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# Cyst morphology and sequence analysis of the small subunit rDNA and *ef1α* identifies a novel *Giardia* genotype in a quenda (*Isoodon obesulus*) from Western Australia

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**Abstract**

Sequence analysis of the small subunit ribosomal DNA (SSU-rDNA) and elongation factor 1 alpha (*ef1α*) was performed on *Giardia* cysts isolated from faeces collected from a quenda (*Isoodon obesulus*) in the southwest of Western Australia. The SSU-rDNA and *ef1α* were sequenced in their entirety and correspondingly aligned with the published sequence information of other known species and genotypes of *Giardia*. Phylogenetic analysis of the SSU-rDNA and *ef1α* sequences identified the quenda isolate as a novel genotype of *Giardia* not previously reported. We believe that this quenda *Giardia* isolate constitutes a distinct species, which may be endemic within the Australian native fauna.

## 1. Introduction

Species of *Giardia* inhabit the intestinal tracts of virtually all classes of vertebrates (Thompson, 2000), with *Giardia duodenalis* (syn. *Giardia intestinalis*; *Giardia lamblia*) being the species found in most mammals ( O'Handley et al., 2000, Thompson et al., 1993, Thompson et al., 1998 and Thompson et al., 2000b). Studies on *Giardia* trophozoite morphology have recognised five species: *G. duodenalis* (occurring in most mammals), *G. agilis* (amphibians), *G. muris* (rodents) and *G. psittaci* and *G. ardeae* (birds) (Thompson et al., 2000a). This taxonomy is largely based on the shape of the trophozoite, the size of the ventral adhesive disc relative to the cell length and the shape of the median bodies (Kulda and Nohynkova, 1996).

Phylogenetic analysis of *G. duodenalis* has split the species into six different assemblages of isolates ( Hopkins et al., 1999, McIntyre et al., 2000, Monis, 1999 and Monis et al., 1996). Some of these appear to be host restricted while others have a broad host range ( Thompson et al., 2000a and Thompson et al., 2000b). The four other species: *G. muris*, *G. agilis*, *G. ardeae* and *G. psittaci* appear to maintain a reasonable level of host-specific infectivity. However, these other species have been little studied and are represented by only a handful of isolates.

A sixth species, *G. microti*, has been described on the basis of trophozoite and cyst morphology as well as small subunit ribosomal DNA gene (SSU-rDNA) sequence analysis (van Keulen et al., 1998). Based primarily on the analysis of the SSU-rDNA gene, van Keulen et al. (1998) concluded that the *Giardia* they isolated from voles and muskrats was a

new species that they called *G. microti*. The SSU-rDNA is a well conserved group of genes widely recognised as a means of detecting genetic diversity among species of organisms (Appels and Honeycutt, 1986). The different regions of the ribosomal DNA provide varying levels of phylogenetically useful information. The coding regions (18S, 5.8S and 28S) are highly conserved whilst the internal and external transcribed spacers are moderately conserved (Hillis and Davies, 1986). Of particular interest is the 5′ end of the 18S gene, which has been used extensively for genotyping and identifying isolates of *Giardia*.

It is widely accepted that analysis of the rDNA can be used to differentiate between strains or species of organisms (McManus and Bowles, 1996). Indeed, sequence analysis of the SSU-rDNA has supported the distinctiveness between the assemblages of *G. duodenalis*, as well as that of *G. ardeae* and *G. muris* ( Hopkins et al., 1997, Monis et al., 1999 and Thompson et al., 2000a). Additionally, the use of the glutamate dehydrogenase (*gdh*), triose phosphate isomerase (*tpi*) and elongation factor 1 alpha (*ef1α*) genes has been widely accepted as a reliable means for the characterisation of both *Giardia* species and genotypes ( Hopkins et al., 1999, Hopkins et al., 1997, McIntyre et al., 2000, McRoberts et al., 1996, Monis, 1999, Monis and Andrews, 1998, Monis et al., 1999, Monis et al., 1998 and Traub et al., 2004).

As such, these loci are useful tools for the characterisation of novel *Giardia* isolates as well as phylogenetic studies. Here we investigate and characterise a novel isolate of *Giardia* found in a southern brown bandicoot (quenda) (*Isoodon obesulus*) in the southwest of Western Australia using both SSU-rDNA and *ef1α* gene sequence analysis.

## 2. Materials and methods

### 2.1. Detection and morphological analysis

Faecal samples were collected from 72 quenda from Jarrah forest approximately 30 km east of Manjimup which is located 300 km south of Perth, Western Australia, four quenda from Batalling, situated 50 km east of Collie, Western Australia, and one quenda from the Perth metropolitan region. Faecal samples were examined by direct stool microscopy stained with 5% malachite green stain and by both sodium nitrate and zinc sulphate flotation (Bartlett et al., 1978). Samples positive for *Giardia* cysts were subsequently purified via saturated salt and glucose gradient purification (Meinema, unpublished). Purified *Giardia* cysts were measured using the Optimas Image Analysis Package version 5.2 at 1000× magnification. The area of analysis was set to enclose each individual cyst in order to minimise computational time. Threshold was optimised by eye to differentiate the cyst background. Once the optimal threshold was reached, the software was set to recognise the cyst as an area object. All measurements (length, width, area and circularity) were transferred to a Microsoft Excel spreadsheet.

### 2.2. DNA extraction and amplification

DNA was extracted from purified and concentrated *Giardia* cysts using glassmilk (Biorad) as per manufacturer's instructions. Preliminary genetic characterisation of the extracted *Giardia* DNA was performed by amplifying a 292 bp region of the 5′ end of the small subunit ribosomal RNA (SSU-rRNA) gene (Hopkins et al., 1997), before amplifying the entire SSU-rRNA gene as well as *ef1*α using the conditions set out below.

## 2.3. SSU-rDNA PCR

Amplification of the SSU-rDNA was performed using conditions previously set out by Hopkins et al. (1997), and the primers RH11 (5′-CATCCGGTCGATCCTGCC-3′) and RH4 (5′-AGTCGAACCCTGATTCTCCGCCAGG-3′) to generate a 292 bp fragment for genotyping *G. duodenalis* isolates and primers RH11 and RM3′ (5′-CAGGTTCACCTACGGATACC-3′) for amplifying the entire 1.4 kb SSU-rRNA gene. In short, PCRs contained 1–50 ng of extracted *Giardia* DNA; 1× Taq DNA polymerase reaction buffer consisting of 67 mM Tris–HCl, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100 and 0.2 mg/ml gelatin at pH 8.8 (Biotech International Ltd., Perth, Western Australia); 2 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphate, 5% DMSO, 12.5 pmol of both the forward and reverse primers, 0.5 U of Taq polymerase (Biotech International Ltd., Perth, Western Australia) and 0.5 U of Taq Extender™ PCR Additive (Stratagene, Integrated Sciences, Sydney). Ultra pure water was used to make up the final volume of each reaction to 25 μl. PCRs were performed in 0.2 ml thin walled PCR tubes on a Perkin-Elmer GeneAmp PCR System 2400 thermocycler. Reaction conditions consisted of a pre-incubation at 96 °C for 2 min and an initial 3 cycles of 96 °C for 20 s, 45 °C for 30 s and 72 °C for 2 min, followed by 32 cycles of 96 °C for 20 s, 55 °C for 30 s and 72 °C for 1 min with a final 72 °C extension for 7 min. Due to the size of the SSU-rDNA gene (1433 bp), amplified products were cloned prior to sequencing.

## 2.4. ef1α PCR

Amplification of *ef1*α was performed as previously described by Monis (1999) and generated a band of approximately 750 bp. In brief, each PCR contained 1–50 ng of extracted *Giardia* DNA; 1× Taq DNA polymerase reaction buffer consisting of 67 mM Tris–HCl, 16.6 mM

($NH_4$)$_2$$SO_4$, 0.45% Triton X-100 and 0.2 mg/ml gelatin at pH 8.8 (Biotech International Ltd., Perth, Western Australia); 4 mM $MgCl_2$; 200 μM of each deoxyribonucleotide triphosphate; 5% dimethyl sulphoxide (DMSO); 12.5 pmol of both the forward (GLongF 5′-GCTCSTTCAAGTACGCGTGG-3′) and reverse (EF1AR 5′-AGCTCYTCGTGRTGCATYTC-3′) primers; 0.5 U of Taq polymerase (Biotech International Ltd., Perth, Western Australia); and 0.5 U of Taq Extender™ PCR Additive (Stratagene, Integrated Sciences, Sydney). Ultra pure water was used to make up the final volume of each reaction to 25 μl. PCRs were performed in 0.2 ml thin walled PCR tubes on a Perkin-Elmer GeneAmp PCR System 2400 thermocycler. Reaction conditions consisted of a pre-incubation at 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final 72 °C extension for 7 min.

## 2.5. Cloning and automatic sequencing

Multiple SSU-rDNA gene products were amplified in isolation from each other under the conditions described above. These PCR fragments, originating from unique PCR master mixes, were excised from their gels using sterile techniques and purified using spin columns (Qiagen, Hilden, Germany) as per manufacturers instructions. Purified PCR products were cloned into TOP 10 F′ competent cells using the TOPO™ Cloning Kit (Invitrogen, Carlsbad, California) as per manufacturer's instructions. Transformed cells were plated onto LB (bacto-tryptone, 10 g/l; bacto-yeast extract, 5 g/l; NaCl, 10 g/l; pH 7.0) plates containing 50 μg/ml of ampicillin. Colonies were grown up overnight (16 h) at 37 °C. Sequencing of *ef1α* was performed directly from the amplified product without cloning as the gene (750 bp in length) could be sequenced in a single step.

PCR templates were purified using spin columns (Qiagen, Hilden, Germany). Sequencing reactions were performed using an ABI Prism™ Dye Terminator Cycle Sequencing Core kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions. Reactions were electrophoresed through an ABI 373 automatic sequencer and sequencing profiles analysed using SeqEd v1.0.3 (Applied Biosystems). Three independent sequences for both the SSU-rDNA and *ef1*α were obtained in both the forward and reverse direction.

## 2.6. Sequence comparison and phylogenetic analysis

Sequence information for the SSU-rDNA and *ef1*α loci was obtained from Genbank for comparison with the sequence data from the quenda *Giardia*. Accession numbers for the SSU-rDNA sequences are as follows: quenda (AY309064); Assemblage A (group I, X52949; group II, AF199446); Assemblage B (AF199447); Assemblage C (AF199443); Assemblage D (AF199449); Assemblage E (AF199448); Assemblage F (AF199444); Assemblage G (AF199450); *G. ardeae* (Z17210); *G. muris* (X65063); *G. psittaci* (AF473853) and *G. microti* (AF006676, AF006677). Accession numbers for these *ef1á* sequences are as follows: quenda (AY309065); Assemblage A (group I, D14342; group II, AF069573); Assemblage B (group III, AF069569; group IV, AF069570); Assemblage C (AF069574); Assemblage D (AF069575); Assemblage E (AF069571); Assemblage F (AF069572); Assemblage G (AF069568); *G. ardeae* (AF069567); *G. muris* (AF069566).

These sequences were aligned using the computer software package Clustal X (Thompson et al., 1997) and the alignments manually adjusted by eye as required. Subsequent phylogenetic analysis of these sequences was performed using MEGA 2.1 (Kumar et al., 2001). Based on

the results of Monis et al. (1999), which found congruence between distance-based, parsimony and maximum likelihood analyses for *Giardia ef1*α and SSU-rDNA sequences, only distance-based analysis was conducted. Distances were estimated using the Tamura–Nei model with pair-wise deletion of missing data ( Table 1 and Table 2). Trees were constructed using the neighbour joining method and support for nodes was estimated by bootstrap analysis using 1000 replicates.

## 3. Results

### 3.1. Diagnosis and morphology

*Giardia* cysts were only detected in one faecal sample from a quenda collected approximately 30 km east of Manjimup, Western Australia. Measurements of 63 quenda *Giardia* cysts at 1000× magnification had a size range of 10.4–14.3 μm in length with an average of 12.5 ± 0.87 μm, and 6.9–8.8 μm in width with an average of 7.7 ± 0.47 μm. No trophozoites were detected. Attempts to culture the purified quenda *Giardia* cysts using the methods outlined in Hopkins et al. (1997) failed. Whether this was due to the *Giardia* cysts being non-viable or due to the inability of the isolate to grow under the specified culture conditions is unknown.

### 3.2. Sequence confirmation and phylogenetic analysis

DNA sequences were obtained for the *Giardia* quenda isolate for both the SSU-rDNA and *ef1*α loci. The sequence data obtained from the quenda *Giardia* was confirmed to be *Giardia* SSU-rDNA and *ef1*α sequence via a BLAST search of the GenBank database. Phylogenetic analysis of the SSU-rDNA (Fig. 1) and the *ef1*α (Fig. 2) sequences identified the quenda isolate as a novel genotype of *Giardia* not previously reported. In both cases it was placed as

a lineage external to all of the previously described assemblages of *G. duodenalis*. In the case of the SSU-rDNA data, which has more published sequences for a greater range of *Giardia* species, the quenda isolate was placed external to the cluster including *G. duodenalis*, *G. microti* and *G. psittaci*. The placement of the quenda isolate in both analyses was highly supported by bootstrap analysis (100% for SSU-rDNA, 85% for *ef1α*).

## 4. Discussion

The detection of *Giardia* cysts in only one of 77 (1.3%) faecal samples examined suggests an extremely low prevalence of this parasite in quendas in Western Australia. However, McGlade et al. (2003) showed that microscopy is a highly insensitive technique for detecting low levels of *Giardia* in faecal samples. The shedding of *Giardia* cysts is typically intermittent and repeat sampling of individuals is generally required to obtain an accurate assessment of prevalence ( Meloni et al., 1993 and O'Handley et al., 1999). For this study, it was not possible to obtain repeat samples from individual animals and it has been estimated that 15–50% of *Giardia* infections can go undetected if only one stool sample is examined for the presence of cysts ( Danciger and Lopez, 1975 and Goka et al., 1990). The detection of *Giardia* in 62% of bandicoots tested by Bettiol et al. (1997) in Tasmania could be attributed to their use of an antigen detection test in addition to microscopy, which is a more sensitive method for detecting *Giardia* infections (McGlade et al., 2003). As such it is not unreasonable to assume that the prevalence of *Giardia* in both native and introduced animals in Western Australia may be higher than reported in this study.

Cyst measurements of the quenda *Giardia* isolate (10.4–14.3 μm) fall within those previously recorded for *G. duodenalis* (Garcia, 1998). However, the size of *Giardia* cysts (and presumably the size of trophozoites as well) in both humans and dogs have been shown to vary significantly from day to day according to host diet as well as different environmental conditions ( Tsuchiya, 1931 and Tsuchiya, 1930). Therefore, whilst cyst morphology may be able to distinguish between populations of *Giardia* in two hosts of the same species, it is inadvisable to use differences in size alone as a means of species distinction within the genus *Giardia* (Filice, 1952). Indeed it is the lack of reliably consistent differences between the *G. duodenalis* assemblages A and B that is retarding their recognition as distinct species (Thompson et al., 2000b), even though it has been demonstrated that the genetic distance separating them exceeds that used to delineate other species of protozoa ( Andrews et al., 1989, Mayrhofer et al., 1995 and Monis et al., 1996). This suggests that genetic and biological data is more reliable than morphology when delineating species of *Giardia*.

Analysis of nucleotide differences at both the SSU-rDNA and *ef1*α loci support the distinction of the quenda *Giardia* isolate from the other known Giardia genotypes and species ( Table 1 and Table 2). Similarly, phylogenetic analysis of the quenda *Giardia* isolate also shows it to be distinct from all of the other recognised species *G. microti*, *G. psittaci*, *G. ardeae* and *G. muris* ( Fig. 1 and Fig. 2). This raises the issue of whether or not this isolate should be classified as a new species of *Giardia*. The phylogenetic analysis of the SSU-rDNA data clearly demonstrates that the quenda isolate is more distantly related to *G. duodenalis* than either *G. microti* or *G. psittaci*, suggesting that it should be recognised as a distinct species. The distinctiveness of the quenda *Giardia* isolate raises the question of whether or not this is a new species adapted to Australian native mammals or, given its low frequency of detection in this study, originated from another host. The former situation would

seem more likely, given that it has never before been isolated from humans or domestic animals. The presence of *Giardia* infections in numerous species of Australian marsupials and the potential for cross transmission between domestic and native animals has previously been demonstrated ( Bettiol et al., 1997 and Kettlewell et al., 1998). Domestic animals regularly harbour *Giardia* infective to humans ( Mayrhofer et al., 1995, Meloni et al., 1995, Monis et al., 1996 and Traub et al., 2004), and as such, they may also provide opportunities for infection of native animals (Kettlewell et al., 1998).

There is no epidemiological evidence to suggest that any of the animal-specific genotypes of *Giardia* frequently occur in the human population (Thompson et al., 2000b). Whilst selected wildlife species, such as beavers and muskrats, have been identified as potential sources of human infection ( Erlandsen et al., 1990 and Monzingo and Hibler, 1987), these earlier studies did not genotype the isolates collected and many of these potential reservoir hosts have since been shown to harbour host specific genotypes ( Ey et al., 1997, Hopkins et al., 1999, Hopkins et al., 1997, Monis et al., 1998, Thompson et al., 2000b and van Keulen et al., 1998). Therefore, the role of wildlife in the transmission of *Giardia* should not necessarily be regarded as that of a reservoir for human infection, as host-adapted genotypes, such as the quenda *Giardia* isolate appears to be, have not been identified in humans and consequently do not appear to represent a risk to public health ( Ey et al., 1997, Thompson et al., 2000a and van Keulen et al., 1998). The identification of a novel *Giardia* isolate from a quenda in the present study implies that this may be a new species of *Giardia* endemic to Australian marsupials. This is of particular interest from both a conservational and epidemiological point of view as previous reports of *Giardia* in Australian wildlife are limited and commonly assumed to be zoonotic genotypes of *G. duodenalis*.

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Fig. 1.

Phylogenetic relationships of *Giardia* isolates inferred by distance-based analysis of SSU rDNA sequences.

Bootstrap support (>50% for 1000 replicates) is indicated at each node.



94 | *Assemblage AI*
99 | *Assemblage AII*
*Assemblage F*
86 | *Assemblage E*
79
51 | *Assemblage BIII*
*Assemblage G*
*Assemblage D*
81
61 | *Assemblage C*
100 | *G. microti AF006676*
93 | *G. microti AF006677*
91 | *G. psittaci*
*Quenda*
*G. ardeae*
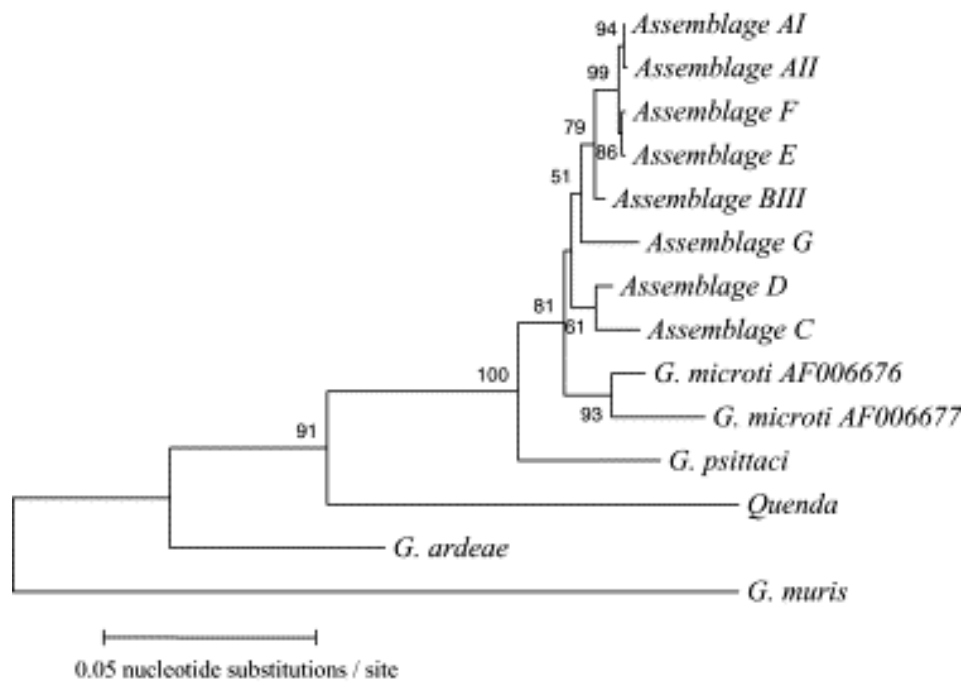*G. muris*

0.05 nucleotide substitutions / site

Fig. 2.

Phylogenetic relationships of *Giardia* isolates inferred by distance-based analysis of *ef1*α sequences. Bootstrap support (>50% for 1000 replicates) is indicated at each node.
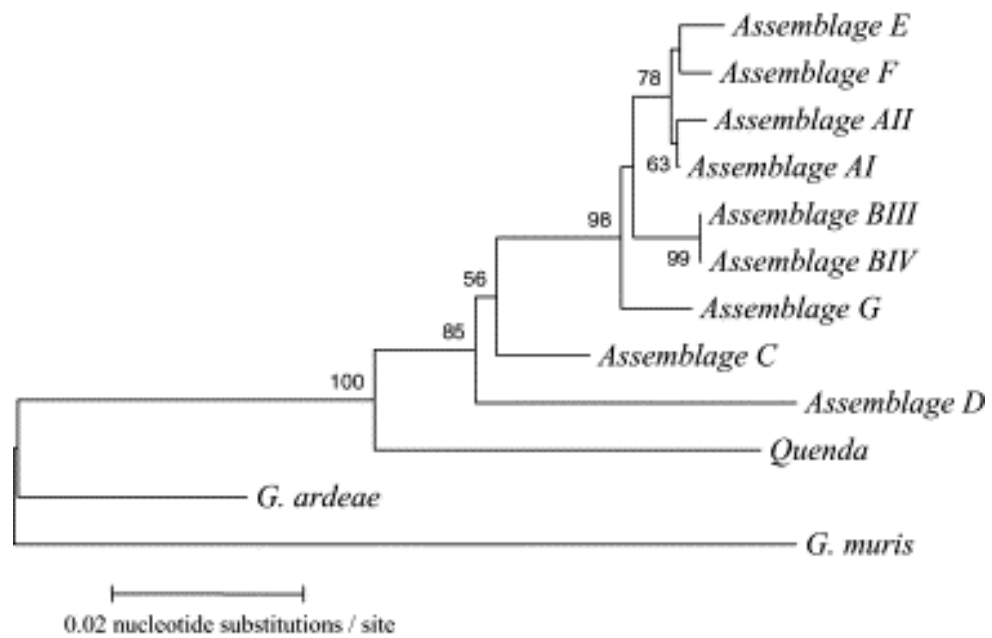


0.02 nucleotide substitutions / site

Table 1.

Tamura–Nei distances estimated from 18S rDNA sequence data

| | AI | AII | B | C | D | E | F | G | G. ardeae | G. microti (AF006676) | G. microti (AF006677) | G. muris | G. psittaci |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AII | 0 | | | | | | | | | | | | |
| B | 0.02 | 0.02 | | | | | | | | | | | |
| C | 0.03 | 0.03 | 0.02 | | | | | | | | | | |
| D | 0.03 | 0.03 | 0.02 | 0.01 | | | | | | | | | |
| E | 0 | 0 | 0.02 | 0.03 | 0.03 | | | | | | | | |
| F | 0 | 0 | 0.02 | 0.03 | 0.03 | 0.01 | | | | | | | |
| G | 0.02 | 0.02 | 0.02 | 0.03 | 0.02 | 0.02 | 0.02 | | | | | | |
| G. ardeae | 0.17 | 0.17 | 0.16 | 0.18 | 0.17 | 0.17 | 0.17 | 0.18 | | | | | |
| G. microti (AF006676) | 0.05 | 0.05 | 0.04 | 0.03 | 0.03 | 0.05 | 0.05 | 0.05 | 0.19 | | | | |
| G. microti (AF006677) | 0.06 | 0.06 | 0.05 | 0.04 | 0.04 | 0.07 | 0.07 | 0.06 | 0.19 | 0.02 | | | |
| G. muris | 0.33 | 0.33 | 0.32 | 0.32 | 0.32 | 0.34 | 0.34 | 0.32 | 0.28 | 0.33 | 0.35 | | |
| G. psittaci | 0.06 | 0.06 | 0.05 | 0.05 | 0.04 | 0.06 | 0.06 | 0.05 | 0.18 | 0.07 | 0.07 | 0.33 | |
| Quenda | 0.17 | 0.17 | 0.17 | 0.17 | 0.17 | 0.18 | 0.18 | 0.18 | 0.29 | 0.18 | 0.18 | 0.31 | 0.19 |

Table 2.

Tamura–Nei distances estimated from *ef1α* sequence data

| | AI | AII | BIII | BIV | C | D | E | F | G | G. ardeae | G. muris |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AII | 0 | | | | | | | | | | |
| BIII | 0.01 | 0.01 | | | | | | | | | |
| BIV | 0.01 | 0.01 | 0 | | | | | | | | |
| C | 0.03 | 0.03 | 0.03 | 0.03 | | | | | | | |
| D | 0.05 | 0.05 | 0.06 | 0.06 | 0.04 | | | | | | |
| E | 0 | 0.01 | 0.02 | 0.02 | 0.03 | 0.05 | | | | | |
| F | 0 | 0.01 | 0.02 | 0.02 | 0.03 | 0.05 | 0.01 | | | | |
| G | 0.01 | 0.01 | 0.01 | 0.01 | 0.02 | 0.06 | 0.02 | 0.02 | | | |
| G. ardeae | 0.09 | 0.09 | 0.08 | 0.08 | 0.08 | 0.1 | 0.09 | 0.09 | 0.09 | | |
| G. muris | 0.13 | 0.13 | 0.13 | 0.13 | 0.12 | 0.14 | 0.13 | 0.13 | 0.14 | 0.09 | |
| Quenda | 0.07 | 0.07 | 0.07 | 0.07 | 0.06 | 0.08 | 0.07 | 0.07 | 0.07 | 0.09 | 0.14 |