# Natural infection with two genotypes of *Cryptosporidium* in red squirrels (*Sciurus vulgaris*) in Italy

# Martin Kváč<sup>1,2</sup>, Lada Hofmannová<sup>3</sup>, Sandro Bertolino<sup>4</sup>, Luc Wauters<sup>5</sup>, Guido Tosi<sup>5</sup> and David Modrý<sup>1,3</sup>

<sup>1</sup>Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic;

<sup>2</sup>Faculty of Agriculture, University of South Bohemia in České Budějovice, Studentská 13, 370 05 České Budějovice, Czech Republic;

<sup>3</sup>Department of Parasitology, University of Veterinary and Pharmaceutical Sciences, Palackého 1–3, 612 42 Brno, Czech Republic;

<sup>4</sup>DIVAPRA Entomology and Zoology, University of Turin, Via L. da Vinci 44, I-10095 Grugliasco (TO), Italy;

<sup>5</sup>Department 'Environment-Health-Safety', University of Insubria, Varese, Via J.H. Dunant 3, I-21100 Varese, Italy

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Abstract. We investigated the genotypes of *Cryptosporidium* infecting red squirrels (*Sciurus vulgaris* L.) in two areas of the Western Alps in Italy. Examination of 141 faecal samples from 70 red squirrels revealed oocysts of *Cryptosporidium* in 17 animals (24.3%). Based on 18S rRNA gene sequencing, two genotypes of *Cryptosporidium* species were found: 15 squirrels were positive for the *Cryptosporidium* ferret genotype and 2 for the *Cryptosporidium* chipmunk genotype I. The occurrence and intensity of *Cryptosporidium* infection did not differ between the study areas or sex. More than 85% of the positive animals were adults; however no difference was found between *Cryptosporidium* infection in the juvenile and adult age groups. Oocysts of the *Cryptosporidium* ferret genotype measured  $5.5 \pm 0.3 \times 5.2 \pm 0.2 \mu m$  (shape index 1.06) and the *Cryptosporidium* chipmunk genotype I  $5.8 \pm 0.3 \times 5.4 \pm 0.3 \mu m$  (shape index 1.07). Neonatal and adult CD1 and BABL/c mice inoculated with  $1 \times 10^3$  fresh oocysts of both genotypes did not produce detectable infection.

Cryptosporidiosis is considered one of the most prevalent parasitic infections in domestic, caged and wild animals. Increased interest in their genetic variability and intense research in the past years has revealed the remarkable diversity of these apicomplexan parasites. The number of valid species is under considerable discussion, with several samples waiting to be described as species (Xiao et al. 2004). In addition to the species described, dozens of different genotypes were reported, in many cases with different host and geographical affinities, and some of them with zoonotic potential.

Cryptosporidiosis is classified as a water-borne disease and oocysts of various *Cryptosporidium* genotypes represent important environmental contaminants. Only some *Cryptosporidium* species/genotypes contaminating water and food were described to be infectious to humans (Jiang et al. 2005, Ryan et al. 2005). Contrary to prevailing opinion, many of the environmental genotypes have been found in wildlife (Zhou et al. 2004, Feng et al. 2007). Wild animals represent an important source of environmental contamination with *Cryptosporidium* oocysts, but only a limited amount of studies have focused on these hosts. Thus, research focused on the occurrence of various genotypes of *Cryptosporidium* in wild animals is significant in elucidating the origin of *Cryptosporidium* in water. Recent studies have shown that natural cryptosporidiosis in the family Sciuridae could be caused by various species and genotypes of both gastric and intestinal *Cryptosporidium* (Matsui et al. 2000, Perz and Blancq 2001, Bertolino et al. 2003, Hůrková et al. 2003, Atwill et al. 2004, Feng et al. 2007). The presence of *Cryptosporidium* in red squirrels (*Sciurus vulgaris* L.) was previously reported from populations in the Western Italian Alps; however, the genotypes of these *Cryptosporidium* were not investigated (Bertolino and Canestri-Trotti 2001, Bertolino et al. 2003).

On the basis of previous work, the purpose of this study was to further document the occurrence of *Cryptosporidium* in alpine populations of red squirrels, to genotype the *Cryptosporidium* collected and to test *in vivo* the infectivity in laboratory mice.

#### **MATERIALS AND METHODS**

**Sample collection.** We monitored red squirrels in two study areas in the Cogne and Rhemes Valleys, located 21 km apart but within a continuous belt of mature secondary subalpine conifer forests of the Gran Paradiso National Park, Western Italian Alps. *Cryptosporidium* was previously detected in red squirrels by Bertolino et al. (2003) in these two localities. Squirrel densities in the study areas in the years 2000 to 2005 ranged from 0.20 to 0.42 ind./ha at Rhemes and between 0.18

Address for correspondence: M. Kváč, Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic; Phone: +420 387 775 419; Fax: +420 385 310 388; E-mail: kvac@centrum.cz

and 0.45 ind./ha at Cogne (Bertolino et al. 2003, Wauters et al. 2007).

Squirrels were live-trapped in September-October 2004 and bimonthly thereafter from April to October 2005, using 25-30 ground-positioned Tomahawk traps (models 201 and 202, Tomahawk Live Trap Co., Wisconsin, USA). Each trapped squirrel was placed into a zipper-tube or wire-mesh 'handling cone' to minimize stress during handling and individually marked using numbered metal ear-tags (type 1003 S, 10 by 2 mm, National Band and Tag Co. Newport, Kentucky, USA). Animals were weighed to the nearest 5 g using a Pesola spring-balance (Pesola AG, Baar, Switzerland) and the length of the right hind foot (without nail) was measured (0.5 mm) with a thin ruler (Wauters et al. 2005, 2007). The sex, age and reproductive condition of each subject was recorded following Wauters and Dhondt (1989, 1995). After a trapped animal was released, faeces were collected from traps and placed in vials containing 2.5% (w/v) aqueous potassium dichromate  $(K_2Cr_2O_7)$  and stored at 4°C.

**Sample examination.** Individual samples were concentrated in modified Sheather's sugar solution (specific gravity 1.30), examined using an Olympus AX 70 light microscope at 200-fold magnification and the number of oocysts was scored semiquantitatively (Table 1). *Cryptosporidium* oocysts were purified, using sucrose gradient and cesium chloride gradient centrifugation (Arrowood and Sterling 1987, Kilani and Sekla 1987), and measured (n = 50) by morphometrical analysis based on digital image analysis (software M.I.S. Quick-PHOTO Pro; camera – Olympus Camedia C-5060WIDE ZOOM, 5.1 mega pixels). Pure oocysts were stored in distilled water in the dark at 4°C.

**DNA extraction.** Total DNA was extracted from 200–300 mg faeces of *Cryptosporidium*-positive samples by bead disruption homogenisation of oocysts, using the Mini-BeadBeater (Biospec Products, USA) for 120 s at 5,000 rpm, and subsequent isolation with the QIAamp<sup>®</sup> DNA Stool Mini Kit (QIAGEN), according to the manufacturer's instructions. The obtained DNA was stored at  $-20^{\circ}$ C.

PCR and DNA sequencing. An approximately 830 bp long fragment of the 18S rRNA gene was amplified by nested PCR as previously described (Jiang et al. 2005). The purified PCR products were sequenced in both directions on an ABI3130 sequencer analyzer (Applied Biosystem, Forest City, CA), using internal sequencing primers and the BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem). Sequences were assembled using Chromas Pro (http://www. technelysium.com.au/chromas.html) and aligned with reference sequences using ClustalX (ftp://ftp-igbmc.u-strasbg.fr/ pub/ClustalX/). The 18S rRNA gene nucleotide sequences were deposited in GenBank under Acc. Nos. EU250844 and EU250845.

**Infectivity assay.** Groups of eight-week-old CD1 and BALB/c mice and seven-day-old CD1 and BALB/c mice (ANLAB, Czech Republic), each consisting of three animals, were used for experimental infection trials with the *Cryptosporidium* isolate. Each animal was inoculated orally with a stomach tube with a dose of  $1 \times 10^3$  oocysts. Each group was housed separately in standard plastic cages and fed with commercial rodent food and water *ad libitum*. Faecal samples were obtained daily from the 4th day post infection (DPI) and ex-

amined using modified Sheather's flotation method and aniline-carbol-methyl violet staining (Miláček and Vítovec 1985). Entire smears covering the whole slide area were microscopically examined by light microscopy at 1,000-fold magnification. All animals were euthanized 30 DPI.

**Statistic analyses.** The significance of any difference between oocyst morphometry (length and width) of *Cryptosporidium* genotypes was evaluated by analysis of variance with Tukey's post-hoc tests. The difference in the prevalence of *Cryptosporidium* according to location, sex and age was assessed by the Chi-square test. All statistical tests were performed using Statistica<sup>®</sup> 6.0 software (StatSoft CR, Praha, Czech Republic).

**Table 1.** Scale for semiquantitative evaluation of infection intensity by light microscopy at a  $\times 200$  magnification.

In	fection intensity	Microscopy finding				
+	Weak infection	Sporadic occurrence of oocysts per sample				
++	Medium infection	1-30 oocysts in 10 fields				
+++	Severe infection	31-50 oocysts in 10 fields				
++++	Intense infection	More than 50 oocysts in 10 fields				

#### RESULTS

In total, 141 faecal samples were collected from 70 red squirrels: 33 at Cogne and 37 at Rhemes. Seventeen squirrels were positive for *Cryptosporidium* (total prevalence = 24.3%); the prevalence did not differ between the study areas (Table 2, Cogne = 24.2%, Rhemes = 24.3%,  $\chi^2 = 0.000$ , d.f. = 1, P = 0.99). Fourteen positive squirrels were trapped more than once; 8 of them were positive during the first trapping. Ten positive squirrels lost the infection during the study period. One animal was detected positive for the same *Cryptosporidium* genotype twice at a 2-month interval, and was negative one month later (Table 2).

Per age-class, 15 out of 55 adults (27.3%) and 2 of 15 juveniles (13.3%) were found positive ( $\chi^2 = 1.25$ , d.f. = 1, P = 0.33). Prevalence was also similar between males (14.3%) and females (10%,  $\chi^2 = 0.29$ , d.f. = 1, P = 0.59).

We found two different *Cryptosporidium* genotypes in both populations of red squirrels (Table 2). Our genotypes were identical to the *Cryptosporidium* ferret genotype (GenBank Acc. No. AF112572) and the *Cryptosporidium* chipmunk genotype I (No. EF641026). Overall, 15 animals were positive for the *Cryptosporidium* ferret genotype and another two for the *Cryptosporidium* chipmunk genotype I, with no squirrel found infected with both genotypes simultaneously.

Oocyst morphology of both genotypes fell in the assemblage of intestinal *Cryptosporidium* spp. Oocysts of the *Cryptosporidium* ferret genotype were 5.5 (4.9–6.0) × 5.1 (4.7–5.6)  $\mu$ m, shape index 1.07 (1.02–1.29), similar to oocysts of *C. parvum* (Vítovec et al. 2006) (P = 0.41). In contrast, they were significantly smaller than those of the *Cryptosporidium* chipmunk genotype I, 5.8 (5.3–6.6) × 5.4 (4.7–5.9)  $\mu$ m, shape index 1.08 (1.02– 1.20) (P <0.001).

Isolate	Area	Trapped					Sav	1 ao*	Cryptosporidium	
		Ι	II	III	IV	V	VI	BUX	ngu	genotype
SV20	lemes	NE	NE	_	-	+	NE	F	Adult	ferret
SV22		NE	NE	NE	+	NE	NE	М	Adult	ferret
SV23		NE	NE	NE	+++	+	_	М	Adult	ferret
SV26		NE	NE	NE	+	_	NE	М	Adult	ferret
SV27		NE	NE	NE	+	_	NE	F	Adult	ferret
SV30	R	NE	NE	NE	-	+++	-	F	Adult	ferret
SV32		NE	NE	NE	++	_	-	М	Adult	ferret
SV33		NE	NE	NE	NE	+++	NE	F	Juvenile	chipmunk 1
SV37		NE	NE	NE	NE	NE	+++	М	Juvenile	ferret
SV38		-	-	-	NE	+++	NE	F	Adult	ferret
SV40		-	NE	_	NE	+++	NE	М	Adult	ferret
SV41	Cogne	-	-	NE	NE	++	-	М	Adult	ferret
SV58		NE	NE	NE	_	+	_	F	Adult	ferret
SV59		NE	NE	NE	-	+++	NE	М	Adult	chipmunk 1
SV65		NE	NE	NE	+++	-	NE	F	Juvenile	ferret
SV66		NE	NE	NE	NE	++	-	М	Adult	ferret
SV67		NE	NE	NE	NE	+	_	М	Adult	ferret

**Table 2.** Frequency of *Cryptosporidium* genotypes and their infection intensity according to study areas (Cogne and Rhemes), season of trapping, and sex and age of red squirrels.

NE – not examined; – negative; + positive (for key to infection intensity see Table 1); I – trapped in September 2004; II – October 2004; III – May 2005; IV – July 2005; V – September 2005; VI – October 2005; \*age at first trapping.

Transmission studies revealed no infectivity of any of these two *Cryptosporidium* genotypes for neonatal and adult CD1 or BALB/c mice.

### DISCUSSION

This study corroborates published data indicating that wild sciurid rodents serve as an important source of cryptosporidia. So far, squirrels were reported to be susceptible to several *Cryptosporidium* species/geno-types of various host specificities and oocyst morphologies (Matsui et al. 2000, Atwill et al. 2001, Bertolino et al. 2003, Hůrková et al. 2003, Feng et al. 2007). In 17 separate cases of infection, we found two intestinal *Cryptosporidium* genotypes that had not been previously observed in red squirrels. These genotypes shared 100% homology with the *Cryptosporidium* ferret genotype and *Cryptosporidium* chipmunk genotype I. Both genotypes were originally reported from the Nearctic Region, while their occurrence in the Palaearctic Region is reported here for the first time.

The *Cryptosporidium* ferret genotype has only been found in ferrets in the eastern USA (Xiao et al. 1999, Sulaiman et al. 2000), and Gómez-Couso et al. (2007) described a genotype closely related to the *Cryptosporidium* ferret genotype (with one nucleotide of difference) from farmed American mink (*Mustela vison*) in Spain. The *Cryptosporidium* chipmunk genotype I was first reported in water after a storm in New York State (Jiang et al. 2005). The eastern gray squirrel (*Sciurus carolinensis*), eastern chipmunk (*Tamias striatus*) and deer mouse (*Peromyscus* sp.) were recently suggested to be sources of water contamination in New York State (Feng et al. 2007). Interestingly, two human infections with the *Cryptosporidium* chipmunk genotype I have been described in Wisconsin (Feltus et al. 2006).

In western USA (California), *C. parvum* was reported in California ground squirrels (*Spermophilus beecheyi*); the majority of isolates matched a bovine-murine genotype, with a few isolates resembling a porcine genotype (Atwill et al. 2001). Nevertheless, these results solely based on PCR-RFLP are not sufficient for genotyping. In another study on the same host species, Atwill et al. (2004) isolated up to three potentially different genotypes (Sbey03a, Sbey03b and Sbey03c). These genotypes differ from the genotypes we observed and those (cervine genotype, deer mouse genotype III, skunk genotype, chipmunk genotype II) described in other hosts of the family Sciuridae (Feng et al. 2007).

These data, along with the simultaneous prevalence of both Cryptosporidium genotypes in two distant squirrel populations in our study, suggest the endemic and natural character of infection in particular squirrel populations. The biogeography of different Cryptosporidium genotypes is far from being clarified. However, the increasing rate of worldwide species introductions could reshuffle the natural pattern before we start to understand the mechanisms of evolution and segregation of different genotypes. Long (2003) reported more than 20 introduced species of Sciuridae worldwide, with hundreds of mostly successful releases. Different subspecies of red squirrels have been widely translocated to the Russian Federation and adjacent independent republics (Long 2003). This human-mediated movement of animals could also facilitate the introduction of parasites to new areas. In Great Britain, for example, the competitive exclusion of the native red squirrel by the introduced American grey squirrel (Sciurus carolinensis) is also mediated by poxvirus infecting the native species, probably imported into the country via the introduced animals (Rushton et al. 2006).

The prevalence of *Cryptosporidium* in red squirrels recorded in this study (24.3%) is similar to the values reported by Bertolino et al. (2003) for the same areas in previous years (2000–2001: 19.2–27.3%), except for a peak at Rhemes in 2000 (63.6%). However, in three other areas in the Central Alps, *Cryptosporidium* was recorded only once in 59 animals examined (Bertolino et al. 2003). Very recently, Feng et al. (2007) reported seven different genotypes in sciurid rodents from the New York region, with prevalence reaching 37.5%.

We found no difference in the prevalence between juveniles and adults. Technically, it is difficult to sample wild squirrels before they leave the nests and to obtain data about the occurrence of cryptosporidiosis in suckling squirrels. Bertolino et al. (2003) reported that the probability of infection increased at higher densities and in young red squirrels. However, in this and other studies on squirrels, Cryptosporidium was commonly found also in adults (Atwill et al. 2004). In our experiments, neither the Cryptosporidium ferret genotype nor the chipmunk genotype I was infectious for adult CD1 and BALB/c mice, which is in accordance with the noninfectivity of Sbey03b and Sbey03c genotypes for BALB/c mice (Atwill et al. 2004). On the contrary, adult SCID or BALB/c mice were susceptible to C. parvum-type oocysts and C. muris that originated from the Siberian chipmunk (Matsui et al. 2000, Hůrková et al. 2003). Furthermore, our failure to infect suckling CD1 and BALB/c mice confirms a certain level of host specificity of the isolates studied.

Despite the fact that oocyst morphology and the size of intestinal *Cryptosporidium* spp. has only limited value for distinguishing species/genotypes, differences between various species and genotypes were repeatedly mentioned (Xiao et al. 2004). The *Cryptosporidium* ferret genotype oocysts were morphologically similar to oocysts of *C. parvum*. Oocysts of the chipmunk genotype I found in this study were significantly larger than both oocysts previously mentioned, and smaller than *C. suis*, as described by Vítovec et al. (2006).

Cryptosporidiosis is a disease reported in many countries worldwide (Dillingham et al. 2002). Transmission of infection occurs by contact with infected persons or animals, or by consumption of contaminated food or water. Cryptosporidium hominis and C. parvum are the primary species infecting humans: the first is almost exclusively a human parasite, while the second is reported mostly in wild and domestic animals. Squirrels are sold as pet animals and are often translocated and released in urban parks (Long 2003). Many species can also colonize green parts of cities and animals may become accustomed to humans. Considering their appeal and vicinity to urban areas, squirrels have many occasions to enter into contact with people. Thus the possible impact of cryptosporidiosis on wild populations, as well as the epidemiological significance of these infections for public health should be further investigated.

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