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Molecular characterisation of *Giardia duodenalis* in captive non-human primates reveals mixed assemblage A and B infections and novel polymorphisms ☆

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ABSTRACT

Giardia is frequently detected in stools of non-human primates (NHP). However, a molecular identification has been rarely applied to *Giardia* isolates from NHP, and the distribution of the zoonotic assemblages A and B remains unclear. Moreover, little is known about the genetic variability among the isolates, although this may contribute to the elucidation of the different transmission pathways, including the role of NHP as a reservoir for human giardiasis. Therefore, 258 *Giardia* samples from 31 NHP species housed in nine zoological gardens and one sanctuary in Belgium and The Netherlands were characterised based on an assemblage-specific PCR targeting the *triose phosphate isomerase* (*tpi*) gene to identify both assemblage A and B infections. In addition, a multi-locus sequencing approach based on the *glutamate dehydrogenase*, the *tpi* and the *β-giardin* genes was used to examine both the genetic variability and the ability to allocate these isolates to different NHP groups. Overall, assemblage B was the most prevalent (78.6%), but mixed assemblage A and B infections occurred in 32.7% of the samples. Sequencing of the isolates revealed the presence of new polymorphisms for both assemblages and at the three loci examined. The majority of the assemblage B isolates could not be grouped into recently described sub-assemblages, particularly at the *tpi* gene. Isolates could only be allocated to a specific group when polymorphisms of the three loci were combined. The results confirm that NHP are a potential reservoir for zoonotic transmission and advocate the use of assemblage-specific primers in molecular epidemiological surveys, as mixed infections are likely to be underestimated. The high level of heterogeneity within assemblages indicates that a revised nomenclature of these sub-assemblages is needed, but points out the potency of a multi-locus sequencing approach to unravel the complex epidemiology of *Giardia duodenalis*.

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1. Introduction

Giardia in non-human primates (NHP) is of noteworthy importance both from a veterinary and a public health point of view. The parasite is commonly found in stools of wild and captive NHP (Ghandour et al., 1995; Hope et al., 2004; Salzer et al., 2007; Levecke et al., 2007) and is a significant cause of diarrhoea and failure to thrive, particularly in young animals (Hamlen and Lawrence, 1994; Kalishman et al., 1996). In addition, NHP might be a potential reservoir for zoonotic transmission, as these animals harbour the zoonotic *Giardia duodenalis* assemblages A (Monis et al., 1996; Graczyk et al., 2002; Vitazkova and Wade, 2006) and B (Monis et al., 1996; Karanis and Ey, 1998; Vitazkova and Wade, 2006; Cacciò et al., 2008). However, the distribution of these assemblages within NHP remains unclear. The majority of these studies examined only a limited number of isolates and/or animal species. Moreover, mixed assemblage A and B infections could not be ruled out, as the standard PCR approach will preferentially amplify the most abundant assemblage (Weiss et al., 1992; Geurden et al., 2007).

Furthermore, little is known about the genetic variation among isolates originating from different NHP populations, as a molecular characterisation was often based on a single genetic locus which frequently differed between studies. Yet, recent molecular analysis of *Giardia* isolates at the *glutamate dehydrogenase* (*gdh*),

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™ under the accession numbers: FJ890942–78.

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triose phosphate isomerase (*tpi*) and the *β-giardin* (*bg*) genes indicates a high degree of genetic variability within both assemblages A and B (Wielinga and Thompson, 2007), which may contribute to the elucidation of different transmission pathways, including the role of animals as a reservoir for human giardiasis. In this study, we have conducted a molecular survey to determine the distribution of *G. duodenalis* assemblage A and B in NHP. *Giardia* samples previously found in NHP housed at nine zoological gardens and one sanctuary (Levecke et al., 2007; unpublished observations) were characterised based on an assemblage-specific PCR targeting the *tpi* gene. In addition, a multi-locus sequencing approach based on the *gdh*, *tpi* and *bg* genes was performed to examine the genetic variability and the ability to allocate these isolates to different NHP groups.

2. Materials and methods

2.1. Selection of the Giardia samples

A total of 258 *Giardia* samples from 47 NHP groups belonging to 31 animal species were withheld for further molecular analysis (Table 1). The samples were obtained from previously conducted epidemiological surveys at nine zoological gardens and one sanctuary in Belgium and The Netherlands (Levecke et al., 2007; unpublished observations) and were positive for at least one of the two techniques used (microscopic examination and a commercial immunofluorescence assay (MERIFLUOR® *Cryptosporidium*/*Giardia* immunofluorescence assay, Meridian Diagnostics Inc., Cincinnati, OH, USA)).

2.2. Molecular identification

DNA was extracted using the QIAamp® Stool Mini Kit (Qiagen) according to the manufacturer's instructions, incorporating an initial step of three freeze-thaw cycles (freezing in liquid nitrogen for 5 min and heating at 95 °C for 5 min) in the protocol to maximise rupture of the cysts.

The molecular identification was based on two approaches: assemblage-specific amplification and multi-locus sequencing.

2.3. Assemblage-specific amplification

The assemblage-specific amplification was performed on all selected *Giardia* isolates and consisted of a nested PCR targeting the *tpi* gene. The first reaction was based on the internal set of primers described by Sulaiman et al. (2003), followed by two separate assemblage-specific nested PCRs. The amplification of *G. duodenalis* assemblage A was according to Geurden et al. (2007). For the detection of assemblage B, new primers (AssBF: 5′ GTT GTT GTT GCT CCC TCC TTT 3′ and AssBR: 5′ CCG GCT CAT AGG CAA TTA CA 3′) were designed based on Genbank accession no. L02120 (assemblage A), AY228628 (assemblage BIII), AY228632 (assemblage BIII-like), AF069560 (assemblage BIV), AY228634 (assemblage BIV-like), AF069563 (assemblage C), DQ246216 (assemblage D), AY655705 (assemblage E), AF069558 (assemblage F) and AF069562 (assemblage G). The GoTaq® Flexi DNA Polymerase kit (Promega) was used for each of the PCR mixtures and consisted of 2.5 μl DNA, 0.5 μl of each primer (10 μM), 1 μl $MgCl_2$ (25 mM), 5 μl GoTaq® Flexi Buffer, 14.875 μl PCR H_2O and 0.125 μl GoTaq® Flexi DNA polymerase. Identical conditions were

Table 1
The number of isolates and mono-and mixed infections of *Giardia duodenalis* assemblage A and B in 31 non-human primate species based on assemblage-specific PCR targeting the *tpi* gene.

Common name	Country	Isolates	Assemblage			NA
			A	B	A & B	
Alaotran lemur	The Netherlands	2	0	2	0	0
Barbary macaque	The Netherlands	20	1	1	5	13
Black-and-white ruffed lemur	Belgium	3	0	1	0	2
Black-capped squirrel monkey	The Netherlands	12	3	1	4	4
Black-headed spider monkey	Belgium/The Netherlands	12	2	3	0	7
Brown howler	The Netherlands	29	7	4	0	18
Brown woolly monkey	The Netherlands	8	2	0	0	6
Chimpanzee	Belgium	15	1	14	0	0
Common squirrel monkey	Belgium	9	1	4	0	4
Crab-eating macaque	The Netherlands	2	1	0	1	0
Eastern gorilla	Belgium	1	0	0	0	1
Golden lion tamarin	The Netherlands	1	0	0	0	1
Golden-bellied capuchin	Belgium	1	0	0	0	1
Grivet/Tantalus monkey	The Netherlands	1	0	0	0	1
Hamadryas baboon	Belgium/The Netherlands	7	2	3	2	0
Javan lutung	Belgium/The Netherlands	4	0	4	0	0
Mantled guereza	Belgium	12	0	10	0	2
Northern white-cheeked gibbon	The Netherlands	1	0	0	0	1
Pygmy marmoset	The Netherlands	1	0	0	0	1
Red ruffed lemur	Belgium/The Netherlands	7	0	0	0	7
Rhesus monkey	The Netherlands	1	1	0	0	0
Ring-tailed lemur	Belgium/The Netherlands	85	8	20	36	21
Ring-tailed lemur/White-fronted lemur	Belgium	2	0	0	0	2
Siamang	Belgium	1	0	1	0	0
Silvery woolly monkey	The Netherlands	1	0	0	0	1
Sunda pig-tailed macaque	The Netherlands	6	2	2	1	1
Vervet monkey/Tantalus monkey	The Netherlands	5	1	1	1	2
Western gorilla	The Netherlands	4	1	2	0	1
White-handed gibbon	Belgium	2	0	0	0	2
White-headed geoffroyi	The Netherlands	3	1	0	2	0
Total		258	34	73	52	99

NA: no amplification.

used for both assemblage-specific PCRs: 35 cycles (95 °C for 45 s, 62 °C for 45 s and 72 °C for 1 min), with an initial step of 2 min at 95 °C and a final extension step at 72 °C for 5 min. All PCR products were electrophoresed on 1.5% agarose gels and visualised with ethidium bromide. In addition, the specificity of the assemblage-specific primers was evaluated by sequencing 20 samples for each assemblage. The PCR products were purified using the QIAquick® PCR purification kit (Qiagen) and fully sequenced using the BigDye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were analysed on a 3100 Genetic Analyzer (Applied Biosystems) and assembled with Seqman II (DNASTAR, Madison, WI, USA). Subsequently, the fragments were aligned using MegAlign (DNASTAR, Madison, WI, USA) and compared with reference sequences summarised in recent studies (Cacciò et al., 2008; Wielinga and Thompson, 2007).

2.4. Multi-locus sequencing

The multi-locus sequencing approach was performed on the first 25% of the selected samples using previously described nested PCR protocols targeting the *gdh* gene (Cacciò et al., 2008), the *tpi* gene (Sulaiman et al., 2003) and the *bg* gene (Cacciò et al., 2002; Lalle et al., 2005). The PCR products were purified and sequenced as described above. In addition to the references, unique sequences of NHP available in the GenBank™ database were included in the alignment. Isolates were grouped into (sub)-assemblages based on the substitution patterns proposed by Wielinga and Thompson (2007) and a comparison was made both at the level of assemblages and sub-assemblages. The occurrence of novel substitution positions was examined. To this end, only novel substitutions which were found in at least two isolates were considered for further analysis. Finally, the ability to allocate the isolates to the different NHP groups was examined.

3. Results

3.1. Assemblage-specific amplification

In 159 (61.6%) samples, an amplification was obtained by one or both PCRs performed. Assemblage B was the most prevalent (78.6% of the samples). However, mixed assemblage A and B infections were observed in 32.7% of the samples (Table 1). The specificity of the primers was confirmed by sequencing 40 random samples.

3.2. Multi-locus sequencing

The first 25% (66) of the samples originating from 13 groups (13 NHP species) were analysed at three loci. Each PCR resulted in a comparable percentage of amplicons, ranging from 71.2% for *tpi* to 78.8% for *gdh* and *bg*. Sequence information for at least one of the loci was collected from 35 samples (Table 2). Only *G. duodenalis* assemblage A or B were identified, the latter being more prevalent (28 samples). Mixed infections based on heterogeneous templates at assemblage-specific positions were found in three samples for the *bg* gene (CH41, R360 and R1127) and in one sample for both the *tpi* (R1127) and the *gdh* (R1127) locus. Yet, the assemblage-specific PCRs revealed the occurrence of mixed assemblages in nine samples. The assignment of isolates to assemblages was not influenced by the locus investigated. However, 'assemblage swapping' was observed in one sample (SQ694). This sample was identified as assemblage A at the *tpi* gene and as assemblage B at the *bg* gene. The assemblage-specific PCRs revealed a mixed assemblage A and B infection.

3.2.1. Assemblage A isolates

The four assemblage A isolates (CH35, SQ678, SQ681 and SQ694) found in the present study could be grouped into sub-assemblages AI (SQ678, SQ681, SQ694) and AII (CH35) based on the specific substitution patterns previously described by Wielinga and Thompson (2007) (Table 3, substitutions positions are highlighted in bold). Samples SQ678 and SQ681 were identified as sub-assemblage AI at all loci examined.

With the exception of isolate SQ694 at the *tpi* gene and isolate CH35 at the *bg* gene, which had identical substitution patterns compared with reference isolates, the isolates SQ678 and SQ681 contained novel substitution positions (Table 3, highlighted in bold italics), and this occurred for all three genes examined. These included two substitutions at both the *gdh* gene (position (p) 510 and 756) and the *bg* gene (p 228 and 480). At the *tpi* gene novel substitutions were observed at p 126, 268, 393 and 453. For this gene, two different isolates of sub-assemblage AI (SQ694, SQ678/SQ681) were found in a group of squirrel monkeys housed at a Dutch zoo.

Table 2
A comparison of three loci for the identification of 35 *Giardia* isolates from non-human primates at the level of assemblage and (sub-assemblage).

Isolate	Host (common name)	*gdh*	*tpi*	*bg*	*tpi*-mixed
SQ678, 681	Black-capped squirrel monkey	A(I)	A(I)	A(I)	A+B
RA, B	Ring-tailed lemur	B(IV)	B(IV)	B(IV)	B
R352, 371	Ring-tailed lemur	B(IV)	B(IV)	B(IV)	A + B
F3, 19, 24, 27–30	Mantled guereza	B(central/IV-like)	B(IV)	B(IV)	B
M	Mandrill	B(central/IV-like)	B(IV)	B(IV)	B
SL2,7	Black-headed spider monkey	B(central/IV-like)	B(IV)	B(IV)	B
CH37	Chimpanzee	B(central/IV-like)	B	B(III)	B
CH53, 55, 56	Chimpanzee	B(central/IV-like)	B	B(IV)	B
R763	Ring-tailed lemur	B(central/IV-like)	B	B(IV)	A + B
CH41	Chimpanzee	B(central/IV-like)	B	A+B	B
R360	Ring-tailed lemur	B(IV)	B(IV)	A+B	A + B
R1127	Ring-tailed lemur	A+B	A+B	A+B	A + B
BW200	Black-and-white ruffed lemur	B(IV)	B	_	B
CH7	Chimpanzee	B(central/IV-like)	B	_	B
R1108	Ring-tailed lemur	B(central/IV-like)	B	_	A + B
CH54	Chimpanzee	B(central/IV-like)	B	_	B
CH1	Chimpanzee	B(central/IV-like)	_	B(IV)	B
SQ694	Black-capped squirrel monkey	_	A(I)	B(IV)	A + B
CH35	Chimpanzee	A(II)	_	_	_
F1	Mantled guereza	_	B(IV)	_	B
R21	Ring-tailed lemur	_	_	B(IV)	B
F4	Mantled guereza	_	_	B(IV)	B
MU6	Javan lutung	_	_	B(IV)	B

Table 3
Assemblage A isolates, positions and breakdowns of intra-sub-assemblage substitutions for *glutamate dehydrogenase* (*ghd*), *triose phosphate isomerase* (*tpi*) and *β-giardin* (*bg*) genes. The isolates found in the present study and substitution patterns proposed by Wielinga and Thompson (2007) are highlighted in bold. Novel substitution positions are highlighted in bold italics. Nucleotide sequences identical to reference isolates are indicated by '.'. Nucleotide sequences not available for comparison are indicated by '_'.

Glutamate dehydrogenase

Assemblage	Isolate	Host/Country	GenBank™ Acc N°	237	246	249	258	270	309	372	390	396	*510*	522	525	531	534	570	603	621	633	636	672	687	693	699	720	723	753	*756*	771	786	807	831	861	867	870	894	902	1080	1266
AI	Portland 1	Human/Australia	M84604	C	C	T	C	T	C	C	T	A	*C*	G	C	C	A	C	T	C	C	T	C	T	C	T	T	C	C	*C*	C	C	C	C	T	T	T	C	C	T	G
	SQ678, 681	**B.-c. squirrel monkey/ The Netherlands**	FJ890951	_	_	_	_	_	_	_	_	_	*T*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*T*	.	.	.	.	.	.	.	.	.	_	_
AII	NLH45	Human/The Netherlands	AY826195	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
A III	NLR118	Roe deer/ The Netherlands	DQ100288	.	.	C	G	C	T	A	C	G	.	C	T	T	G	T	C	.	T	C	T	C	T	C	C	T	.	.	A	T	.	.	.	C	C	_	_	_	_

Triose phosphate isomerase

Assemblage	Isolate	Host/Country	GenBank™ Acc N°	93	108	111	117	120	*126*	129	133	144	162	174	189	231	*268*	352	*393*	394	399	*453*
AI	WB	Human/Afghanistan	L02120	T	C	G	C	C	*C*	T	G	G	A	A	A	C	*A*	C	*C*	T	C	*T*
	SQ694	**B.-c. squirrel monkey/ The Netherlands**	FJ890962	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	SQ678, 681	**B.-c. squirrel monkey/ The Netherlands**	FJ890961	.	.	.	.	.	*T*	.	.	.	.	.	.	.	*G*	.	*A*	.	.	*C*
AII	JH	Human/USA	U57897	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	T	.
AIII	_	Fallow deer/Italy	DQ650648	C	T	A	T	T	.	.	T	A	G	G	G	T	.	T	.	C	.	.

β-giardin

Assemblage	Isolate	Host/Country	GenBank™ Acc N°	*228*	294	393	417	450	460	468	*480*	483	501	541	573	603	606	621	729
AI	Portland 1	Human/UK	X85958	*G*	C	T	T	C	C	T	*C*	T	A	G	G	T	C	A	A
	SQ678, 681	**B.-c. squirrel monkey/ the Netherlands**	FJ890978	*A*	.	.	.	.	.	.	*T*	.	.	.	.	.	.	.	_
AII	ISSGF7	Human/Italy	AY072724	.	.	.	.	.	T	C	.	.	.	.	.	.	T	.	G
	CH35	**Chimpanzee/Belgium**		.	.	.	.	.	T	C	.	.	.	.	.	.	T	_	_
AIII	_	Fallow deer/Italy	DQ650649	.	T	C	C	.	.	C	.	C	G	.	A	C	.	G	_

B.-c. squirrel monkey: black-capped squirrel monkey.

3.2.2. Assemblage B isolates

In 25 samples, clear sequence data were obtained for both the *gdh* and *tpi* genes (Table 2). Only five (R352, R360, R371, RA and RB) isolates could be grouped into the proposed polymorphisms using these two genes (Table 4, substitution positions highlighted as bold); all five were identified as sub-assemblage BIV. Ten sam-

Table 4
Assemblage B isolates, positions and breakdowns of intra-sub-assemblage substitutions for *glutamate dehydrogenase* (*gdh*), *triose phosphate isomerase* (*tpi*) and *β-giardin* (*bg*) genes. The isolates found in the present study and substitution patterns proposed by Wielinga and Thompson (2007) are highlighted in bold. Novel substitution positions are highlighted in bold italics. Nucleotide sequences identical to reference isolates are indicated by '.'. Nucleotide sequences not available for comparison are indicated by '_'.

Glutamate dehydrogenase (Nucleotide position from start of gene)

Assemblage	Isolate	Host/Country	GenBank™ Acc N°	219	297	309	357	360	429	447	519	540	561	570	576	582	597	612	642	690	699	705	723	756	786	807	825	876	891	896	921	969	1077	1143	1251	1254
BIII	FCQ21	Human/Mexico	AY178756	T	C	Y	Y	G	T	T	Y	C	C	C	G	G	C	G	C	G	C	T	C	T	C	G	A	G	T	G	G	T	T	T	C	Y
BIII-like	gd-ber1	Human/Norway	DQ090532	_	.	C	T	.	.	.	C	T	.	.	.	.	.	.	.	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
B-central	NLH25	Human/Netherlands	AY826193	.	T	C	T	.	C	C	C	T	.	.	.	.	.	.	.	.	.	.	T	.	.	T	G	.	.	C	_	_	_	_	_	_
B-central/ BIV-like	F3, 19, 24, 27- 30	Mantled guereza/Belgium	FJ890942	_	_	_	_	_	_	_	C	T	.	.	.	.	.	A	T	.	.	.	T	.	.	T	G	.	.	.	A	_	_	_	_	_
	M	Hamadryas baboon/Belgium	FJ890943	_	_	_	_	_	_	_	C	T	.	.	.	.	.	A	T	.	.	.	T	.	.	T	G	.	.	.	A	_	_	_	_	_
	SL2, 7	B.-h. spider monkey/Belgium	FJ890944	_	_	_	_	_	_	_	C	T	.	.	.	.	.	A	T	.	.	.	T	.	.	T	G	.	.	.	A	_	_	_	_	_
	CH37, 41, 54-56	Chimpanzee/Belgium	FJ890945	_	_	_	_	_	_	_	C	T	.	.	.	.	.	A	T	.	.	.	T	.	.	T	G	.	.	.	A	_	_	_	_	_
	CH7, 53	Chimpanzee/Belgium	FJ890950	_	_	_	_	_	_	_	C	T	.	.	.	.	.	A	Y	.	.	.	T	.	.	T	G	A	.	.	A	_	_	_	_	_
	CH1	Chimpanzee/Belgium	FJ890946	_	_	_	_	_	_	_	C	T	.	.	.	.	.	A	.	.	.	.	T	.	.	T	G	A	.	.	A	_	_	_	_	_
	R763, 1108	R.-t. lemur/The Netherlands	FJ890947	_	_	_	_	_	_	_	C	T	.	.	.	.	.	A	.	.	.	.	T	.	.	T	G	.	.	.	A	_	_	_	_	_
BIV-like	gd-ber8	Human/Norway	DQ090539	_	.	T	C	.	C	C	C	T	.	.	.	.	.	.	.	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
BIV	CZ:D41	Dog/Czech. Rep.	AY178749	C	.	T	C	.	C	C	C	T	T	.	.	.	.	A	.	.	.	.	T	.	T	T	G	.	.	.	A	C	C	C	T	C
	R352, 360, 371, A, B	R.-t. lemur/Belgium	FJ890948	_	_	_	_	_	_	_	C	T	T	.	.	.	.	A	.	.	.	.	T	.	.	T	G	.	C	.	A	_	_	_	_	_
	BW200	B. & w. ruffed lemur/Belgium	FJ890949	_	_	_	_	_	_	_	C	T	T	.	.	.	.	A	.	.	.	.	T	C	.	T	G	.	.	.	.	_	_	_	_	_

Triose phosphate isomerase

Assemblage	Isolate	Host/Country	GenBank™ Acc N°	39	45	91	162	165	168	189	210	216	297	402	429	471	483	534
BIII	2436	Water/USA	AY368163	G	C	C	G	C	C	A	G	T	A	A	G	C	G	C
BIII-like	3920	Water/USA	AY368166	.	T	.	.	T	.	.	.	C	.	G	.	A	.	.
B	ISSGdA711	Barbary macaque/Italy	EU637591	.	.	T	.	T	T	.	A	C	.	.	.	A	A	T
	CH37, 41, 53- 56	Chimpanzee/Belgium	FJ890952	.	.	T	.	T	T	.	A	C	.	.	.	A	A	_
	CH7	Chimpanzee/Belgium	FJ890953	.	.	T	.	T	Y	R	A	C	.	.	.	A	A	_
	BW200	B. & w. ruffed lemur/Belgium	FJ890954	.	.	T	.	T	T	.	A	C	.	.	.	A	A	_
	R763, 1108	R.-t. leumur/The Netherlands	FJ890955	A	T	T	.	T	.	.	.	C	.	.	.	A	A	_
BIV-like	2901	Human/Peru	AY228635	_	_	T	.	T	T	.	.	C	.	.	.	A	A	.
BIV	2100	Water/USA	AY368170	A	T	T	.	T	T	.	A	C	.	.	.	A	A	.
	R352, 360, 371, A, B	R.-t. lemur/Belgium	FJ890956	A	T	T	.	T	T	.	A	C	.	.	.	A	A	_
	M	Hamadryas baboon/Belgium	FJ890957	A	T	T	.	T	T	.	A	C	.	.	.	A	A	_
	SL2, 7	B.-h. spider monkey/Belgium	FJ890958	A	T	T	.	T	T	.	A	C	.	.	.	A	A	_
	F1, 3, 19, 24, 28, 29	Mantled guereza/Belgium	FJ890959	A	T	T	.	T	T	R	A	Y	.	.	.	A	A	_
	27, 30	Mantled guereza/Belgium	FJ890960	A	T	T	.	T	T	R	A	Y	.	.	R	A	A	_

β-giardin

Assemblage	Isolate	Host/Country	GenBank™ Acc N°	105	210	228	234	285	327	354	369	378	426	438	495	510	564	648
BIII	BAH8	Human/Australia	AY072727	C	C	A	G	T	C	C	C	C	C	C	C	G	T	G
	CH37	Chimpanzee/Belgium	FJ890963	_	.	.	.	.	.	.	.	.	.	.	.	.	.	_
BIV	BG-Ber2	Human/Norway	DQ090523	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
	R21	R.-t. lemur/Belgium	FJ890964	_	.	.	.	.	.	T	.	.	.	.	.	.	.	_
	SL2, 7	B.-h. spider monkey/Belgium	FJ890965	_	.	.	.	.	.	T	.	.	.	.	.	.	.	_
	SQ694	B.-c. squirrel monkey /the Netherlands	FJ890966	_	T	.	.	.	.	T	.	.	.	.	.	.	.	_
	R763	R.-t. lemur/the Netherlands	FJ890967	_	T	.	.	.	.	T		.	.	.	.	.	.	_
	M	Hamadryas baboon/Belgium	FJ890968	_	T	.	.	.	.	T	.	.	.	.	.	.	.	_
	ISSGd722	Barbary macaque/Italy	EU637581	_	T	.	.	.	.	T	.	.	.	T	.	.	.	A
	F4	Mantled guereza/Belgium	FJ890969	_	T	.	.	.	.	T	.	.	.	T	.	.	.	_
	F27	Mantled guereza/Belgium	FJ890970	_	T	.	.	.	.	T	.	.	.	Y	.	.	.	_
	F30	Mantled guereza/Belgium	FJ890971	_	T	.	.	.	.	T		.	.	Y	.	.	.	_
	F3	Mantled guereza/Belgium	FJ890972	_	T	.	.	.	Y	T	.	.	Y	.	T	.	.	_
	F28, 29	Mantled guereza/Belgium	FJ890973	_	T	.	.	.	Y	T	.	.	Y	Y	Y	.	.	_
	F24, 19	Mantled guereza/Belgium	FJ890974	_	T	.	.	.	T	T	.	.	T	.	T	.	.	_
	MU6	Javan lutung	FJ890975	_	T	.	R	.	T	T	.	.	T	.	T	.	.	_
	CH1, 53, 55, 56	Chimpanzee/Belgium	FJ890976	_	T	.	.	.	.	T	T	.	.	.	.	.	.	.
	R352, 371, A, B	R.-t. lemur/Belgium	FJ890977	_	T	.	A	.	.	T	T	.	.	.	.	A	.	.

B.-h. spider monkey: black-headed spider monkey; B. & w. lemur: black-and-white ruffed lemur; R.-t. lemur: ring-tailed lemur; B.-c. squirrel monkey: black-capped squirrel monkey.

ples (F3, F19, F24, F27–30, M, Sl2, SL7) were characterised as BIV at the *tpi* gene. However, these samples could not be assigned to a sub-assemblage at the *gdh* gene, due to the limited sequences information at position 219 distinguishing B-central from BIV-like (Table 4). Isolate BW200 was classified as BIV at the *gdh* gene, but consisted of a combination of BIII/III-like (p 39: guanine, 168: cytosine), BIV-like (p 210: guanine) and BIV (p 210: adenine) substitution patterns at the *tpi* gene. The remaining nine isolates (CH7, CH37, CH41, CH53, CH54, CH55, CH56, R763 and R1108) could not be classified due to limited sequence information (*gdh*) and because isolates consisted of a combination of previously described sub-assemblages (*tpi*).

For the *bg* gene, clear sequences were obtained for 24 assemblage B isolates (Table 4). At present, polymorphisms are not available to link the *bg* locus heterogeneity to the *gdh* and *tpi* gene sub-assemblage classifications, but position 354 seems to be a promising candidate, separating the sub-assemblage BIII (cytosine) from BIV (thymine) (Table 4, substitution position highlighted as bold). Based on this polymorphism, only one (CH37) out of 24 isolates was identified as sub-assemblage BIII (Table 2). At this gene, both sub-assemblages were found in a group of chimpanzees housed at a Belgian zoo (BIII: CH37 and BIV: CH1, 53, 55, 56).

For 19 samples clear sequences were obtained at all three loci. The multi-locus comparison at the level of sub-assemblages revealed only a perfect agreement at all loci in four samples (R352, R371, RA and RB) (Table 2). In 10 samples (F3, F19, F24, F27–30, M, Sl2, SL7) there was a perfect agreement between *tpi* and *bg*, but not with the *gdh* gene. In the remaining four samples (CH37, CH53, CH55, CH56 and R763) a different sub-assemblage at each of the genes was found.

For 31 samples clear sequences were available at either the *gdh*, *tpi* or *bg* gene (Table 4, highlighted as bold), revealing a high genetic variability among the different isolates at all loci. Twenty-two samples had identical polymorphisms to previously described isolates at one or two of the examined loci (*tpi*: ISSGdA711 (Cacciò et al., 2008), 2100 (Sulaiman et al., 2004); *bg*: BAH8 (Cacciò et al., 2002), BG-Ber2 (Robertson et al., 2006)). Three new polymorphisms were found at the *gdh* (p 642, 876 and 891). At the *tpi* gene, a heterogeneous template (R = adenine/guanine) was noted at position 189. Novel polymorphisms at the *bg* gene were observed at position 234, 426, 495 and 510 (Table 4, highlighted in bold italics). Different isolates of sub-assemblage BIV were found in a group of mantled guerezas (F4, F19/F24) at the *bg* gene and in a group of chimpanzees (CH1, CH37/41/54/55/56) at the *gdh* gene. Moreover, heterogeneous templates found among the isolates of these two groups indicate a mixture of isolates within one sample or a mixture of alleles within one isolate.

3.2.3. Allocation of the isolates to NHP groups

Despite the genetic variation found among the assemblage B isolates, polymorphisms found in the present study could not be allocated to a specific group of NHP based on a single locus. At the *gdh* gene, an identical substitution pattern was found in isolates from the mantled guerezas (F3, F19, F24, F27–30), black-headed spider monkey (SL2 and SL7), Hamadryas baboon (M) and chimpanzees (CH37, 53, 55 and 56) which were all housed at the same zoo in Belgium. At the *tpi* gene, the isolates of the chimpanzees, Hamadryas baboon and black-headed spider monkey also had the comparable patterns as black-and-white ruffed and ring-tailed lemurs at another zoo in Belgium. In addition, no difference was observed in substitution pattern at the *bg* gene between isolates obtained from Hamadryas baboons in Belgium and ring-tailed lemurs (R763) in The Netherlands. Yet, a combination of the three loci examined resulted in a substitution pattern which was unique for each of the NHP groups.

4. Discussion

We believe this is the first study where *Giardia* isolates from a large and diverse population of captive NHP were characterised based on both an assemblage-specific PCR and a multi-locus sequencing approach. The results confirm that NHP harbour the zoonotic assemblages of *G. duodenalis*, with assemblage B being the most prevalent. This finding is in contrast to other animal species where assemblage A appears more prevalent than assemblage B (Xiao and Fayer, 2008), but mirrors the results obtained in human samples (Amar et al., 2002; van der Giessen et al., 2006). The high prevalence of mixed infections detected by the assemblage-specific PCR illustrates the limitations of PCR followed by sequencing and advocates the use of assemblage-specific primers in molecular studies for three reasons. Firstly, prevalence data obtained by a standard PCR protocol are biased as illustrated by a previous study in cattle (Geurden et al., 2007) and confirmed in the present study. Secondly, the detection of mixed infections may give supplementary insights into the incongruent assignment to *G. duodenalis* assemblages based on sequences of different loci ('assemblage swapping'). This phenomenon has previously been described both in human (Cacciò et al., 2008; unpublished observations) and animal isolates (Read et al., 2004; Traub et al., 2004) and was also observed in one of the presented samples (SQ694). Although various explanations for 'assemblage swapping' such as mixed infections and/or meiotic recombination (Cooper et al., 2007; Teodorovic et al., 2007; Wielinga and Thompson, 2007) have been suggested, its underlying mechanism(s) remains unclear. However, the high prevalence of mixed infections in the present study, including in sample SQ694, suggests that sexual reproduction (Birky, 2005; Logsdon, 2008) is likely to be less important as a cause of 'assemblage swapping'. Thirdly, an assemblages-specific approach is also necessary to confirm the host specificity of the other assemblages of *G. duodenalis* (assemblage C to G). In contrast to assemblages A and B, these assemblages are restricted to specific hosts (C/D: dogs and other canids; E: cattle and other hoofed livestock, F: cats, G: rats). Mixed infections of assemblages C, D, E, F and/or G outside their specific hosts have never been considered. Yet, this may have an impact on the newly proposed taxonomy of *Giardia* (Thompson and Monis, 2004), in which the assemblages are revised into *Giardia canis* (assemblage C/D), *Giardia bovis* (assemblage E), *Giardia cati* (assemblage F) and *Giardia simondi* (assemblage G).

The multi-locus sequencing approach confirmed the high genetic variability at the three loci examined (Wielinga and Thompson, 2007; Cacciò et al., 2008). Moreover, the isolates sequenced displayed mainly novel polymorphisms. The assemblage A isolates were identified as either AI or AII. In a previous study in brown howler monkeys (Volotão et al., 2008), only sub-assemblage AI was found, suggesting that sub-assemblage AI is more prevalent. However, sequence data are too limited to draw robust conclusions. Only a minority of the assemblage B isolates could be grouped into the substitution patterns described, and were exclusively identified as sub-assemblage BIV. This is in contrast to other studies were isolates of NHP were also identified as BIII, BIII-like and/or BIV-like at either the *gdh* or the *tpi* gene (Itagaki et al., 2005; Cacciò et al., 2008; unpublished observations). The remaining assemblage B isolates could not be classified due to shortness of sequence data (*gdh*) and/or because isolates were a combination of the previously described BIII-like, BIV-like and BIV polymorphisms (*tpi*). Although it is not clear whether these *tpi* polymorphisms are novel polymorphisms or the result of meiotic recombination among isolates from different B sub-assemblages (Cooper et al., 2007; Teodorovic et al., 2007), this finding illustrates the complexity of the sub-assemblage classification.

The classification of Wielinga and Thompson (2007) is mainly based on sequence data of single loci, because the availability of multi-locus sequences in GenBank™ is limited. This obviously impedes a correct comparison of sub-assemblages identified at different loci, as indicated by the incongruent assignment in the present study. Therefore, it is necessary to further revise and to adapt the grouping into sub-assemblages based on additional multi-locus sequences. The high heterogeneity within assemblage B suggests that it may be better to limit the number of polymorphisms used for the sub-assemblage classification, consequently also reducing the number of sub-assemblages, since the variety in sequences from various hosts and geographical origins will increase with further studies. At present, substitution patterns in analogy with *tpi* and *gdh* are not available for the *bg* gene. In the past, various B sub-assemblages (B1–9, 19) have been proposed for this gene (Cacciò et al., 2002; Lalle et al., 2005; Gelanew et al., 2007). However, the sub-assemblages identified were difficult to link to sub-assemblages of the other genes. Moreover, a different nomenclature was used (B + number instead of the Roman numeral III/IV). In the present study, a substitution pattern was proposed by analogy with the two other genes, separating the sub-assemblage BIII (cytosine) from BIV (thymine) at position 354. The validity of this substitution pattern needs to be confirmed in future studies, but the multi-locus comparison already suggests its robustness.

Due to the various polymorphisms within sub-assemblages, the assessment of transmission patterns based solely on the identification of sub-assemblages is questionable (Graczyk et al., 2002). Furthermore, the origin of isolates could rarely be determined based on a single locus, yet combining the polymorphisms of multiple loci was highly discriminatory. The mixture of polymorphisms within the same group of animals in the present study indicates the need for multi-locus sequencing of more than one isolate to identify the infection source. Although these findings need to be confirmed in epidemiological surveys where isolates of susceptible hosts in localised endemic foci are characterised, the polymorphisms found in NHP emphasise the potency of this multi-locus sequencing approach as a tool for the assessment of zoonotic transmission between NHP and the animal caretakers or visitors to the study sites examined (van der Giessen et al., 2006; unpublished observations).

In conclusion, the results indicate that NHP are a potential reservoir for zoonotic transmission. The present study further emphasises the need for assemblage-specific PCRs in molecular surveys as mixed infections were frequently diagnosed. The results of the multi-locus sequencing suggest that the classification of genetic heterogeneity within an assemblage should be approached with caution and should be completed based on more multi-locus sequencing data. However, this approach holds promise for the assessment of the complex epidemiology of *Giardia*.

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