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Enterocytozoon bieneusi **Genotypes in Cattle on Farms Located within a Water Catchment Area**

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ABSTRACT

Enterocytozoon bieneusi is a microsporidian found in humans and other animals around the world. Investigations in some countries, such as the USA, have indicated the importance of *E. bieneusi* as a zoonotic water- and food-borne pathogen. However, there is scant epidemiological information on *E. bieneusi* in animals in many countries including Australia. Here, we conducted the first molecular epidemiological study of *E. bieneusi* in farmed cattle in Victoria, Australia, to assess whether these bovids are carriers of 'zoonotic' genotypes of *E. bieneusi*. A total of 471 individual faecal samples were collected from calves of < 3 months and of 3 - 9 months of age. Genomic DNAs were extracted from individual faecal samples and then subjected to nested PCR-based sequencing of the internal transcribed spacer (*ITS*) of nuclear ribosomal DNA to identify *E. bieneusi* and define genotypes. *Enterocytozoon bieneusi* was detected in 49 of the 471 samples (10.4%). An analysis of *ITS* sequence data revealed three known genotypes (BEB4, I and J) and three novel genotypes (designated TAR_fc1 to TAR_fc3). Phylogenetic analysis showed that genotypes BEB4, I, J, TAR_fc1 and TAR_fc2 clustered with genotypes identified previously in humans, indicating that cattle are carriers of *E. bieneusi* with zoonotic potential.

Keywords
Australia; genetic diversity; genotypic characterisation; Microsporidia; phylogenetic analysis; prevalence; risk factors.

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SEVENTEEN species of Microsporidia are reported to be opportunistic pathogens of humans (e.g. Fayer and Santín-Durán 2014). *Enterocytozoon bieneusi* is the commonest

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species contributing to most cases of intestinal microsporidiosis in humans, causing chronic or acute diarrhoea, malabsorption and/or wasting (Santín-Durán 2015); it can also infect various species of animals. This microorganism is mainly transmitted through a faecal-oral route from *E. bieneusi* spore-contaminated water, food or the environment (Mathis et al. 2005), and has been classified as a Category B Priority Pathogen in the USA (Didier and Weiss 2006).

Due to the small size of its spores (0.5 x 1.5 µm) (Santín and Fayer 2011), it is challenging to identify *E. bieneusi* using microscopic methods (Garcia 2002). Therefore, the molecular method of PCR-based sequencing of the internal transcribed spacer (*ITS*) of the nuclear ribosomal DNA has been widely used for genetic identification and characterisation (Santín and Fayer 2011). Using such molecular techniques, more than 200 *E. bieneusi* genotypes have been identified in water, food and faecal samples from humans and various species of animals including artiodactyls, carnivores, diprotodontia, lagomorphs, perissodactyls, primates and rodents (Santín-Durán 2015). Some genotypes (e.g. B, CAF3 and DeerEb9) have been found exclusively in humans or in other animals, indicating that they are host-specific. However, other genotypes (e.g. D, J and Type IV) have been characterised from both humans and animals, indicating that they have zoonotic potential.

To date, using PCR-based sequencing of *ITS*, more than 50 distinct genotypes of *E. bieneusi* have been identified in cattle in 11 countries (cf. Santín-Durán 2015). However, surprisingly, there is no record of *E. bieneusi* in cattle in numerous countries including Australia. Nonetheless, in the latter country, there have been some recent studies in other animals. For instance, Zhang et al. (2018a, b) identified and characterised ten genotypes of *E. bieneusi* in wild sambar deer and native marsupials (i.e. D, J, NCF2, Type IV, MWC_d1, MWC_d2, MWC_m1 - m4) inhabiting water catchments supplying drinking water to the city of Melbourne, Australia. These studies highlight the relevance of exploring the genetic composition of *E. bieneusi* populations in these water catchments, in order for the water industry and health authorities to be informed about the *E. bieneusi* genotypes present and about their zoonotic potential. Overall, these studies (Zhang et al. 2018a, b) showed a relatively low prevalence (0.1 - 4.1%) of *E. bieneusi* genotypes with zoonotic potential in

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catchment areas, to which no access by humans or domesticated animals is permitted. Of Melbourne's ten main water catchments, the only "open" catchment, in which domesticated animals including beef and dairy cattle are extensively farmed, is the Tarago area. Because previous studies have demonstrated that particularly young calves can represent carriers of zoonotic microbes (e.g. Li et al. 2016), it was important for us to investigate the prevalence and genetic composition of *E. bieneusi* in calves on farms located near the Tarago water reservoir. In this study, we surveyed faecal samples from calves on dairy and beef farms in this catchment area for *E. bieneusi* genotypes utilising a PCR-based sequencing approach.

MATERIALS AND METHODS

In total, 471 faecal samples were collected from calves of two age-groups: < 3 months (*n* = 292) and 3 - 9 months (*n* = 179) from three dairy farms (A, B and C) and two beef farms (D and E) located within the Tarago water catchment area in November 2011 (Spring; *n* = 261) and April 2012 (Autumn; *n* = 210) (Table 1). All calves were maintained together on individual farms (A to E). Calves from all farms were born *in situ*, apart from farm C, on to which calves (3 months of age) were introduced from multiple dairy farms. Freshly deposited faecal samples were collected from enclosures where calves were reared. Genomic DNA was extracted directly from 0.1 g to 0.4 g of each of the 471 faecal samples using the PowerSoil kit (MoBio, Carlsbad, CA, USA).

Individual genomic DNA samples were subjected to nested PCR-coupled sequencing of the *ITS* region using an established technique (Zhang et al. 2018a). *ITS* sequences obtained (GenBank accession nos. MH899203 – MH899203) were inspected for quality and compared with reference sequences acquired from the GenBank database (Table S1). Genotypes of *E. bieneusi* were named according to the recommendations by Santín and Fayer (2009a, 2011).

All *ITS* sequences obtained were aligned with the references and subjected to phylogenetic analysis using the methods from Zhang et al. (2018c). *Enterocytozoon bieneusi* Groups were assigned using an established classification system (Karim et al. 2015; Li et al. 2015b). The Chi-square and Fisher's exact tests were utilised to compare *E. bieneusi* test-positives (faecal samples) with each risk factor (age and season) separately,

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and to test the association between the prevalence of *E. bieneusi* DNA and possible risk factors. The strength of association between *E. bieneusi* prevalence and a univariate risk factor was measured using the odds ratio (OR) calculated with 95% confidence intervals (95% CI). A *P*-value of < 0.05 was considered statistically significant. IBM SPSS Statistics 25.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses (Zhang et al. 2018c).

RESULTS

Enterocytozoon DNA was detected in 49 of the 471 (10.4%) faecal samples from calves from the five farms, including 24 calves of < 3 months of age (8.2%; 24/292) and 25 calves of 3 - 9 months of age (14.0%; 25/179) by nested PCR of *ITS* (Table 2). Of the 49 test-positive samples, 35 were detected in Spring (13.4%; 35/261) and 14 in Autumn (6.7%; 14/210). There was no association of *E. bieneusi*-positivity with either age-group (P = 0.061), but there was with season (P = 0.022), showing that cattle in Spring had a 2.17-times higher risk of *E. bieneusi* contamination than in Autumn (OR = 2.168; 95% CI [1.133 - 4.148]) (Table 2).

The sequencing of the 49 *ITS* amplicons (241 - 243 bp) and their subsequent comparisons with reference sequences from the GenBank database revealed that three known genotypes of *E. bieneusi* (called BEB4, I and J) and three novel genotypes (designated here as TAR_fc1 to TAR_fc3) represented 46 amplicons (Table 3); three ambiguous sequences were derived from three amplicons each containing multiple genotypes. All of the 46 amplicons (sequences) unequivocally represented genotypes I (39.1%; 18/46), J (30.4%; 14/46), followed by BEB4 and TAR_fc2 (both 13.0%; 6/46), TAR_fc1 and TAR_fc3 (both 2.2%; 1/46).

The *ITS* sequences from amplicons representing genotypes BEB4 (synonym: CHN1), I (synonyms: BEB2 and CEbE) and genotype J (synonyms: BEB1, CEbB and PtEb X) were identical (over 243 bp) to those with accession nos. MF592788 (derived from cattle), AF135836 (rex rabbit) and MF693833 (sambar deer), respectively (Table S2). The sequences from amplicons representing TAR_fc1, TAR_fc2 and TAR_fc3 were 99.6%,

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99.6% and 95.1% similar (over 243 bp) to those with accession nos. KX383627 (genotype JLD-II), MF592788 (genotype BEB4) and KY706126 (genotype CHY1), respectively.

The *ITS* sequences for all six genotypes defined herein were aligned with sequences representing all nine established Groups of *E. bieneusi* (Karim et al. 2015; Li et al. 2015b) and then subjected to phylogenetic analysis (Fig. 1). In this analysis, Groups 1 to 9 were each strongly supported (pp = 0.99 to 1.00). Based on this analysis, genotype TAR_fc1 was assigned to Group 1 (pp = 0.97) and genotypes BEB4, I, J and TAR_fc2 were linked to Group 2 (pp = 1.00), with strong statistical support. Novel genotype TAR_fc3 formed a new, unique branch (Fig. 1).

DISCUSSION

This study revealed a prevalence of *E. bieneusi* of 10.4% (49/471) in calves on farms located in the Tarago water catchment in Victoria, Australia. The total prevalence of *E. bieneusi* in cattle worldwide is reported to range from 3.1% to 37.6% (see Table S3). The prevalence of > 10% is higher than that recorded in many previous studies of *E. bieneusi* of cattle (e.g. Fayer et al. 2007 [4.1%]; Jiang et al. 2015 [6.0%]; Sulaiman et al. 2004 [6.3%]) (see Table S3). These results indicate a relatively high prevalence of *E. bieneusi* infection, although the passage of *E. bieneusi* spores through the gastrointestinal tract (pseudoparasitism) cannot be entirely excluded, as the specific amplification of *E. bieneusi* DNA from faecal samples is not direct evidence of infection.

Analyses between *E. bieneusi* prevalence in cattle and season revealed a significant association with Spring ($P < 0.05$). The present findings are similar to those of a study by Buckholt et al. (2002), who investigated pigs and found a higher prevalence of *E. bieneusi* infection in Summer and late Spring than in other seasons ($P > 0.05$). However, no significant association between *E. bieneusi* prevalence and age was found here ($P > 0.05$), although a higher prevalence of *E. bieneusi* was observed in post-weaned (3 - 9 months of age; 14.0%, 25/179) than in pre-weaned calves (< 3 months of age; 8.2%, 24/292). A similar observation has been made in some investigations (Fayer et al. 2007; Santín and Fayer 2009b), but other studies have reported the opposite (da Silva Fiuza et al. 2016; Jiang et al. 2015; Juránková et al. 2013; Li et al. 2016; Qi et al. 2017). This variation might relate

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to a multitude of possible factors, including sex, immune and health status of hosts, livestock management, and environmental temperature, humidity and sunlight.

The analysis of *ITS* sequences data revealed three known *E. bieneusi* genotypes, i.e., BEB4, I and J. Genotype I (synonyms: BEB2 and CEbE) was the predominant type (36.7%; 18/49), similar to results for most other studies of *E. bieneusi* in cattle (Fayer et al. 2012), followed by genotype J (28.6%; 14/49) and BEB4 (12.2%; 6/49). All three genotypes were found in humans (Table S2), indicating that cattle may act as a source of *E. bieneusi* infections in humans. Moreover, genotype I and J have been found previously in water (Table S2), suggesting that these genotypes might be transmissible to other susceptible hosts via spore-contaminated water or the environment. In addition, genotypes I and J have a relatively broad host range, with genotype I having been reported in 11 mammal species, and genotype J recorded in 15 animal species, compared with BEB4 recorded previously in five species of mammals (black-capped capuchin, pileated gibbon, white-handed gibbon, yak and pig; see Table S4). This relatively broad host-range suggests that cross-species transmission of these three genotypes is likely. Previously, we investigated *E. bieneusi* from humans (Zhang et al. 2018c), alpacas (Koehler et al. 2018) and marsupials (Zhang et al. 2018b) in Australia, but none of the three abovementioned genotypes was detected here. However, we have detected genotype J in sambar deer in one of Melbourne's water catchments (O'Shannassy) (Zhang et al. 2018a), suggesting that this genetic variant of *E. bieneusi* might be transmissible to humans.

To assess the zoonotic potential of *E. bieneusi* genotypes in the present study, our phylogenetic analysis included *ITS* sequences of six genotypes and representatives from nine established *E. bieneusi* Groups (Fig. 1). The analysis of these sequence data sets revealed that novel genotype TAR_fc1 was inferred to be in Group 1 (with zoonotic potential) (Fig. 1). Novel genotypes TAR_fc1 and TAR_fc2 clustered with genotypes BEB4, I and J, identified previously in humans, linking them to Group 2 recognised to represent genotypes with zoonotic potential. Interestingly, previous studies (Abu Samra et al. 2012; Santín and Fayer 2009b) have inferred that some genotypes (e.g. BEB1, BEB2 and BEB4) within Group 2 are specific to cattle; however, after more genotypes from this group were discovered in humans, accumulating evidence indicates that these genotypes

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more likely represent a zoonotic group than a cattle-specific group. The identification of potentially zoonotic genotypes in cattle in the present study does indicate that cattle might be a source for *E. bieneusi* infection to humans and to other susceptible hosts, such as sambar deer (Zhang et al. 2018a). Clearly, more investigations of *E. bieneusi* from humans and other animals are needed to address this aspect.

The zoonotic potential or host specificity of novel genotype TAR_fc3 is challenging to evaluate (cf. Fig. 1), since it formed a unique branch which did not conform to the proposed scheme of nine distinct Groups (Karim et al. 2015; Li et al. 2015b). A similar situation was found in a number of other published studies (Deng et al. 2018; Deng et al. 2017; Li et al. 2015a). For instance, some genetically divergent genotypes (e.g. CHY1, CHK1 and SCC-1), which have been recorded in yak, red kangaroo and common chipmunk (cf. Karim et al. 2015; Li et al. 2015b), did not conform to any of the nine Groups, making it challenging to associate particular genotypes with particular host species or host groups, and/or to reliably infer zoonotic potential. In some cases, the unexpected finding of previously-assigned, apparently animal host-adapted genotypes in humans in subsequent studies (cf. Santín-Durán 2015) confuses the issue even more. Additionally, discrepancies in the findings among some publications can make the assignment of genotypes to "Groups" challenging. For instance, genotype WW6 clustered within Group 4 in the studies by Guo et al. (2014) and Stensvold et al. (2014), but it was assigned to Group 6 in another study (Li et al. 2012). Therefore, future work should include a critical re-appraisal of all current sequence data sets as well as epidemiological and biological information published in the peer-reviewed literature, to reconstruct the phylogenetic relationships of *E. bieneusi* genotypes using multiple tree-building methods and re-assess the validity of Groups.

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FIGURE LEGEND

Fig. 1. Phylogenetic analysis of internal transcribed spacer (*ITS*) of nuclear ribosomal DNA sequence data (Table S1) by Bayesian inference (BI). Included here are *ITS* sequences of (i) *E. bieneusi* genotypes representing all currently recognised Groups (1 to 9) from the published literature, (ii) six genotypes of *Enterocytozoon* identified in the present study (bold-type) and (iii) two outgroups. Group 10 has been proposed, based on a previous study (Zhang et al. 2018b). Statistically significant posterior probabilities (pp) are indicated on branches. The scale-bar represents the number of substitutions per site.

SUPPORTING INFORMATION

Table S1. GenBank accession numbers of all internal transcribed spacer (*ITS*) of nuclear ribosomal DNA sequences used for phylogenetic analysis (Fig. 1), and associated information. Included here are *ITS* sequences of (i) *E. bieneusi* genotypes representing currently recognised Groups (1 to 10) from the published literature and genotypes without group assignment; (ii) six genotypes of *Enterocytozoon* identified/defined in the present study; and (iii) the outgroups CD8 (KJ668735) and PtEbIX (DQ85585)

Table S2. Genotypes BEB4 (synonym CHN1), BEB8 (synonym CM19), I (synonyms BEB2 and CEbE) and J (synonyms BEB1, CEbB, PtEb X) of *Enterocytozoon bieneusi* recorded in different host species and water samples in previous studies

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Table S3. Prevalence of *Enterocytozoon bieneusi* recorded previously in cattle in 11 countries. Genotyping studies using previously confirmed *E. bieneusi* samples were excluded

Table S4. All *Enterocytozoon bieneusi* genotypes recorded previously in four species of Bovinae worldwide

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Table 1. The information regarding faecal samples collected from calves (mixed age) raised on three dairy farms (A - C) and two beef farms (D and E) located within Tarago water catchment, Victoria, Australia, in November 2011 (Spring) and April 2012 (Autumn), corresponding to consecutive calving seasons.

Season	Age group (months)		
Farm	≤ 3	3 - 9	Total
Autumn	159	51	210
A	27	na	27
B	36	na	36
C	na	51	51
D	39	na	39
E	57	na	57
Spring	133	128	261
A	50	na	50
B	38	12	50
C	na	57	57
D	45	20	65
E	na	39	39
Total	292	179	471

na = not available.

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Table 2. Association analysis of the risk factors, cattle age (< 3 mo and 3 - 9 mo) and season (Spring and Autumn), with Enterocytozoon bieneusi test-positivity (by PCR-based sequencing of the internal transcribed spacer, ITS), assessed using the Chi-square and Fisher's exact tests. The strength of association was measured using an odds ratio calculated with 95% confidence intervals (95% CI), and statistical significance was given as a P-value

Risk factors (age/season)	No. of samples tested	No. of test-negative samples	No. of test-positive samples (%)	Odds ratio (95% CI)	P-value
Age group					
< 3 mo	292	268	24 (8.2)	1.813 (1.001 - 3.284)	0.061
3 - 9 mo	179	154	25 (14.0)		
Season					
Spring	261	226	35 (13.4)	2.168 (1.133 - 4.148)	0.022*
Autumn	210	196	14 (6.7)		
Total	471	422	49 (10.4)		

* = statistically significant ($P < 0.05$).

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Table 3. Genotypes of Enterocytozoon bieneusi characterised by nested PCR-based sequencing of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA from 49 individual faecal samples (sample codes given) from cattle (different age) from five farms (A to E) located in Tarago water catchment in Victoria, Australia, in Spring (November 2011) and Autumn (April 2012)

Genotypic designation	GenBank accession no.	Sample code	Farm	Age (mo)	Season
TAR_fc2[a]	MH899207	2	A	< 1	Spring
BEB4	MH899203	6	A	< 1	Spring
BEB8/TAR_fc5[b]	MH899210	14	A	< 1	Spring
TAR_fc2[a]	MH899207	15	A	< 1	Spring
TAR_fc2[a]	MH899207	18	A	< 1	Spring
TAR_fc2[a]	MH899207	19	A	< 1	Spring
BEB8/TAR_fc5/ TAR_fc6/ TAR_fc7[b]	MH899211	20	A	< 1	Spring
TAR_fc2[a]	MH899207	29	A	1 - 3	Spring
TAR_fc2[a]	MH899207	45	A	1 - 3	Spring
I	MH899204	51	B	< 3	Spring
I	MH899204	53	B	< 3	Spring
I	MH899204	56	B	< 3	Spring
I	MH899204	61	B	< 3	Spring
I	MH899204	76	B	< 3	Spring
I	MH899204	79	B	< 3	Spring
I	MH899204	82	B	< 3	Spring
BEB4	MH899203	111	C	3 - 6	Spring
BEB4	MH899203	119	C	3 - 6	Spring
I	MH899204	129	C	3 - 6	Spring
BEB4	MH899203	130	C	3 - 6	Spring
J	MH899205	144	C	3 - 6	Spring
BEB4	MH899203	148	C	3 - 6	Spring
I	MH899204	149	C	3 - 6	Spring
BEB4	MH899203	150	C	3 - 6	Spring

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J	MH899205	206	E	3 - 6	Spring
I	MH899204	209	E	3 - 6	Spring
J	MH899205	228	E	3 - 6	Spring
I/TAR_fc4[a]	MH899209	237	E	3 - 6	Spring
J	MH899205	241	E	3 - 6	Spring
J	MH899205	244	D	3 - 6	Spring
I	MH899204	248	D	3 - 6	Spring
I	MH899204	252	D	3 - 6	Spring
J	MH899205	256	D	3 - 6	Spring
I	MH899204	261	D	3 - 6	Spring
J	MH899205	262	D	3 - 6	Spring
TAR_fc1[a]	MH899206	317	B	< 1	Autumn
I	MH899204	326	C	5 - 9	Autumn
J	MH899205	328	C	5 - 9	Autumn
J	MH899205	334	C	5 - 9	Autumn
I	MH899204	351	C	5 - 9	Autumn
J	MH899205	353	C	5 - 9	Autumn
J	MH899205	373	C	5 - 9	Autumn
I	MH899204	391	D	< 3	Autumn
I	MH899204	407	D	< 3	Autumn
I	MH899204	436	E	< 3	Autumn
J	MH899205	440	E	< 3	Autumn
J	MH899205	447	E	< 3	Autumn
J	MH899205	459	E	< 3	Autumn
TAR_fc3[a]	MH899208	473	E	< 3	Autumn

[a] novel genotype. [b] mixed (indeterminant) genotypes.

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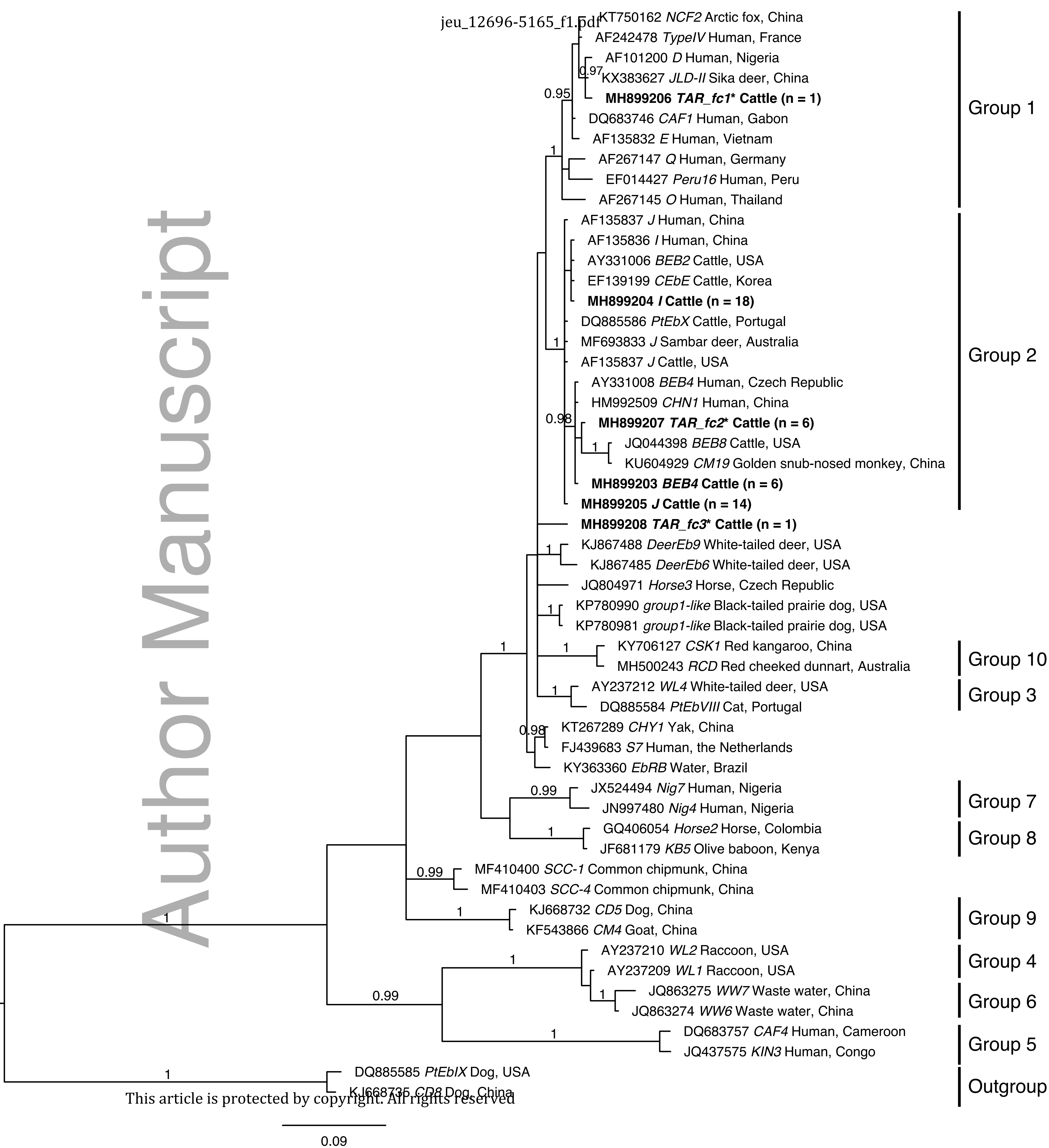

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