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Description and molecular characterisation of *Cloacina johnstoni* sp. nov. (Nematoda: Strongyloidea) from the wallaroo, *Macropus robustus* (Marsupialia: Macropodidae) and relationships with the synhospitalic species *C. macropodis*.

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Electronic supplementary material The online version of this article contains supplementary material, which is available to authorized users.

Abstract *Cloacina johnstoni* sp. nov. (Nematoda: Strongyloidea) is described from the sacculated forestomach of the wallaroo, *Macropus robustus* (Marsupialia: Macropodidae) from Australia. It resembles the synhospitalic species *C. macropodis*, but differs from it principally in the shape of the cephalic papillae. The two species are also distinguishable based on differences in the first and second internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA). *Cloacina johnstoni* commonly co-occurs in the same individual host as *C. macropodis*, but has a more restricted geographical distribution.

Keywords *Cloacina* · nematode · new species · wallaroo · marsupial · internal transcribed spacers

Introduction

The nematode genus *Cloacina* is restricted to the sacculated forestomachs of kangaroos and wallabies (family Macropodidae) in Australia and Papua New Guinea, and is currently composed of 116 species (Beveridge 2014; Beveridge et al. 2014a,b), with additional species remaining to be described (Spratt and Beveridge 2015). The common wallaroo and its three mainland subspecies (*M. robustus robustus*, *M. r. woodwardi* and *M. r. erebescens*) harbour 22 species of *Cloacina*, the largest number found in any macropodid host (Beveridge 1998; Beveridge et al. 2014b). Recent collections of *Cloacina* spp. from the common wallaroo in Western Australia, South Australia, the Northern Territory and Queensland have revealed a new species of *Cloacina* similar to, but distinguishable from the synhospitalic species *C. macropodis*.

The new species is described here and is differentiated from *C. macropodis* using both morphological and molecular methods. In addition, the geographical distributions of the two species are compared.

Materials and methods

Morphological identification

Specimens of *Cloacina* spp. were collected opportunistically from fresh, road-killed common wallaroos (*Macropus robustus*) or from hosts that had been shot for commercial harvesting. Individual or pooled nematodes were placed in Eppendorf tubes and kept at -80 °C until required. Additional nematodes were fixed in Berland's fluid (Gibson 1979) and then transferred to 70% ethanol prior to morphological identification.

Specimens were cleared in lactophenol prior to morphological examination and illustrations were made using a drawing tube attached to an Olympus BH-2 microscope (Olympus Optical Co., Shinjuku-ku, Tokyo, Japan). All measurements were made with an ocular micrometer and are presented in millimetres (mm) with the range for 15 specimens followed by the mean in parentheses. In illustrations of the apical views of the cephalic ends of nematodes, the dorsal aspect is oriented towards the top of the page. In the case of the bursa, the ventral lobes are uppermost. The format of the description and the use of morphological terminology for the genus follow Beveridge (1998).

Holotype and allotype specimens of the new species have been deposited in the South Australian Museum, Adelaide, South Australia (SAM). Paratypes have been deposited in SAM and in The Natural History Museum, London (BMNH), UK. Host nomenclature follows van Dyck and Strahan (2008). The distribution of the subspecies shown in van Dyck and Strahan (2008) is approximate only (Clancy and Croft 2008).

Molecular characterisation

Specimens of *C. macropodis* and of the new species were selected for molecular investigations. The anterior and posterior ends of each nematode were excised and cleared in lactophenol for morphological identification before being stored in 70% ethanol and retained as voucher specimens (SAM 47616-47621 and 47638-47640, respectively). The remaining midsection of each worm was stored in Eppendorf tubes containing approximately 0.5 µl of H₂O and frozen until molecular identification.

Genomic DNA (gDNA) was isolated by suspending worms in a 200µl of 20mM Tris-HCL (pH 8.0), 100 mM EDTA and 1% sodium dodecyl-sulphate containing 10 mg/ml proteinase K (Amresco Inc., Solon, Ohio, USA) and incubating at 37 °C for 16 hours. Genomic DNA was column-purified from the homogenised suspension using the Wizard®

DNA Clean-Up Kit (Promega, Madison, WI, USA), following the manufacturer's recommendations.

The first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) of nuclear ribosomal DNA (rDNA) were amplified from genomic DNA using the polymerase chain reaction (PCR). The primers used were NC16/NC13R for ITS-1, and NC1/NC2 for ITS-2 (Chilton, 2004). PCRs were conducted in 50 µl volumes containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl (Promega, USA), 3.5 mM MgCl₂, 250 µM of each deoxynucleotide triphosphate (dNTP), 25 pmol of each primer, and 1 U of *GoTaq* polymerase (Promega). PCR cycling conditions were 94 °C for 5 min, then 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec, followed by 72 °C for 5 min. Negative and positive controls were included in each PCR.

PCR products were then treated with shrimp alkaline phosphatase and exonuclease I (Germentas Inc., USA) (Werle et al. 1994), and subjected to bi-directional, automated sequencing (BigDye® Terminator v.3.1, Applied Biosystems, California, USA) using (separately) the PCR primers. The quality of nucleotide sequences was appraised using the program Geneious Pro 5.6.5 (Biomatters Ltd., Auckland, New Zealand) (Drummond et al. 2011). Sequences were aligned using Clustal X version 2.0.10 (Larkin et al. 2007) and adjusted manually using the programs BioEdit (Hall, 1999) and MEGA 6.0 (Tamura et al. 2013). Pairwise differences were calculated in MEGA using default settings.

Geographical distribution

Due to their close morphological similarities, distributions of both the new species and *C. macropodis* were mapped onto the host range, with localities for *C. macropodis* being obtained from specimens deposited in SAM (Supplementary Table 1). Abbreviations and

contractions of Australian state and territory names used were: NT: Northern Territory; Qld: Queensland; SA: South Australia; WA: Western Australia.

Results

Cloacina johnstoni sp. nov. (Figs. 1-12)

Description

Robust, medium-sized nematodes; cuticle scarcely inflated to level of excretory pore; sub-median papillae minute (0.006), projecting anteriorly from peri-oral cuticle; distal segment ovoid, short, 0.002 long; cylindrical proximal segment short, 0.004 long; lips absent; buccal capsule shallow, heavily sclerotized, sub-symmetrical in lateral and dorsal views, sub-circular in cross-section, without striations; 6 leaf crown elements, not striated; dorsal oesophageal gland duct opens in buccal capsule in conical projection; each ventral sector of oesophagus with small sclerotized cone projecting into buccal capsule; oesophagus simple, claviform, without pre-neural swelling, lining with faint sclerotized bosses extending from anterior extremity to level of nerve ring, terminating in faint, comb-like pattern; denticles absent; nerve ring in mid-oesophageal region; deirid in anterior oesophageal region; excretory pore in region of oesophago-intestinal junction.

Male. Length 6.20–7.58 (6.89); maximum width at mid-body 0.19–0.31 (0.25), buccal capsule 0.075–0.120 (0.098) wide, 0.030–0.042 (0.036) deep; oesophagus 0.39–0.46 (0.43) long; nerve ring 0.20–0.25 (0.23), excretory pore 0.36–0.44 (0.40) and deirid 0.08–0.18 (0.10) from anterior end; spicules 2.95–4.10 (3.53) long; gubernaculum 0.03–0.07 (0.05) long. Bursa sub-circular in apical view, divisions between lobes subtle; ventro-ventral and ventro-lateral rays apposed, reaching margin of bursa; externo-lateral ray divergent, not

reaching margin of bursa; medio-lateral and postero-lateral rays apposed, reaching margin of bursa; externo-dorsal ray arising from lateral trunk, not reaching margin of bursa; dorsal ray variable, divides at one-quarter of length into 2 arcuate branches, second division mid-length; major internal branches elongate and slender, almost reach bursal margin; gubernaculum sub-triangular in dorso-ventral view; spicule tips sharp, with transversely striated ala diminishing in width towards tip; anterior lip of genital cone conical; posterior lip genital cone with paired lateral inflations.

Female. Length 7.34–9.7 (8.52); maximum width in mid-body 0.31–0.41 (0.36); buccal 0.075–0.120 (0.098) wide, 0.030–0.042 (0.036) deep; oesophagus 0.46–0.58 (0.52) long; nerve ring 0.22–0.27 (0.25), excretory pore 0.34–0.47 (0.41) and deirid 0.08–0.13 (0.10) from anterior end; tail 0.17–0.22 (0.20) long; vulva 0.37–0.43 (0.40) from posterior end; vagina vera 1.15–1.58 (1.37), elongate and recurrent; eggs 0.11–0.17 (0.14) long, 0.060–0.080 (0.070) wide. Female tail short, conical; vagina opens anterior to anus; eggs ellipsoidal.

Types: Holotype and allotype, Mulga Park Station via Alice Springs, Northern Territory, Australia (25°63'S 131°79'E) in SAM (47659–47660); paratypes same data, 78 ♀♀, 43 ♂♂, SAM 47661, BMNH 2015.9.10.1–2.

Host: *Macropus robustus* Gould, 1840 (Marsupialia: Macropodidae)

Other material examined: From *Macropus robustus*: Queensland: 23 ♀, 10 ♂, 76 km west of Cloncurry (SAM 47650–47651); 30 ♀, 17 ♂, 70 km west of Cloncurry (SAM 47653); 2 ♀, 52 km west of Cloncurry (SAM 47645); 14 ♀, 15 ♂, Devoncourt Station via Cloncurry (SAM

47649, 47657-47658); 1♀, 1♂, Hillgrove Station via Charters Towers (SAM 47654); Western Australia: 12♀, 4♂, Kalgoorlie (SAM 47647-47648); 18♀, 18♂, Menzies (SAM 47643); 9♀, 2♂, Yalgoo (SAM 47644-47646); 1♀, 1♂, 27 km north of Fortescue River Roadhouse (SAM 47655); 1♀, 6♂, 6 km N of Fortescue Roadhouse (SAM 47652); South Australia: 4♀, Port Augusta (SAM 47656).

Site in host: Sacculated forestomach.

Etymology: This species is named after T.H. Johnston, formerly Professor of Zoology at The University of Adelaide, South Australia, who made a number of collections of *Cloacina* in central Australia under difficult conditions in 1932 and 1933 which were subsequently described by Johnston and Mawson (1938).

Molecular characterisation

The ITS-1 and ITS-2 regions revealed the sequence lengths of ITS-1 for *C. macropodis* and *C. johnstoni* were 379 and 380-2 bp, respectively, and for the ITS-2 region were 218 and 220 bp, respectively (Supplementary Fig. 1). There were a number of nucleotide variations between the two species, with the most notable differences being at positions 127, 200, 236, 256, 349, 351, 356 and 357 for ITS-1 and positions 56, 86, 153, 162, 177 and 179 for ITS-2 (Table 1; Supplementary Fig. 1); *C. johnstoni* had additional nucleotides when compared to *C. macropodis* (Table 1). Pairwise distances calculated between the two species revealed a difference of 3.7% and 5.0% for the ITS-1 and the ITS-2, respectively.

Geographical distribution

Cloacina johnstoni was found in the NT, WA, SA and Qld and was less prevalent and more restricted in its range than *C. macropodis* (Fig. 13). Both species were found in the host subspecies *M. r. erubescens* and *M. r. robustus* but *C. johnstoni* was absent from the two collections from *M. r. woodwardi* containing *C. macropodis*.

Cloacina macropodis was more prevalent along the eastern coast of Australia when compared with *C. johnstoni* with the latter species being equally widespread across the inland and western regions of the continent. *C. johnstoni* was abundant at Cloncurry, Qld, Mulga Park Station, NT and at Kalgoorlie, Menzies and Yalgoo in WA. At each of these localities, it was co-parasitic with *C. macropodis* (16 of a total of 17 wallaroos examined). In north-eastern Queensland, only two specimens of *C. johnstoni* were found in one individual wallaroo near Charters Towers. Eighteen host individuals were examined in this region, suggesting that the parasite is extremely uncommon. Likewise, *C. johnstoni* was found only in a single individual host in the Port Augusta region of SA, in spite of the examination of 15 host individuals. Only small numbers of *C. johnstoni* ($n = 9$) were recovered from wallaroos in the Fortescue River region of WA with only two of ten individual hosts were infected. Here it was not synhospitalic with *C. macropodis*.

Discussion

The new species can be differentiated from all congeners except *C. australis*, *C. aedon*, *C. annulata*, *C. ares*, *C. digitata*, *C. eos*, *C. ips*, *C. macropodis*, *C. papillata*, *C. papillatissima* and *C. polyxo* by its lack of denticles in the oesophagus and lacking lips around the mouth opening. It can be differentiated from *C. annulata*, *C. ares*, *C. digitata*, *C. ips*, *C. papillatissima* and *C. polyxo* by its recurrent vagina. It differs from *C. australis* in lacking elongate bodies lining the anterior oesophagus and from *C. papillata* and *C. eos* in the sub-

symmetrical shape of the buccal capsule. It is possible to differentiate the new species from *C. aedon* by the anterior position of deirid in *C. johnstoni* and the ornamentation in the lining of oesophagus; faint bosses are present in the anterior third in *C. johnstoni*. Similarly, it is possible to differentiate *C. johnstoni* from *C. ares* by the position of the deirid at the level of the nerve ring and the non-recurrent vagina in *C. ares*. The species is most similar to the synhospitalic species *C. macropodis*. However, they differ notably, in the size and shape of the cephalic papillae which are significantly smaller in the new species (0.006 in new species, 0.011 in *C. macropodis*) and with a relatively smaller terminal segment, making the anterior region readily distinguishable between the species. Their morphological similarity therefore suggests that the two species are closely related.

Johnston and Mawson (1938) in their original description of *C. macropodis* included a figure (Fig. 49) of the anterior end showing very short cephalic papillae, somewhat shorter than those illustrated in the redescription of the species by Beveridge (1998) and more closely resembling those of *C. johnstoni*. Beveridge (1998) examined the type specimens of *C. macropodis* but noted that they were in poor condition, with no cephalic papillae on the holotype and with small but very widely spaced papillae on the allotype. Many other morphological features were not discernible. However, examination of the original collection of gastric nematodes from this host animal from Mt Liebig, NT (SAM 2905, 3071) revealed five specimens of *C. macropodis* (SAM 24591), all of which were consistent with the redescription by Beveridge (1998). No specimens of *C. johnstoni* were found. The specimen illustrated by Johnston and Mawson (1938) may have been part of the variation seen in the cephalic papillae of *C. macropodis* or it may have been a specimen of *C. johnstoni* which was mistakenly considered by Johnston and Mawson (1938) to be *C. macropodis*. However, no specimens exist in SAM to verify the latter contention.

The morphological distinction between *C. johnstoni* and *C. macropodis* is further supported by differences in the ITS-1 and the ITS-2 regions of rDNA. They have been shown to be reliable species markers for stronglyloid nematodes (Chilton 2004) and have been utilised in descriptions of *Cloacina* spp. (Beveridge et al. 2014c) previously as well as in genetic analyses of the genus (Chilton et al. 2009; Lott et al. 2014; Shuttleworth et al. 2014). Based on these earlier studies, the current differences in ITS-1 and ITS-2 sequences provide additional evidence for the distinctiveness of the new species.

The biological interest of the species pair, *C. johnstoni* and *C. macropodis*, lies in the fact that, apart from being very similar morphologically, *C. johnstoni* was almost always found in association with *C. macropodis*. The only exception was in the north west of Western Australia (Fortescue River), although *C. macropodis* was present at a nearby site (Marble Bar). *Cloacina macropodis* had a wider geographical distribution and was the commoner species along the eastern coast of Australia. *C. johnstoni* was primarily parasitic in *M. r. erubescens* in the central and western parts of the continent. However, in north-eastern Queensland, the division between the two sub-species *M. r. robustus* and *M. r. erubescens* is not clear (Clancy and Croft 2008) and the single collection of *C. johnstoni* within the nominal range of *M. r. robustus* may simply represent a case of host introgression as there are no geographical barriers between the kangaroo two sub-species.

Sympatric or synxenic speciation has been predicted to be common in parasitic nematodes (Price 1980) but has proved very difficult to demonstrate (McCoy 2003). *Cloacina johnstoni* and *C. macropodis* may represent a pair of species which have evolved by this mechanism. They are essentially restricted to a single host species (*M. robustus*), *C. johnstoni* usually co-occurs with *C. macropodis* both in terms of collection locality and within the same individual host animal, and both species appear to be closely related morphologically and genetically. However, further evidence demonstrating that they are

sister species would be needed to support this argument, and such evidence is not available from the present study. In addition, given the widespread nature of the host and the small number of collections made thus far, additional collecting is needed to confirm the hypotheses presented here.

Acknowledgements

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Compliance with ethical standards Yes.

Conflict of interest The authors declare that they have no conflict of interest.

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Caption to figures

Figs. 1-12. *Cloacina johnstoni* sp. nov. from the wallaroo, *Macropus robustus* (Marsupialia: Macropodidae). **1** Anterior end, lateral view; **2** Cephalic extremity, lateral view; **3** Cephalic extremity, dorsal view; **4** Cephalic papilla, lateral view; **5** Mouth opening apical view showing leaf-crown elements; **6** Transverse optical section through buccal capsule showing dorsal tooth; **7** Gubernaculum, ventral view; **8** Gubernaculum, lateral view. **9** Spicule tip, lateral view; **10** Bursa, apical view; **11** Female tail, lateral view; **12** Vagina and ovejector, lateral view. Scale bars: Figs. 1, 10-12, 0.1mm; Figs. 2-9, 0.01 mm; Figs 7 and 8 to same scale.

Fig. 13. Distribution of *Cloacina johnstoni* (open circles) and *C. macropodis* (closed circles), both gastric parasites of *Macropus robustus*, shown with the distributions of the mainland subspecies of *M. robustus*. Collection data for *C. macropodis* are based on specimens held in SAM (Supplementary Table 1). Localities have been simplified to indicate the closest town to the actual site of collection. Numerals for collection localities of *C. johnstoni* indicate those mentioned in the text: 1 Mulga Park Station; 2 Cloncurry; 3 Devoncourt Station; 4, Charters Towers; 5 Yalgoo; 6 Menzies; 7 Kalgoorlie; 8, 9 regions around Fortescue River Roadhouse; 10, Port Augusta.

Table 1 Variable positions in the aligned ITS-1 and ITS-2 rDNA sequences of *Cloacina macropodis* and *C. johnstoni*. Alignment numbers are given based on the length of the new species sequence.

ITS-1 (alignment position)																						
3 3 3 3																						
5 5 6 7																						
6 7 9 2																						
<i>C. macropodis</i> (n = 9)*		G/R	-	R	C	G	C	G	C	T	T	G	C/Y	G	R	T	C	S	A	C/G	T	
A G A R/W																						
<i>C. johnstoni</i> (n = 1)		A		T	A	T	-	T	A	T	C	C	A	T		C	A	C	-	T	G	T
G A T G																						
ITS-2 (alignment position)																						
<i>C. macropodis</i> (n = 7)		A	C	A	G	C	G	G	-	-	T/K	G	G	G	C	T						
<i>C. johnstoni</i> (n = 5)		T	A	G	C	T	A	C	G	C	G		A	A	A	T	G					

*indicates number of sequences

Electronic Supplementary Material

Description and molecular characterisation of *Cloacina johnstoni* sp. nov. (Nematoda: Strongyloidea) from the wallaroo, *Macropus robustus* (Marsupialia: Macropodidae) and relationships with the synhospitalic species *C. macropodis*.

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Supplementary Table 1 Specimens of *Cloacina macropodis* from *Macropus robustus robustus* and *M. r. erubescens* deposited in the South Australian Museum, Adelaide.

Localities	Voucher nos.
New South Wales	
Coonabarabran	24310, 24646, 25171
Rivertree	25285
Kingstown	25286, 25297
Menindee	10990, 25069, 31991
Thackaringa Stn* via Broken Hill	31975
Enngonia	33848
Northern Territory	
Alice Springs	23546
Mount Liebig	24591, 41356
Queensland	
Charters Towers	25262-3, 25273, 25280, 25287
Warrawee Stn via Charters Towers	24112, 25170, 25267, 25281, 25302, 27533
Harvest Home Stn via Charters Towers	25121, 25266, 25269, 25276-9
Fletcher View Stn via Charters Towers	25274
Pallamana Stn via Charters Towers	25278
Hillgrove Stn via Charters Towers	32532
30 km N of Charters Towers	32486
Barcaldine	24113, 24112, 31617
Augathella	24115, 25252
Mingela	24181, 25271, 25283
Charleville	24308
Jericho	24309, 31593, 31609
Morven	24029
Georgetown	25117
Bluewater Springs	25131
Warwick	25275, 25284
Mount Surprise	25290-1
Oban Stn via Mount Isa	25837-40
Blackall	31641, 31647-8
Devoncourt Stn via Cloncurry	31697, 32502, 32513, 32559
Prairie	32579
Woodbine Stn via Prairie	32359, 32375
Konkool Stn via Hughenden	32391, 32526
Cloncurry	32396, 32406, 32415, 32425
Kangaroo Hills Stn via Lyndhurst	32737

South Australia

Moralana Stn via Hawker	8174
Wonoka Stn via Hawker	25264
Myrtle Springs Stn via Leigh Creek	8319, 252306
Port Augusta	24030, 32010
Spear Creek Stn via Port Augusta	24117, 27534
Nonning Stn via Iron Knob	25270, 25293-4
Melrose	25277
Horrock's Pass, Port Augusta	25282, 25295
Hookina Stn via Hawker	25296
Mount Lyndhurst Stn via Lyndhurst	25298

Western Australia

Marble Bar	4355
Yalgoo	31760, 31774, 32850
Kalgoorlie	31801, 31828
Menzies	45561
Fitzroy Crossing	25292
Lissadell Stn via Kunanurra	25301

*Stn = station

A

	102030405060708090100
<i>C. johnstoni</i> (KU853837)	TCGTGGAACCTTATGGTTCCCTTGATCTTGAGAACCAACATGCTTGTAGCTTCAGGACTTTGTCCGTTGAAGAGTTGGGAGTATCAGCCACCCCTTTT
<i>C. macropodis</i> (KU853834)	R.....
<i>C. macropodis</i> (KU853842)	CG
<i>C. macropodis</i> (KU853856)	
<i>C. macropodis</i> (KU853870)	
<i>C. macropodis</i> (KU853879)	R.....R.....
<i>C. macropodis</i> (KU853881)	
<i>C. macropodis</i> (KU853883)	
<i>C. macropodis</i> (KU853884)	
<i>C. macropodis</i> (KU853885)	G.....
	110120130140150160170180190200
<i>C. johnstoni</i> (KU853837)	AATCCGTTTGAGGTGTCTACGTACAAACATGAGCCGTATCGGATGGCGTCCGTGATTGTGTGCAAGTTGCGGCATTTCATGTGCTGAGCTTCAGACTC
<i>C. macropodis</i> (KU853834)	C.....G.....
<i>C. macropodis</i> (KU853842)	G.....
<i>C. macropodis</i> (KU853856)	T
<i>C. macropodis</i> (KU853870)	G.....
<i>C. macropodis</i> (KU853879)	G.....
<i>C. macropodis</i> (KU853881)	G.....
<i>C. macropodis</i> (KU853883)	G.....T.....
<i>C. macropodis</i> (KU853884)	C.....
<i>C. macropodis</i> (KU853885)	G.....C.....T
	210220230240250260270280290300
<i>C. johnstoni</i> (KU853837)	GATGAGCACTGCATGAGTGCCGCTCTAACTTTGTACTGTGGTTAGGCATTAGGTTGCTTGCAGCCCTTCGCAACACCTCTTGTGAGAAATCTCAAT
<i>C. macropodis</i> (KU853834)	
<i>C. macropodis</i> (KU853842)	
<i>C. macropodis</i> (KU853856)	
<i>C. macropodis</i> (KU853870)	G.....C.....
<i>C. macropodis</i> (KU853879)	G.....C.....
<i>C. macropodis</i> (KU853881)	G.....C.....
<i>C. macropodis</i> (KU853883)	G.....C.....G.....R.....
<i>C. macropodis</i> (KU853884)	G.....C.....G.....
<i>C. macropodis</i> (KU853885)	G.....C.....
	310320330340350360370380
<i>C. johnstoni</i> (KU853837)	GATTGCGCTT-CACGCGAGCGCAGTGCAGATGAACATTAACTTTGTAATGATTGAGAAACCGTGACTTTTAGTCMCAATCGA
<i>C. macropodis</i> (KU853834)	A.....C.T.....AG.....A.....
<i>C. macropodis</i> (KU853842)	S.....C.T.....AG.....A.....R.....
<i>C. macropodis</i> (KU853856)	C.....G.T.....AG.....A.....W.....
<i>C. macropodis</i> (KU853870)	C.T.....AG.....A.....
<i>C. macropodis</i> (KU853879)	C.T.....AG.....A.....
<i>C. macropodis</i> (KU853881)	C.T.....AG.....A.....
<i>C. macropodis</i> (KU853883)	C.T.....AG.....A.....
<i>C. macropodis</i> (KU853884)	C.T.....AG.....A.....
<i>C. macropodis</i> (KU853885)	C.T.....AG.....A.....

B

	102030405060708090100
<i>C. johnstoni</i> (KU853919)	TTCAATATACTACAGTGTGGCCTGTGTACACTGTTGTGCAATGACGCTGTGGCGCTGCACAATCCTCATTCCAGATGAGAAGTAACGCAACATGTAC
<i>C. johnstoni</i> (KU853920)	
<i>C. johnstoni</i> (KU853921)	C.....
<i>C. johnstoni</i> (KU853923)	A.....
<i>C. johnstoni</i> (KU853946)	
<i>C. macropodis</i> (KU853922)	A.....G.....
<i>C. macropodis</i> (KU853932)	A.....G.....
<i>C. macropodis</i> (KU853937)	A.....G.....
<i>C. macropodis</i> (KU853938)	A.....G.....
<i>C. macropodis</i> (KU853940)	A.....G.....
<i>C. macropodis</i> (KU853952)	A.....GC.....
<i>C. macropodis</i> (KU853953)	C.....A.....G.....
	110120130140150160170180190200
<i>C. johnstoni</i> (KU853919)	ATCGAGGTTTCCCAATGTACACGATGTCTATGCTGCGTTGTCACTGTGAGAGTATCTAGCGAGATTGATACTTGGGAGGGCATGTTTGACAACG
<i>C. johnstoni</i> (KU853920)	T.....
<i>C. johnstoni</i> (KU853921)	
<i>C. johnstoni</i> (KU853923)	K.....
<i>C. johnstoni</i> (KU853946)	T.....G.....C.T.....
<i>C. macropodis</i> (KU853922)	T.....G.....C.T.....
<i>C. macropodis</i> (KU853932)	G.....C.T.....
<i>C. macropodis</i> (KU853937)	T.....G.....C.T.....
<i>C. macropodis</i> (KU853938)	T.....G.....C.T.....
<i>C. macropodis</i> (KU853940)	TG.....CT.....
<i>C. macropodis</i> (KU853952)	T.....G.....
<i>C. macropodis</i> (KU853953)	C.T.....
	210220
<i>C. johnstoni</i> (KU853919)	TCGATTCAATTTCATTGCA
<i>C. johnstoni</i> (KU853920)	
<i>C. johnstoni</i> (KU853921)	
<i>C. johnstoni</i> (KU853923)	
<i>C. johnstoni</i> (KU853946)	
<i>C. macropodis</i> (KU853922)	
<i>C. macropodis</i> (KU853932)	
<i>C. macropodis</i> (KU853937)	
<i>C. macropodis</i> (KU853938)	
<i>C. macropodis</i> (KU853940)	
<i>C. macropodis</i> (KU853952)	
<i>C. macropodis</i> (KU853953)	

Supplementary Fig. 1. Alignment of the first (A) and second (B) internal transcribed spacers for *Cloacina macropodis* and *Cloacina johnstoni*. Figures in parentheses show a unique GenBank accession no. for each sequence. A dot indicates an identical nucleotide with respect to the sequence of *C. macropodis*; a dash indicates an insertion/deletion (indel) event. N indicates a polymorphic position within the sequence.

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Author/s:

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Title:

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