Genetic polymorphism and population structure of *Echinococcus ortleppi*

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SUMMARY

The zoonotic cestode *Echinococcus ortleppi* (Lopez-Neyra and Soler Planas, 1943) is mainly transmitted between dogs and cattle. It occurs worldwide but is only found sporadically in most regions, with the notable exception of parts of southern Africa and South America. Its epidemiology is little understood and the extent of intraspecific variability is unknown. We have analysed in the present study the genetic diversity among 178 *E. ortleppi* isolates from sub-Saharan Africa, Europe and South America using the complete mitochondrial *cox1* (1608 bp) and *nad1* (894 bp) DNA sequences. Genetic polymorphism within the loci revealed 15 *cox1* and six *nad1* haplotypes, respectively, and 20 haplotypes of the concatenated genes. Presence of most haplotypes was correlated to geographical regions, and only one haplotype had a wider spread in both eastern and southern Africa. Intraspecific microvariance was low in comparison with *Echinococcus granulosus* sensu stricto, despite the wide geographic range of examined isolates. In addition, the various sub-populations showed only subtle deviation from neutrality and were mostly genetically differentiated. This is the first insight into the population genetics of the enigmatic cattle adapted *Echinococcus ortleppi*. It, therefore, provides baseline data for biogeographical comparison among *E. ortleppi* endemic regions and for tracing its translocation paths.

Key words: *Echinococcus ortleppi*, microdiversity, haplotype, *cox1*, *nad1*, Africa, Brazil, France.

INTRODUCTION

Cystic echinococcosis (CE) was formerly considered to be caused by one polymorphic species, *Echinococcus granulosus*. Data on morphology, host range and other biological parameters had eventually lead to a subdivision of *E. granulosus* (sensu lato) into several strains, most of them named after the principal intermediate host species (Thompson and Lymbery, 1988, 1990; Bowles *et al.* 1992; Thompson and McManus, 2002). More recently, gene sequence data confirmed the distinctness of some of these strains, and led to the recognition of (at least) five cryptic species, namely *E. granulosus* sensu stricto, *Echinococcus equinus*, *Echinococcus ortleppi*, *Echinococcus canadensis* and *Echinococcus felidis* (Nakao *et al.* 2013; Romig *et al.* 2015). A comparatively large body of information on distribution, host range and genetic diversity is available for the most widespread and frequent of these species, *E. granulosus* s.s., which has recently been shown to be responsible for 88% of human cases (Alvarez Rojas *et al.* 2014). Based on genetic data, hypotheses have been established to trace back the routes of distribution of this parasite, pinpointing the Middle East as its most probable origin (Nakao *et al.* 2010; Casulli *et al.* 2012; Yanagida *et al.* 2012). This led to hypotheses regarding historical introduction routes on continental scales (Alvarez Rojas *et al.* 2016).

Far less data are available for the remaining species of the *E. granulosus* sensu lato cluster. Two recent studies found little intraspecific mitochondrial sequence diversity among *E. equinus* isolates from Europe and Africa (Boufana *et al.* 2015; Wassermann *et al.* 2015). No comprehensive study has been done so far on the global genetic structure of the *E. canadensis* cluster, and available data are still insufficient to resolve the taxonomic uncertainty concerning proposed subdivisions of *E. canadensis* (Thompson, 2008; Nakao *et al.* 2013, 2015;

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Lymbery *et al*. 2015*a*, *b*). No data on intraspecific variation are available for *E. felidis* (due to the lack of isolates suitable for analysis) and for *E. ortleppi* (Lopez-Neyra and Soler Planas, 1943).

Formerly known as the cattle strain of *E. granulosus*, or genotype G5, *E. ortleppi* is well adapted to cattle as intermediate hosts, although other host species may also be affected (Thompson and McManus, 2002). In contrast to other agents of CE, cysts of *E. ortleppi* in cattle are often large and fertile, and occur predominantly in the lungs (Kamenetzky *et al*. 2002; Pednekar *et al*. 2009; Balbinotti *et al*. 2012; Grenouillet *et al*. 2014; Mbaya *et al*. 2014; Tigre *et al*. 2016). The parasite is globally widespread, but even in cattle it is usually rare or of sporadic occurrence (Romig *et al*. 2011; Cardona and Carmena, 2013; Mbaya *et al*. 2014). This has been tentatively explained by the fact, that cattle (in contrast e.g. to sheep) are usually not slaughtered at home even in traditional pastoralist societies, which causes a barrier to transmission to local dogs as definitive hosts (Addy *et al*. 2012). The very few human CE cases caused by this species are scattered throughout the world (Netherlands, South Africa, Mexico, Brazil and Argentina) (Alvarez Rojas *et al*. 2014). It is unknown whether the scarcity of human infections reflects the infrequency of the parasite or increased resistance of humans against *E. ortleppi*.

The present study describes the intraspecific microdiversity within and between sub-populations of this enigmatic parasite from sub-Saharan Africa, Brazil and France, in order to provide baseline data for studies on dispersal routes of the parasite.

MATERIALS AND METHODS

Echinococcus ortleppi *isolates*

In total, we examined 178 *E. ortleppi* cyst isolates from Kenya (54 in cattle, three in goat, two in camel, one in sheep), Zambia (52 in cattle, one in pig), Namibia (35 in cattle, three in oryx), Ethiopia (seven in cattle), Brazil (seven in cattle), France (seven in cattle) and Sudan (five in cattle, one in camel). Some of these isolates were from previous reports made available for the present study including 48 from Mbaya *et al*. (2014), seven from Grenouillet *et al*. (2014), and one from Addy *et al*. (2012). The isolates were obtained from slaughterhouse surveys, except three Namibian isolates taken from euthanized *Oryx gazella* antelopes (see supplemental file for description of isolates). All cyst isolates (germinal layer and/or protoscoleces) were stored in 70% ethanol until use.

DNA extraction, amplification and sequencing

DNA of the African isolates was obtained by lysing single protoscoleces or tissue pieces in 10 μL 0·02 M NaOH as described by Nakao *et al*. (2003). This method was modified in some cases by using more tissue and lysing it in 50–100 μL 0·02 M NaOH at 99 °C for 30 min. The lysate was centrifuged at 8000 ***g*** for 1 min and the supernatant used directly as template in the polymerase chain reaction (PCR). Where the above approaches yielded inadequate DNA for PCR, and for the Ethiopian isolates, cyst tissues were cut into pieces and treated with proteinase K and DNA was extracted by phenol-chloroform as described previously (McManus *et al*. 1985; Dinkel *et al*. 1998). The French isolates were extracted using the iPrep purification instrument (Invitrogen, iPrep ChargeSwitch gDNA Tissue Kit) and the Brazilian isolates were extracted using QIAamp DNA mini kit, Qiagen USA.

The NADH dehydrogenase subunit 1 (*nad1*) (894 bp) was obtained by nested PCR in a 50 μL reaction mixture containing: 10 mM Tris–HCl (pH 8·3), 50 mM KCl, 2 mM $MgCl_2$, 20 pmol of each external/internal primer, 0·2 mM dNTPs, 1·25 U Ampli-*Taq* Polymerase (Applied Biosystems) and 2 μL crude lysate/DNA in primary reaction and 1 μL DNA template in the nested reaction. PCRs were thermal cycled 35 times involving a denaturation step at 95 °C for 30 s, annealing at 50 °C for 30 s and elongation at 72 °C for 60 s. The previously designed primer pairs by Hüttner *et al*. (2008) were used including forward 5′-TGGAACTCAGTTTGAGCTTTACTA-3′/reverse 5′-ATATCAAAGTAACCTGCTATGCAG-3′ in the primary reaction, and forward 5′-TATTAAAAATATTGAGTTTGCGTC-3′/reverse 5′-TCTTGAAGTTAACAGCATCACGAT-3′ in the nested reaction.

The cytochrome c oxidase subunit 1 (*cox1*) (1608 bp) gene was also obtained by nested PCR in one fragment including the complete gene or in two overlapping fragments. In both cases, primary PCR was performed in a 50 μL reaction mixture containing 10 mM Tris–HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, 20 pmol of each external primer, 0·2 mM dNTPs, 1·25 U Ampli-*Taq* Polymerase (Applied Biosystems) and 1 μL crude lysate/DNA. PCR was cycled for 25 times involving a denaturation step at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C step for 90 s. In the nested PCR, the $MgCl_2$ was reduced to 1·375 mM to improve fidelity and reaction cycled for 35 cycles under same conditions as primary PCR. Primers used in amplifying the *cox1* gene in one piece include forward 5′-GTGGAGTTACTGCTAATAATTTTTG-3′/reverse 5′-TACGACTYACTTATCAC-3′ (Wassermann *et al*. 2015) in the primary reaction, and forward 5′-TTACTGCTAATAATTTTGTGTCAT-3′/reverse 5′-GCATGATGCAAAAGGCAAATAAAC-3′ (Hüttner *et al*. 2008) in the nested reaction. When amplifying the *cox1* gene in two overlapping fragments, the five prime part was obtained using the primers forward 5′-GTGAA

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GTTACTGCTAATAATTTTG-3′ /reverse 5′-ACRTAATGAAAATGAGC-3′ (Ebi *et al*. 2014 personal communication) in the primary reaction, and forward 5′-TTACTGCTAATAATTTT GTGTCAT-3′ (Hüttner *et al*. 2008)/reverse 5′-CAAGTAAACACCTTTATAC-3′ (Ebi *et al*. 2014 personal communication) in the nested reaction. While the three prime part was obtained using the primers forward 5′-TTTGCTATGTTTTCT ATAG-3′ (Ebi *et al*. 2014 personal communication)/reverse 5′-TACGACTYACTTATCAC-3′ (Wassermann *et al*. 2015) in the primary reaction, and forward 5′-CATCATATGTTTACTGTT GG-3′ (Ebi *et al*. 2014 personal communication)/reverse 5′-GCATGATGCAAAAGGCAAATAA AC-3′ (Hüttner *et al*. 2008) in the nested reaction.

Nested PCR amplicons of both genes were purified using the High Pure PCR Product Purification Kit (Roche, Mannheim-Germany) following the manufacturer's protocol and sequenced (GATC Biotech AG, Konstanz-Germany).

Data analyses

DNA chromatographs were viewed using GENtle v. 1.9 (Manske M. 2003, University of Cologne, Germany) and manually edited in case of nucleotide base misreads. Isolates sequenced in two overlapping fragments were joined. Amino acid sequences were inferred from the nucleotide sequences by echinoderm mitochondrial genetic code (Nakao *et al*. 2000). Percentage divergence of DNA sequences were determined using Kimura 2-parameter model (Kimura, 1980) in MEGA v 6 (Tamura *et al*. 2013). Single locus and concatenated *cox1 – nad1* haplotypes were estimated based on statistical parsimony (Templeton *et al*. 1992) and network drawn at 95% connection limit using TCS v 1.8 (Clement *et al*. 2000). DnaSP v 5 (Librado and Rozas, 2009) was used to estimate the population indices: number of haplotypes, haplotype diversity and nucleotide diversity. Analysis of molecular variance (AMOVA), degree of genetic differentiation (pairwise fixation index – Fst) and neutrality indices of Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) were calculated using the Arlequin v 3.5 software (Excoffier *et al*. 2005). The identified *cox1-nad1* concatenated haplotypes and representative sequences of *E. canadensis* G6-10 genotypes and *E. oligarthra* were aligned using Clustal Omenga in EMBL-EBI before used to construct a maximum likelihood phylogenetic tree in MEGA v 6.

RESULTS

Diversity and neutrality indices

Intraspecific variations were observed in both mitochondrial loci. Nucleotide exchanges could be observed at 13 and five sites in the *cox1* and *nad1* sequences, respectively, yielding 15 (*cox1*) and six (*nad1*) haplotypes (Table 1). Most of these sites, 54% in *cox1* and 60% in *nad1* were parsimony informative. Synonymous substitutions exceeded nonsynonymous substitutions (4 *vs* 1) in the *nad 1* coding region while half (seven) of the changes in the *cox1* coding region were synonymous. A point mutation, thus site 933, showed multiple evolutionary paths whereby the transverse mutation, T – G, was nonsynonymous in Hp5 but the transition mutation, T – A, was synonymous in Hp6. No insertion or deletion was observed within either genes, thus the nucleotide numbers were stable. A pairwise divergence computed among all isolates indicates maximum values of 0·4% in *cox1* and 0·3% in *nad1*. Using the concatenated sequences of the two loci (*cox1-nad1*), the seven sub-populations were organised into four hierarchical groups consisting of eastern Africa (Ethiopia, Kenya, Sudan), southern Africa (Namibia, Zambia), Brazil and France. Analysis of molecular variance (AMOVA) using these groupings revealed that 53·61% of observed variance was among individuals within sub-populations ($F_{SC} = 0{\cdot}424$, $P = 0{\cdot}000$) and 39·51% among sub-populations ($F_{ST} = 0{\cdot}464$, $P = 0{\cdot}000$) and only 6·88% of the molecular variance was among the groups ($F_{CT} = 0{\cdot}069$, $P = 0{\cdot}191$).

Haplotype and nucleotide diversities for each subpopulation and groups were calculated. The eastern African population showed higher nucleotide diversity, but lower haplotype diversity, than the southern African population (Table 2). Most of the eastern African observed diversity was in the (few) isolates from Sudan. The Ethiopian isolates were the least polymorphic (0.00034) but had similar (0·524) haplotype diversity as in Kenya (0·521). The two southern African sub-populations also had similar haplotype diversity (0·644 in Namibia and 0·616 in Zambia) but the nucleotide diversity in isolates from Namibia was only 0.00038 compared with 0 00050 in isolates from neighbouring Zambia. Pulling all the isolates from Africa (sub-Saharan) together revealed relatively higher haplotype diversity of 0·714 and nucleotide diversity of 0.00071. Tajima's D and Fu's Fs shown here (Table 2) indicate rather insignificant deviation of sub-populations and the sub-regional groups from neutrality. The more polymorphic Kenyan, Sudan and Zambian isolates had positive neutrality values (yet insignificant), which indicates observed deficiency of haplotypes. In contrast, the Ethiopian and Namibian sub-populations, that kept less polymorphic mtDNA but relatively high isolates variance, gave negative values, likewise the average polymorphic sum of African isolates (with significant Fu's Fs). These negative values indicate frequent occurrence of some rare haplotypes than would be expected under neutrality. Diversity and neutrality indices of the non-African isolates

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Table 1. Nucleotide substitutions in the mitochondrial *cox1* and *nad1* genes in 20 *cox1-nad1* concatenated genes haplotypes of *E. ortleppi;* substitution sites are numbered from the initiation codon.

	cox1 substitution sites													*nad1* substitution sites					*cox1* and *nad1* haplotypes	
	27 0*	36 8	49 8*	78 3*	84 9*	86 7*	93 3**	97 1	10 0	10 4*	13 6 4	13 8 5	14 2 9	22 5*	25 4	30 3*	81 0*	88 5*	*cox1*	*nad1*
Hp1	C	C	T	C	A	T	T	T	C	A	C	C	A	A	T	T	C	T	Eo02	Eo01[a]
Hp2	T	.	.	T	G	.	.	.	.	.	G	.	.	.	.	.	T	.	Eo06	Eo02
Hp3	.	.	.	T	.	.	.	.	.	.	G	.	.	.	.	.	T	.	Eo03	Eo02
Hp4	.	.	.	T	.	.	.	.	.	G	G	.	.	.	.	.	T	.	Eo07	Eo02
Hp5	.	.	.	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	Eo09	Eo01[a]
Hp6	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	Eo04	Eo01[a]
Hp7	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	Eo08	Eo01[a]
Hp8	.	T	.	T	.	.	.	.	.	.	G	.	.	.	.	.	T	.	Eo11	Eo02
Hp9	.	.	.	T	.	.	.	.	.	.	G	.	G	.	.	.	T	C	Eo10	Eo03
Hp10	.	.	.	.	.	.	.	.	.	.	G	.	.	G	.	.	T	.	Eo01	Eo04
Hp11	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	.	Eo01	Eo02
Hp12	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	Eo01	Eo01[a]
Hp13	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	T	.	Eo02	Eo04
Hp14	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Eo12	Eo01[a]
Hp15	T	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	.	Eo05	Eo02
Hp16	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Eo13	Eo01
Hp17	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	Eo13	Eo05
Hp18	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	Eo12	Eo06
Hp19	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	Eo14	Eo01[a]
Hp20	.	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	T	.	Eo15	Eo02

Numbers are read vertically. All haplotypes reported here were deposited in the GenBank database under the accession numbers KU743915–KU743926, KX010903, KX138067, KX138068 for *cox1* haplotypes Eo01 – E012, Eo13, Eo14, Eo15, and KX010904, KU842044 – KU842047, KX138069 for *nad1* haplotypes Eo01, Eo02 – Eo05, Eo06, respectively. Hp, haplotype.
*Synonymous substitution. **Synonymous substitution in Hp6/replacement change in Hp5.
[a & b] Haplotypes were 100% identical to AB235846 *cox1* and *nad1* sequences, respectively.

Table 2. Diversity and neutrality indices of *E. ortleppi* populations estimated from concatenated mitochondrial *cox1* and *nad1* genes (2502 bp)

			Diversity indices		Neutrality indices	
Countries	*n*	Hn	Hd ± SD	π ± SD	D	Fs
Ethiopia	7	3	0·524 ± 0·209	0·00034 ± 0·00016	−1·358	−0·237
Kenya	59	5	0·521 ± 0·045	0·00060 ± 0·00005	0·403	1·272
Sudan	5	2	0·600 ± 0·175	0·00072 ± 0·00021	1·573	2·429
(Eastern Africa)	71	9	0·624 ± 0·039	0·00077 ± 0·00006	−0·630	−0·767
Namibia	35	6	0·644 ± 0·050	0·00038 ± 0·00008	−0·936	−1·568
Zambia	53	5	0·616 ± 0·045	0·00050 ± 0·00003	1·797	0·563
(Southern Africa)	88	9	0·700 ± 0·037	0·00056 ± 0·00005	0·017	−1·723
Total (Africa)	159	17	0·714 ± 0·032	0·00071 ± 0·00004	−1·101	−6·252*
Brazil	7	2	0·286 ± 0·196	0·00011 ± 0·00008	−1·006	−0·095
France	7	1	0·000	0·00000	0·000	0·000

n, number of isolates; Hn, number of haplotype; Hd, haplotype diversity; π, nucleotide diversity; D, Tajima's D; Fs, Fu's Fs.
*Significant *P*-value = 0·025.

included here were estimated for the limited Brazilian isolate set giving the lowest diversity indices and negative insignificant neutrality indices. The very low diversity indices here were due to lopsided haplotype or sequence frequencies of Hp16 and Hp17, 6 *vs* 1, respectively (Fig. 1C). The seven French isolates were all identical.

Parsimony networks of mtDNA Haplotypes

Generally, polymorphism of the *cox1* locus resulted in 13 haplotypes (*cox1* haplotypes Eo01 – 04, Eo06 – 12, 14 and 15) from the sub-Saharan African isolates, one haplotype (Eo05) from France and another haplotype (Eo13) from Brazil

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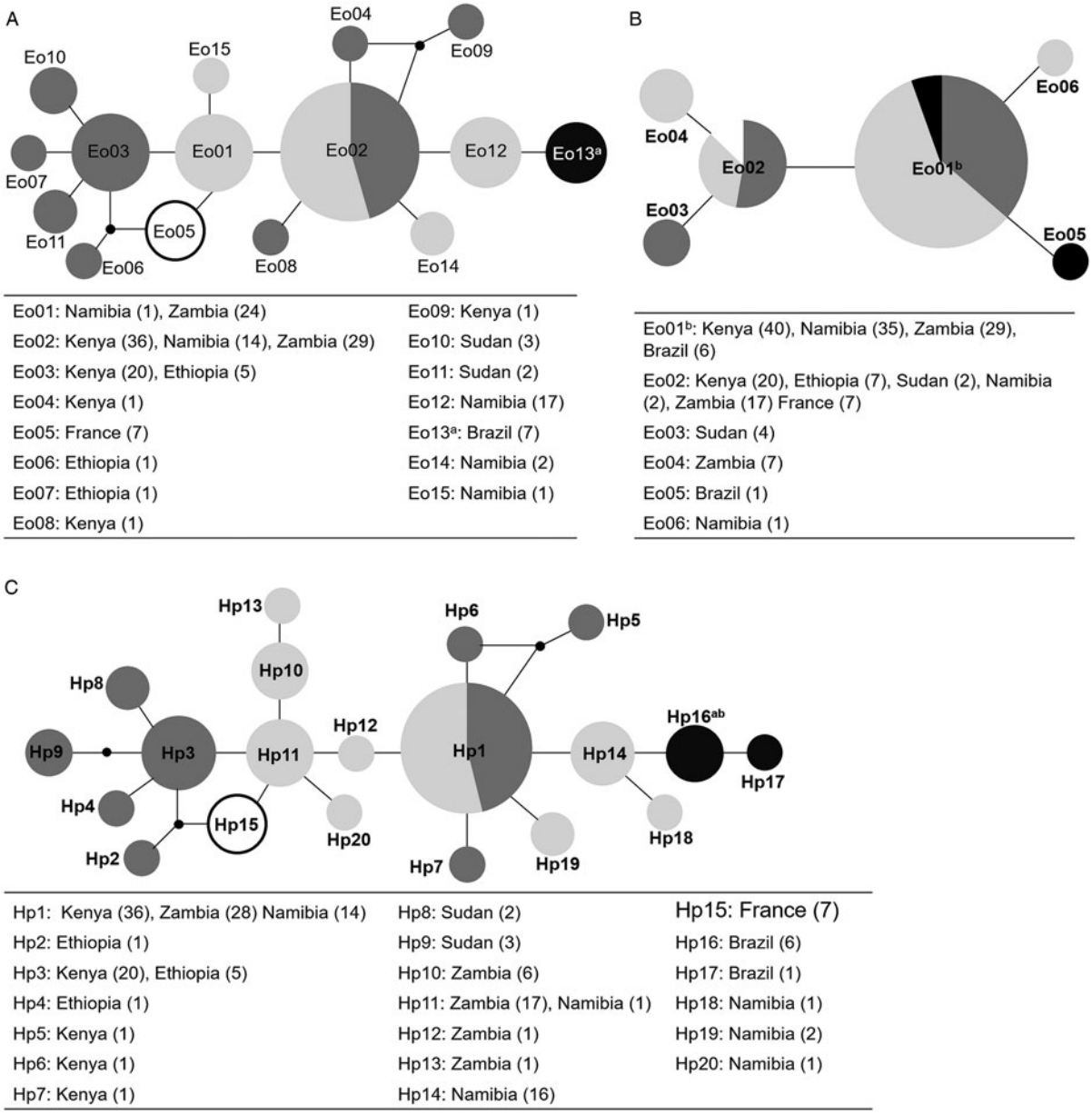

Eo01: Namibia (1), Zambia (24)
Eo02: Kenya (36), Namibia (14), Zambia (29)
Eo03: Kenya (20), Ethiopia (5)
Eo04: Kenya (1)
Eo05: France (7)
Eo06: Ethiopia (1)
Eo07: Ethiopia (1)
Eo08: Kenya (1)
Eo09: Kenya (1)
Eo10: Sudan (3)
Eo11: Sudan (2)
Eo12: Namibia (17)
Eo13[a]: Brazil (7)
Eo14: Namibia (2)
Eo15: Namibia (1)

Eo01[b]: Kenya (40), Namibia (35), Zambia (29), Brazil (6)
Eo02: Kenya (20), Ethiopia (7), Sudan (2), Namibia (2), Zambia (17) France (7)
Eo03: Sudan (4)
Eo04: Zambia (7)
Eo05: Brazil (1)
Eo06: Namibia (1)

Hp1: Kenya (36), Zambia (28) Namibia (14)
Hp2: Ethiopia (1)
Hp3: Kenya (20), Ethiopia (5)
Hp4: Ethiopia (1)
Hp5: Kenya (1)
Hp6: Kenya (1)
Hp7: Kenya (1)
Hp8: Sudan (2)
Hp9: Sudan (3)
Hp10: Zambia (6)
Hp11: Zambia (17), Namibia (1)
Hp12: Zambia (1)
Hp13: Zambia (1)
Hp14: Namibia (16)
Hp15: France (7)
Hp16: Brazil (6)
Hp17: Brazil (1)
Hp18: Namibia (1)
Hp19: Namibia (2)
Hp20: Namibia (1)

Fig. 1. Statistical parsimony networks of *E. ortleppi* mtDNA haplotypes derived from sequences of cox1 (1608 bp) (A), nad1 (894 bp) (B) and concatenated cox1/nad1 (2502 bp) (C). Isolates number in (A) is less five cattle cysts (one in Kenya, one in Sudan and three in Namibia) whose cox1 gene could not be amplified. Circle and wedge sizes and numbers in parenthesis represent haplotype frequency; black spots represent hypothetical haplotypes. Regional/sub-regional haplotypes are shaded dark grey (eastern Africa), light grey (southern Africa), black (southern America) and white (France). [a, b] = cox1 and nad1 haplotypes 100% identical to cox1 and nad1 sequences of previous deposit from Argentinian cattle (AB235846).

(Fig. 1A). The Brazilian haplotype in this study was 100% identical to the *cox1* sequence of a previous deposit from an Argentinian cattle cyst under the accession number AB235846. Haplotype Eo02 was the most predominant, occurring in Kenya, Namibia and Zambia, whereas Eo03 was found only in Kenya and neighbouring Ethiopia. All other *cox1* haplotypes were restricted to individual countries. The *nad1* locus gave only six haplotypes, five (*nad1* haplotypes Eo01 – 04 and 06) in sub-Saharan Africa, two in Brazil (Eo01 and Eo05) and one in France (Eo02) (Fig. 1B). The most frequent *nad1* haplotype Eo01 was also widely distributed from eastern to southern Africa and in South America (Brazil) and is 100% identical to the *nad1* sequence of the previous Argentinian cattle isolate (AB235846). The second most frequent haplotype Eo02 was found both in sub-Saharan Africa and Europe (France). Concatenating the mtDNA sequences (2502 bp) of 173/178 isolates yielded 20 haplotypes (Table 1). Seventeen of them were found in sub-Saharan Africa (Hp1 – 14, 18–20), one in France (Hp15) and two (Hp16–17) in Brazil (Fig. 1C). Due to the higher discriminatory power,

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subsequent analyses were based on the concatenated sequences.

The *cox1-nad1* combined network (Fig. 1C) showed rather a complex divergence genealogy of the participating haplotypes. No common haplotype was found on the three continents, and only one of the 20 haplotypes (Hp1) had a wider geographical spread within sub-Saharan Africa (in Kenya, Zambia and Namibia). All other haplotypes grouped according to country. The single haplotype from France was positioned far closer to African than to the Brazilian samples. All the haplotypes reported here could be found in cattle (refer to supplemental file for detail descriptions). In addition, Hp1 was found in camel and goat from Kenya and in oryx from Namibia, Hp9 in camel from Sudan and Hp10 in pig from Zambia.

Phylogenetic tree of mtDNA haplotypes

A rooted cladogram was inferred from the concatenated *cox1-nad1* genes sequences with *E. oligarthra* as outgroup (Fig. 2). The *E. canadensis* genotypes were included to show the relation among the *E. ortleppi* haplotypes in the clade. Topology of the cladogram depicts a loose grouping of the various haplotypes into the individual countries or subregion following the limited number of shared haplotypes, but lacks geographic structuring.

Genetic differentiation index

A pairwise fixation index (Fst) was computed using the mtDNA data to estimate the degree of genetic differentiation of *E. ortleppi* sub-populations in sub-Saharan Africa and outside Africa. Fst index has 0–1 value range, where 0 denotes complete identity of sub-populations and 1 denotes fixed sub-populations. In our analysis, Fst values ranged from 0·089 to 0·955 (Table 3). These values denote significant genetic differentiation between sub-populations except between those in Kenya and Zambia.

DISCUSSION

After the split of the *Echinococcus granulosus* sensu lato complex into its various species as recognized today, intraspecific variation within these species has received attention with a view to obtain information on biogeographical dynamics (including anthropogenic translocation of the livestock hosts) and possible genetic markers for differences in host adaptations, including pathogenicity to humans. Several studies have tried to elucidate the diversity of *E. granulosus* s.s. sub-populations (Nakao *et al*. 2010; Yanagida *et al*. 2012; Casulli *et al*. 2012; Alvarez Rojas *et al*. 2016), but other species of the *E. granulosus* s.l. cluster have received far less attention. Here we report the intraspecific variation of two mt genes for *E. ortleppi* in eastern and southern Africa, together with data on isolates from western Europe (France) and southern America (Brazil and Argentina). The complete sequences of the mitochondrial *cox1* (1608 bp) and *nad1* (894 bp) genes showed 99–100% identity with the previously deposited sequence of the accession number AB235846.

In comparison with intraspecific diversity indices of other *Echinococcus* species that have been published so far, polymorphism (nucleotide diversity) within *E. ortleppi* detected here is lower than that found in *E. granulosus* s.s. elsewhere. Using partial sequences of *cox1* Casulli *et al*. (2012) and Nakao *et al*. (2010), reported a polymorphism range of 0·0017–0·0051 among different *E. granulosus* s.s. populations in China, Eastern Europe and Italy. Outside the *E. granulosus* s.l., Santos *et al*. (2012) found 0·0044 in the Brazilian *Echinococcus vogeli* populations while Nakao *et al*. (2010) observed 0·0055 in *Echinococcus shiquicus* from China. The low polymorphism within *E. ortleppi* may be an indication for a rather recent differentiation from other members of the *E. canadensis/ortleppi* clade, which appears to contain other taxa in the process of speciation (Lymbery *et al*. 2015*a*). In addition, the worldwide dispersal of domestically transmitted *Echinococcus* species may have facilitated low genetic diversity through introduction of small founder populations and extinctions of ancestral transmission systems involving wild mammals. In contrast, species transmitted in wildlife cycles, for example *E. shiquicus* and *E. vogeli*, show higher variation due to conservation of ancient polymorphisms (Nakao *et al*. 2010; Santos *et al*. 2012). The limited polymorphism among isolates of *E. ortleppi* is reflected by only subtle deviations from neutrality. Collectively, the African *E. ortleppi* population might be expanding (based on Fu's Fs) but no single sub-population recorded any significant deviation from neutrality. The least polymorphic Ethiopia and Namibia isolates showed some marginal expansion following their comparably high haplotype diversities.

Parsimony networks and the phylogenetic tree presented in this study showed no geographic structuring between the different investigated countries or geographic groups. Indeed, there was more molecular variance within and between sub-population than among the geographic groupings. This is a common phenomenon observed also in *E. granulosus* populations in Eastern Europe (4% among populations, 96% within populations) (Casulli *et al*. 2012), Brazil, Australia and Europe (−0·8% among metpopulations, 96% within meta-populations) (Haag *et al*. 1999). Santos *et al*. (2012) found a contrary feature in *E. vogeli* (39%, $F_{CT} = 0{\cdot}38$, $P = 0{\cdot}01$) indicating significant geographic structure between populations of the eastern and western Amazon in Brazil, 2500 km apart from each other, which was

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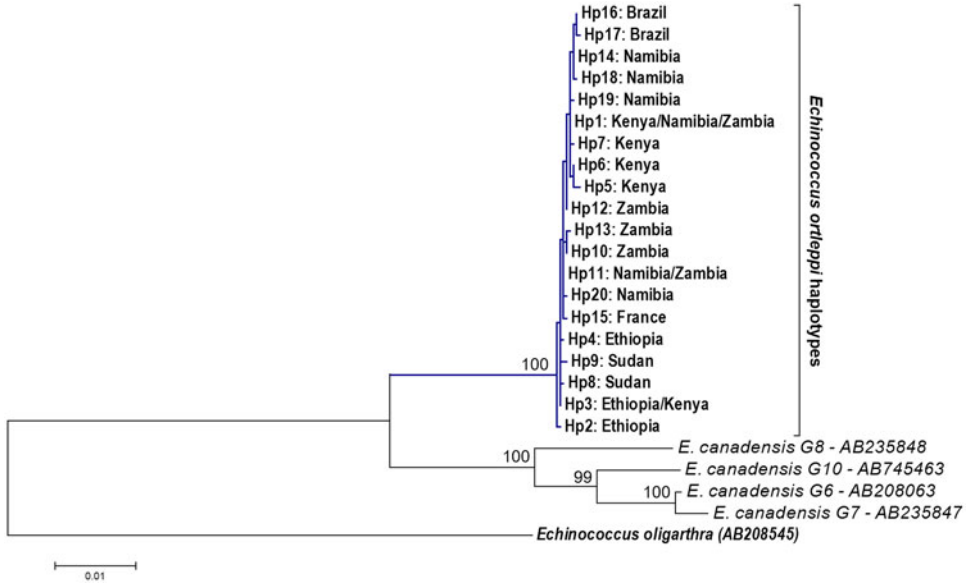

Fig. 2. A maximum likelihood tree of 20 *E. ortleppi* haplotypes and their relations to genotypes on the *E. canadensis*/*E. ortleppi* clade. ML phylogram was inferred based on the Kimura 2-parameter model with 1000 bootstrap replications, using the concatenated (2502 bp) dataset of mitochondrial *cox1* (1608 bp) and *nad1* (894 bp) genes and the *Echinococcus* basal species, *E. oligarthra*, used as an outgroup. Hp1 – 20 were haplotypes found in this study.

Table 3. Pairwise fixation index (Fst values) between *E. ortleppi* sub-populations based on concatenated mtDNA sequences of *cox1*/*nad1* genes (2502 bp)

	1	2	3	4	5	6	7	8
1. Ethiopia								
2. Kenya	0·466							
3. Sudan	0·372	0·571						
4. Zambia	0·568	0·089	0·647					
5. Namibia	0·760	0·272	0·783	0·325				
6. France	0·800	0·580	0·793	0·578	0·783			
7. Brazil	0·892	0·619	0·866	0·673	0·587	0·955		
8. Eastern Africa	nd	nd	nd	nd	nd	nd	nd	
9. Southern Africa	nd	nd	nd	nd	nd	nd	nd	0·147

All values were statistically significant at $P < 0{\cdot}01$. n d, not determined.

believed to have been caused by demographic and historic factors such as host vagility. In our study, we observed significant genetic differentiation (*F*st), or lack of gene flow, between most *E. ortleppi* sub-populations, which is an indication of lack of host vagility, except between Kenya and Zambia sub-populations. Nonetheless, geographic structuring was absent, likely due to recent colonization of the endemic regions. The lack of increased diversity within any of the sub-populations included in this study precludes conclusions on a geographical origin of *E. ortleppi*. It is however important to note that isolate numbers used in this study were very uneven (very small sample panels from Brazil and France) and may demonstrate only partially the actual demographic dynamics.

The majority of the haplotypes, 17/20, found in our study were restricted to individual sub-populations. The eastern African group had Hp3 occurring in the neighbouring Kenya and Ethiopia while Hp11 was found in the southern African neighbours Namibia and Zambia. The most frequent haplotype Hp1 was also the most widely distributed, occurring in livestock of Kenya, Namibia and Zambia, and in two wild *Oryx gazella* from Namibia. To the best of our knowledge, this is the first account of *E. ortleppi* occurring in any wild bovid host. Contrary to the star-like distribution of haplotypes of *E. granulosus* s.s. (Nakao *et al.* 2010, 2013; Casulli *et al.* 2012; Yanagida *et al.* 2012; Boufana *et al.* 2014) and *E. canadensis* G6/7 (Addy *et al.* 2016 unpublished results), haplotypes of *E. ortleppi* showed a divergent structure. It is only in the Tibetan *E. shiquicus*, where a divergent genetic structure was observed (Nakao *et al.* 2010) and the authors attributed this feature to a long-term geographical segregation to the Tibetan Plateau. Following the wide distribution of *E. ortleppi*,

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geographic segregation cannot be the cause of the observed genetic structure. The incomplete assemblage of isolates from all endemic regions in the current study prevents further conclusions on how the *E. ortleppi* parasite haplotypes might have developed.

It is widely assumed that livestock, particularly cattle, sheep and goats, were domesticated in the fertile crescent of the Middle East and globally distributed *via* colonization and trade. This assumption presupposes that the cattle adapted form of *Echinococcus* was introduced with its intermediate host into Africa and other endemic regions. The situation in Africa is, however, more complex, since domesticated cattle originating from the Middle East appear to have mixed with wild northern African aurochs (*Bos primigenius*) populations on their route to sub-Saharan Africa (Decker *et al*. 2014), making the region of parasite origin uncertain. The low level of genetic diversity of *E. ortleppi* might be due to a loss of polymorphism during the domestication of cattle, or due to introduction events of the parasite into the studied regions from elsewhere. More genetic data is clearly needed from other *E. ortleppi* endemic regions globally, and particularly the Middle East, to be able to conclude on the origin of this parasite.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0031182016001840.

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REFERENCES

Addy, F., Alakonya, A., Wamae, N., Magambo, J., Mbae, C., Mulinge, E., Zeyhle, E., Wassermann, M., Kern, P. and Romig, T. (2012). Prevalence and diversity of cystic echinococcosis in livestock in Maasailand, Kenya. *Parasitology Research* **111**, 2289–2294.

Alvarez Rojas, C. A., Romig, T. and Lightowlers, M. W. (2014). *Echinococcus* granulosus sensu lato genotypes infecting humans–review of current knowledge. *International Journal for Parasitology* **44**, 9–18.

Alvarez Rojas, C. A., Ebi, D., Gauci, C. G., Scheerlinck, J. P., Wassermann, M., Jenkins, D. J., Lightowlers, M. W. and Romig, T. (2016). Microdiversity of *Echinococcus granulosus* sensu stricto in Australia. *Parasitology* 1–8.

Balbinotti, H., Santos, G. B., Badaraco, J., Arend, A. C., Graichen, D. Â. S., Haag, K. L. and Zaha, A. (2012). *Echinococcus ortleppi* (G5) and *Echinococcus granulosus* sensu stricto (G1) loads in cattle from Southern Brazil. *Veterinary Parasitology* **188**, 255–260.

Boufana, B., Lahmar, S., Rebai, W., Safta, Z. B., Jebabli, L., Ammar, A., Kachti, M., Aouadi, S. and Craig, P. S. (2014). Genetic variability and haplotypes of *Echinococcus* isolates from Tunisia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **108**, 706–714.

Boufana, B., Lett, W. S., Lahmar, S., Buishi, I., Bodell, A. J., Varcasia, A., Casulli, A., Beeching, N. J., Campbell, F., Terlizzo, M., McManus, D. P. and Craig, P. S. (2015). *Echinococcus equinus* and *Echinococcus granulosus* sensu stricto from the United Kingdom: genetic diversity and haplotypic variation. *International Journal for Parasitology* **45**, 161–166.

Bowles, J., Blair, D. and McManus, D. (1992). Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Molecular and Biochemical Parasitology* **54**, 165–173.

Cardona, G. A. and Carmena, D. (2013). A review of the global prevalence, molecular epidemiology and economics of cystic echinococcosis in production animals. *Veterinary Parasitology* **192**, 10–32.

Casulli, A., Interisano, M., Sreter, T., Chitimia, L., Kirkova, Z., La Rosa, G. and Pozio, E. (2012). Genetic variability of *Echinococcus granulosus* sensu stricto in Europe inferred by mitochondrial DNA sequences. *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases* **12**, 377–383.

Clement, M., Posada, D. and Crandall, K. A. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657–1659.

Decker, J. E., McKay, S. D., Rolf, M. M., Kim, J. W., Molina Alcalá, A., Sonstegard, T. S., Hanotte, O., Götherström, A., Seabury, C. M., Praharani, L., Babar, M. E., Correia de Almeida Regitano, L., Yildiz, M. A., Heaton, M. P., Liu, W. S., Lei, C. Z., Reecy, J. M., Saif-Ur-Rehman, M., Schnabel, R. D. and Taylor, J. F. (2014). Worldwide patterns of ancestry, divergence, and admixture in domesticated Cattle. *PLoS Genetics* **10**, 1–14.

Dinkel, A., Von Nickisch-Rosenegk, M., Bilger, B., Merli, M., Lucius, R. and Romig, T. (1998). Detection of *Echinococcus multilocularis* in the definitive host: coprodiagnosis by PCR as an alternative to necropsy. *Journal of Clinical Microbiology* **36**, 1871–1876.

Excoffier, L., Laval, G. and Schneider, S. (2005). Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**, 47–50.

Fu, Y. X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**, 915–925.

Grenouillet, F., Umhang, G., Arbez-Gindre, F., Mantion, G., Delabrousse, E., Millon, L. and Boué, F. (2014). *Echinococcus ortleppi* infections in humans and cattle, France. *Emerging Infectious Diseases* **20**, 2100–2102.

Haag, K. L., Araújo, A. M., Gottstein, B., Siles-Lucas, M., Thompson, R. C. and Zaha, A. (1999). Breeding systems in *Echinococcus granulosus* (Cestoda; Taeniidae): selfing or outcrossing? *Parasitology* **118**, 63–71.

Hüttner, M., Nakao, M., Wassermann, T., Siefert, L., Boomker, J. D. F., Dinkel, A., Sako, Y., Mackenstedt, U., Romig, T. and Ito, A. (2008). Genetic characterization and phylogenetic position of *Echinococcus felidis* Ortlepp, 1937 (Cestoda: Taeniidae) from the African lion. *International Journal for Parasitology* **38**, 861–868.

Kamenetzky, L., Gutierrez, A. M., Canova, S. G., Haag, K. L., Guarnera, E. A., Parra, A., García, G. E. and Rosenzvit, M. C. (2002). Several strains of *Echinococcus granulosus* infect livestock and humans in Argentina. *Infection, Genetics and Evolution* **2**, 129–136.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111–120.

Librado, P. and Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics (Oxford, England)* **25**, 1451–1452.

Lopez-Neyra, C. R. and Soler Planas, M. A. (1943). Revision del genero *Echinococcus* Rudy description de una especie nuéva Parasita intestinal del porro en Almeria. *Revista ibérica de parasitología* **3**, 169–194.

Lymbery, A. J., Jenkins, E. J., Schurer, J. M. and Thompson, R. C. A. (2015*a*). *Echinococcus canadensis*, *E. borealis*, and *E. intermedius*. What's in a name? *Trends in Parasitology* **31**, 23–29.

Lymbery, A. J., Jenkins, E. J., Schurer, J. M. and Thompson, R. C. A. (2015*b*). Response to Nakao *et al*. –is *Echinococcus intermedius* a valid species? *Trends in Parasitology* **31**, 343–344.

Mbaya, H., Magambo, J., Njenga, S., Zeyhle, E., Mbae, C., Mulinge, E., Wassermann, M., Kern, P. and Romig, T. (2014). *Echinococcus* spp. in central Kenya: a different story. *Parasitology Research* **113**, 3789–3794.

McManus, D. P., Knight, M. and Simpson, A. J. G. (1985). Isolation and characterisation of nucleic acids from the hydatid organisms, *Echinococcus* spp. (cestoda). *Molecular and Biochemical Parasitology* **16**, 251–266.

Nakao, M., Sako, Y., Yokoyama, N., Fukunaga, M. and Ito, A. (2000). Mitochondrial genetic code in cestodes. *Molecular and Biochemical Parasitology* **111**, 415–424.

Nakao, M., Sako, Y. and Ito, A. (2003). Isolation of polymorphic microsatellite loci from the tapeworm *Echinococcus multilocularis*. *Infection, Genetics and Evolution* **3**, 159–163.

Nakao, M., Li, T., Han, X., Ma, X., Xiao, N., Qiu, J., Wang, H., Yanagida, T., Mamuti, W., Wen, H., Moro, P. L., Giraudoux, P., Craig, P. S. and Ito, A. (2010). Genetic polymorphisms of *Echinococcus* tapeworms in China as determined by mitochondrial and nuclear DNA sequences. *International Journal for Parasitology* **40**, 379–385.

Nakao, M., Yanagida, T., Konyaev, S., Lavikainen, A., Odnokurtsev, V. A., Zaikov, V. A. and Ito, A. (2013). Mitochondrial phylogeny of the genus *Echinococcus* (Cestoda: Taeniidae) with emphasis on relationships among *Echinococcus canadensis* genotypes. *Parasitology* **140**, 1625–1636.

Nakao, M., Lavikainen, A. and Hoberg, E. (2015). Is *Echinococcus intermedius* a valid species? *Trends in Parasitology* **31**, 342–343.

Pednekar, R. P., Gatne, M. L., Thompson, R. C. A. and Traub, R. J. (2009). Molecular and morphological characterisation of *Echinococcus* from food producing animals in India. *Veterinary Parasitology* **165**, 58–65.

Romig, T., Omer, R. A., Zeyhle, E., Huettner, M., Dinkel, A., Siefert, L., Elmahdi, I. E., Magambo, J., Ocaido, M., Menezes, C. N., Ahmed, M. E., Mbae, C., Grobusch, M. P. and Kern, P. (2011). Echinococcosis in sub-Saharan Africa: emerging complexity. *Veterinary Parasitology* **181**, 43–47.

Romig, T., Ebi, D. and Wassermann, M. (2015). Taxonomy and molecular epidemiology of *Echinococcus granulosus* sensu lato. *Veterinary Parasitology* **213**, 76–84.

Santos, G. B., Soares, M. do C. P., Elisabete, E. M., Rodrigues, A. L., Siqueira, N. G., Gomes-Gouvêa, M. S., Alves, M. M., Carneiro, L. A., Malheiros, A. P., Póvoa, M. M., Zaha, A. and Haag, K. L. (2012). Mitochondrial and nuclear sequence polymorphisms reveal geographic structuring in Amazonian populations of *Echinococcus vogeli* (Cestoda: Taeniidae). *International Journal for Parasitology* **42**, 1115–1118.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**, 585–595.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–2729.

Templeton, A. R., Crandall, K. A. and Sing, C. F. (1992). A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**, 619–633.

Thompson, R. C. A. (2008). The taxonomy, phylogeny and transmission of *Echinococcus*. *Experimental Parasitology* **119**, 439–446.

Thompson, R. C. A. and Lymbery, A. J. (1988). The nature, extent and significance of variation within the genus *Echinococcus*. *Advances in Parasitology* **27**, 209–258.

Thompson, R. C. A. and Lymbery, A. J. (1990). *Echinococcus*: biology and strain variation. *International Journal for Parasitology* **20**, 457–470.

Thompson, R. C. A. and McManus, D. P. (2002). Towards a taxonomic revision of the genus *Echinococcus*. *Trends in Parasitology* **18**, 452–457.

Tigre, W., Deresa, B., Haile, A., Gabriël, S., Victor, B., Pelt, J. V., Devleesschauwer, B., Vercruysse, J. and Dorny, P. (2016). Molecular characterization of *Echinococcus granulosus* s.l. cysts from cattle, camels, goats and pigs in Ethiopia. *Veterinary Parasitology* **215**, 17–21.

Wassermann, M., Aschenborn, O., Aschenborn, J., Mackenstedt, U. and Romig, T. (2015). A sylvatic lifecycle of *Echinococcus equinus* in the Etosha National Park, Namibia. *International Journal for Parasitology: Parasites and Wildlife* **4**, 97–103.

Yanagida, T., Mohammadzadeh, T., Kamhawi, S., Nakao, M., Sadjjadi, S. M., Hijjawi, N., Abdel-Hafez, S. K., Sako, Y., Okamoto, M. and Ito, A. (2012). Genetic polymorphisms of *Echinococcus granulosus* sensu stricto in the Middle East. *Parasitology International* **61**, 599–603.

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