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Echinococcus granulosus genotype G1 dominated in cattle and sheep during 2003–2006 in Buenos Aires province, an endemic area for cystic echinococcosis in Argentina

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ABSTRACT

Cystic echinococcosis (hydatidosis) is a severe and widespread disease, caused by the larval stage of the tapeworm *Echinococcus granulosus*; it affects large numbers of humans and farm animals annually, causing serious health and economic problems. Molecular studies have identified large genetic variation within the *E. granulosus* complex, with various hosts displaying different susceptibility to different genotypes. For the effective management of the disease, one of the most pressing tasks is to combine epidemiological and genetic data to better understand the role of different hosts and genotypes in the transmission of the parasite. The aim of the present study was to describe the epidemiology of cystic echinococcosis in cattle and sheep, and to characterise the genotypes of *E. granulosus* present in these farm animals. The study was carried out in the Pampa region of Argentina, with a particular focus on Buenos Aires province, where cystic echinococcosis represents an important human and veterinary health problem.

Among 513 cattle and 792 sheep, 11.9% and 4.0%, respectively, were infected with *E. granulosus*. Genetic characterisation of 42 isolates from cattle and 34 isolates from sheep was carried out by sequencing mitochondrial *cox1* and *nad1* genes. The vast majority of isolates were identified as genotype G1, except for a single sheep isolate determined as genotype G2, and a single cattle isolate that corresponded to genotype G5. Genotype G1 has previously been found to be the most infectious genotype to humans. As G1 was also the genotype principally responsible for cystic echinococcosis in Buenos Aires province, these results have important implications for developing effective disease control programmes to improve human and animal healthcare in this region.

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1. Introduction

Cystic echinococcosis (CE) is an acute zoonotic disease caused by the larval stage (hydatid cyst) of the tapeworm *Echinococcus granulosus*. It is one of the most widespread zoonoses of veterinary and medical importance (McManus et al., 2003; Craig et al., 2007; Brunetti et al., 2010). The life cycle of *E. granulosus* includes dogs and wild carnivores as definitive hosts in which adult tapeworms develop following ingestion of infected tissue from an intermediate host. A wide range of domestic and wild mammals, including humans, can serve as intermediate hosts, in which tapeworm larvae develop after oral infection with parasite eggs (Thompson, 2008). *E. granulosus* is well adapted to a wide range of host species and exhibits considerable intraspecific variability in terms of its host specificity, genetic background, epidemiology, morphology, biochemistry and physiology (Thompson and McManus, 2002).

Epidemiological studies in combination with genetic characterisation have demonstrated that prevalence of CE and of particular *E. granulosus* genotypes varies in relation to the type of

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livestock host analysed. Moreover, it has been argued that human susceptibility to CE also depends on the genotype of *E. granulosus* (*e.g.* Nakao et al., 2007). Therefore, understanding levels of genetic variability and phylogenetic relationships within and among *E. granulosus* genotypes has important implications for human safety and development of efficient disease control programmes (Rosenzvit et al., 1999). Molecular studies have identified a number of genotypes/species within the *E. granulosus* complex (Bowles et al., 1992, 1994; Bowles and McManus, 1993; Lavikainen et al., 2003). However, their phylogenetic relationships, as well as the phylogeny of the whole genus, have remained controversial: analysis based on mitogenomes (Nakao et al., 2007) and on three nuclear genes (Knapp et al., 2011) indicate that the *E. granulosus* complex is not monophyletic but paraphyletic, whereas analysis based on five nuclear genes suggests that *E. granulosus* complex is monophyletic (Saarma et al., 2009).

South America is one of the regions where CE is most prevalent, with about 2000 human cases per year. *E. granulosus* is widespread in Argentina and is particularly abundant in Buenos Aires province, where CE represents an important human and veterinary health problem (Andresiuk et al., 2009; Dopchiz, 2006; Dopchiz et al., 2007, 2009; Elissondo et al., 2002, 2003): the annual incidence in Buenos Aires province was 0.3/100,000 in the year 2000 and 0.4/100,000 in 2001 (Ministero de Salud de la Nación). Moreover, Argentina is currently the world's leading beef consumer on a per capita basis and one of the largest beef exporters in the world. In 2006, up to 55 million head of cattle were kept by farmers, mostly in the fertile pastures of the Pampa region and especially in the provinces of Buenos Aires and Santa Fe. Though cattle have historically been recognised as the main farm animal, Buenos Aires is also the top producer of sheep meat in the country.

Several studies have previously been carried out in Argentina, with the main objective of characterising the genetic variants involved in different *E. granulosus* life cycles. These have revealed the presence of the following genotypes: G1, G2, G3, G5, G6 and G7 (Badaraco et al., 2008; Haag et al., 2004; Kamenetzky et al., 2000, 2002; Rosenzvit et al., 1999, 2001; Santos et al., 2003; Soriano et al., 2010; Zanini et al., 2006). Nevertheless, data from one of the most important provinces with respect to CE, Buenos Aires province, remain scarce, with only 10 isolates so far analysed from this province (Kamenetzky et al., 2002). Due to Argentina's extensive geographic and climatic diversity, the different types of livestock occurring in each region, and due to the complex genetic structure of *E. granulosus*, it is important to evaluate the genetic variation and proportion of different genotypes at a fine regional scale. It is highly plausible that different genotypes of *E. granulosus* prevail locally depending on the epidemiological conditions in each region (Kamenetzky et al., 2002; Rosenzvit et al., 1999).

The aim of the study was to describe the epidemiology of CE in cattle and sheep, and to characterise the genotypes of *E. granulosus* infecting these farm animals in Buenos Aires province.

2. Materials and methods

2.1. Epidemiological study

Viscera from 513 cattle slaughtered at an abattoir located in Mar del Plata city (Buenos Aires province, Argentina) were obtained between September 2003 and July 2004, and viscera from 792 sheep slaughtered at an abattoir in Tres Arroyos (Buenos Aires province, Argentina) were obtained during 2006 (Fig. 1). The majority of the slaughtered animals were from Buenos Aires province, which covers the largest area in the Pampa region. Several samples from La Pampa and Entre Ríos provinces were also included in the epidemiological analysis. For each sample, the date of slaughter and the identity of the animal (cattle, bull, calf, heifer, old sheep and lamb) were provided by abattoirs. Animals were divided into two categories depending on their age: (1) 'young', which included calf and heifer for cattle, and lamb for sheep, and (2) 'adults', which included cow and bull for cattle, and old female sheep. Viscera were inspected visually and through palpation to detect and collect hydatid cysts. Each cyst was processed as an individual isolate. Cysts were removed from infected organs and their number, size and typology were registered. Cysts were classified as either fertile or sterile (acephalocysts). Fertility was assessed by determining the percentage of viable protoscoleces through microscopic examination of flame cell movements, the presence of calcareous corpuscles and negative methylene blue staining (Andresiuk et al., 2009). Membranes and protoscoleces were stored in 70% ethanol at 4 °C until further use.

2.2. Statistical analyses

Proportional differences in the prevalence and fertility of hydatid cysts in different species and anatomical locations were analysed using chi-square tests. Groups were compared with the Kruskal–Wallis test for two groups. Tests were carried out using Epi Info 2002. Statistical significance was assessed at $P \leq 0.05$.

2.3. Molecular analyses

Protoscoleces or cyst membranes of 76 isolates from cattle (42) and sheep (34) from Buenos Aires province were used for DNA extraction. Total genomic DNA was isolated using previously described procedures (Kamenetzky et al., 2000) or using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Two target sequences of mitochondrial DNA coding for *nad1* (NADH dehydrogenase subunit 1) and *cox1* (cytochrome c oxidase subunit 1) were amplified using previously described primers (Bowles et al., 1992; Bowles and McManus, 1993). In addition, *nad1* sequences of some samples were amplified using the novel primers eNDf1 (TCGTTTTACACGCGATTGAACT) and eNDr1 (ACCTGCTATGCAGCCCTATT). Lengths of the amplified gene segments were 366 bp for *cox1*, 466 bp for *nad1*, and 1271 bp for *nad1* with eND primers. PCRs were performed in a total volume of 20 μl containing 1 × BD Advantage 2 PCR buffer, 1U BD Advantage 2 Polymerase mix (BD Biosciences, Franklin Lakes, NJ, USA), 0.2 mM dNTP (Fermentas, Vilnius, Lithuania), 4 pmol of each primer and 20–80 ng of purified DNA. The cycling parameters were: 95 °C for 1 min, 40 cycles at 95 °C for 30 s, 50 °C for 30 s and 68 °C for 90 s followed by a final extension step at 68 °C for 7 min. PCR products were resolved on 1.2% agarose gels (Fermentas, Vilnius, Lithuania) stained with ethidium bromide. Individual PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) or by adding one unit of both shrimp alkaline phosphatase and exonuclease I (Fermentas, Vilnius, Lithuania) to the PCR mixture, which was then incubated for 30 min at 37 °C and heated to 80 °C for 15 min to inactivate the enzymes. Sequencing was performed using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA USA), following the manufacturer's protocols. Initial denaturing was at 96 °C for 1 min, followed by 27 cycles at 96 °C for 10 s (denaturation), 50 °C for 15 s (annealing) and 60 °C for 4 min (extension). Sequences were resolved on an ABI3130xl automated DNA sequencer (Applied Biosystems) or in an automatic DNA sequencer (ABI PRISM Model 377 PerkinElmer). Both DNA strands were sequenced with the primers used in the primary amplification. Consensus sequences were created using the programme Consed (Gordon et al., 1998). Sequences were aligned using Clustal W (Thompson et al., 1994) and corrected in BioEdit (Hall, 1999). All

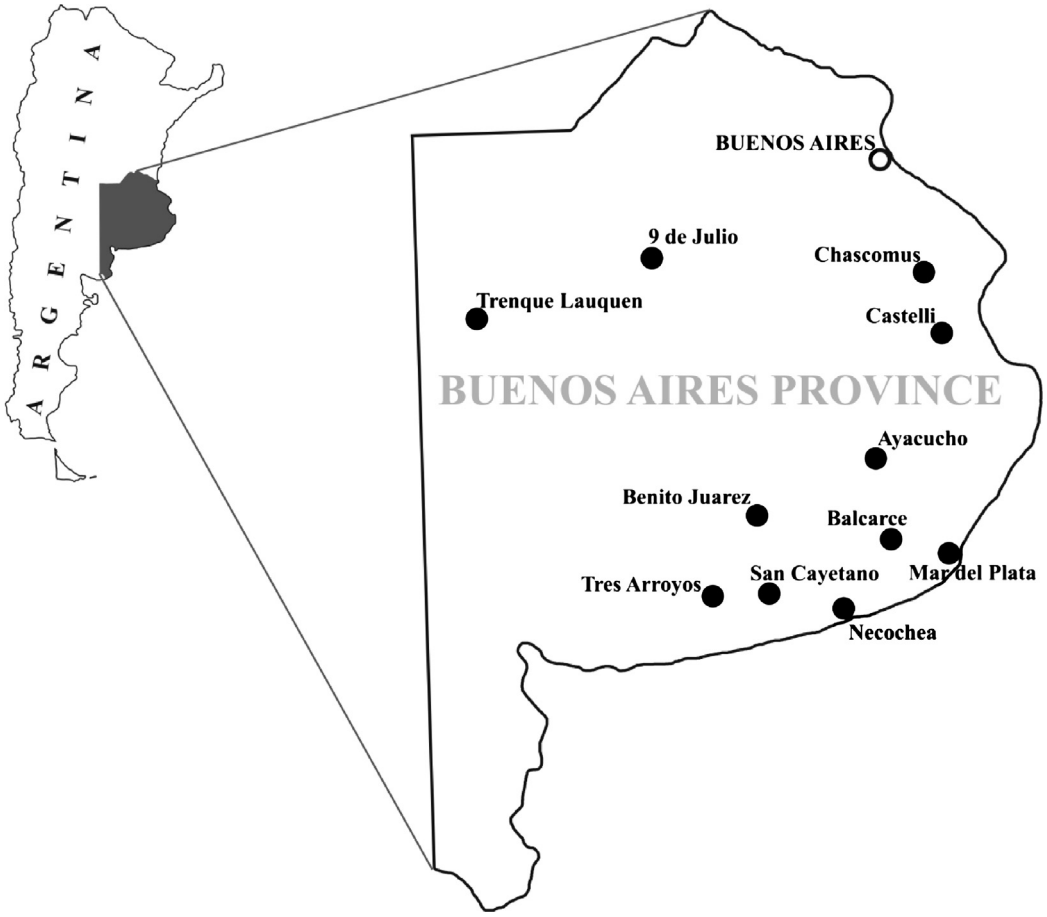

Fig. 1. Map of *E. granulosus* sampling locations in Buenos Aires province (filled black circles).

sequences were aligned with homologous sequences of genotypes G1–G10 available in GenBank.

2.4. Phylogenetic analysis

For analysis of phylogenetic relationships between isolates of *E. granulosus sensu stricto* (*i.e.*, mitochondrial genotypes G1–G3), the dataset was expanded to include additional mitochondrial *cox1* and *nad1* sequences for *E. granulosus* s.s. available in Genbank. Median joining networks were calculated with Network 4.510 (Bandelt et al., 1999) using the default settings. The network analyses were performed on alignments of 339 bp for *cox1*, and 871 bp and 343 bp for *nad1* sequences (shorter sequences were used for the large network which included GenBank sequences).

3. Results

3.1. Epidemiological study

3.1.1. Cattle

A total of 747 hydatid cysts were isolated from 513 cattle slaughtered at the Mar del Plata abattoir (Table 1). The prevalence of CE among cattle was 11.9% and the overall average intensity of infection was 12.2 hydatids per infected animal. The majority of animals (85.4%) were adults and most infected animals were also adults (98.4%). The average intensity of infection in adults was 13.5 hydatids per infected animal. The vast majority of hydatid cysts were recovered from lungs (698), with a minority recovered from livers (47). In addition, two cysts were found in the heart of one animal.

3.1.2. Sheep

A total of 81 cysts were obtained from 792 sheep slaughtered at the Tres Arroyos abattoir and at a farm near Mar del Plata city (Table 1). Four percent of sheep were infected with *Echinococcus* sp. While the great majority (96%) of slaughtered animals belonged to the younger category (lambs), the majority of infected animals (68.8%) were adults (difference in proportions of infected animals by age class; $\chi^2 = 4.5$; $P < 0.05$). The average intensity of infection was 3.0 hydatids per infected animal, with intensity significantly higher among adults: on average, 1.0 cysts per infected young animal versus 3.9 cysts per infected older animal (Kruskal–Wallis $H = 6.5$; $P < 0.05$). The majority (67.9%) of hydatids were located in lungs and a minority in livers (32.1%).

The prevalence of CE was significantly higher in cattle than in sheep ($\chi^2 = 29.1$; $P < 0.05$). The average intensity of infection, both overall and in adult animals alone, was higher in cattle than in sheep (overall: Kruskal–Wallis $H = 22.5$; $P < 0.05$ and adults: Kruskal–Wallis $H = 10.7$; $P < 0.05$). Additional information for cysts recorded in cattle and sheep can be found in Supplementary content.

Table 1
Numbers and characteristics of hydatid cysts recorded in cattle and sheep.

	Cattle	Sheep
Total no of animals	513	792
Total no of infected animals	61 (11.9%)	32 (4%)
Total no of cysts	747	81
Fertile cysts	86 (11.5%)	34 (42%)

3.2. Genotype identification and phylogenetic relations

In total, 47 *nad1* and 69 *cox1* sequences were obtained from 76 *E. granulosus* isolates, yielding seven *cox1* and four *nad1* haplotypes (GenBank accession numbers KC579441–KC579451). Sequencing and comparative analysis with *Echinococcus* sequences from GenBank showed that most of the isolates were G1. However, based on *cox1* (339 bp) a single sheep isolate represented genotype G2. Network analysis based on *cox1* revealed that genotypes G1 and G2 are divided into seven haplotypes (C1–C7 in Fig. 2A; note that haplotype C6 corresponds to genotype G2). The majority of cattle (31 out of 38) and sheep (24 out of 33) belonged to the central haplotype C1, while other haplotypes were less numerous and separated from C1 by one to three mutations. The G1 sequences belonging to haplotype C1 were identical to the sequence taken as reference for the G1 genotype (M84661 in GenBank; Bowles et al., 1992), which is the most numerous G1 haplotype worldwide and has previously been recorded among Argentinian sheep, cattle, dogs, and humans (Soriano et al., 2010). Other haplotypes (C2–C7 in Fig. 2A) were considerably less numerous, though two of them matched haplotypes previously described in Argentina: C2 with a haplotype recorded in sheep, goats and dogs (Soriano et al., 2010), and C3 with a haplotype recorded in sheep, humans (Kamenetzky et al., 2002) and dogs (Soriano et al., 2010). Other *cox1* haplotypes found in this study (C4–C7) had not previously been described in Argentina or elsewhere. We also attempted to place *cox1* haplotypes from this study into a worldwide context and aligned them with homologous *E. granulosus* s.s. (G1–G3) sequences available in GenBank. However, this yielded an unresolved and extremely confusing network with many reticulations and median vectors (data not shown).

Analysis of *nad1* (871 bp) yielded three haplotypes (N1–N3 in Fig. 2B). The vast majority of cattle (21 out of 23) and sheep (20 out of 23) isolates from this study belonged to haplotype N1, while only two cattle and two sheep isolates were assigned as N2. Both haplotypes were determined as genotype G1. A single sheep haplotype (N3) from Mar del Plata was identified as G2/G3, and one cattle isolate as genotype G5. To place the *E. granulosus* s.s. (G1–G3) *nad1* haplotypes from this study into a worldwide context we aligned these with homologous sequences from GenBank. As the majority of the *nad1* sequences from GenBank were considerably shorter, the final alignment consisted of 294 sequences that were 343 bp long. Network analysis demonstrated that these sequences were divided into 54 distinct haplotypes that could be allocated into two haplogroups (see the inset in Fig. 3): one comprising sequences belonging to genotype G1; the other formed by sequences belonging to genotypes G2 and G3. The most frequent haplotype (N1) in this study formed part of the most numerous and central haplotype globally (I in Fig. 3). Haplotypes N2 and N3 had not previously been described. N2 was closely related to the central haplotype (I), while N3 formed part of the G2/G3 haplogroup.

4. Discussion

Although several genetic studies have previously been carried out on *E. granulosus* in Argentina (Badaraco et al., 2008; Haag et al., 2004; Kamenetzky et al., 2000, 2002; Rosenzvit et al., 1999, 2001; Santos et al., 2003; Soriano et al., 2010; Zanini et al., 2006) they have rarely been accompanied by epidemiological data (Soriano et al., 2010). Here we present data from both epidemiological and molecular analysis. The prevalence of CE in cattle reported in the present study (approximately 12%) is similar to those registered in previous studies in the south east of Buenos Aires province (9–12%; Andresiuk et al., 2009; Dopchiz, 2006), Iran (16%; Dalimi et al., 2002) and Algeria (14%; Bardonnet et al., 2003), where cattle also play an important role in CE transmission. In contrast, the prevalence of CE in sheep in this study (4%) was significantly lower than that recorded in cattle from the Pampa region or in sheep in regions where they represent the main intermediate host for *E.* granulosus: Iran (11–74%; Dalimi et al., 2002; Daryani et al., 2007), Italy (75%; Scala et al., 2006; Varcasia et al., 2006) and Greece (30%; Varcasia et al., 2007). However, the prevalence of CE in sheep in this study (4%) is very similar to that recorded in Brazil (3%; de la Rue et al., 2006) where, just as in the Pampa region, cattle are the most important intermediate host involved in the transmission of CE (Andresiuk et al., 2009; Dopchiz, 2006). Adult animals were more likely to have CE than younger ones. Such an age-dependent increase in infection rate is to be expected given the shorter time of exposure of young animals (Muñoz and Sievers, 2005).

Although previous studies have revealed several genotypes of *E. granulosus* in Argentina, data for one of the most important provinces with respect to CE (and also meat production and export), Buenos Aires province, remained scarce (only 10 isolates from several host species had previously been described; Kamenetzky et al., 2002). While studies in various regions of Argentina have found a wide variety of *E. granulosus* genotypes, namely G1, G2, G3, G5, G6 and G7, here we found only three of them: G1 from both sheep and cattle, G2 from a single sheep and G5 from a cattle isolate. The absence of G6 and G7 genotypes in this study is probably explained by the fact that in Argentina these genotypes are specific to species that were not included in this study, such as goats, pigs, dogs and humans (*e.g.* Kamenetzky et al., 2002; Soriano et al., 2010). Most of the isolates analysed in this study belonged to genotype G1, which is the most widespread genotype not only in Buenos Aires province, but throughout Argentina – where it occurs in sheep, cattle, goats, dogs and humans (Badaraco et al., 2008; Haag et al., 2004; Kamenetzky et al., 2000, 2002; Rosenzvit et al., 1999, 2001; Santos et al., 2003; Soriano et al., 2010; Zanini et al., 2006) – and worldwide.

The most predominant *cox1* haplotype (C1 in Fig. 2A) has previously been found elsewhere in Argentina: in sheep and cattle, but also in dogs and humans (Soriano et al., 2010). However, this is the first time that this haplotype has been described in Buenos Aires province. Of other *cox1* haplotypes in this study, C2 has been recorded in Buenos Aires province in cattle and sheep and in sheep, goats, and dogs in other provinces of Argentina (Soriano et al., 2010); while C3 has been recorded in humans, sheep (Kamenetzky et al., 2002) and dogs (Soriano et al., 2010). C1–C3 haplotypes have been recorded in other countries of South-America: C1 from cattle in Brazil, cattle and sheep in Chile, and humans, sheep, cattle, pigs and alpaca in Peru (Moro et al., 2009; Yanagida et al., 2012; Sánchez et al., 2010, 2012); C2 from humans in Chile (Kamenetzky et al., 2002); and C3 from cattle in Brazil. Haplotypes C4–C7 are described for the first time here.

Our results highlight once more that G1 is one of the most significant *E. granulosus* genotypes, with its global distribution and wide range of intermediate hosts. The fact that G1 is also the most frequently implicated genotype in human infections means that it merits particularly close attention (*e.g.* Eckert and Thompson, 1997; McManus and Thompson, 2003). Indeed, when the *nad1* haplotypes from sheep and cattle of this study were analysed together with homologous sequences from GenBank, the resultant network revealed that out of 42 G1 haplotypes 18 (43%) have been described in humans (Fig. 3). Based on *cox1* data, human isolates have been described in Argentina, corresponding to haplotype C1 (Soriano et al., 2010) and to C3 (Kamenetzky et al., 2002). Moreover, human isolates belonging to G1 (and to G6) genotype have been found in Buenos Aires province, whereas of domesticated animals in the same province, G1 has been found in cattle and pigs, and G7 in pigs only (Kamenetzky et al., 2002). The variation within G1 described in the present study confirms that this is one of the most variable genotypes within the complex. Its variability is presumably

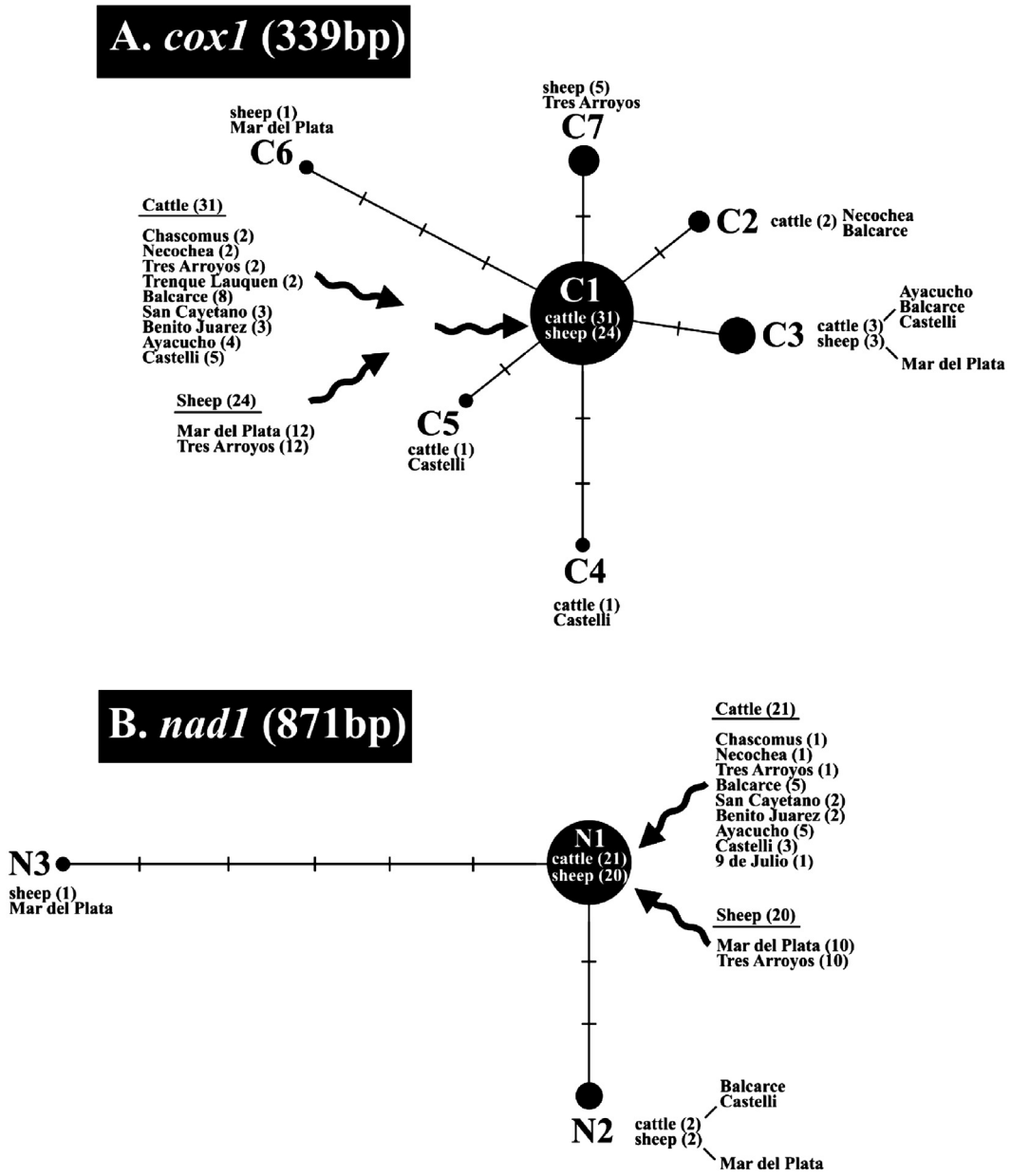

Fig. 2. Median joining networks illustrating phylogenetic relationships between *E. granulosus* s.s. isolates of cattle and sheep from Buenos Aires province, Argentina. (A) A network based on seven haplotypes (71 sequences in total: 38 cattle and 33 sheep) of mitochondrial *cox1* gene fragment (339 bp); (B) a network based on three haplotypes (46 sequences: 23 cattle and 23 sheep) of mitochondrial *nad1* gene fragment (871 bp).

connected to its very low intermediate host specificity and global distribution.

The G2 and G5 genotypes found in cattle and sheep in this study are the first reports of these genotypes from Buenos Aires province. Of other provinces in Argentina, G2 has been previously reported in sheep, cattle and humans only in Tucuman (Kamenetzky et al., 2002), whereas G5 has been found in cattle, dogs and humans in Santa Fe, Catamarca and Tucuman, respectively (Kamenetzky et al., 2002; López et al., 2002).

Haplotypes of *nad1* in *E. granulosus* s.s. from Argentina do not cluster together (Fig. 3), but are present in both haplogroups, indicating that *E. granulosus* s.s. might have a complex evolutionary history in Argentina. There are three plausible explanations for this pattern: (1) *E. granulosus* s.s. was originally introduced to Argentina from a single source population which was already genetically quite diverse; (2) *E. granulosus* s.s. was originally introduced to Argentina from different source populations; (3) extensive livestock trading between Argentina and other countries in recent times has introduced additional haplotypes from populations different from the original source.

The differences observed between cattle and sheep – in the mean intensity of infection and in the percentage of animals with fertile cysts (nine times greater in cattle) – may indicate that cattle are more suitable hosts than sheep and play a more important role in CE transmission in the Pampa region. Nevertheless, the fact that G1 was by far the most abundant genotype in both cattle and sheep samples suggests that G1 is probably the most prevalent and the principal genotype responsible for CE in all hosts (including cattle, sheep, pigs and humans) in the Pampa region. The result that cattle are principally infected with G1 and not with *Echinococcus ortleppi* (G5) is significant for assessment of the risk of human infection with *E. granulosus* in the region. In neighbouring Brazil, a recent study

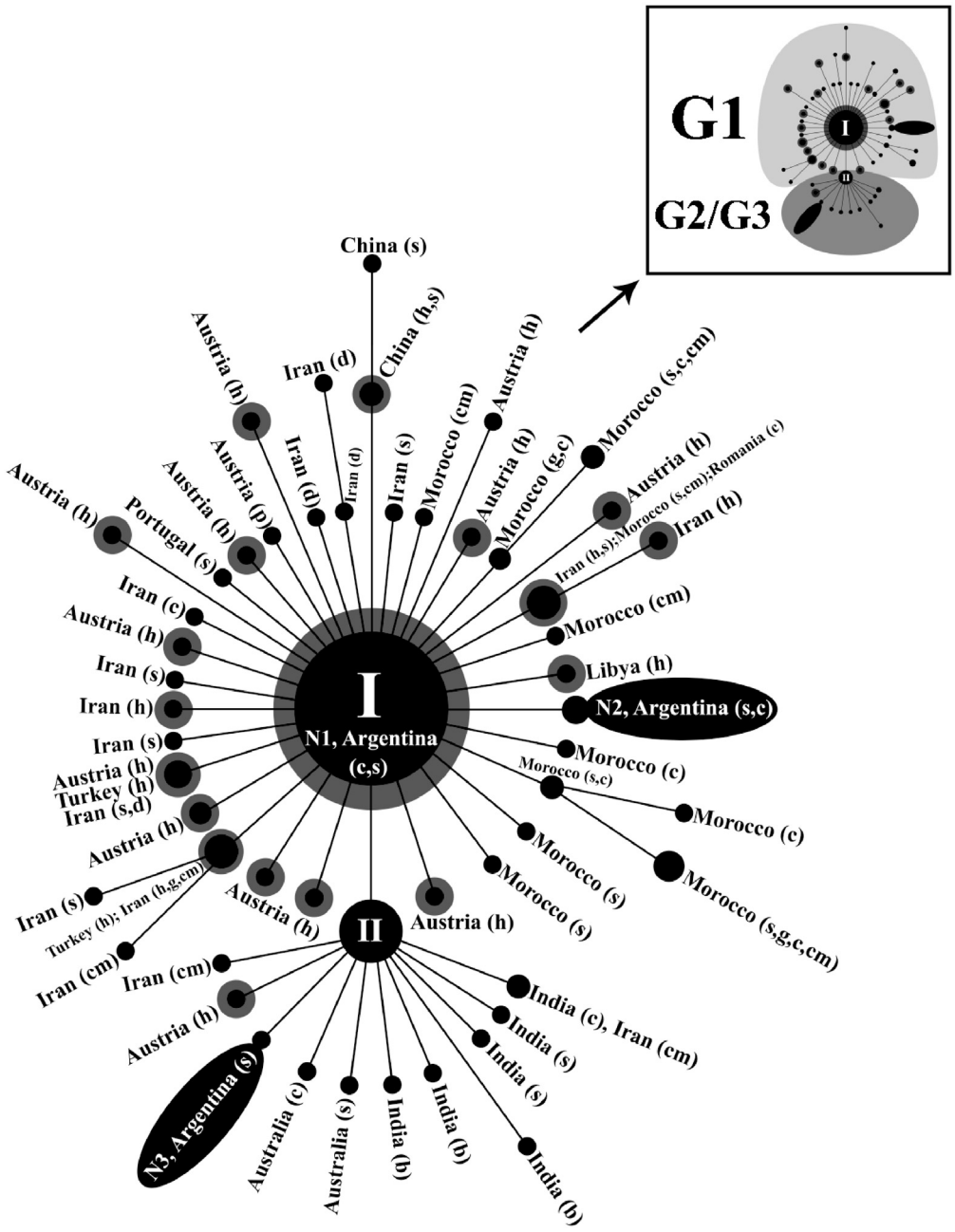

Fig. 3. Median joining network of mitochondrial *nad1* gene fragment (343 bp) illustrating relationships of three *E. granulosus* s.s. haplotypes from Buenos Aires province together with 51 homologous sequences from GenBank (54 haplotypes in total). All haplotypes are divided into two haplogroups: one comprising sequences of genotype G1 and another of G2/G3 (scheme in the box). Three haplotypes from Buenos Aires region are designated as N1–N3 (see also Fig. 2B). Two major haplotypes are designated as I and II. Haplotypes surrounded by a grey circle include human sequences. Host species are indicated with small letters (c – cattle, cm – camel, b – buffalo, d – dog, g – goat, h – human, p – pig, s – sheep). Haplotype I, countries and hosts: Algeria (s); Argentina (c and s; this study); Austria (h and s); China (c, h and s); France (c and s)M; Greece (s); India (c, g and s); Iran (c, cm, g, h and s); Italy (s); Libya (h); Mongolia (h); Morocco (c, cm, g, h and s); Peru (alpaca, c, h, p and s) and Turkey (c). Haplotype II: Iran (cm and s); India (b, c, g and s); Morocco (c and s); France (c and s); Greece (s); Romania (s); Tasmania (s).

by de la Rue et al. (2011) showed that *E. granulosus* s.s. exhibited higher parasite loads in both humans and dogs compared to G5.

In conclusion, the fact that the majority of cattle are infected with genotype G1, which is easily transmissible from dogs to humans, is an important parameter to consider in a risk assessment of human contamination by *E. granulosus* in Argentina. It should alert veterinary authorities to focus their attention on control of sheep and cattle health, slaughtering and the disposal of animal remains. Further investigations are necessary to unravel the transmission potential of different genotypes in different hosts in Buenos Aires province. Therefore, in a complex epidemiological situation where various hosts interact to advance the parasite lifecycle, genotyping of *E. granulosus* isolates is an essential epidemiological tool to fully understand the genetic diversity and transmission modes of the parasite, as well as the main hosts and genotypes involved in human contamination. This knowledge can be used to inform an effective regional control strategy.

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