Genetic diversity among Canadienne, Brown Swiss, Holstein and Jersey cattle based on mitochondrial D-loop sequence variation

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Hansen, C., Shrestha, J. N. B., Parker, R. J., Crow, G. H., McAlpine, P. J. and Derr, J. N. 2003. **Genetic diversity among Canadienne, Brown Swiss, Holstein and Jersey cattle based on mitochondrial D-loop sequence variation.** Can. J. Anim. Sci. **83**: 39–44. Polymorphisms creating 36 unique haplotypes were observed within breeds at 55 sites in the displacement loop (D-loop) region of the mitochondrial DNA (mtDNA) consisting of 814 bp. The majority (56%) of the differences observed were the result of nucleotide substitution events with 19 transitions, 12 transversions, 11 deletions, 12 insertions and 1 inversion. In all cases, the insertions and deletions were of a single nucleotide. Canadienne cattle were found to have 60% unique haplotypes within the population compared to 89% in Brown Swiss, 90% in Holstein and 100% in Jersey cattle, possibly reflecting the narrow genetic base in the Canadienne breed. The degree of sequence divergence in the D-loop region of mtDNA was based on samples from 20 Canadienne, 9 Brown Swiss, 10 Holstein and 10 Jersey cattle and a phylogenetic analysis showed that these cattle (*Bos taurus*) were not evolutionarily distinct. All four breeds grouped together when a strict consensus tree was generated. Intra-breed variability proved to be high for the Canadienne, Holstein and Jersey breeds (57–73%) but not the Brown Swiss breed (29%). The Canadienne and Brown Swiss (45%), and Brown Swiss and Holstein (43%) showed the lowest degree of inter-breed variability. The greatest variability among the four breeds was between Canadienne and Jersey (80%) cattle. These findings question the validity of phenotypic assessment of genetic diversity, such as Canadienne cattle being described as "Black Jersey".

Key words: Genetic distance, phylogenetic analysis, D-loop sequence, cattle

Hansen, C., Shrestha, J. N. B., Parker, R. J., Crow, G. H., McAlpine, P. J. et Derr, J. N. 2003. **Diversité génétique des races Canadienne, Suisse brune, Holstein et Jersey d'après la variation des séquences sur la boucle D des mitochondries**. Can. J. Anim. Sci. **83**: 39–44. Les auteurs ont dénombré 36 haplotypes uniques attribuables au polymorphisme intra-racial à 55 endroits de la boucle D de l'ADN mitochondrial (ADNmt), sur laquelle on retrouve 814 paires de bases. La plupart des variations (56 %) observées résultent de la substitution de nucléotides, en l'occurrence une transition (19 cas), une transversion (12 cas), une suppression (11 cas), une insertion (12 cas) ou une inversion (1 cas). Les insertions et les suppressions n'affectent toujours qu'un nucléotide. La race Canadienne présente 60 % d'haplotypes uniques au sein de sa population, contre 89 % pour les Suisse brune, 90 % pour les Holstein et 100 % pour les Jersey, ce qui illustre peut-être son étroite base génétique. Le degré de divergence des séquences sur la boucle D de l'ADNmt a été établi au moyen des échantillons prélevés de 20 sujets Canadienne, 9 Suisse brune, 10 Holstein et 10 Jersey. Une analyse phylogénétique révèle que les animaux (*Bos taurus*) ne sont pas distincts sur le plan de l'évolution. En effet, les quatre races se retrouvent ensemble quand on produit un arbre de consensus strict. Il existe bien une forte variation intra-raciale pour les sujets Canadienne, Holstein et Jersey (de 57 à 73 %), mais pas pour ceux de la race Suisse brune (29 %). Les sujets Canadienne et Suisse brune (45 %) ainsi que Suisse brune et Holstein (43 %) présentent le plus faible degré de variation inter-raciale. Des quatre races, ce sont les races Canadienne et Jersey qui montrent la plus grande variabilité entre elles (80 %). Ces résultats remettent en question l'utilité du phénotype pour évaluer la diversité génétique de certains animaux tels les bovins de la race Canadienne appelés « Jersey noirs ».

Mots clés: Distance génétique, analyse phylogénétique, séquences de la boucle D, bovins

Many of the 780 cattle breeds that have been characterized world-wide (Scherf 2000) are today threatened with extinction and, on a global basis, one-third of mammals and poultry breeds are endangered. This fact has led to growing concern over the narrowing of the genetic base and, following the formal ratification in December 1993 of the Convention on Biodiversity, a global effort for the conservation of domestic animal diversity was initiated. Canadienne cattle, one of the oldest breeds in the western hemisphere (St-

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Abbreviations: **D-loop**, displacement loop; **mtDNA**, mitochondrial DNA

Pierre 1936) having recorded only 105 purebred registrations in 1995 (Shrestha and Hansen 1997), suffer from an alarming decline in population size. The ancestors of the Canadienne breed, which came mainly from the Normandy and Brittany regions of France, can be traced to the very first settlements in the early 1600s in what is today the province of Québec. Over the years, their relative isolation in Québec, combined with the effects of natural selection in a harsh environment complemented by creative human activity, resulted in the development of an extremely hardy, self-sufficient breed of small, compact dairy cattle that were well suited to pioneer life (Grisdale 1909). These cattle, known to have played an important role in the colonization of French Canada, not only provided draught power for ploughing and transportation, but also produced both milk and meat for human consumption. In 1883, census results showed that 75% of the cattle in Québec were of the Canadienne breed (Couture 1909). Later, in 1901, Canadienne cattle gained recognition as the most profitable dairy breed at the Pan-American show in Buffalo, New York (Fortin 1940). Canadienne cattle are an important candidate for a national conservation effort and must be characterized on a more fundamental level so as to establish the unique nature of the breed (Shrestha 1990; Bernier 1995; Hansen 1997). It is widely believed that Canadienne cattle stem from the same general lineage as Jersey, Guernsey and Kerry cattle.

While several approaches can be used to tackle this problem, the analysis of sequence variation in the mtDNA displacement loop (D-loop) region can be used to determine inter-breed diversity. The D-loop is 900 base pairs in length in cattle and this region has been shown to contain a significant proportion of variation. By amplifying the mtDNA D-loop region using specific flanking primers, sequencing the amplified portion and determining the sequence variation that exists, it is possible to estimate the genetic distance among breeds. This is the first study in the Canadian conservation effort to determine genetic diversity based on the extent of the mitochondrial DNA D-loop sequence variation among the Canadienne, Brown Swiss, Holstein and Jersey cattle breeds. The genetic distances among these breeds were estimated and the phylogenetic analysis was utilized to determine evolutionarily distinct populations.

MATERIAL AND METHODS

Animals

Pedigree records provided by Canadian Livestock Records Corporation and Holstein Canada were edited before computing the additive relationship coefficients for individual animals (Cruden 1949; Emik and Terrill 1949; Van Vleck 1993). Animals showing the least relationship with each other were chosen for sampling. Concurrently, a reasonable driving distance from the laboratory in Ottawa was also considered while selecting unrelated animals for sampling. In total, 21 Canadienne, 20 Brown Swiss and 20 Jersey cows were sampled. The average relationship among the selected animals was 2.20% for Canadienne, 1.05% for Brown Swiss, and 2.10% for Jersey cows. The 20 Holstein bulls sampled had no common ancestor for three generations. The D-loop sequences of the mtDNA were determined for 20 Canadienne, 9 Brown Swiss, 10 Jersey and 10 Holstein cattle. In terms of the average effective population size the sample represents 16% for Canadienne, 0.4% for Brown Swiss and 0.2% for Jersey cattle. Corresponding values in terms of annual registrations in 1994 represent 8, 0.9 and 0.5%, respectively. The care and handling of bulls used in this study conformed to the guidelines established by the Canadian Council of Animal Care.

Extraction of Total Genomic DNA from Whole Blood Samples

Blood samples were collected from individual animals in 8.5-mL ACD-Vaccutainer tubes (VWR Scientific, Ville Mont-Royal, Quebec, Canada; ACD: 22.0 g L^{-1} trisodium citrate, 8.0 g L^{-1} citric acid and 24.5 g L^{-1} dextrose) and kept at 4°C until the time of the DNA extraction. Total genomic DNA was extracted from individual blood samples using the Super Quik-Gene DNA isolation kit (Analytical Genetic Testing Center, Inc., Denver, Colorado, USA) following the manufacturer's instructions. Every effort was made to extract the DNA from the blood samples within a day or two of collection.

Polymerase Chain Reaction Procedure

The D-loop region of the bovine mtDNA was amplified with a set of primers that generated a distinct polymerase chain reaction (PCR) product of approximately 1100 bp in size. The set consisted of primers complementing the bovine sequence in the conserved threonine tRNA region of mtDNA (forward 5'- AGAGAAGGAGAACAACTAAC-CTCC- 3'; position 15695) and the 12S rRNA gene (reverse 5'AACAGGAAGGC TGGGACC- 3'; position 457). All of the primer position numbers indicated correspond to Anderson et al. (1982) and the primers were designed to work under identical PCR conditions.

The PCR was carried out in a 90-μL reaction volume using 66.5 μL of sterile, deionized, distilled water, 9.0 μL of 10X PCR buffer, 6.0 μL of 25 mM $MgCl_2$, 2.5 μL of a mixture containing 10 mM of each of the dNTPs, 2.5 μL of DMSO, 1.5 μL of each of the two primers and 0.5 μL of AmpliTaq DNA polymerase (Perkin Elmer, Applied Biosystems Canada Inc., Mississauga, Ontario, Canada). A total of 35 cycles consisting of 94°C for 1 min, 55°C for 1 min. and 74°C for 1 min were used. Following purification using the QIAquick PCR Purification Kit (QIAGEN Inc., Chatsworth, California, USA), the purity and quantity of amplified DNA were estimated by running the samples with a ΦX174 HAE III standard on a 1.5% Agarose gel. Occasionally, the low quantity of amplified product found in some of the samples necessitated the reamplification and purification of the fragments under the same conditions described above.

Sequencing of all amplified D-loop fragments was performed using an ABI Prism 377 automatic sequencer (Perkin-Elmer, Foster City, California, USA). The actual sequencing reaction was carried out using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit.

Four primers were used in the sequencing process: (1) *THR* (forward, 5' GGAGAACAACTA ACCTCC-3'), (2) *Loftus et al. (1994a)* (forward, 5'-CTGCAGTCTCACCAT-CAACC-3'), (3) *1975* (reverse, 5' -CTGGACTTA ACTG-CA TCTTGAGC-3') and (4) *G-Jump* (reverse, 5'-CAATTTAGCACTCCAA ACAAAGTC-3'). All were designed to work under identical conditions. Sequences were verified through quality control and visually. Specifically, each sequence trace was carefully checked to make sure that each base that was called was done so unequivocally. Any sequences where doubts existed were redone and any portion of the sequence (i.e., end) that was not of the highest quality was discarded. D-loop sequences were submitted to GenBank and can be retrieved through accession numbers AF016060 to AF016097.

Sequence Processing and Phylogenetic Analysis

The D-loop sequences obtained through the automatic sequencing protocol described above were aligned using the Clustal V computer program (Higgins et al. 1992). The genetic distances calculated were corrected for multiple hits by the two-parameter method of Kimura (1980) and sites representing gaps were excluded from the analysis. Trees were constructed under the criterion of maximum parsimony using the "branch and bound" search method in the PAUP computer package (Swofford 1993). In the latter analysis, it was necessary to decrease the number of animals included in the data because constraints in computer resources did not facilitate solving the complete set of equations. All unique sequences found in Canadienne cattle were thus included along with two unique sequences from each of the remaining three breeds. According to the PAUP website (http://paup.csit.fsu.edu/paupfaq/faq.html), the exact time and computing power required to complete a heuristic search cannot be estimated based on the size of a data set. There are several reasons for this including the quality of the data (i.e., how homoplastic the data are) and the fact that there is no simple expression for calculating the number of tree bisection-reconnection (TBR) or subtree pruning-regrafting (SPR) rearrangements that will be made on a given tree. As the PAUP site points out, "the problem is further complicated by the fact that it is not known how many suboptimal trees will be found during a search before optimal trees are found, and what portion of potential rearrangements of a given tree will be performed before a better tree is found." The sequences for the N'Dama and Tharparkar breeds, published by Loftus et al. (1994a), were used as the outgroup for the analysis.

RESULTS

Mitochondrial DNA Variation

The D-loop sequences of the mtDNA consisting of 814 bp were determined for 20 Canadienne, 9 Brown Swiss, 10 Jersey and 10 Holstein cattle. Among the animals within the four breeds studied, 36 unique mitochondrial haplotypes were identified. Of the 20 Canadienne cattle, 12 possessed specific haplotypes that differed from all others in the breed. Similarly, 8 of the 9 Brown Swiss cattle, all 10 Jersey cattle, and 9 of the 10 Holstein cattle carried haplotypes that were unique within the breed. When the populations were considered together, 11 of the Canadienne, 6 of the Brown Swiss, 9 of the Jersey and 8 of Holstein cattle possessed haplotypes that were unique both within breed and within the general population. One haplotype was shared between the Canadienne and Brown Swiss breeds, and one haplotype was shared between the Brown Swiss, Jersey and Holstein breeds. Polymorphisms creating these 36 unique haplotypes were observed at 55 sites. The majority (56%) of the differences seen were the result of nucleotide substitution events. Most of these (19) were the result of transitions. Also observed were 12 transversions, 11 deletions, 12 insertions and 1 inversion. In all cases the insertions and deletions were of a single nucleotide.

Table 1. Percent Inter-breed and intra-breed (on diagonal) variability estimated from the D-loop sequences of four cattle breeds

Breed	Canadienne	Brown Swiss	Jersey	Holstein
Canadienne	0.57	0.45	0.80	0.64
Brown Swiss		0.29	0.56	0.43
Jersey			0.73	0.68
Holstein				0.61

Table 2. Average variability (%) among Canadienne, Brown Swiss, Jersey and Holstein D-loop sequences and representative published[z] sequences from six other breeds

Breed	Friesian	Hereford	Butana	N'Dama	Hariana	Tharparkar
Canadienne	0.69	0.87	0.95	1.19	5.63	5.77
Brown Swiss	0.48	0.65	0.77	1.04	5.48	5.66
Jersey	0.95	1.13	1.25	1.54	5.68	5.80
Holstein	0.74	0.92	1.02	1.28	5.69	5.84

[z]Loftus et al. (1994a).

Average pairwise sequence divergence estimates among breeds and within breeds were generated (Table 1). Intra-breed variability ranged from 29 to 73%, while the inter-breed variability ranged from 43 to 80%. The intra-breed variability observed was generally of the same magnitude as the inter-breed variability. Only Brown Swiss (29%) cattle were an exception to this rule. The variability seen within this breed was much lower than the variability observed between the Brown Swiss and the other three breeds (43 to 56%) studied.

The mtDNA D-loop sequences that were generated in this study were also compared to published D-loop sequences of the Friesian, Hereford, Butana, N'Dama, Hariana and Tharparkar breeds (Loftus et al. 1994a). The results of this comparison are shown in Table 2. As expected, the Canadienne, Brown Swiss, Holstein and Jersey breeds were shown to be most closely related to the Friesian breed of dairy cattle. The beef breed Hereford was the next most closely related breed followed by the African Butana and N'Dama breeds. The greatest variability in D-loop sequence was seen between the four breeds (*Bos taurus*) in the present study and the Hariana and Tharparkar breeds (*Bos indicus*) from India.

Phylogenetic Analysis

The results of the phylogenetic analysis using all the unique mtDNA D-loop sequences from Canadienne cattle and two

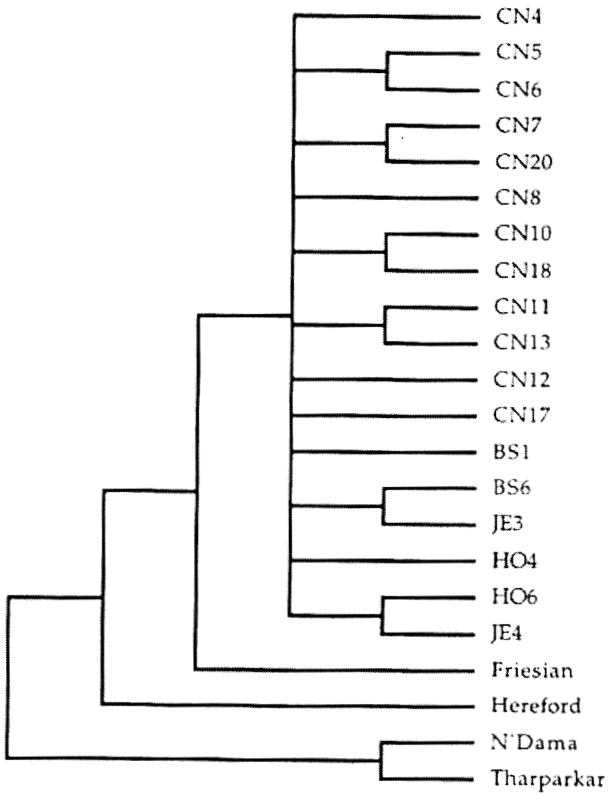

Fig. 1. Strict consensus tree generated from the 264 maximum parsimony trees found using the "branch and bound" search method in the PAUP computer program.

unique sequences randomly selected from those of each of the other three breeds are presented in Fig. 1. In total, 264 possible maximum parsimony trees were generated using the "branch and bound" search method in the PAUP computer package. Relevant statistics for the trees were as follows: number of steps: 91, homoplasy and consistency indices: 0.143 and 0.857, respectively, homoplasy and consistency indices excluding uninformative characters: 0.325 and 0.675, respectively, and retention index: 0.723. Specifically, Fig. 1 shows the strict consensus tree generated from the 264 possible trees. No distinct separation was observed between the animals of the Canadienne breed and those from the Brown Swiss, Holstein and Jersey breeds. As expected, N'Dama and Tharparkar cattle, which were used as the outgroup for this analysis, were most distinct from the other populations.

DISCUSSION

Most previous studies examining differences in bovine mtDNA, with the exception of studies such as those by Loftus et al. (1994a), Mannen et al. (1998, 2000) and Cymbron et al. (1999), have traditionally used polymorphism in restriction enzyme cleavage pattern (e.g., Watanabe et al. 1985, 1989; Bhat et al. 1990; Kikkawa et al. 1995; Parfitt and Huismans 1998). These studies were successful in revealing breed differences but, as RFLP analysis cannot identify variation that is present at sites other than those specific for restriction enzyme cleavage, a fair amount of variation was probably missed by these studies. While Loftus and co-workers, for example, failed to show distinct phylogenetic lineages within the European cattle breeds, the pairwise distances were able to demonstrate that variation does exist among breeds. As a result, it was of interest to examine the variation that exists in the D-loop sequence of several dairy cattle breeds in Canada, one of which, the Canadienne, is considered to be endangered.

The nature of the polymorphisms seen in the various sequences in the present study shows that there was a transitional bias in nucleotide substitutions. This finding is in agreement with that reported by Loftus et al. (1994a), although their data favoured transitions to an even greater extent, and was characteristic of mammalian mitochondrial evolution (Wilson et al. 1985). The present data set also showed a larger number of insertion-deletion events compared to those reported. The significance of this observation may relate to incidence of rate of mutation.

As can be seen from Table 1, the inter-breed variability or distance between Canadienne and Brown Swiss (45%) cattle was found to be lower than between Canadienne and Jersey (80%) or Canadienne and Holstein (64%) cattle. In the beginning of the year 1969, the crossbreeding of Canadienne cows with Brown Swiss bulls was initiated and as a result, today, many of the cattle comprising the Canadienne breed share germplasm with the Brown Swiss breed. This is also true for the animals sampled. A lower amount of variation in the D-loop sequence between the two breeds is, therefore, likely to result from the introduction of Brown Swiss maternal lineages into the Canadienne breed.

Another interesting observation that can be made from Table 1 is that among the breeds, the most variability appears to occur between Jersey and a specific breed assessed. This is particularly noteworthy for the Canadienne breed. Canadienne cattle are believed to have originated from animals brought to North America from the Normandy and Brittany regions of France. It has, therefore, been postulated as having come from the same general lineage as Jersey cattle, among others. It would, therefore, have been reasonable to expect the D-loop of Canadienne cattle to be somewhat less variable when compared to Jersey cattle. In fact, the opposite appears to be true. Of the three breeds studied, the Canadienne shows the greatest amount of variation in its D-loop sequence in relation to the Jersey. This tends to support a difference based on genetic and phenotypic diversity. Studies based on 15 bovine microsatellite markers in the same breeds support the findings in the present study (Hansen et al. 2002).

Table 2 presents the average variability in D-loop sequence between the four Canadian breed populations and representative published sequences for the Friesian, Hereford, Butana, N'Dama, Hariana and Tharparker breeds. The magnitude of the individual estimates reported in this table is comparable to those published (Loftus et al. 1994a). The variability shown suggests that the Holstein and Friesian breeds have differentiated somewhat from one another at the mtDNA level. In fact, the variability between the D-loop of Holstein and Friesian cattle was greater than those between Holstein and either of the Canadienne, Jersey, or Brown Swiss breeds. The Holstein cattle were only recognized as a separate breed from the Friesian nearly 80 yr ago; therefore the variability between the two breeds was expected to be narrow.

Bhat et al. (1990) and Watanabe et al. (1985) characterized variations in mtDNA restriction enzyme cleavage patterns within and between various European, North American, Indian and Asian cattle breeds. Similarly, Loftus et al. (1994a, b) have demonstrated, using both restriction fragment length polymorphism (RFLP) analysis of mtDNA and mtDNA D-loop sequence variation, that while numerous differences among and within breeds can be identified, sequence variation between European and African (*Bos taurus*) cattle breeds is not significant enough to point to more than one major mitochondrial lineage. These results are confirmed by the present study. All of the Canadienne, Brown Swiss, Jersey and Holstein cattle studied group together on the phylogenetic tree (Fig. 1). Only the Tharparker (*Bos indicus*) breed shows any major separation. Furthermore, Canadienne cattle do not appear to possess any mitochondrial variation that would uniquely separate them from the remaining *Bos taurus* breeds in the form of a minor lineage.

The number of different mitochondrial haplotypes found in Canadienne cattle gives some reasons for concern about this breed. As was mentioned earlier, 60% of Canadienne animals carried haplotypes that were unique within the breed. This compares with 89% of Brown Swiss, 100% of Jersey and 90% of Holstein cattle. It is thus evident that the decrease in the size of the Canadienne population may have resulted in a decrease in the percentage of animals carrying unique maternal lineages compared with the much more populous Jersey and Holstein breeds.

Further studies with larger numbers of animals will help validate the findings from the present study. Suggestions have been made in the past as to the best sample sizes to use to resolve questions of phylogenetic relationship [Takezaki and Nei (1996) for example]. When dealing with rare breeds, however, it is not always possible to adhere to such guidelines, as the availability of animals is severely limited and questions of breed specificity such as encountered here may again be dependent on factors other than phylogenetic relationship. Furthermore, the techniques used in the present study were developed to discriminate species rather than breeds within species. This is the first study in Canada to determine genetic diversity among cattle breeds and it will be interesting to further examine Canadienne cattle with and without Brown Swiss parentage for comparison with Jersey cattle and a number of other breeds established in the country.

In conclusion, the distance estimated between the Canadienne and Jersey breeds based on mitochondrial D-loop sequence variation underlines the fact that assumptions about relationship based on lineage may not concur with phenotypic diversity. At the very least, multiple sources of information should be included in any evaluation and molecular evidence may help crystallize specific facts that may otherwise be ignored by grass-roots organizations that have been associated with the conservation of phenotypic diversity.

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