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Genetic Diversity and Population Structure of *Escherichia coli* from Neighboring Small-Scale Dairy Farms

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The genetic diversity and population structure of *Escherichia coli* isolates from small-scale dairy farms were used to assess the ability of *E. coli* to spread within the farm environment and between neighboring farms. A total of 164 *E. coli* isolates were obtained from bovine feces, bedding, cow teats and milk from 6 small-scale dairy farms. Ward's clustering grouped the isolates into 54 different random amplified polymorphic DNA (RAPD) types at 95% similarity, regardless of either the sample type or the farm of isolation. This suggests that RAPD types are shared between bovine feces, bedding, cow teats, and milk. In addition, transmission of RAPD types between the studied farms was suggested by the Ward grouping pattern of the isolates, Nei's and AMOVA population analyses, and genetic landscape shape analysis. For the first time, the latter analytical tool was used to assess the ability of *E. coli* to disseminate between small-scale dairy farms within the same producing region. Although a number of dispersal mechanisms could exist between farms, the genetic landscape shape analysis associated the flow of *E. coli* RAPD types with the movement of forage and milking staff between farms. This study will aid in planning disease prevention strategies and optimizing husbandry practices.

***Keywords*:** *Escherichia coli*, small-scale dairy farms, RAPD, population structure, gene flow, genetic landscape shape analysis

Escherichia coli is primarily a commensal organism in the digestive tracts of a variety of mammals, including humans (Mariat *et al.*, 2009); however, several strains of *E. coli* cause diverse intestinal and extraintestinal diseases (Kaper *et al.*, 2004). In addition, *E. coli* is one of the bacterial species implicated in bovine mastitis (Hogan and Smith, 2003), a condition resulting in the inflammation of the mammary glands that negatively impacts milk production and profitability of dairy production (Seegers *et al.*, 2003). Knowledge of the persistence of *E. coli* in secondary habitats (soil, feces, and bodies of water) within a dairy farm has been restricted to specific pathotypes, in particular, Shiga toxin-producing *E. coli* (STEC) (Fremaux *et al.*, 2008); however, farm environments may contain a wide range of potentially pathogenic *E. coli* genotypes (Wenz *et al.*, 2006). Therefore, effective human and bovine disease prevention requires analyzing *E. coli* populations in dairy farm environments as a whole rather than limiting studies to specific pathotypes present in these environments. Bovine feces are one of the main sources of milk contamination in dairy farms (Oliver *et al.*, 2005). Transmission of *E. coli* to humans may occur by direct contact with contaminated feces during the management of manure or by the ingestion of milk contaminated during milking or dairy products derived from contaminated milk (McGarvey *et al.*, 2004). A genetic characterization of *E. coli* strains isolated from dairy farms and an evaluation of their ability to spread are essential for the sanitary risk assessment of bovine mastitis and human diarrheic outbreaks (Jayarao *et al.*, 2006). This type of analysis is particularly relevant to small-scale farms in developing countries because locally consumed milk presents a high sanitary risk. Milk from small-scale farms in developing countries may contain pathogenic bacteria because it is often extracted and sold unrefrigerated; raw milk is also used for cheese production (Oliver *et al.*, 2005, 2009). In addition, the risk to public health is increased in developing countries by the low level of applied technology and by poor management practices on small-scale dairy farms (Kivaria *et al.*, 2006; Lakew *et al.*, 2009).

The presence of the same genotype of *E. coli* has been noted in different ecological niches or environmental samples from dairy farms (e.g., Schouten *et al.*, 2005; Cho *et al.*, 2006; Wetzel and LeJeune, 2006; Fremaux *et al.*, 2008; Son *et al.*, 2009). These studies identified genotypes and their relative frequencies in different environments, within and between farms, and were primarily based on cluster analyses. Genetic landscape shape analysis (Manel *et al.*, 2003) is commonly

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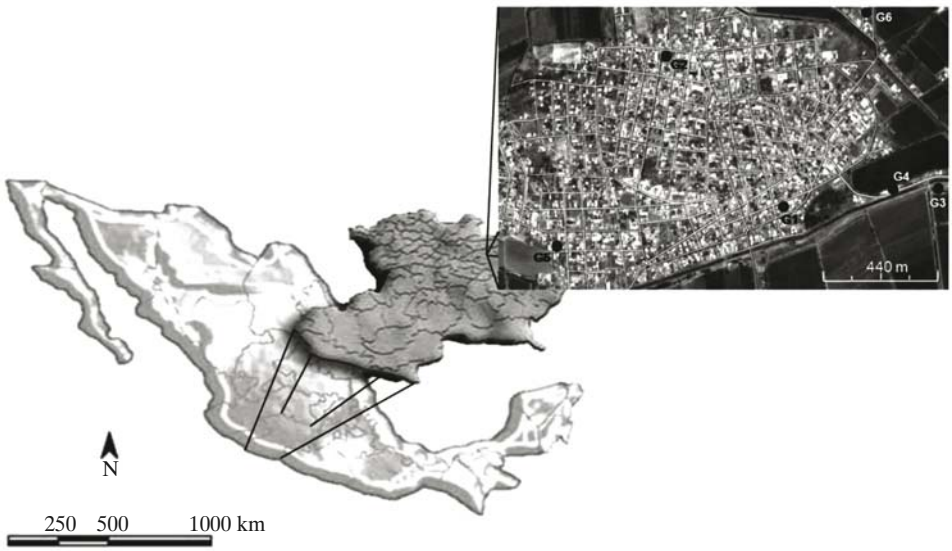

Fig. 1. Locations of the studied small-scale dairy farms. The location of the state of Michoacán in Mexico is shown. The image to the right shows an aerial view of Téjaro and Cotzio with the locations of the studied farms (Image from Google Earth).

used to evaluate gene flow in eukaryotic populations but it has not been used to assess the dispersal of *E. coli* genotypes in dairy farms. Landscape genetics has been defined as an "amalgamation of molecular population genetics and landscape ecology" (Manel *et al.*, 2003) that applies statistics to genetic and geographic variations between individuals. Spatial patterns of genetic structure and possible regions of gene flow continuity and discontinuity can be identified. Landscape genetic analysis has been widely used in studies of eukaryotic ecology, mainly in animals (Miller *et al.*, 2006; Holzman *et al.*, 2009; Kenchington *et al.*, 2009), but it has not been applied to studies of prokaryotic ecology.

The RAPD assay continues to be used to analyze the genetic diversity of *E. coli* strains (Aslam *et al.*, 2003; Vidovic and Korber, 2006; Tristao *et al.*, 2007; Holley *et al.*, 2008) due to its speed, low cost, ease of implementation, and efficient detection of differences throughout the genome (Foxman *et al.*, 2005). RAPD discriminates between *E. coli* isolates as effectively (Aslam *et al.*, 2003) or better (Cagnacci *et al.*, 2008; Machado *et al.*, 2008) than pulse field gel electrophoresis (PFGE), which is considered the gold standard for subtyping foodborne bacterial diseases (Foley *et al.*, 2009). RAPD is also commonly used to assess gene flow between populations using landscape genetics in several eukaryotic species, including algae (Bouza *et al.*, 2006), trees (Colling *et al.*, 2010), amphibians (Bernal *et al.*, 2005), and insects (Rocha *et al.*, 2007). Random amplified polymorphic DNA (RAPD) data has also been used to assess genetic discontinuities across geographic space by means of a Delaunay triangulation to establish a connectivity network between collection sites of different populations of an amphibian species (Telles *et al.*, 2007).

In this study, we used RAPD to assess the population diversity of *E. coli* strains in samples from small-scale dairy farms within a production region of west-central México. Using Nei's classical population analysis approach, we also evaluated the dispersal of isolates within the farm environment. Finally, we used genetic landscape shape analysis to evaluate the ability of *E. coli* strains to disseminate between small-scale dairy farms separated by relatively short (<1.6 km) geographic distances. To the best of our knowledge, this is the first reported use of RAPD markers to evaluate gene flow by means of landscape genetics in bacterial populations from dairy

Table 1. Management and husbandry practices at the studied small-scale dairy farms and the relationships between farms

Farm	Management and technological characteristics				Relationships between farms
	Herd size[a]	Milking	Bedding	Frequency of feces removal	
G1	18	Mechanical	Dry feces and forage	Daily	Stores forage for G2 and G6
G2	9	Manual	Dry feces	Annually	Occasionally receives forage from G1
G3	18	Mechanical	Dry feces and forage	Annually	Shares forage with G4 and G6
G4	10	Mechanical	Wood chips and forage	Monthly	Shares milking staff with G3
G5	21	Mechanical	Dry feces and forage	Monthly	Shares milking staff with G6
G6	10	Mechanical	Dry feces and forage	Annually	Shares forage transportation vehicle with G5

[a] Only productive females were counted.

farms.

Materials and Methods

Farms studied

The study included six small-scale dairy farms (G1 to G6) in the rural communities of Téjaro and Cotzio in the municipality of Tarímbaro in the state of Michoacán, located in west-central México (Fig. 1). The six farms chosen had similar management and husbandry practices, with herds of 9 (G2) to 21 (G5) Holstein milk cows (Table 1). Milking was primarily mechanical and performed twice daily, yielding an average of 168 L/day. The cows were fed corn, sorghum, and alfalfa hay. The bedding was mostly a mixture of hay and dry bovine feces. Waste management involved the periodic removal of bovine feces at frequencies ranging from daily to yearly. To analyze the spatial structure of the genetic relationships between isolates by farm of isolation (see below), all farms were georeferenced, and the geographical pairwise distances between all of the farms were measured. The distances between the dairy farms ranged from 208.5 m between farms G3 and G4 to 1543.4 m between farms G3 and G5.

Sampling and *E. coli* isolation

The point prevalence (multiple farms analyzed at approximately the same time) of *E. coli* was performed by collecting samples on two days of the same week; farms G1, G2, and G3 were sampled on October 22, and the other three farms were sampled on October 24 (2007). Three samples per farm were randomly selected for each sample type (milk, feces, bedding material, and cow teats) from the same day from each farm, and each sample was processed individually. All samples were placed in sterile containers and processed within 24 h after collection. At each farm, 100 ml milk samples were obtained from cow teats in the four quarters of the udders of three different cows, and undiluted 50 μl aliquots were used to inoculate solid Luria-Bertani (LB) and MacConkey (MC) media. Three 5 g samples of fresh bovine feces were randomly taken at each farm, from which 1 g was resuspended and homogenized in 10 ml of saline solution (0.9% NaCl) before serial dilutions of the homogenate were made. Solid LB and MC media were inoculated with 50 μl aliquots of the 1:100 dilution. For isolates from bedding material, three 5 g samples were taken from each farm and homogenized, then 2 g of each homogenate were resuspended in 20 ml of LB medium, and 50 μl of the resulting suspension were used to inoculate solid sorbitol MC medium. Teat samples were obtained by rubbing a sterile cotton swab on the surface of each of the four teats of three cows per herd, and the cotton swabs were used to inoculate individual solid MC plates by the streak technique. For all samples, the inoculated plates were incubated at 37°C for 24 h, and sorbitol-fermenting colonies (small red/pink) with *E. coli* morphology were inoculated on tryptic soy slants and incubated for 24 h at 37°C before storage at 4°C. A total of 234 well-differentiated colonies were transferred to LB plates, and 164 of these were identified as *E. coli* by phenotypic characterization and conventional biochemical activity assays (Gram, catalase, oxidase, indole, methyl red/Voges-Proskauer, citrate, and urease). The total number of isolates and the *E. coli* isolates obtained by farm and sample type are shown in Table 2.

Molecular genotyping

Genomic DNA was extracted from the 164 isolated *E. coli* strains by the phenol-chloroform method (Sambrook and Russell, 2001). Isolates were identified by sequencing PCR products obtained under previously described conditions with highly specific primers that targeted the *E. coli* 16S rRNA gene, ECA75F (5′-GGAAGAAGCTTGC TTCTTTGCTGAC-3′) and ECR619R (5′-AGCCCGGGGATTTCA CATCTGACTTA-3′) (Sabat *et al.*, 2000). The amplification products were sequenced in an AbiPrism Genetic Analyzer ABI310 (Applied Biosystems, USA) using the BigDye Terminator Ver. 3.1 kit (Applied Biosystems) according to the manufacturer's instructions. The obtained sequences (400 bp average length) were compared to sequences in GenBank with the Blastn algorithm. The sequences were submitted to GenBank with the accession numbers GU646040 to GU646203.

For RAPD amplification assays, the primers Ecorapd 1252 (5′-GC GGAAATAG-3′), Ecorapd 1254 (5′-CCGCAGCCAA-3′), and Ecorapd 1290 (5′-GTGGATGCGA-3′) (Berg *et al.*, 1994) were used. The total volume of the reaction mix was 25 μl, which contained 25 ng DNA, 10 mM Tris-HCl pH 8.5, 1.5 mM $MgCl_2$, 0.5 mM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 0.5 μM each oligonucleotide and 0.5 U *Taq* DNA recombinant polymerase (Invitrogen, USA). The amplification program included one cycle of 5 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C, with a final extension at 72°C for 7 min. The amplification products were visualized in a 2% agarose gel (Sambrook and Russell, 2001) with a ChemiDoc (Bio-Rad, USA) imaging system using a 1 kb ladder (Invitrogen, USA) as a molecular weight marker. All RAPD amplification assays were carried out at least twice to confirm their reproducibility in our lab.

Analysis of data

The gels containing the RAPD amplification patterns were analyzed with Quantity One 4.4.1 software (Bio-Rad) following the instructions in the manufacturer's user guide, which allowed for the following: generation of densitometric curves for each lane in the gel, depuration of the background, and overlapping of densitometric curves

Table 2. Number of total isolates, *E. coli* isolates and RAPD types obtained by farm and by sample type

Farm	Sample[a]				Total[b]
	Milk	Cow teats	Bedding	Feces	
G1	4/8 (4)	4/12 (4)	6/12 (5)	5/12 (5)	19/44 (11)
G2	5/8 (3)	9/9 (8)	5/10 (4)	9/9 (9)	28/36 (18)
G3	10/10 (9)	10/11 (9)	1/10 (1)	4/8 (3)	25/39 (20)
G4	10/11 (9)	7/10 (3)	10/10 (7)	9/11 (6)	36/42 (21)
G5	8/9 (7)	10/12 (9)	7/10 (4)	4/8 (4)	29/39 (22)
G6	6/6 (6)	8/9 (6)	5/10 (4)	8/9 (6)	27/34 (18)
Total	43/52 (31)	48/63 (34)	34/62 (23)	39/57 (27)	164/234 (54)

[a] Number of *E. coli* isolates/number of total isolates. The corresponding number of RAPD types shown in parentheses.
[b] Because some RAPD types are shared between samples and between farms, the totals differ from the sums of the RAPD types in farms and in sample types.

between lanes for comparison of peak patterns. Once the densitometry analysis was completed, the MATCH function analyzed all of the lanes in the gel to build a binary matrix with values of 1 or 0, indicating band presence or absence, respectively. All bands above 4 kb were removed from this analysis. Before the binary matrix was built, the software allowed the adjustment of the difference in band weight tolerance for identifying bands as being the same. In this study, we adjusted the band weight tolerance to 3%. All of these considerations and the data processing significantly curtail the subjectivity involved in the RAPD analysis to generate reliable band patterns. The resulting binary matrix was used in all of the subsequent analyses.

RAPD data were processed by four different analytical criteria to evaluate the genetic diversity of the *E. coli* isolates, establish the relatedness between groups of isolates according to farm provenance, and establish probable strain mobilization patterns (gene flow) between studied farms. In the first approach, all isolates were analyzed without regard to the farm of isolation. In this case, a hierarchical clustering analysis was performed with Ward's method with the PAST version 1.42 software package (Hammer *et al.*, 2001). PAST calculates a Euclidean distance matrix from the original binary matrix and minimizes the increment in the total sum of squares error every time a new group is added to a pre-existing cluster. Major clusters were defined at an 80% similarity level, and strains with a similarity of at least 95% were included in the same RAPD subtype. The numerical index of discrimination, based on the Simpson's index of diversity (Hunter and Gaston, 1988), was used to assess the capacity of the RAPD assay to differentiate *E. coli* isolates. To evaluate the population structure of the studied *E. coli* strains according to farm of provenance and type of sample, the genetic differentiation parameters G_{ST} and ϕ_{ST} were computed by Nei's standard (1972) approach. The coefficient of genetic differentiation (G_{ST}) was estimated for haplotypes using POPGENE version 1.31 (Yeh *et al.*, 1999). The molecular analysis of variance (AMOVA) was analyzed with GenAlex version 6.0 (Peakall and Smouse, 2006); AMOVA is commonly used to estimate the genetic differentiation among populations when using dominant markers. The software computes the parameter ϕ_{PT} as the proportion of the variance between populations in relation to the total variance according to the equation $\phi_{PT} = V_{AP}/ (V_{AP} + V_{WP})$, in which V_{AP} is the between-population variance and V_{WP} is the within-population variance. The parameter f_{PT} represents the correlation between individuals within a population relative to the total and is analogous to F_{ST} when the analyzed data are binary, as was the case for the RAPD data. To determine the G_{ST} and ϕ_{ST} values, a sub-population structure by farm of provenance or by sample type was *a priory* assumed, but acceptance or rejection of this assumption depended on the obtained values. Finally, strain mobilization between the studied farms was assessed. The spatial structure of the genetic relationships between the isolates by farm of provenance was visualized with AIS software (Miller, 2005) by generating a 3D graphic representation of the genetic landscape shape, in which the X- and Y-axes represented longitude and latitude in UTM coordinates and the Z-axis represented genetic distance. The analysis with AIS was achieved in three steps: first, genetic distances between all of the individuals were generated by computing a simple allelic similitude index; second, a linear regression of isolation by distance (IBD) was obtained from the previously calculated genetic distances and Euclidean geographical distances for all pairs of individuals; finally, a genetic surface was interpolated using the residuals of the linear regression and a Delaunay triangulation establishing a connectivity network between collection sites. In the present work, a grid size of 50×50 and a weight of distance value of $\alpha=1.0$ were used.

Results

For all of the isolates studied, partial sequencing (400 bp average length) of the 16S rRNA gene confirmed the species identity previously assigned by conventional microbiological and biochemical protocols. In all cases, Blastn searches in GenBank matched the 16S rRNA gene sequences of different *E. coli* strains with 95% or greater identity and E values ranging from e^{-74} to 0.0 (data not shown).

RAPD analyses using primer Ecorapd 1252 produced a minimum of one and a maximum of 10 bands per isolate, with sizes ranging from 237 to 6,073 bp. Using primer Ecorapd

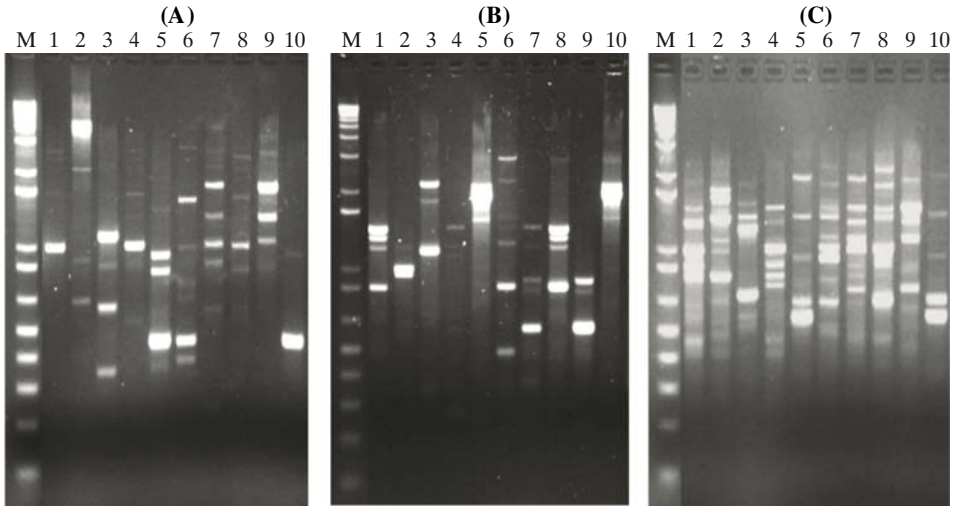

Fig. 2. Examples of ethidium bromide-stained agarose (2% w/v) gels showing RAPD profiles obtained from *E. coli* isolates using the primers Ecorapd 1252 (A), Ecorapd 1254 (B), and Ecorapd 1290 (C). Lanes: M, 1 kb plus DNA ladder molecular weight marker (Invitrogen); Lanes 1 to 10, banding patterns of isolates G1T6, G1T7, G1T8, G1F9, G1F10, G1F11, G1F12, G1F13, G1B14, and G1B15, respectively.

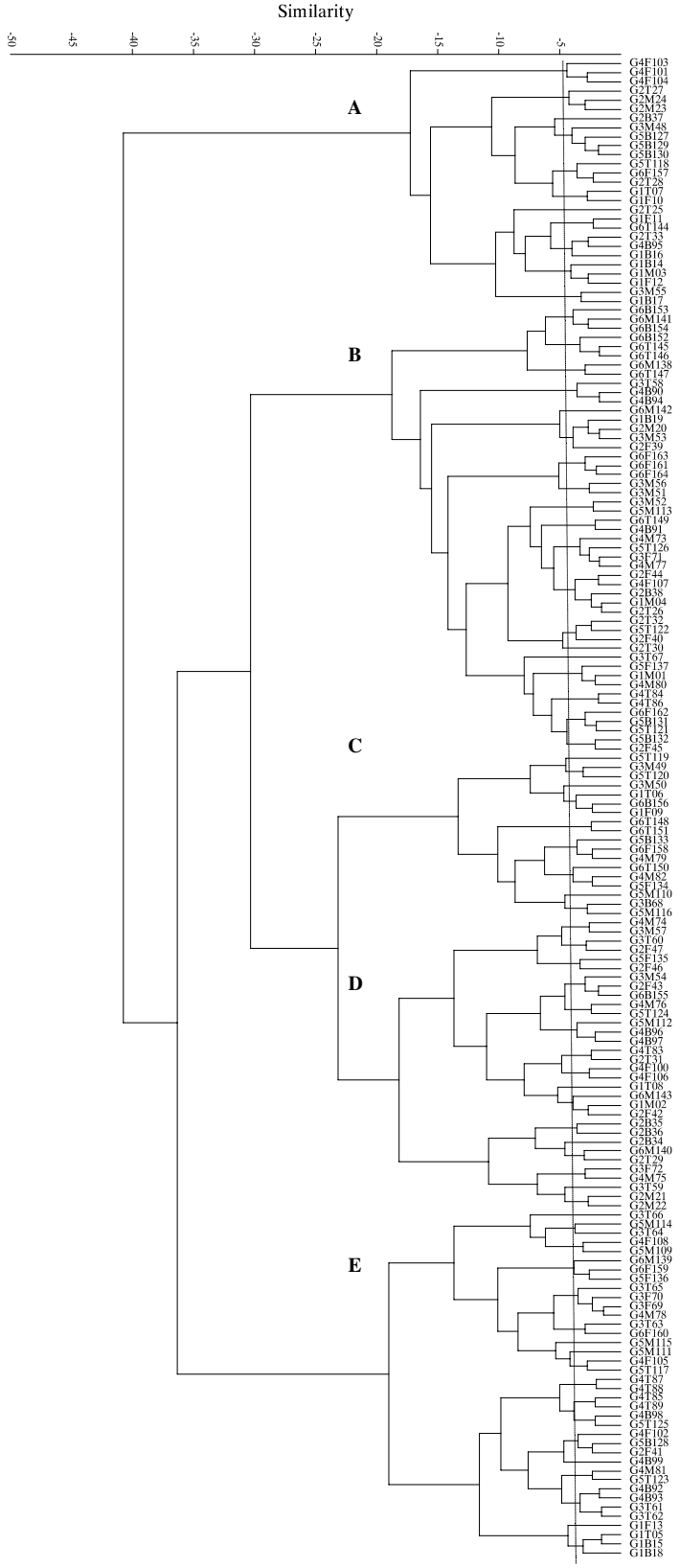

Fig. 3. Clustering of the studied *E. coli* strains by Ward's method using the Euclidean distances calculated from the RAPD patterns generated with the primers Ecorapd 1252, Ecorapd 1254, and Ecorapd 1290. The strains are identified by codes denoting farm of provenance (G1 to G6), type of sample (M, milk; T, cow teats; B, bedding; F, feces), and an identification number. The dashed line represents 95% similarity.

Table 3. Unique and shared RAPD *E. coli* types by farm and by sample type

by farm[a]							by type of sample[b]				
	G1	G2	G3	G4	G5	G6		M	T	B	F
G1		4	4	3	1	4	M		16	13	17
G2			4	6	6	6	T			12	16
G3				7	8	5	B				9
G4					11	4					
G5						6					
Unique	3	4	2	2	1	4		2	5	2	2

[a] G1 to G6 farms as numbered in Table 1.
[b] M=Milk, T=cow teats, B=Bedding material; F=Feces.

1254, up to 11 bands with sizes between 339 and 6,835 bp were observed for some isolates. Finally, primer Ecorapd 1290 generated banding patterns of 1 to 9 bands per isolate, with sizes between 360 and 5,891 bp (Fig. 2). A total of 120 loci were amplified with the three RAPD primers, which were used to generate a clustering pattern.

The clustering analysis resulted in a dendrogram with five main genetic clusters with a similarity value of 80%, which were further subdivided into subclusters (Fig. 3). Cluster B was the largest and contained 29.8% (49) of the studied strains, while cluster C was the smallest with 10.9% (18) of the strains. Clusters A, D, and E included 16.4% (27), 19.5% (32), and 23.2% (38) of the analyzed strains, respectively. The dendrogram did not cluster strains by type of sample or by location. At a similarity level of 95%, 54 RAPD types were discriminated among the 164 analyzed strains (Fig. 3). The numerical index of discrimination (*D*) of the RAPD assay that was calculated for all of the isolated strains was 0.981, indicating that the markers used permitted adequate discrimination between the isolates.

When clustered according to sample type, 31 RAPD types were found in 43 isolates from milk, 34 RAPD types were present in 48 isolates from teats, 23 RAPD types were found in 34 isolates from bedding material, and 27 RAPD types were detected in 39 isolates from feces (Table 2). When clustered according to farm provenance, the farm with the fewest RAPD types (11) was G1; this was also the farm with the fewest isolated strains (19). The largest number of RAPD types (22) was isolated from farm G5 (Table 2).

The analysis of RAPD type distribution by farm of provenance and by sample type showed that 16 RAPD types were exclusive to an individual farm and 11 RAPD types were present in only one of the four types of analyzed samples. Seventy-nine RAPD types were shared between farms, while eighty-three were shared between at least two sample types. Because all possible combinations of farm provenance and sample type were considered, the total number of shared RAPD types was greater than 54. Only one RAPD type was common to five of the studied farms, while no RAPD type was common to all six sampled farms. Three RAPD types were present in all of the analyzed sample types. Of the 54 RAPD types found, farms G2 and G6 had four unique RAPD types, the largest number of unique RAPD types found (7.4%). Farm G5 had only one unique RAPD type (Table 3). Farm G5 shared a single RAPD type with farm G1 but 11 with farm G4. With respect to sample type, 17 RAPD types (31.5%) were present both in milk and feces; 16 (29.6%) RAPD types were common to milk and cow teats, while 13 (24.0%) RAPD types were

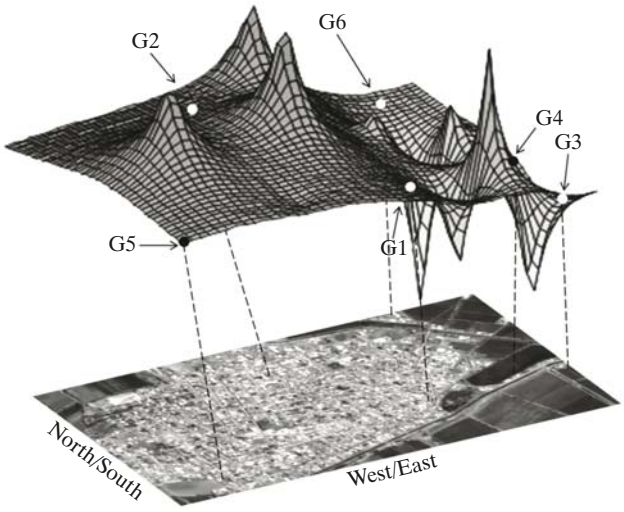

Fig. 4. Genetic landscape shape analysis of *E. coli* isolates from the studied dairy farms. The x- and y-axes represent geographic coordinates, and the z-axis represents the genetic distance. Positive value peaks indicate large genetic distances; negative peaks denote areas with high genetic similarity and high gene flow (image from Google Earth).

Table 4. Results of Nei's population analysis of *E. coli* isolates by farm and by type of sample[a]

	G_{ST}	AMOVA						
		d.f.	SS	MS	Variance	% total variance	ϕ_{PT}	P[b]
By farm								
among farms	0.058	5	92.147	18.429	0.313	3		
within farms		158	1571.219	9.944	9.944	97		
Total		163	1663.366		10.257	100	0.030	0.001
By sample								
among samples	0.026	3	41.370	13.790	0.090	1		
within samples		160	1621.996	10.137	10.137	99		
Total		163	1663.366		10.227	100	0.009	0.001

[a] G_{ST} was calculated with the POPGENE 3.2 software bundle, and the AMOVA was calculated with the GenAlex ver. 6.0 software package. See 'Materials and Methods' for details.
[b] P, probability of obtaining larger or equal values than those observed based on 9,999 permutations.

shared between milk and bedding material (Table 3). The sample type with the largest number of unique RAPD types was cow teats, which had five (9.2%), while each of the other sample types had only two (3.7%) unique RAPD types.

The comparison of diversity partition statistics obtained by Nei's classical method shows consistency between the G_{ST} and ϕ_{PT} parameters. The value of G_{ST} between populations (farms) was 0.058, while the AMOVA analysis estimated a ϕ_{PT} value of 0.030 (Table 4). According to the G_{ST} determination, 5.7% of the observed diversity was due to between-farm genetic differences, and 94.3% of the measured genetic diversity was due to intra-population genetic variation, i.e., within a particular farm of provenance (Table 4). In the same way, the ϕ_{PT} value showed that only 3% of the observed variation was due to between-farm variation and 97% of the genetic differences were found within farms.

Further evidence of the mobilization of RAPD types between the studied farms was provided by genetic landscape shape analysis. Examination of the genetic landscape shape clearly revealed two contrasting migration zones. On one side, three main discontinuities of genetic flow between the studied isolates of *E. coli* were observed as three positive value peaks toward the northwest portion of the study area (farms G2, G5, and G6) (Fig. 4). On the other side, three negative value peaks were seen toward the southeast, corresponding to farms G1, G3, and G4 (Fig. 4) and indicating a significant flow of RAPD types between isolates from these farms. However, the second migration area also displayed a positive value peak as a potential barrier for gene flow between farms located in that geographical area.

Discussion

The presence of common RAPD types among different types of samples is an indicator of the ability of *E. coli* to spread within the dairy farm environment. Clustering of individual isolates failed to discriminate them based on sample type. Thus, our results clearly demonstrate the substantial ability of several RAPD types of *E. coli* to mobilize between several dairy farm environmental compartments. Because milk samples had only two unique RAPD types (3.7% of the total) and shared 17 RAPD types (31.5%) with feces and 13 (24.0%) with bedding material, the source of milk contamination under the conditions studied here is clearly bedding material and ovine feces. Our results contrast with those of Kagkli *et al.* (2007b), who did not find *E. coli* strains derived from bovine feces in milk, and also with those of Son *et al.* (2009), who found that few genotypes were shared by milk, compost, and bovine feces. Most of the isolates from milk in these two studies had unique genotypes, suggesting other sources of contamination. However, our data are in agreement with a diverse array of studies in which bovine feces were reported to be the main source of milk contamination by *E. coli* (Oliver *et al.*, 2005), which can mainly be attributed to deficient production practices and poor hygiene in the dairy farm environment (Oliver *et al.*, 2005, 2009).

We found a significantly low percentage of sample-specific RAPD types, indicating that different RAPD types mobilize between the different sample types analyzed. Interestingly, 9 RAPD types were shared between bedding material and bovine feces, which agrees with the data reported by Son *et al.* (2009) in which only 11 of the 130 analyzed isolates had the same genotypes in compost and fresh bovine feces. This reflects a change in the composition of the *E. coli* population in both types of sample. Although the bedding material may contain a large proportion of bovine feces mixed with other materials, our results show that not all of the RAPD types initially present in the bovine feces are capable of enduring once they are incorporated into the bedding material. Because udders are in frequent contact with the bedding material, we investigated whether the RAPD types present on cow teats were also present in feces and in bedding material. *E. coli* genotypes that are common in feces and on cow teats but are absent from milk have been identified previously (Gelsomino *et al.*, 2001; Kagkli *et al.*, 2007a, 2007b). While Kagkli *et al.* (2007a) recognized that cow teats are contaminated by contact with bedding material, management by appropriate husbandry practices could prevent the milk from becoming contaminated. In contrast with these results, we found 16 RAPD types that were shared between bedding material, milk and teats, 13 that were shared between milk and bedding, and 17 that were common to milk and feces. In addition, some RAPD types were common to all four samples (data not shown). These results indicate that a primary route by which *E. coli* strains can contaminate milk is from feces to bedding material and finally to cow teats. Such a contamination pathway could easily be avoided by cleaning cow teats prior to milking, which is evidently not a part of current husbandry practices.

E. coli populations in dairy farm environments have previously been characterized with similar numbers of isolates (Vidovic and Korber, 2006) or even fewer (Stanford *et al.*, 2005; Wetzel and LeJeune, 2006) than the 164 isolates used in our study. Son *et al.* (2009) found 155 genotypes in 570 studied commensal strains of *E. coli*, and Holley *et al.* (2008) found 209 genotypes from a total of 796 such strains, which were analyzed from different years and sample types. Vidovic and Korber (2006) isolated 194 strains of the O157:H7 serotype and identified 39 genotypes that grouped into three main clusters. The above-mentioned studies differ from the present study with respect to the isolation methods, the types of samples analyzed, the markers used, the number of analyzed isolates and the threshold values for defining the number of genotypes. Despite these differences, in all of these studies, the ratio of genotypes (PFGE profiles or RAPD types) to the total number of analyzed strains fell within a relatively narrow range, 0.21-0.27.

Despite differences in the types of markers used, the geographic scale sampled and, in some cases, the mathematical methods applied for the data analysis, our values from Nei's population analysis were in close agreement with previous reports. On a broad geographic scale, initial studies of genetic differentiation between *E. coli* populations were performed using multilocus enzyme electrophoresis (MLEE), which showed that the mean value of G_{ST} between the Australian, Mexican, and ECOR strain collections was 0.047 (Souza *et al.*, 1999). On an intermediate geographic scale, an analysis of *E. coli* isolates from several Australian mammals revealed a ϕ_{PT} value of 0.045 (Gordon and Lee, 1999). On a finer geographical scale, a more recent study that used a combination of DNA markers with MLEE to study isolates from freshwater beaches

found a G_{ST} value of 0.011 (Walk *et al.*, 2007). The values of G_{ST} were low for all of these studies, including the present study. The values of G_{ST} and ϕ_{PT} that we obtained, 0.058 and 0.030, show that the greatest diversity was observed within farms rather than between farms. G_{ST} and ϕ_{PT} values for the sample types were even lower, clearly suggesting a constant flow of RAPD types between sample types and between farms and, as a consequence, a lack of division of farms and sample types into subpopulations.

Seemingly identical genotypes of *E. coli* O157:H7 have been found in different bovine farms globally (Davis *et al.*, 2003), at a regional scale in farms separated by a few hundred kilometers (Rice *et al.*, 1999; Van Donkersgoed *et al.*, 2001; Vidovic and Korber, 2006) and at distances of less than 100 km between studied farms (Rice *et al.*, 1999; Stanford *et al.*, 2005; Vidovic and Korber, 2006; Wetzel and LeJeune, 2006). The number of unique RAPD types varied among the farms in our study, which was performed on a further restricted geographical scale in which the maximum separation between the sampled farms was approximately 1.5 km. Each farm shared a different number of RAPD types with the remaining farms, and a variety of genotypes was found within each farm. This heterogeneous pattern of genotype distribution has been observed previously for *E. coli* O157:H7 in bovine farms separated by longer distances than those evaluated here (Stanford *et al.*, 2005; Vidovic and Korber, 2006; Wetzel and LeJeune, 2006). Based on these previous studies, the sharing of genotypes between farms when samples are taken over a short period of time is considered evidence of the dissemination ability of these genotypes within the studied geographic area, and the presence of a large variety of genotypes within the same farm has been associated with the continuous arrival of new genotypes (Rice *et al.*, 1999).

The recorded dispersion vectors of *E. coli* O157:H7 include birds (Wallace *et al.*, 1997; Shere *et al.*, 1998; Nielsen *et al.*, 2004), other wild and domestic animals (Rice *et al.*, 1995; Shere *et al.*, 1998; Nielsen *et al.*, 2004), and domestic flies (Rahn *et al.*, 1997). Because of the proximities of the studied dairy farms, the above-mentioned vectors likely have a dominant role in the exchange of *E. coli* strains within and between the studied farms. Other factors that are associated with the dissemination of *E. coli* on dairy farms are the movement of people (Davis *et al.*, 2003; Wetzel and LeJeune, 2006) and forage (Fairbrother and Nadeau, 2006), which should also be taken into consideration in the studied farms. *E. coli* strains may be disseminated between farms G1 and G6 through forage because both farms store their forage stock in the same storehouse. Farms G5 and G6 share a forage transportation vehicle, while farms G3 and G4 share the same milking staff, where transmission by human contact is most likely. *E. coli* isolates would have to be obtained from the milking staff members and from forage to corroborate these dispersal mechanisms. However, the results obtained from both the clustering patterns by farm and the genetic landscape shape analysis support the possibility of the above-mentioned dispersal mechanisms.

The present work incorporated population genetic tools to analyze *E. coli* isolates from dairy farms. One of these tools was Nei's approach for determining G_{ST} and ϕ_{PT}, as discussed above. Another tool was genetic landscape shape analysis, which explicitly quantifies the effects of landscape composition, configuration and matrix quality on gene flow and spatial genetic variation (Storfer *et al*., 2007). With respect to the epidemiological applications of this latter tool, it has recently been proposed that it should be defined in a broader context including "spatially implicit studies as well as phylogeographic approaches", in which *landscape* is interpreted as a "heterogeneous space that can influence microevolutionary processes in parasite populations across scales, from within individual hosts to global species distributions (Biek and Real, 2010)."

The application of genetic landscape shape analysis to molecular epidemiology is still just beginning (Biek and Real, 2010); the published studies of bacterial populations are limited, representing about 3% (5 papers) of the 174 analyzed publications on genetic landscape analysis between 1998 and 2008 (Storfer *et al*., 2010). In the present study, given the pathogenic potential of certain strains of *E. coli* for humans and cattle, genetic landscape shape analysis has contributed to a study of public health and contagious infectious diseases, which are related to epidemiology.

The results of the genetic landscape shape analysis partially agreed with the observed relationships between farms with respect to forage transportation and storage and the mobility of the milking staff. For example, genetic landscape shape analysis indicated the existence of gene flow between farms G1-G6, which shared forage storage, and between farms G3-G4, which shared milking staff. Additionally, genetic landscape shape analysis showed barriers to the exchange of RAPD types between farms G2-G6, which did not share forage or milking staff. Not all of the RAPD type exchanges can be explained by forage and milking staff mobility, and other dispersion vectors need to be evaluated in the future to arrive at more robust conclusions. Genetic landscape shape analysis was also congruent with the values of G_{ST} and ϕ_{PT}, revealing that there was no genetic differentiation between *E. coli* isolates according to farm of provenance, which is indicative of exchange of RAPD types between farms.

Although genetic landscape shape analysis aims to correlate gene flow with physical aspects such as rivers, mountains, and roads, the present work indicates that, in the small-scale dairy farms studied, gene flow may also be correlated with socioeconomic factors in the production system. The role of these factors is particularly evident in the results of the genetic landscape shape analysis from farms G1-G6. Although these farms are further away from one another and are separated by a large stretch of houses and roads, these farms shared RAPD types and showed evidence of gene flow associated with the mobility of farm staff and forage. Given the above socioeconomic aspect, the present work contributes a new application of genetic landscape shape analysis to public health issues in dairy farms, in which this tool may contribute to an understanding of the dispersion patterns of pathogenic bacteria within a dairy-producing region and aid in the identification of physical (landscape features), environmental (e.g., temperature and humidity), biological (e.g., birds and insects) or socioeconomic (cattle exchange, people, and forage flow) factors that favor or hamper pathogen dispersion on various geographic scales.

Genetic landscape shape analysis at a wider geographic range should better resolve gene flow patterns. This knowledge

would aid in planning and implementing measures to prevent the dissemination of pathogenic bacteria such as *E. coli* between and within dairy farms, thus reducing the risk of transmission and disease surges and improving management and husbandry practices.

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