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GENETICS, EVOLUTION, AND PHYLOGENY - SHORT COMMUNICATION

# Isolation and molecular characterization of four novel *Neospora caninum* strains

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## Abstract

*Neospora caninum* causes neosporosis, a leading cause of bovine abortion worldwide. Uruguay is a developing economy in South America that produces milk to feed seven times its population annually. Naturally, dairy production is paramount to the country's economy, and bovine reproductive failure impacts it profoundly. Recent studies demonstrated that the vast majority of infectious abortions in dairy cows are caused by *N. caninum*. To delve into the local situation and contextualize it within the international standing, we set out to characterize the Uruguayan *N. caninum* strains. For this, we isolated four distinct strains and determined by microsatellite typing that these represent three unique genetic lineages, distinct from those reported previously in the region or elsewhere. An unbiased analysis of the current worldwide genetic diversity of *N. caninum* strains known, whereby six *typing clusters* can be resolved, revealed that three of the four Uruguayan strains group closely with regional strains from Argentina and Brazil. The remaining strain groups in an unrelated genetic cluster, suggesting multiple origins of the local strains. Microsatellite typing of *N. caninum* DNA from fetuses opportunistically collected from local dairy farms correlated more often with one of the isolates. Overall, our results contribute to further understanding of genetic diversity among strains of *N. caninum* both regionally and worldwide.

**Keywords** Apicomplexa · Bovine abortion · *Neospora* · Microsatellite · Genetic diversity · Animal health

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**Abbreviations**

| | |
|---|---|
| AMOVA | Analysis of molecular variance |
| DAPC | Discriminant analysis of principal components |
| DMEM | Dulbecco's modified Eagle's medium |
| HFF | Human foreskin fibroblast |
| MS | Microsatellite |
| PCA | Principal component analysis |

## Introduction

*Neospora caninum* is an obligate intracellular protozoan parasite of the phylum Apicomplexa, which preferably infects cattle as intermediate hosts, causing neosporosis, one of the main causes of cattle abortion and reproductive failure worldwide (Dubey and Schares 2011). Cattle acquire the parasite by ingesting oocysts shed in feces by the definitive hosts, wild canids, or domestic dogs, developing, in most cases, a chronic asymptomatic disease (Dubey and Schares 2011). Canids become subsequently infected by ingestion of tissue cysts lodged in the intermediate hosts, hence perpetuating the transmission cycle (Cavalcante et al. 2011). *N. caninum* can also be transmitted transplacentally (Gondim et al. 2004). Infected fetuses can display irreversible tissue damage, resulting in fetal death and abortion, or be born asymptomatic and chronically infected. In chronically infected bovine herds, abortions are normally associated with transplacental transmission following reactivation of a dormant infection. Epidemic abortion outbreaks, known as "abortion storms," in naive pregnant cows can also occur and are normally associated with oocyst-derived infection (exogenous transplacental transmission) (Dubey and Schares 2011).

*N. caninum* abortion in livestock is estimated to generate millions of dollars in losses worldwide (Reichel et al. 2013). For instance, studies in the UK estimated that 12.5% of annual abortions in dairy cattle are caused by this agent (Davison et al. 1999). Meanwhile, in China, this number varies between 26 and 40% (Jia et al. 2014). In Argentina, *N. caninum* has been estimated to produce economic losses of up to 44 million USD (Moore et al. 2013). Uruguay produces milk to feed more than twenty million people annually, and the number of cows triples the human population. Thus, dairy production is of paramount importance to the country's economy. Seroepidemiological studies dated over 10 years ago estimated that virtually all dairy farms in Uruguay had at least one seropositive cow for *N. caninum* (Bañales et al. 2006; Piaggio 2006), and that about a third of the diagnosed abortions were caused by this agent (Easton 2006). Currently, the epidemiological situation has remained unchanged; a large share of current abortions in dairy cattle, diagnosed at the laboratory level, is caused by *N. caninum* (Macías-Rioseco M., personal communication[1]).

Previous studies have shown that genetic diversity exists among *N. caninum* isolates from geographically adjacent regions, including those present in South America (Al-Qassab et al. 2009; Regidor-Cerrillo et al. 2013). However, genetic diversity among *N. caninum* in South America is understudied with respect to worldwide diversity. More recently, an isolate from Argentina (Campero et al. 2015) and two novel Brazilian isolates (Locatelli Dittrich et al. 2018) have been reported. To deepen our understanding of both the national and regional situations, here we set out to characterize, at the molecular level, *N. caninum* isolates in Uruguay and to compare them with recent regional and worldwide isolates. We isolated four strains from congenitally infected dairy calves originating from three distinct geographical regions in Uruguay. Multilocus microsatellite typing of genomic DNA revealed a unique genetic pattern, resulting in the distribution of the Uruguayan isolates into two distinct genetic groups. Importantly, our results indicate that greater genetic variability exists among regional *N. caninum* populations than previously appreciated, contributing to further understanding this parasite's genetic diversity.

## Material and methods

### Strain isolation

To increase the chances of isolating distinct genetic backgrounds, we selected individuals from dairy farms with different neosporosis histories and from distinct geographical locations within the country's dairy-producing area. Three of the isolated strains (NcUru1–NcUru3) originated from *La Estanzuela*, in Colonia Department (Fig. S1A), an experimental dairy farm with approximately two hundred Holstein cows, located at the National Institute of Agricultural Research (INIA), whereby abortions occur sporadically. This herd presented chronically infected individuals. The seroprevalence of *N. caninum* in this herd is about 25%, and abortions have a sporadic occurrence. Here, we identified ten of thirty-six pregnant cows/heifers (28.5%) to be seropositive for *N. caninum* by ELISA. One of them had been recently acquired from a commercial dairy farm in the department of Soriano and was already pregnant when brought over to La Estanzuela (Fig. S1A). Six asymptomatic, congenitally infected male calves, with precolostral antibodies for *N. caninum*, were selected to pursue isolation, including the calf born to the heifer from Soriano. The isolate NcUru4 was derived from a dairy farm located in Durazno (Fig. S1A). This is a large farm with over ten thousand cows, which had experienced a laboratory-confirmed

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epizootic outbreak of *N. caninum* abortion affecting several hundred dams. Here, four newborn asymptomatic male calves with precolostral antibodies to *N. caninum* were selected to pursue isolation. Pregnant cows were diagnosed positive for *N. caninum* using a commercial ELISA kit (PrioCHECK™ Bovine Neospora Ab 2.0 Serum Kit, Thermo Fisher Scientific, Waltham, MA, USA) and followed to term.

Precolostral serum samples were obtained from newborn calves, and *N. caninum* antibodies were determined by the same ELISA kit. Seropositive calves were selected to attempt parasite isolation. For a scheme, refer to Supplementary Figure 1. Parasite isolation was carried out following the procedure described previously (Pena et al. 2007; Rojo-Montejo et al. 2009; Campero et al. 2015). In short, brain samples of the congenitally infected calves were homogenized in a phosphate-buffered saline (PBS) solution containing antibiotics, DNA was extracted, and PCR assay was carried out to identify areas of the brain harboring *N. caninum* DNA. PCR-positive homogenates were processed and inoculated into interferon-γ knockout mice B6.129S7-Ifngtm1Ts/J (Jackson Laboratory, Bar Harbor, Maine, USA) by intraperitoneal injection (200 μl). Mice were evaluated daily for the onset of clinical signs compatible with neosporosis (ataxia, forelimb paralysis, emaciation, and/or lethargy). Symptomatic mice were humanly euthanized at the post-inoculation times indicated in Supplementary Material Figure 2. Brain samples were aseptically obtained, tested by PCR to identify *N. caninum*-infected regions, and inoculated onto human foreskin fibroblasts (HFF-1, SCRC-1041) (ATCC, Manassas, VA, USA). Parasites were observed in tissue culture, at the times indicated in Supplementary Material Figure 2, for each strain.

All experiments involving animals were done following pre-approved protocols by institutional ethic committees (CEUA no. 010-17) at the Institut Pasteur de Montevideo or the National Institute for Agricultural Research (INIA).

## Cell culture and strain maintenance

Cell culture was maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) solution and 5% fetal bovine serum (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and 5% $CO_2$. All isolates were serially passaged in human foreskin fibroblasts (HFFs). Isolates were cryopreserved in liquid nitrogen on 10% DMSO (Sigma-Aldrich, Merck, Darmstadt, Germany).

## DNA extraction, polymerase chain reaction, and microsatellite typing

Genomic DNA was extracted using a commercial kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. *N. caninum*-specific detection by PCR was carried out by amplifying the *N. caninum*-specific gene Nc5, using primers Np6 and Np21, as described previously (Yamage et al. 1996). Coccidian and *Toxoplasma gondii*-specific DNA detection was carried out as described previously (Michael et al. 1996; Homan et al. 2000). A mammalian nuclear gene was amplified as a DNA extraction control, as described previously (Murphy et al. 2001). Microsatellite analysis was performed on *N. caninum* PCR–positive samples extracted from isolated strains in vitro and on bovine samples. The genetic markers MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12, and MS21 were amplified using specific primers and PCR conditions previously described (Regidor-Cerrillo et al. 2013). PCR products were analyzed by 1% agarose gel electrophoresis stained with SYBR safe (Invitrogen, Carlsbad, CA, USA), purified (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany), and sequenced in house. Sequences were analyzed using BioEdit Sequence Alignment Editor v.7.0.1 software (Ibis Therapeutics, Carlsbad, CA, USA). Allele assignment was performed as described previously (Regidor-Cerrillo et al. 2013). Fifteen aborted fetuses recovered from the field, with *N. caninum*-compatible lesions or with an undiagnosed cause of abortion, were processed for DNA extraction and subjected to PCR analysis for *N. caninum* DNA detection, microsatellite amplification, and analysis, as described above. All fetuses came from the dairy-producing region of the country (Fig. 1a). Particularly, 4 of them (Fig. 1a, samples 8, 10, 12, and 14) originated from regions adjacent to the dairy farm where NcUru1 and NcUru2 were isolated. Sample 7 originated in the same region as NcUru3. The remaining 10 samples were obtained from other localities, as shown in Fig. 1 a.

## Immunofluorescence assay

Immunofluorescence assay of intracellular tachyzoites grown on HFFs was carried out using a commercial anti-*N. caninum* antiserum raised in goat (1:100) (VMRD, Pullman, WA, USA). Donkey anti-goat IgG H&L-FITC conjugated (Abcam, Cambridge, UK) was used at a 1:1000 dilution as secondary antibody. Acquisition was carried out in an epifluorescence microscope (Olympus Life Science, Tokyo, Japan) using a × 100 oil objective.

## Histopathology and immunohistochemistry

Brain samples from the *N. caninum*-congenitally infected neonate calves and liver samples from the inoculated mice were collected postmortem, immersion fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, sectioned at 4–5μm, mounted on glass slides, and stained with hematoxylin and eosin for routine histologic examination. Additionally, selected formalin-fixed paraffin-embedded (FFPE) sections of the liver from the mice were processed for *N. caninum* antigen detection by immunohistochemistry. Briefly, the slides were immersed in citrate buffer and heat-induced antigen retrieval was performed in

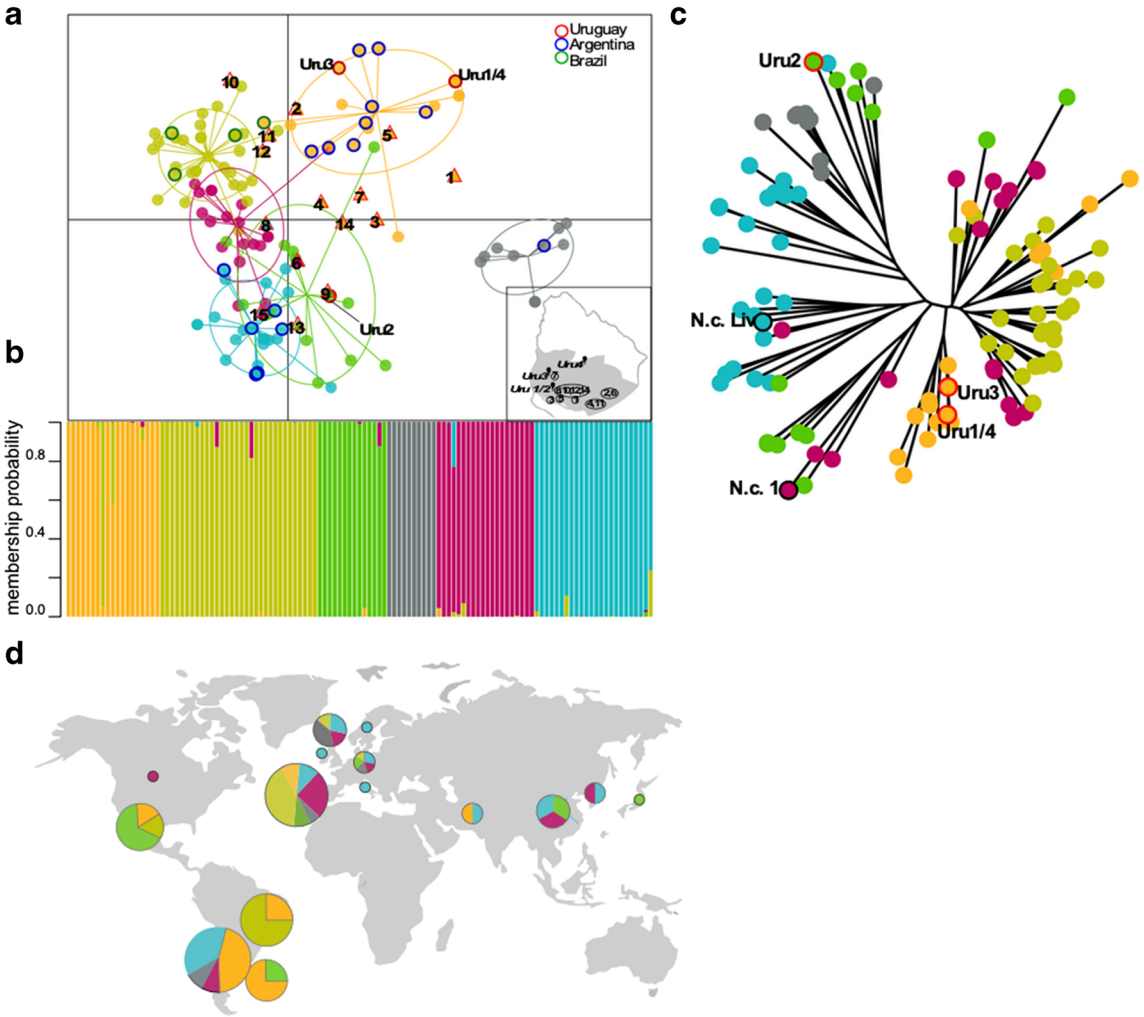


**Fig. 1** **a** Strain assignment to a genetic group by discriminant analysis of principal components (DAPC). Strains were grouped according to their combination of 8 microsatellite alleles into 6 distinct genotypic groups. Argentinean strains are circled in blue, Brazilian strains in green, and Uruguayan strains in red. See Supplementary Table 1 for details on the strains used in this analysis. Strains identified in aborted fetuses were assigned to a given genetic group according to their combination of microsatellite alleles and are represented with a triangle. Each strain was color coded as per their assigned group and numbered to match its geographical origin specified in the inset. The probability of each strain belonging to a given group identified in abortion samples is detailed in Supplementary Table 2. The gray-shaded area represents the dairy-producing region of the country. **b** Likelihood of genotype assignment per strain. The likelihood of each strain to be assigned to a given genotype is shown. Color codes correspond to the ones used in **a**, for each group. **c** Neighbor-joining tree. Evolutionary distances among strains sampled are graphically represented. **d** Geographical distribution of genotypes. Genotypes are color coded to match those in **a**

a decloaking chamber (Biocare Medical, Pacheco, CA, USA) at 110 °C for 30 min, after quenching the endogenous peroxidase with 3% hydrogen peroxide for 15 min. Goat polyclonal antibody against *N. caninum* (VMRD, Pullman, WA, USA) was applied as a primary antibody for 60 min. Horse anti-goat IgG horseradish peroxidase (HRP)–labeled polymer (Vector Laboratories, Burlingame, CA, USA) was used as the detection system (30-min incubation), with 3-amino-9-ethylcarbazole (Dako, Santa Clara, CA, USA) as the chromogen substrate solution. Archived FFPE brain from a dog infected with *N. caninum* was used as a positive control. As negative controls, duplicate sections of all slides were processed as mentioned above, but the primary antibody was replaced by normal goat serum (Vector Laboratories, Burlingame, CA, USA). Slides were counterstained with hematoxylin and visualized under an Axio Scope.A1 trinocular optical microscope (Zeiss, Oberkochen, Germany), coupled with an Axiocam 512 digital camera (Zeiss, Oberkochen, Germany).

## Principal component analysis and discriminant analysis of principal components

Genetic structure of samples from Argentina, Brazil, and Uruguay was first assessed by a principal component analysis (PCA). To perform the principal component analysis, a combination of nine microsatellites (MSs) was used to generate a

genetic distance matrix using the allele-sharing coefficient using *princomp* in R Studio (RStudio Team 2015). The nine MSs analyzed resulted from splitting MS10 in three independent satellites, as this previously showed better resolution. Argentinian and Brazilian strains were included ($n$ = 25). The results were not affected by missing data, since the results did not change using NA or imputation by mean. Then, the discriminant analysis of principal components (DAPC) in the *adegenet* package (Jombart 2008), implemented in R, was used to investigate the genetic structure of samples from Asia, Europe, and the Americas. DAPC does not make assumptions of the Hardy–Weinberg equilibrium, so all microsatellite loci were included in the analysis. The optimal number of clusters was determined using the *find.clusters* function of adegenet by $k$-means clustering and run sequentially with an increasing value of $k$ ($k$ = 1 to $k$ = 15), and the best number of clusters was chosen by the lowest value of the Bayesian information criterion (BIC) (Jombart et al. 2010). We ran the find.clusters with 1000 starting points and 1,000,000 iterations, and the results were consistently convergent over 10 independent trials. The assignment probability of the *N. caninum* strains isolated from abortions was estimated with the *predict.dapc* function over the previously identified clusters in the adegenet package (Jombart 2008). The distribution of the genetic variability within and between clusters, and $\Phi_{ST}$, was estimated by the analysis of molecular variance (AMOVA) (Excoffier et al. 1992) in the *poppr* package (Kamvar et al. 2013) implemented in R. The genomic accumulation curve and neighbor-joining tree with Provesti's distance were done with the poppr package (Kamvar et al. 2013)

## Results and discussion

### Genetic characterization of four novel Uruguayan *N. caninum* isolates from congenitally infected calves

In order to characterize *N. caninum* isolates in Uruguay, we obtained four *in vitro* isolates herein referred to as four distinct isolates, NcUru1 and NcUru2 (from the region of Colonia), NcUru3 (from the region of Soriano), and NcUru4 (from the region of Durazno) (Fig. S1A). In short, *N. caninum* PCR–positive regions of the brain from congenitally infected calves were inoculated in IFN-γ-KO or nude mice. Tachyzoites were isolated post inoculation of PCR-positive mouse brain regions onto HFFs (Fig. S1B, 4 and Fig. S2). Species identity was confirmed by PCR (Fig. S1C).

MSs are highly variable molecular markers which allow genotyping individuals within a population, and the study of phylogenetic relationships (Field and Wills 1996). We characterized well-known MS markers on our in vitro isolates as to be able to compare their genetic diversity with previously characterized strains (Al-Qassab et al. 2009; Pedraza-Díaz et al. 2009; Rojo-Montejo et al. 2009; Regidor-Cerrillo et al. 2013; Campero et al. 2015, 2018; Medina-Esparza et al. 2016).

All, but NcUru1 and NcUru4, exhibited a distinct pattern of microsatellites, suggesting that they represented at least three distinct strains (Table 1). The only microsatellite allele shared among all four isolates was MS8, consisting of 13 repeats. This allele is also found in Brazilian isolates and has been detected in samples obtained from abortions caused by *N. caninum* in Argentina (Table 1). NcUru2 only shared MS8 with NcUru1, NcUru3, and NcUru4 and was distinct for all other MSs. Moreover, NcUru2 shared little identity at the MS level with previously reported isolates from the region, exhibiting a combination of MS7 and MS10 (13 and 6.14.7 repeats, respectively) which has not been described in regional strains (i.e., Argentina and Brazil) before. Moreover, its individual combination of microsatellites was unique and has not been previously reported. We speculate that this scenario could have emerged by the introduction to Uruguay of strains with two different genetic origins.

### The *N. caninum* species groups in six typing clusters

We sought to determine how NcUru1–NcUru4 fit among the genetic variabilities reported for *N. caninum* strains identified worldwide (Table S1). By iterating all MS sequence combinations available (found in Table S1), a genotype accumulation curve revealed that a combination of 8 MSs was sufficient to achieve the maximum number of genotypes able to be resolved among unique individuals given a random sample of *n loci* (Fig. S4). Adding the genetic information available for 115 other worldwide strains, we ran DAPC that statistically supported the assemblage of six distinct genetic groups, arbitrarily named 1 through 6 (Fig. 1a and Fig. S5). Through AMOVA, we observed that ~ 30% of the variation was due to differences between clusters with $\Phi_{ST}$ = 0.3 ($p < 0.001$), and that ~ 70% was due to genetic variability within clusters. Cluster 2 grouped ~ 27% of the total samples, and cluster 6 (grouping ~ 20% of the total samples) was the most widely distributed, composed of strains originating in 10 different countries (Fig. 1a). As previously reported, this analysis showed a tendency for genotypes to group by geographical origin (Al-Qassab et al. 2009; Regidor-Cerrillo et al. 2013). At the continental scale, it was observed that ~ 63% of cluster 1 was from the Americas, and ~ 84% of cluster 2, 90% of cluster 4, 80% of cluster 5, and ~ 63% of cluster 6 were from Europe. Cluster 3 was distributed ~ 50% in the Americas and ~ 50% in Europe (Fig. 1c). Nonetheless, all six typing units were present in the Americas and Europe. Of note, cluster 2 was the only one not found in Asia. At a country level in South America, three of the four reported Brazilian isolates grouped together in cluster 2. Argentinean strains grouped in cluster 1 or 6, with a single exception in cluster 4. Neither Brazilian nor Argentinean strains were found elsewhere. Likewise, this

**Table 1** Comparison of four Uruguayan *N. caninum* isolates with regional strains by microsatellite typing

| STRAIN | ORIGIN | HOST | MICROSATELLITE | | | | | | | | | SOURCE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | MS4 | MS5 | MS6A | MS6B | MS7 | MS8 | MS10 | MS12 | MS21 | |
| N. c. Uru1 | URUGUAY | Cattle | 11 | 14 | 15 | 12 | 9.1 | 13 | 6.15.9 | 16 | 6 | This study |
| N. c. Uru2 | | | 14 | 9 | 12 | 11 | 13 | | 6.14.7 | 15 | 5 | |
| N. c. Uru3 | | | 13 | 14 | 15 | 12 | 9.1 | | 6.15.9 | 16 | 6 | |
| N. c. Uru4 | | | 11 | | | | | | | | | |
| N. c. 1 | USA | Dog | 12 | 12 | 12 | 17 | 16 | 16 | 7.12.9 | | | Regidor-Cerrillo, 2013 |
| N. c. Liv | UK | | 15 | 9 | 15 | 12 | 14 | 17 | 6.26.10 | | | Regidor-Cerrillo, 2006 |
| N. c. Goias | BRAZIL | Cattle | 13 | 18 | 14 | 12 | 9.1 | 12 | 6.16.9 | | 6 | García-Melo, 2009<br>Regidor-Cerrillo, 2006<br>Oliveira, 2017<br>Locatelli, 2018 |
| N. c. Bahia | | | | 13 | 15 | | | 14 | 5.14.9 | | | |
| N. c. PSP1 | | | | 19 | | | | 13 | 6.14.10 | | | |
| N. c. BNC-PR4 | | | 14 | 15 | | | | | 6.13.9 | | | |
| N.c. ARG-04-1 | ARGENTINA | | 13 | 17 | | | | | 6.15.8 | | | Regidor-Cerrillo, 2013 |
| N.c. ARG-04-2 | | | 11 | 10 | 11 | 11 | NA | NA | | | | |
| N.c. ARG-05-3 | | | 13 | | NA | 13 | 10 | 14 | 6.12.7 | | 5 | |
| N.c. ARG-05-4 | | | | 16 | 16 | 12 | 9.1 | 13 | 6.15.9 | | 6 | |
| N.c. ARG-05-5 | | | | 9 | 13 | 11 | 15 | 16 | 6.17.10 | 16 | | |
| N.c. ARG-05-6 | | | | | | | | | 6.17.9 | | | |
| N.c. ARG-05-7 | | | | | | | 14 | 13 | 6.18.10 | | | |
| N.c. ARG-05-8 | | | | NA | 16 | 12 | 9.1 | | 5.15.9 | | | |
| N.c. ARG-07-9 | | | | 9 | 14 | 11 | 14 | 16 | 6.17.11 | | | |
| N.c. ARG-07-10 | | | 11 | 14 | 15 | 12 | 9.1 | 12 | 6.15.9 | | | |
| N.c. ARG-07-11 | | | 13 | 11 | NA | NA | NA | 16 | 6.16.9 | | | |
| N.c. ARG-07-12 | | | | 9 | 13 | 11 | 16 | | 6.18.10 | | | |
| N.c. ARG-07-13 | | | 11 | NA | 14 | 12 | NA | 12 | 6.15.9 | | NA | |
| N.c. ARG-08-14 | | | 13 | 9 | 16 | | 16 | 13 | | | 6 | |
| N.c. ARG-08-15 | | | 12 | 15 | 14 | | 9.1 | 12 | | | | |
| N.c. ARG-08-16 | | | 13 | 14 | NA | | NA | | | | | |
| N.c. ARG-LP1 | | | NA | 16 | 16 | 13 | 10 | NA | | | | Campero, 2015 |
| N.c. ARG-Goat | | Goat | 14 | 15 | | 12 | 9.1 | 13 | 5.14.8 | | 7 | Campero, 2018 |

Microsatellite allele configuration of each Uruguayan strain is shown. The number of repeats within the microsatellite is shown. The microsatellite configurations previously characterized in regional isolates as well as in circulating *N. caninum* strains from aborted fetuses are shown. Commonly used *reference* strains N. c. Liverpool and Nc-1 are included. Coinciding alleles for each MS are shaded in the same color for clarity. Uncolored alleles appear only once for each column. *NA* denotes uncharacterized microsatellite sequences

analysis showed that the Uruguayan strains were found in two clusters: NcUru1, NcUru3, and NcUru4 in cluster 1 and NcUru2 in cluster 3. Interestingly, the latter cluster included strains identified in abortions, but no representative isolate has been reported. Lastly, much like the Argentinean "outlier" strain grouping in cluster 4, NcUru2 did not group with any other South American strain. Instead, it grouped with strains from Mexico, Spain, Germany, Japan, and China and was more closely related to the Argentinean strains present in cluster 6 than to its Uruguayan counterparts (Fig. 1a, Table S1). A neighbor-joining tree, based on Provesti's distance, supported the previous clustering analysis in which individuals that were grouped by DAPC tended to be more closely related (Fig. 1c). Incongruences observed between the tree and DAPC analysis were consistent with the degree of admixture among clusters, as shown by the membership probability plot (Fig. 1b).

The fact that three out of the four isolates clustered by geographical origin, while still grouping closely to genotypes present in Europe, may suggest that minimal genetic drift has occurred from founding strains originating from the founding cow import waves. This could indicate that strain mixes by sexual replication and recombination occurs at low frequency, or alternatively, that the strains experiencing recombination are rarely transmitted to cows.

## Molecular examination of *N. caninum* strains causing bovine abortions reveals additional local variability

Lastly, we wondered whether our in vitro isolates were representative of strains causing abortions. To test this, we amplified and sequenced the same combination of

**Table 2** Microsatellite typing of *N. caninum* strains found in aborted bovine fetuses

| CASE | MICROSATELLITE | | | | | | |
|---|---|---|---|---|---|---|---|
| | MS4 | MS5 | MS6A | MS6B | MS7 | MS8 | MS10 |
| 1 | 11 | * | 12 | * | * | 13 | 6.15.9 |
| 2 | 13 | | * | 12 | 9.1 | 14 | |
| 3 | * | * | 12 | * | * | * | |
| 4 | | | * | * | 9.1 | 13 | * |
| 5 | | | | | | | 6.15.9 |
| 6 | 12 | | 12 | | | | |
| 7 | 11 | | * | 12 | | * | * |
| 8 | 13 | 14 | 12 | | * | | |
| 9 | * | * | * | 11 | | | |
| 10 | 13 | 15 | 16 | 12 | 9.1 | 13 | 6.14.9 |
| 11 | | 14 | 15 | | | | |
| 12 | | 15 | 16 | | | * | |
| 13 | 19 | 9 | 14 | 11 | 10 | 14 | * |
| 14 | * | * | 13 | 12 | 9.1 | * | |
| 15 | 12 | | * | * | 10 | | |

NcUru1/4 allele NcUru2 allele NcUru3 allele

Asterisk denotes an allele failed to be amplified by PCR

nine microsatellites, used to type the isolates, from 15 aborted fetuses, confirmed by PCR for the presence of *N. caninum*. Due to the variable states of conservation of the DNA in these samples, we succeeded in amplifying a variable number of MS per fetus (Pedraza-Díaz et al. 2009). MS12 and MS21 could not be amplified in any of the samples. Interestingly, the multiple combinations of MS identified suggest that the abortions were caused by multiple strains of *N. caninum*, and that we have not yet saturated our sampling, as clearly there is still a rich pool of uncharacterized genetic diversity among strains present in the country. Only samples 4, 5, 9, and 11 exhibited a combination, albeit incomplete, potentially compatible with our isolates, whereby only sample 9 could possibly correspond to an NcUru2 genotype (Table 2). On the other hand, 11 other strains detected seemed to differ from NcUru1 through NcUru4, from each other, and from previously reported regional isolates (Table 2 and Table S2).

Despite the validity of microsatellites as an epidemiological approximation, it is well established that MS variation does not correlate, *per se*, with phenotypic traits. This discordance is consistent with NcUru1 and NcUru4 being genetically identical by MS typing but isolated from farms with very different histories. NcUru1 originated from a dairy farm with a history of sporadic abortions. In stark contrast, NcUru4 was isolated from a dairy farm where approximately 700 abortions were registered within a weeks' time. Complete genomic sequences of multiple isolates from distinct geographical origins would be required to perform comprehensive functional inferences by mapping genes responsible for distinct virulence phenotypes.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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