Morphological and molecular characterization of *Isospora neochmiae n. sp.* in a captive-bred red-browed finch (*Neochmia temporalis*) (Latham, 1802)

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Abstract

A new *Isospora* (Apicomplexa:Eimeriidae) species is described from a single red-browed finch (*Neochmia temporalis*) (subspecies *N. temporalis temporalis*), that was part of a captive population in Western Australia. Sporulated oocysts of this isolate are spherical, 18.3 (18.2-18.9) × 18.2 (18.2-18.6) µm, with a shape index (length/width) of 1.0; and a smooth and bilayered oocyst wall, 1.2 µm thick (outer layer 0.9 µm, inner 0.3 µm). A polar granule is present, but the oocyst residuum and a micropyle are absent. The sporocysts are ovoid-shaped, 13.3 (9.5-16.4) × 8.6 (6.8-10.0) µm, with a shape index of 1.5. An indistinct Stieda body is present, but the substieda body is absent. A sporocyst residuum is present and composed of numerous granules of different size scattered among the sporozoites. Morphologically, the oocysts from this isolate are different from those of all known valid *Isospora* spp. Molecular analysis was conducted at 4 loci; the 18S and 28S ribosomal RNA (rRNA), the mitochondrial cytochrome oxidase (COI) gene and the heat shock protein 70 (*hsp*70) gene. At the 18S locus, this new isolate exhibited 99.9%, 99.8%, 99.7%, and 99.5% similarity to *I. sp.* MAH-2013a from a superb starling (*Lamprotornis superbus*), *I.* MS-2003 from a Southern cape sparrow (*Passer melanurus*), *I.* sp. Tokyo from a domestic pigeon (*Columba livia domestica*) and *I.* MS-2003 from a Surinam crested oropendula (*Psarocolius decumanus*). At the 28S locus, this new isolate exhibited 99.7% similarity to both an *Isospora* sp (MS-2003) from a Northern house sparrow (*Passer domesticus*) and an *Isospora* sp. (MS-2003) from a Southern cape sparrow. At the COI locus, this new isolate exhibited 98.9% similarity to an *Isospora sp. ex Apodemus flavicollis*. At the *hsp*70 locus, this new isolate exhibited 99% similarity to isolate MS-2003 (AY283879) from a wattled starling (*Creatophora cinerea*). Based on morphological and molecular data, this isolate is a new species of *Isospora*, which is named *Isospora neochmiae* n. sp. after its host, the red-browed finch (*Neochmia temporalis*).

Keywords: *Isospora*; red-browed finch; morphology; phylogeny; 18S rRNA; 28S rRNA; COI; *hsp*70.

1. Introduction

The red-browed finch (*Neochmia temporalis*) is an easily recognized grassfinch that is widespread along the east and south east coast of Australia: from Cape York in Queensland to the Mt Lofty Ranges in South Australia (Pizzey and Knight, 2007). The red-browed finch, or the red-browed firetail, as it is also known, has a black tail, a scarlet bill, eyebrow and rump, whilst the upper parts of its body are olive-green. Adult birds are 11-11.5 cm in length (Pizzey and Knight, 2007). The red-browed finch is also found in the Darling ranges in southern Western Australia where it established itself after aviary escapes that took place around 1960 (Pizzey and Knight, 2007).

The red-browed finch is one of four species in the *Neochmia* genus. The three subspecies of *Neochmia temporalis* are: *N. temporalis temporalis*, which is found on much of the east coast (north east Queensland to south Victoria and south east South Australia *N. temporalis minor*, which is found in north east Queensland (Cape York Peninsula) and *N. temporalis loftyi* in the south west corner of South Australia (avibase.bsc-eoc.org). *Neochmia temporalis loftyi* is not always listed as a subspecies due to the relatively small differences between it and the type species (Morcombe, 2003).

Isospora spp. from passerine birds have been reported worldwide (Duszynski et al., 1999), and in recent years especially, several species of *Isospora* have been characterised (Schrenzel et al., 2005; Berto et al., 2011; Berto et al., 2013; Schoener et al., 2013; Yang et al., 2014; Yang et al., 2015a, b and c). In Australia, five species of *Isospora* from passerine birds have been described; (1) *I. lesouefi* from the endangered regent honeyeater (*Xanthomyza phrygia*), which is endemic to south-eastern Australia (Morin-Adeline et al., 2011), (2) *I. anthochaerae* from a red wattlebird (*Anthochaera carunculata*), (3) *I. streperae* from a grey currawong (*Strepera versicolour plumbea*), (4) *I. serinuse* from a domestic canary *(Serinus canaria forma domestica)* and (5) *I. manorinae* from a yellow-throated miner (*Manorina flavigula wayensis*). Of these, the latter four have been reported in Western Australia (Yang et al., 2014; 2015a and b; Yang et al., 2016). To date, no

species of *Isospora* has been characterized from the red-browed finch. In the present study, we characterized a new species of *Isospora* from a red-browed finch, both morphologically and molecularly, and propose the species name *Isospora neochmiae* n. sp.

2. Materials and methods

2.1 Sample collection

Two red-browed finch carcasses were sent to the Kanyana Wildlife Rehabilitation Centre, Perth for investigation. These finches were from a private captive-bred finch collection. These birds had shown signs of discomfort that included plucking feathers from around the vent area. No other finch species seemed to be affected even though they shared an aviary with other species. On examination both birds were found to be underweight and had soiled vents. Faecal matter was collected from the intestine of both birds. Microscopy was performed and both samples were found to contain coccidian oocysts as well as large numbers of tapeworm eggs and tapeworms. The *Isospora* oocysts were morphologically identical in both red-browed finches. PCR amplification was only successful from one of the two samples and therefore only one finch sample was used for this study. No other faecal samples from other finch species were examined.

2.2 Morphological analysis

The presence of oocysts was identified by direct microscopic examination of a faecal suspension in saline. A portion of oocyst-containing faeces was placed in 2% (w/v) potassium dichromate solution ($K_2Cr_2O_7$), mixed well and poured into Petri dishes to a depth of less than 1 cm and kept at room temperature (20-22°C) in the dark to facilitate oocyst sporulation. The sample was checked for sporulation twice per day in the first 48 hours and four times during the third day

(between 48 -72 hours). Sporulated oocysts were observed using the 100 × oil immersion objective of an Olympus CH-2 binocular microscope, in combination with an ocular micrometre.

2.3 DNA extraction

Total DNA was extracted from 200 mg of each faecal sample using a Power Soil DNA Kit (MolBio, Carlsbad, California) with some modifications. Briefly, samples were subjected to four cycles of freeze/thaw by liquid nitrogen and boiling water to ensure efficient lysis of oocysts before being processed using the manufacturer's protocol.

2.4 PCR amplification of four loci

A nested PCR with the primers EiGTF1 5' – TTC ACA GGA CCC TCC GAT C (This study) and EIGTR1 5'- AAC CAT GGT AAT TCT ATG G (this study) was used for the external amplification of the 18S rRNA gene. The expected PCR product was ~1,510 bp. The primers EiGTF2 5' – TTA CGC CTA CTA GGC ATT CC (this study) and EiGTR2 5' – TGA CCT ATC AGC TTT CGA CG were used for the internal reaction. The PCR reaction contained 2.5 µL of 10 × Kapa PCR buffer, 2 µl of 25 mM $MgCl_2$, 1.0 µL of 10mM dNTP's, 10 pM of each primer, 1 unit of KapaTaq (Geneworks, Adelaide, SA), 1 µL of DNA (~50 ng) for the external reaction or 1 µL of external PCR product for the internal reaction, and 16.4 µL of H_2O. PCR cycling conditions both for the external and internal reactions were 1 cycle of 94ºC for 3 min, followed by 40 cycles of 94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 2 min and a final extension of 72ºC for 5 min.

125 The PCR for the 28S rRNA locus was carried out using a nested PCR with the external
126 primers: 28SExF: 5'-TAC CCG CTG AAC TTA AGC and 28SExR: 5'- CMA CCA AGA TCT
127 GCA CTA G as previously described (Schrenzel et al., 2005), which produced a PCR product size
128 of ~1,362 bp. The internal primers (28InF: 5' – ACT ATG TTC CCT AGT AAC G and 28SInR 5'-
129 AAC GCT TCG CCA CGA TCC) produced an amplicon size of 1,420 bp (Yang et al., 2014). The
130 PCR reaction contained 2.5 µL of 10 × Kapa PCR buffer, 2 µL of 25mM $MgCl_2$, 1 µL of 10mM
131 dNTP's, 10 pM of each primer, 1 unit of KapaTaq (Geneworks, Adelaide, SA), 1 µL of DNA (~50
132 ng) and 16.9 µL of H_2O. Both primary and secondary PCR's were conducted using the same
133 cycling conditions; 1 cycle of 94ºC for 3 min, followed by 35 cycles of 94ºC for 30 sec, 60ºC for 30
134 sec and 72ºC for 90 sec and a final extension of 72ºC for 5 min.
135 The partial COI gene sequence (723 bp) was amplified using a nested PCR with the
136 following primers COIF1 (Ogedengbe et al., 2011) and COXR1 (Dolnik et al., 2009) for the
137 external reaction and COIF2 (Yang et al., 2013a) and COXR2 (Dolnik et al., 2009) for the internal
138 reaction. The PCR reaction contained 2.5 µL of 10 × Kapa PCR buffer, 2 µL of 25 mM $MgCl_2$, 1.0
139 µL of 10mM dNTP's, 10 pM of each primer, 1 unit of KapaTaq (Geneworks, Adelaide, SA), 1 µL
140 of DNA (about 50ng) and 13.4 µL of H_2O. PCR cycling conditions were 1 cycle of 94 ºC for 3 min,
141 followed by 40 cycles of 94 ºC for 30 sec, 58 ºC for 30 sec and 72 ºC for 1 min and a final
142 extension of 72 ºC for 5 min. The external and internal PCR cycling conditions were identical.

143 PCR for the *hsp*70 gene was carried out using the primers HSP70F 5' AAY GAY CAR
144 GGW AAY MGD ACR ACH CC 3' and HSP70R 5' CCV BNK CCY TTY TTR TSN ARA CC 3',
145 as described by Schrenzel et al. (2005).

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147 *2.5 Sequence analysis*

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149 The amplicons from the second round PCRs were gel purified using an in house filter tip
150 method as previously described (Yang et al., 2013b). All the PCR products were sequenced using
151 forward and reverse primers in duplicate using amplicons from different PCR runs. An ABI
152 Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) was
153 used for Sanger sequencing according to the manufacturer's instructions.
154 The results of the sequencing reactions were analysed and edited using Finch *TV*® v1.4.0.
155 (http://www.geospiza.com/Products/finchtv.shtml). Sequences were compared to existing *Isospora*
156 and other coccidian parasite sequences available on GenBank using BLAST searches and aligned
157 with reference sequences with BioEditor (http://bioeditor.sdsc.edu/download.shtml).
158
159 *2.6 Phylogenetic analysis*
160
161 Phylogenetic trees were constructed for *Isospora* spp. at the 18S, 28S, COI and *hsp*70 loci
162 with additional isolates from GenBank. Parsimony analyses were conducted using MEGA
163 (Molecular Evolutionary Genetics Analysis software, version 6, Arizona State University, Tempe,
164 Arizona, USA). Neighbor-joining (NJ) and maximum likelihood (ML) analyses were conducted
165 based on the most appropriate model selection using ModelTest in MEGA 6 (Tamura-Nei).
166 Bootstrap analyses were conducted using 1,000 replicates to assess the reliability of inferred tree
167 topologies.

168

169 *2.7 Line drawing*

170

171 Oocyst line drawings were conducted using Inkscape (http://www.inkscape.org/en/).

172

173 **3. Results**

174 *3.1 Description of I. neochmiae* n. sp.

175 Sporulated oocysts of *Isospora neochmiae* n. sp. are spherical, 18.3 (18.2-18.9) × 18.2 (18.2-18.6)
176 µm, with a shape index (length/width) of 1.0; and a smooth and bilayered oocyst wall, 1.2 µm thick
177 (outer layer 0.9 µm, inner 0.3 µm). The sporocysts are ovoid -shaped, 13.3 (9.5-16.4) × 8.6 (6.8-
178 10.0) µm, with a shape index of 1.5. An indistinct Stieda body is present, but the substieda body is
179 absent. A sporocyst residuum is present and composed of numerous granules of different size
180 scattered among the sporozoites. Morphologically, the oocysts from this isolate are different from
181 those of all known valid *Isospor*a spp. (Fig. 1a and 1b and Table 1).
182 Type hosts: the red-browed finch (*Neochmia temporalis*)

183 Type locality: Perth, Western Australia.

184 Prevalence: Unknown

185 Other hosts: Unknown.

186 Prepatent period: Unknown.

187 Patent period: Unknown.

188 Site of infection: Unknown

189 Sporulation time: 48-72 hours (The sample was checked for sporulation twice per day during the
190 first 48 hours and four times during the third day (between 48 -72 hours).

191 Material deposited: DNA sequences have been deposited in GenBank under the accession numbers
192 KT224380, KT224382, KT224378 and KX013543 for the 18S, 28S, COI and *hsp*70 loci
193 respectively.

194 Etymology: This species is named *Isospora neochmiae* n. sp. after its host, *Neochmia*
195 *temporalis* (red-browed finch).
196

197 *3.2 Histological study of the small intestine*

198 Histological studies were conducted on different sections of the small intestine. Coccidian
199 merozites and meronts were found in the intestine in the jejunum region (Fig. 2).

200 *3.2 Phylogenetic analysis of I. neochmiae* n. sp. *at the 18S locus*

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202 A 1,226 bp 18S rRNA sequence of *I. neochmiae* n. sp was aligned with nine other *Isospora*
203 spp. sequences from passerine birds; *I. gryphoni* (AF080613) (Olson et al., 1998), *I. robini*
204 (AF080612) (Carreno and Barta, 1999), *I.* sp. MS-2003 (JX984668), *I.* sp. MS-2003 (JX984668
205 andAY331569), *I.* sp. MS-2003 (AY331571) (Schrenzel et al., 2005), *I. serinuse* (KR477877)
206 (Yang et al., 2015b), *I.* sp. MAH-2013a (KF648870), *I.* sp. MAH-2013b (KF648871), and *I.*
207 *manorinae* (KT224379) (Yang et al., 2016), two *Isospora* spp. sequences from domestic pigeons
208 (*Isospora* sp. Tokyo - AB757860 and AB757862), as well as 17 *Eimeria* 18S rRNA sequences from
209 GenBank. *Toxoplasma gondii* was used as the outgroup.

210 Phylogenetic analysis using distance, parsimony and ML revealed that *I. neochmiae* n. sp.
211 exhibited 99.9%, 99.8%, 99.7% and 99.5% similarity to an *Isospora* sp. (MAH-2013a) (KF648870)
212 from a superb starling (*Lamprotornis superbus*) from Canada, an *Isospora* sp. (MS-2003)
213 (AY33157) from a Southern cape sparrow (*Passer melanurus*) from America, an *Isospora* sp.
214 (Tokyo) (AB75786) from a domestic pigeon (*Columba livia domestica*) from Japan and an *Isospora*
215 sp. (MS-2003) (AY331569) from a Surinam crested oropendola (*Psarocolius decumanus*) from
216 America (Fig. 3a). Further analysis of a subgroup of shorter 18S sequences (300 bp) (n=11),
217 including *I. anthochaerae* and the other four *Isospora* characterized in birds in Western Australia
218 was conducted. The results were similar to the phylogenetic analysis from the longer 18S sequences
219 and showed that *I. neochmiae* n. sp. was 100% identical to two *Isospora* sp.; MAH-2013a
220 (KF648870) and MAH-2013b (AY33171) (Fig. 3b).

221

3.3 Phylogenetic analysis of I. neochmiae n. sp. *at the 28S locus*

A 1,367 bp amplicon from *I. neochmiae* n. sp. was obtained at the 28S rRNA locus. Phylogenetic analysis included thirty-one *Isospora* sequences from the North American passerine birds from a single report by Schrenzel et al., (2005), *I. anthochaerae* (KF766053) from a red wattlebird (Yang et al., 2014), *I. serinuse* (KR477878) (Yang et al., 2015b) and *I. manorinae* (KT224381) (Yang et al., 2016). In this analysis, *I. neochmiae* n. sp. grouped separately but exhibited 99.7% similarity with an *Isospora* sp. (MS-2003) from a Northern house sparrow (*Passer domesticus*, AY283843) and an *Isospora* sp. (MS-2003) (AY283847) from a Southern cape sparrow from America (Fig. 4).

3.4 Phylogenetic analysis of I. neochmiae n. sp. *at the COI locus*

A 725 bp amplicon at the COI locus from *I. neochmiae* n. sp. was obtained. Phylogenetic analysis included 18 sequences from avian *Isospora* isolates available in GenBank and 18 *Eimeria* COI gene sequences. *Toxoplasma gondii* (HM771690) was used as the outgroup (Fig. 5a). *Isospora* neochmiae n. sp. again grouped separately and exhibited the highest similarity (98.9%), with an *Isospora* sp. ex *Apodemus flavicollis* (JQ993711) from a yellow-necked mouse (*Apodemus flavicollis*) from the Czech Republic and 98.7% and 98.6% similarity respectively with *Isospora sp*. MAH-2013b and. MAH-2013a (KF648869 and KF648868) obtained from a superb starling from Canada. A subset of 215 bp long COI gene sequences, including *I. anthochaerae* and another 5 isolates from the Eurasian blackcap (*Sylvia atricapilla*) in Germany were used for further phylogenetic analysis. For this shorter analysis, *I. neochmiae* n. sp. was 100% identical to *Isospora* sp. ex *Apodemus flavicollis* and MAH-2013b (Fig. 5b).

247 *3.5 Phylogenetic analysis of I. neochmiae* n. sp. at the *hsp*70 locus

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249 A 435 bp amplicon at the *hsp*70 locus from *I. neochmiae* n. sp. was obtained. Unfortunately
250 only one *hsp*70 sequence from an isosporoid coccidian was available in GenBank. At this locus, *I.*
251 *neochmiae* n. sp. was 99.0% similar to isolate MS-2003 (AY283879) from a wattled starling
252 (*Creatophora cinerea*).

253

254 **4. Discussion**

255 Sporulated oocysts of *I. neochmiae* n. sp. are morphologically distinct from other
256 characterized *Isospora* species and did not match any *Isospora* species from Passeriformes
257 (http://biology.unm.edu/biology/coccidia/passer1.html (Accessed on 19 January 2015) and other
258 additional species, which were not in the database (Trachta e Silva et al., 2006; Yang et al., 2014;
259 Yang et al., 2015a, 2015b). As shown in Table 1, the dimensions of the *I. neochmiae* n. sp. oocyst
260 are smaller than other identified *Isospora* species with the exception of *I. braziliensis*, which was
261 identified from a lesser seed-finch (*Oryzoborus angolensis*) in Brazil (Trachta e Silva et al., 2006)
262 (Table 1). The oocyst of *I. neochmiae* n. sp. is spherical in shape with a L /W ratio of 1.0 and a
263 polar granule was present. Both *I. neochmiae* n. sp. and *I. braziliensis* have similar oocysts with
264 indistinct Stieda bodies and the absence of a substieda body, however, there was no polar granule
265 present in the oocysts of *I. braziliensis*. Unfortunately, no morphological data is available from the
266 *Isospora* isolates (MAH-2013a and MAH-2013b) from a superb glossy starling in Canada and
267 isolate MS-2003 (AY283879) from a wattled starling.
268 Molecular characterization of *I. neochmiae* n. sp. at the 18S rRNA locus showed that it was
269 most closely related to an *Isospora sp.* (MAH-2013a) from a superb starling in Canada, an *Isospora*
270 (MS-2003) from a Southern cape sparrow, an *Isospora* sp. (Tokyo) from a domestic pigeon and an

Isospora (MS-2003) from a Surinam crested oropendola. At the 28S rRNA locus, *I. neochmiae* n. sp. was most closely related to an *Isospora* sp. MS-2003 (AY283843) from a Northern house sparrow and an *Isospora* sp. MS-2003 (AY283849) from a Southern cape sparrow from America. Phylogenetic analysis of COI gene sequences revealed that *I. neochmiae* n. sp. exhibited the highest similarity (98.9%) with an *Isospora* sp. ex *Apodemus flavicollis,* followed by 98.6% with *Isospora* sp. MAH-2013b and MAH-2013a (KF648869 and KF648868) from a superb starling from Canada. At the *hsp*70 locus, *I. neochmiae* n. sp. was 99.0% similar to isolate MS-2003 (AY283879) from a wattled starling.

Interestingly, *I. neochmiae* n. sp. was isolated from red-browed finches, which were aviary bred birds. No other wild-caught finches were in the collection and none were recently imported from other countries. The red-browed finches were housed in a mixed collection of both Australian and exotic finch species. None of the other finch species showed any signs of illness. The finches were all housed in a large open-air aviary but the feed station was protected from the weather and it is unlikely that the feed could have been contaminated with droppings from wild birds. Wild birds commonly seen in the area include magpie-larks (*Grallina cyanoleuca*), willie wagtails (*Rhipidura leucophrys*), New Holland honeyeaters (*Phylidonyris novaehollandiae*), Australian ravens (*Corvus coronoides*), silvereyes (*Zosterops lateralis*) and various dove species. It is possible that the open flight section may have been contaminated with faeces from wild birds. It is not known if the coccidia were solely responsible for the birds' death. Large numbers of tapeworms and their eggs were seen in the faeces by microscopy and these parasites may well have played a role in their death. It may be useful to screen wild birds for parasites before introducing them into aviaries containing captive populations.

Further investigation and identification of more isolates from wild red-browed finches from the eastern coast of Australia is necessary to track where this *Isospora* species originally came from and whether they are the same species reported from Canada and America.

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Fig. 1a. Nomarski interference-contrast photomicrographs of *Isospora neochmiae* n. sp. Scale bar = 20 µm. **Fig. 1b**. Composite line drawing of *Isospora neochmiae* n. sp. sporulated oocyst. Scale bar = 20 µm.

Fig. 2. H and E stained section of the in jejunum region in the intestine showing (a) merozites and (b) meronts.

Fig. 3.a. Evolutionary relationships of *Isospora neochmiae* n. sp. inferred by distance analysis of 18S rRNA sequences. Percentage support (>50%) from 1000 pseudoreplicates from distance, ML and parsimony analysis, respectively, is indicated at the left of the support node ('_' = Not available). **b.** Phylogenetic relationships of *I. neochmiae* n. sp., and 10 other *Isospora* sequences including *I. anthochaerae* from a red wattlebird in Western Australia (300 bp of 18S rRNA sequence only).

Fig. 4. Evolutionary relationships of *Isospora neochmiae* n. sp. inferred by distance analysis of 28S rRNA sequences. Percentage support (>50%) from 1000 pseudoreplicates from distance, ML and parsimony analysis, respectively, is indicated at the left of the support node ('_' = Not available).

Fig. 5.a. Evolutionary relationships of *Isospora neochmiae* n. sp. inferred by distance analysis of COI sequences. Percentage support (>50%) from 1000 pseudoreplicates from distance, ML and parsimony analysis, respectively, is indicated at the left of the support node ('_' = Not available). **b.** Phylogenetic relationships of *I. neochmiae* n. sp., and 14 other *Isospora* sequences including *I. anthochaerae* from a red wattlebird in Western Australia (215 bp of COI sequence only).