pilbara region of Western Australia can be
305 advanced, and more detailed genetic studies of the host are needed. Although it appears
306 to be an isolated population, much more extensive sampling of the host in Western
307 Australia is required to determine its precise geographical range. The present study is the
308 second investigation in which sibling or sister species of cloacinine nematodes have been
309 identified from highly disjunct distributions using molecular methods. Tan et al. (2012)
310 demonstrated the existence of a sibling species in *Macropoanema comani*, although in this
311 instance, the primary host was the eastern grey kangaroo, *Macropus giganteus*, and the
312 disjunct population of nematodes was found in the northern wallaroo, *M. r. woodwardi*,

in the Northern Territory. The current study underlines the importance of the use of molecular methods when investigating relatively minor morphological differences between populations of strongyloid nematodes, especially where populations are geographically disjunct. In this instance, it was possible to find morphological differences allowing the erection of a new species. This is not always possible as Tan et al. (2012) found no morphological differences in a geographically disjunct population of *Macropodema comani*. However, such molecular studies are increasingly significant in the delineation of species of strongyloid nematodes (Chilton et al, 2012, Beveridge and Jabbar 2013).

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Conflict of interest

The authors declare that they have no conflict of interest.

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Captions to figures

Figs. 1-12 *Cloacina attheis* sp. nov. from the stomach of *Macropus robustus*. **1** Anterior end, lateral view; **2** Cephalic extremity, lateral view, dorsal aspect on right side; **3** Cephalic extremity, dorsal view; **4** Submedian cephalic papilla; **5** Cephalic extremity, apical view; **6** Optical transverse section through buccal capsule at base of leaf crown elements; **7** Genital cone, dorsal lobe of bursa and gubernaculum, lateral view; **8** Spicule tip; **9** Bursa, apical view; **10** Gubernaculum, ventral view; **11** Female tail, lateral view; **12** Vagina vera and ovejector, lateral view. Scale bars: **1-3, 5-7, 9-12**, 0.1 mm; **4, 8**, 0.01 mm.

Fig. 13 Localities in Australia at which one or more specimens of *Macropus robustus* have been examined for parasites, with the distribution of subspecies indicated following Richardson and Sharman (1976), modified by Clancy and Croft (2008). Closed circles represent localities at which *Cloacina clymene* has been found; closed squares represent sites at which *Cloacina atthis* has been found.

Fig. 14 Phylogenetic relationships of *Cloacina atthis* and *Cloacina clymene* based on concatenated ITS-1 and ITS-2 sequence data determined herein, together with a selected reference sequence for *Cloacina cloelia*. Relationships were inferred based on analyses employing Bayesian Inference (BI) and distance-based Neighbour-Joining (NJ) methods. Nodal support is given as a posterior

463 probability for BI (first) and bootstrap value for NJ (second). The scale bar
464 indicates distance. GenBank accession numbers are shown in parentheses.

Table 1 The classification of *Cloacina* nematode specimens based on single-strand conformation polymorphism (SSCP) profiles for two loci (ITS-1 and 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 two *Cloacina* spp. are also provided.

Parasite species	Genotype	ITS-1										ITS-2									
		SSCP profile (no.of samples with unique profile)	Accession no.	Length (bp)	Polymorphism ^a (alignment position)	GC content (%)	Mean nucleotide frequencies (%)				SSCP profile (no. of samples with unique profile)	Accession no.	Length (bp)	Polymorphism ^a (alignment position)	GC content (%)	Mean nucleotide frequencies (%)					
							A	C	G	T						A	C	G	T		
<i>Cloacina attheis</i> <i>Cloacina clymene</i>	A	P1-1 (27)	XXXXXX XXX	380	---	47.1 1					P2-1 (30)	XXXXXX XXX	223	---	42.1 5	25.11	20.18	21.97	32.94		
	B	P1-2 (14)	XXXXXX XXX	380	---	47.8 9	22.8 9	22.3 7	24.7 4	30.0 0	P2-2 (16)	XXXXXX XXX	219	---	42.4 7	25.11	19.63	22.83	32.43		
	C	-do-	-do-	-do-	---	-do-	22.3 7	23.4 2	24.4 7	29.7 4	P2-3 (1)	XXXXXX XXX	219	R (4)	42.4 7	24.66	19.63	22.83	32.43		
	Total (larvae)	41									47										

^aPolymorphism for each sequence type was assessed by aligning these sequences with the reference sequences. R = A/G; K = G/T; S = C/G; Y = C/T