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Single-Nucleotide Polymorphisms Reveal Spatial Diversity Among Clones of *Yersinia pestis* During Plague Outbreaks in Colorado and the Western United States

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Abstract

Background: In western North America, plague epizootics caused by *Yersinia pestis* appear to sweep across landscapes, primarily infecting and killing rodents, especially ground squirrels and prairie dogs. During these epizootics, the risk of *Y. pestis* transmission to humans is highest. While empirical models that include climatic conditions and densities of rodent hosts and fleas can predict when epizootics are triggered, bacterial transmission patterns across landscapes, and the scale at which *Y. pestis* is maintained in nature during inter-epizootic periods, are poorly defined. Elucidating the spatial extent of *Y. pestis* clones during epizootics can determine whether bacteria are propagated across landscapes or arise independently from local inter-epizootic maintenance reservoirs.

Material and Methods: We used DNA microarray technology to identify single-nucleotide polymorphisms (SNPs) in 34 *Y. pestis* isolates collected in the western United States from 1980 to 2006, 21 of which were collected during plague epizootics in Colorado. Phylogenetic comparisons were used to elucidate the hypothesized spread of *Y. pestis* between the mountainous Front Range and the eastern plains of northern Colorado during epizootics. Isolates collected from across the western United States were included for regional comparisons.

Results: By identifying SNPs that mark individual clones, our results strongly suggest that *Y. pestis* is maintained locally and that widespread epizootic activity is caused by multiple clones arising independently at small geographic scales. This is in contrast to propagation of individual clones being transported widely across landscapes. Regionally, our data are consistent with the notion that *Y. pestis* diversifies at relatively local scales following long-range translocation events. We recommend that surveillance and prediction by public health and wildlife management professionals focus more on models of local or regional weather patterns and ecological factors that may increase risk of widespread epizootics, rather than predicting or attempting to explain epizootics on the basis of movement of host species that may transport plague.

Key Words: *Yersinia pestis*—Plague–Single-nucleotide polymorphisms—Spatial diversity—Western United States—Colorado.

Introduction

Yersinia pestis, the causative agent of plague, draws attention because of its documented potential for spillover and devastating human disease outbreaks killing millions (Pollitzer 1954). Today, approximately 3000 human cases occur worldwide annually, mostly in Africa, with smaller numbers occurring in Asia and the Americas. In the United States, the frequency of human disease is low, with an average of eight cases reported annually (range, 1–40) from 1950 to 2008 (World Health Organization 2004, 2010). Nevertheless, case fatality rates are high if appropriate antibiotic

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therapy is delayed or inadequate (Hull et al. 1986), making plague a disease of high consequence. Currently, *Y. pestis* is established in hundreds of natural plague foci around the world where it causes sporadic epizootics, primarily in rodents (Link 1955, Barnes 1982, Anisimov et al. 2004, Zhou et al. 2004). During plague epizootics, humans are at the greatest risk of exposure to *Y. pestis* because mass rodent die offs increase the potential for human exposure to sick or dead animals and infectious fleas (Barnes 1982, Eisen and Gage 2009). Recently, plague also has been identified as a significant concern for the conservation of certain species, including prairie dogs and black-footed ferrets in the western United States where the disease is enzootic in many locations (Matchett et al. 2010).

Despite its historical and public health significance, our understanding of *Y. pestis* maintenance in nature is limited. Although empirical models have linked plague epizootics and climatic conditions, as well as increased rodent and flea densities (Parmenter et al. 1999, Enscore et al. 2002, Davis et al. 2004, Gage and Kosoy 2005, Gage et al. 2008, Ben-Ari et al. 2011, Savage et al. 2011), how *Y. pestis* bacteria are maintained during inter-epizootic periods is still not clearly understood. Alternative (but not mutually exclusive) hypotheses include: (1) Maintenance in off-host fleas (Kartman et al. 1962, Bazanova and Maevskii 1996, Gage and Kosoy 2005), (2) via transmission cycles between ''enzootic'' hosts (Barnes 1982, Gage et al. 1994, Gage and Kosoy 2005, Eisen and Gage 2009), (3) persistence among metapopulations of rodent hosts where epizootics occur in relatively isolated subpopulations (Keeling and Gilligan 2000, Davis et al. 2004, Collinge et al. 2005, George et al. 2013), (4) as a percolation phenomenon highlighting interactions between spatial structure of host populations and abundance (Davis et al. 2008, Salkeld et al. 2010), and (5) bacterial persistence in soil following the death of infected animals (Eisen et al. 2008). Maintenance mechanisms may affect how *Y. pestis* bacteria spread across landscapes and the scope of epizootic activity. Currently, our understanding of how *Y. pestis* spreads across expansive land areas is largely based on observations of epizootics in animals and epidemics in humans (Gage and Kosoy 2005). In reality, we do not know whether increased transmission and epizootics arise from geographically isolated bacterial clones or from a chain of infection propagated through sustained transmission and geographic spread across large landscapes.

Using genetic markers to examine the population structure of *Y. pestis* isolates collected during widespread epizootic activity may elucidate if bacteria are being spread and at what scale, or if they may cause plague locally without transport. Our recent understanding of *Y. pestis* genetic diversity, evolution, and transmission patterns on several geographic scales has increased dramatically and suggests that plague exists as a clonal pathogen (Klevytska et al. 2001, Achtman et al. 2004, Girard et al. 2004, Lowell et al. 2005, Auerbach et al. 2007, Touchman et al. 2007, Vogler et al. 2007, Zhang et al. 2009, Morelli et al. 2010, Cui et al. 2013).

Much of the diversity detected among North American *Y. pestis* isolates has been based on analyses of repetitive genetic elements (variable number of tandem repeats, VNTRs) via multilocus VNTR analysis (MLVA), which is useful for molecular epidemiologic studies on geographically local scales. However, the mutation rates within repetitive genomic regions are too great to accurately reconstruct large-scale movements and transmission patterns (Keim et al. 2003, Girard et al. 2004, Lowell et al. 2007). Whole genome comparisons among globally distributed *Y. pestis* collections have revealed the evolutionary diversification of *Y. pestis* in its historic range in Asia, its worldwide spread, and among North American isolates (Achtman et al. 2004, Chain et al. 2004, 2006, Auerbach et al. 2007, Morelli et al. 2010, Cui et al. 2013, Wagner et al. 2014). However, we still do not know whether repeated epizootics in North America are caused by large-scale clonal spread of bacteria from an initial focus, or whether near-simultaneous eruptions of plague epizootics over large areas are the result of multiple epidemic clones arising at local scales, perhaps facilitated by a combination of high rodent or flea abundance and favorable environmental conditions (Enscore et al. 2002, Davis et al. 2004, Salkeld et al. 2010, Savage et al. 2011).

We used DNA microarray technology (Hinds et al. 2004) to identify SNPs in 34 *Y. pestis* isolates collected in the western United States from 1980 to 2006. A subset of 21 isolates from Colorado was collected during plague epizootics between 1999 and 2006. Single-nucleotide polymorphism (SNP) arrays provide an alternative to genome sequencing for standard laboratories, where the costs, labor, and time associated with whole genome library preparation, sequencing, bioinformatic analysis, and data storage are prohibitive. Furthermore, SNP arrays demonstrate concordance rates of up to 99.86% to SNPs discovered using full genome sequences and have yielded identical phylogenies in studies of *Bacillus anthracis* diversity (Gardner et al. 2013). The objectives of this study were to: (1) test the hypothesis that during widespread epizootic plague activity *Y. pestis* spreads clonally between the mountainous Front Range of northern Colorado and the eastern plains, and (2) provide additional information on the population structure of *Y. pestis* in the United States on local and regional scales. Elucidating *Y. pestis* population structure on local scales may determine how epizootics spread and may provide insight into long-term maintenance of *Y. pestis* during inter-epizootic periods. An understanding of how *Y. pestis* bacteria circulate during epizootics, and at what spatial scales they are maintained, will also facilitate development of predictive surveillance and control measures intended to aid public health professionals, wildlife ecologists, and natural resource managers in implementing focused recommendations during and between plague epizootics.

Materials and Methods

For this study, a total of 21 isolates were available for analysis that represented the northern Colorado mountainous Front Range and the plains of the Pawnee National Grasslands (Fig. 1). Isolates were grouped according to collection from mountain or plains locations (Table 1). All were collected from 1992 to 2006 during routine surveillance and human plague case investigations carried out by the Centers for Disease Control and Prevention's (CDC) Division of Vector-Borne Diseases (DVBD) and by researchers from Colorado State University (CSU) carrying out intensive epidemiologic studies of prairie dogs, fleas, and plague in northern Colorado (Tripp et al. 2009). During collection periods, evidence of plague epizootics, such as animal-to-human transmission and rodent

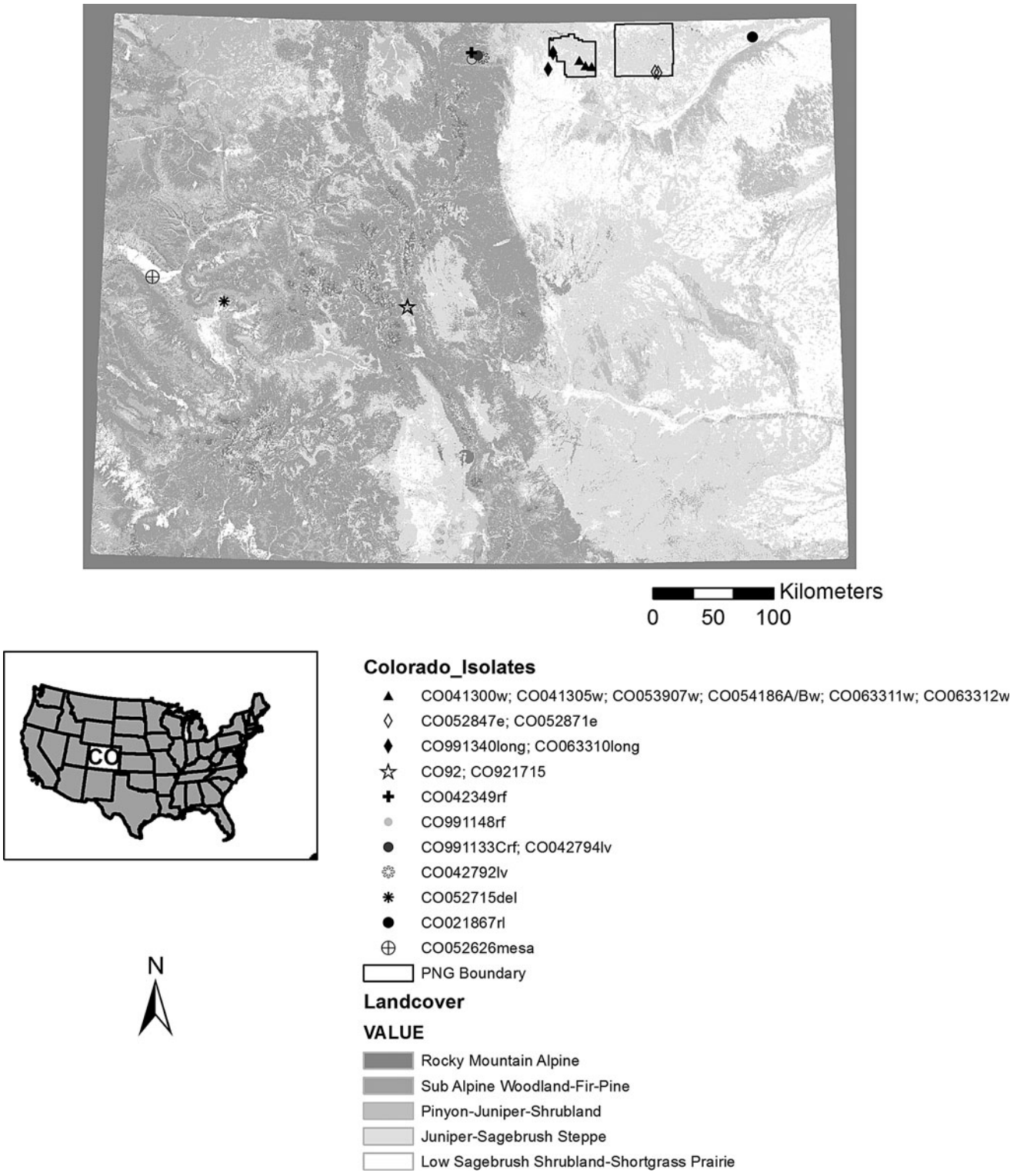


FIG. 1. Colorado isolate collection locations. Symbols represent isolates with matching SNP profiles. CO054186A/Bw represents two isolates—CO054186Aw and CO054186Bw. Corresponding CDC accession numbers are listed in Table 1.

die-offs, were apparent. Colorado isolates collected strictly from 1999 to 2006 were used to compare phylogeographic relationships during widespread epizootic activity that occurred during this interval in the northcentral mountains in Colorado and approximately 70–150 km further east on the plains of the Pawnee National Grasslands.

Epizootic activity was especially apparent during 1999 and 2004 in both the mountains (rodent and human plague cases), and plains (prairie dog die-offs), and during 2005 and 2006 on the plains (prairie dog die-offs) (Table 1). One pair of isolates, which were previously collected about 13 km apart in 1999 (CO991340) and 2006 (CO063310), was included for

Table 1. Origins of *Y. pestis* Isolates Used for Single-Nucleotide Polymorphism Discovery

CDC accession number[a]	*County*	*Isolate origin*	*Collection location (general characteristic of area)*	*CDC collaborator in isolate collection*
CO92	Chaffee	human blood	Mountains	Colorado Department of Public Health and Environment
CO921715	Chaffee	Chipmunk	Mountains	Colorado Department of Public Health and Environment
CO991133Crf	Larimer	human blood	Mountains	Colorado Department of Public Health and Environment
CO991148rf	Larimer	*Cediopsylla inaequalis* (rabbit flea)	Mountains	Colorado Department of Public Health and Environment
CO042349rf	Larimer	*Human*	Mountains	Colorado Department of Public Health and Environment
CO042792liv	Larimer	cat blood	Mountains	Larimer County Health Department, Colorado
CO042794liv	Larimer	cat blood	Mountains	Larimer County Health Department, Colorado
CO052871e	Weld	*Cynomys ludovicianus* (black-tailed prairie dog)	Plains	Colorado State University
CO052847e	Weld	*Cynomys ludovicianus*	Plains	Colorado State University
CO053907w	Weld	*Cynomys ludovicianus*	Plains	Colorado State University
CO054186Aw	Weld	*Pulex sp.* (flea)	Plains	Colorado State University
CO054186Bw	Weld	*Cynomys ludovicianus*	Plains	Colorado State University
CO041300w	Weld	*Cynomys ludovicianus*	Plains	Colorado State University
CO041305w	Weld	*Cynomys ludovicianus*	Plains	Colorado State University
CO063311w	Weld	*Cynomys ludovicianus*	Plains	Colorado State University
CO063312w	Weld	*Cynomys ludovicianus*	Plains	Colorado State University
CO052715del	Delta	unidentified flea species from coyote	Plains	USDA Wildlife Services
CO063310[b]	Larimer	*Cynomys ludovicianus*	Plains	Larimer County Health Department, Colorado
CO991340[b]	Larimer	*Cynomys ludovicianus*	Plains	Larimer County Health Department, Colorado
CO052626mes	Mesa	*Spermophilus variegatus* (rock squirrel)	Mountains	Mesa County Health Department, Colorado
CO021867rl	Sedgwick	*Oropsylla hirsuta* (prairie dog flea)	Plains	Colorado Department of Public Health and Environment
80CA2178	Los Angeles	*Spermophilus beecheyi*	Mountains	California Department of Health Services
CA812298	Shasta	*Spermophilus lateralis*	Mountains	California Department of Health Services
AZ921389	Apache	Human	Mountains	CDC/Indian Health Services
AZ962456	Coconino	Human	Plains	CDC/Indian Health Services
NV870978_51	Clark	*Thrassis bacchi* (antelope ground squirrel flea)	Mountains	CDC/Environmental Health Department, Clark County, Nevada
NM021852	Santa Fe	*Orchopeas sexdentatus* (wood rat flea)	Mountains	New Mexico Department of Health
NM024479	Santa Fe	*Peromyscopsylla hesperomys* (deer mouse flea)	Mountains	New Mexico Department of Health
NE054860	Box Butte	*Cynomys ludovicianus*	Plains	USDA Wildlife Services
WY042773	Goshen	*Sylvilagus audubonii*(desert cottontail rabbit)	Plains	Colorado Department of Public Health and Environment
WY000345	Washakie	Human	Plains	Wyoming Department of Health
MT922215	Big Horn	*Lynx rufus* (bobcat)	Plains	Wyoming Department of Health
SD053915_255	Shannon	*Oropsylla hirsuta*	Plains	USDA Wildlife Services
SD053909	Shannon	*Cynomys ludovicianus*	Plains	USDA Wildlife Services

[a]The CDC accession number consists of the state abbreviation followed by the last two digits of the collection year, except for isolate 80CA2178 in which these two identifiers are reversed. The following digits are unique identifiers. The extension abbreviations on each accession number are as follows: rf, Red Feather; liv, Livermore; e, Pawnee National Grasslands east; w, Pawnee National Grasslands west; rl, Red Lion National Wildlife Refuge; del, Delta County; mes, Mesa County.

[b]Isolates representing temporal comparison.

CDC, Centers for Disease Prevention and Control.

temporal comparison. Another pair, CO92 and CO921715, represented mountain isolates collected ~1 km apart during the same time period but in a different region of Colorado than the other isolates. These were included for additional geographic comparisons (Table 1). An additional 13 isolates from Arizona, California, Nebraska, New Mexico, Nevada, Montana, South Dakota, and Wyoming, collected as part of routine plague surveillance activities and human plague case investigations carried out by the CDC/DVBD and its partners in state and local health departments (Table 1), were genotyped and included in the analysis to provide a regional ecological scale (Fig. 2). These were selected retrospectively because they were historically well characterized by isolate host and place and time of collection, and they provided a broad geographic and temporal representation of *Y. pestis* isolates from across the western United States. Isolates

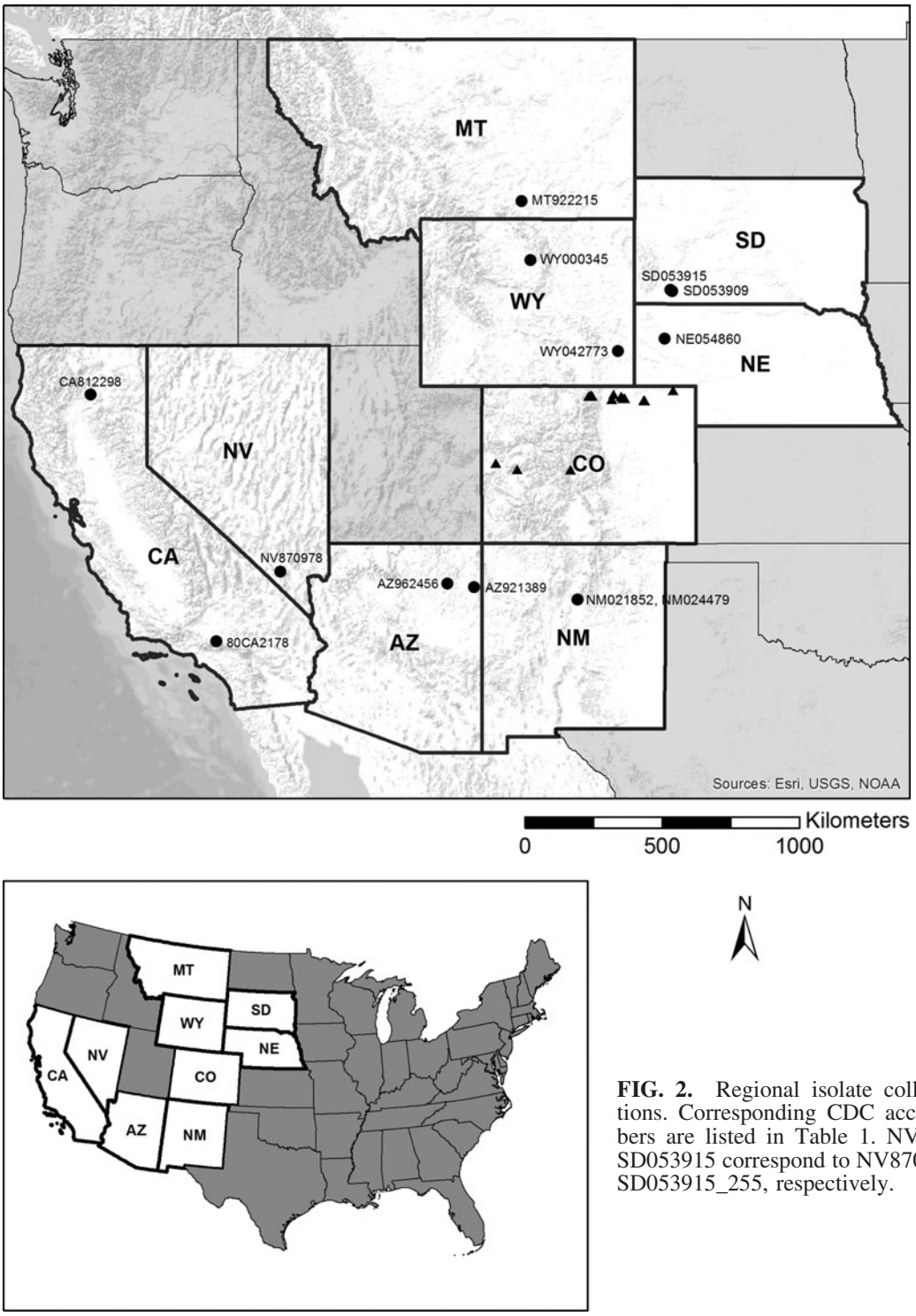


FIG. 2. Regional isolate collection locations. Corresponding CDC accession numbers are listed in Table 1. NV870978 and SD053915 correspond to NV870978_51 and SD053915_255, respectively.

were collected from a variety of sources, including humans, rodents, and fleas (Table 1). These samples were collected during the course of human plague case investigations or routine plague surveillance activities for public health purposes rather than as part of research projects. Because each of the isolates made from these samples was obtained as part of a public health response, their use was not subject to the same Institutional Animal Care and Use Committee (IACUC) or Institutional Review Board (IRB) approvals and restrictions required for isolates derived from research activities. All personal identifiers, however, were removed from samples prior to use. An isolate from Kazakhstan (KZ993829), was included as an outgroup for phylogenetic analysis.

Animal care and welfare protocols were approved by the CSU Animal Care and Use Committee (ACUC) (animal welfare assurance no. A3572-01 and protocol approval no. 03-053A-01). Sample collection permits were issued under the authority of the State of Colorado Department of Natural Resources, Division of Wildlife (permit nos. 06TR983, 07TR983, and 08TR983).

Isolate preparation and DNA extractions

All isolates were grown on Congo Red agar for 48 h at 25°C to screen for the presence of the *pgm* locus (Burrows and Jackson 1956, Surgalla and Beesley 1969). One inoculation loop full of red colonies (*i.e.*, *pgm* positive) was selected for each isolate, transferred to 5 mL of Heart Infusion Broth (HIB) (Archibald and Kunitz 1971) (Becton Dickinson, Franklin Lakes, NJ), and shaken overnight (ON) at 25°C and 160 rpm. One milliliter of each 5-mL ON culture was transferred to 25 mL of HIB, and ON culturing was repeated. Cells were pelleted in Oakridge tubes by centrifugation in a Beckman Coulter J series centrifuge (Fullerton, CA) at 8000 rpm for 10 min. Cell pellets were stored at −80°C until DNA extractions were performed. DNA was extracted using the PureGene DNA Purification Kit™ (Qiagen, Valencia, CA) protocol with the following modifications. Cell pellets from 25-mL ON cultures were used rather than 50-mL cultures. Pellets were resuspended in 1.0 mL of 50 mM Tris/50 mM EDTA and transferred to a 50-mL conical tube prior to the cell lysis step. For RNA digestion, RNase A was added to a final concentration of 200 μg/mL to each sample, mixed by inverting the tubes 25 times, and incubated at 37°C for 30 min. Proteinase K (Invitrogen, Grand Island, NY) was then added to a final concentration of 100 μg/mL, and the samples were incubated at 55°C for 1 h. Following DNA precipitation, the pellets were transferred to 1.5-mL microcentrifuge tubes containing 1.0 mL of 70% ethanol, washed, and centrifuged for 3 min at 6000 rpm. The ethanol was removed, and the pellets were air-dried and resuspended in 1.0 mL of kit-supplied DNA Hydration Solution. DNA was shipped overnight to Lawrence Berkeley Laboratory, (Berkeley, CA) where it was transferred to Perlegen Sciences (Mountain View, CA) for microarray analysis.

SNP discovery array

The SNPs were discovered by resequencing and comparison of 147 strains of global origin. For this study, the resequencing microarray was designed by incorporating unique regions of six reference genomes: CO92 (Parkhill et al. 2001), KIM (Deng et al. 2002), Antiqua, Microtus 91001 (Song et al. 2004), Nepal 516 (Chain et al. 2006), and *Y. pseudotuberculosis* IP32953 (Chain et al. 2004), from which a minimal set of nonredundant contiguous fragments was developed. First, the set of all unique 25-mers present in the source sequences was identified, and the first occurrence of each unique 25-mer by sequence ID and position was chosen. Next, six bases were adjoined to either side of each chosen 25-mer to provide context for SNP discovery. Adjacent sequences were merged into fragments. Unique sequences were tiled. These reference sequences provided 7.85 Mb of potential SNP positions after redundant regions, such as insertion sequence elements and repeat regions, were removed. Bases were called from the resequencing array data using the ABCUS algorithm, a clustering program based on Gaussian mixture models (Cutler et al. 2001). Comparisons were also made between each of the reference sequences to determine base-calling accuracy. For the discovery array, base calling was determined to be 99.8% accurate. Furthermore, similar probe designs have revealed call rates of over 99% when compared to full genome sequencing (Gardner et al. 2013).

Our *Y. pestis* isolate DNAs were compared to the reference isolates by hybridization of samples to the high-density oligonucleotide array containing the reference oligonucleotides. A total of 20 μg of DNA per sample was fragmented using DNase I and biotinylated. The resulting labeled oligonucleotides were hybridized to the arrays according to Hinds et al. (2004). Hybridization of the labeled sample to the microarray was detected using a confocal laser scanner (Patil et al. 2001).

SNP genotyping

To find SNP locations, positions 9, 13, and 17 of each oligonucleotide were queried for a match or mismatch between the reference and queried sequences. SNPs were determined by measuring the ratios of mean intensity of perfect match features to mismatch features on the microarray (Hinds et al. 2004). Each 25-bp oligonucleotide that contained a SNP at the queried position was compared against the published CO92 *Y. pestis* genome (GenBank acc. no. NC_003143), using BLAST (www.ncbi.nlm.nih.gov/BLAST/). SNPs were categorized as intergenic or within an open reading frame, synonymous or nonsynonymous, and as a transition or transversion by generating graphical representations of codons using Codon Plot (Stothard 2000).

Phylogenetic analysis

SNPs were used to compare isolates within Colorado and from around the western United States. Data were entered into PAUP* version 4.0b10 (Swofford 2002). The phylogeny was inferred using equally weighted parsimony and 1000 tree–bisection–reconnection (TBR) searches with a maximum of 20 trees held per search. TBR branch swapping was then performed on all of the most parsimonious trees found with a maximum of 100,000 trees held, from which a strict consensus tree was calculated (Schuh and Polhemus 1980). Jackknife (JK) support (Farris et al. 1996) was inferred using 37% deletion and 1000 replicates, each consisting of 10 TBR searches and a maximum of 20 trees held. A *Y. pestis* isolate (KZ993829) from the Tien Shan region of Kazakhstan, which was included in the microarray analysis, was used as an outgroup (Achtman et al. 2004, Lowell et al. 2007).

Results

SNP discovery

Microarray comparisons of this *Y. pestis* isolate set yielded 40 previously unidentified, and two previously identified SNPs (Gibbons et al. 2012). Of those, 10 (24%) were intergenic, 23 (55%) were nonsynonymous SNPs, and 9 (21%) were synonymous SNPs (Table 2). Six (23%) of the SNPs found in coding regions were in virulence plasmids. Eighteen SNPs (45%) were present in more than one isolate, whereas the remainder represented single isolates (Table 2). Fifteen SNP (36%) mutations caused transitions and 27 (64%) caused transversions.

Phylogenetic analysis

Eighteen parsimony informative SNPs were identified among this isolate collection (Table 3). SNPs that appeared only in single isolates were termed ''singletons,'' and, although interesting for describing the full diversity within this group of isolates, singletons do not provide information about common ancestry among isolates. Phylogenetic analysis showed that unique genotypes corresponded with local geographic scales, and that regionally, genetic differences approximated geographic distance. First, three unique genotypes identified among the Colorado plains isolates signified near simultaneous emergence of several localized epidemic clones during widespread plague activity. Isolates from the western Pawnee National Grasslands, eastern Pawnee National Grasslands, and the temporal comparison (Table 3, Fig. 3) were highly supported by JK consensus, with values of 90%, 95%, and 85%, respectively. Second, two similar genotypes were detected among Colorado mountain isolates. The JK consensus yielded 64% support among isolates from Red Feather and Livermore (C0991133Crf and CO042794lv), collected in 1999 and 2004, respectively, and 61% between CO92 and CO921715 from Chaffee County. The Colorado isolates collected from mountainous Delta and Mesa counties, and the majority of the remaining plains and mountain isolates yielded singleton SNPs and were unresolved in the tree (Fig. 3). One isolate, C0042349rf, yielded no SNPs when compared to the other North American isolates. Among regionally defined samples, the JK consensus supported a unique genotype among the South Dakota isolates (94%) and the two isolates from New Mexico (65%).

When considered as pairs, these comparisons further exemplified localized emergence of unique genotypes. The inferred phylogeny grouped regional isolates collected in 1987 and 1992 from Nevada, Montana, and Arizona (Fig. 3). Whereas this group shared a single common mutation, MT922215 and AZ921389 contained several singleton mutations that may have occurred following regional distribution from a common ancestor (Fig. 3). Interestingly, isolates from Nebraska, Wyoming, and New Mexico fell into a weakly supported group with the geographically nearby isolates from the western Pawnee National Grasslands and shared a previously described SNP from a New Mexico *Y. pestis* collection (Gibbons et al. 2012). A second common ancestral mutation, also previously described by (Gibbons et al. 2012), was shared by the western Pawnee National Grasslands and New Mexico isolates, lending additional support to a historic *Y. pestis* radiation event after long-range spread from a plague focus originally located around Santa Fe, NM (Gibbons et al. 2012). The remaining isolates collected regionally were unresolved at the base of the tree. An ensemble consistency index (CI) (Kluge and Farris 1969) of 0.88 and an ensemble retention index (RI) of 0.95 (Farris 1989) suggested that identical mutations that arise independently (homplasies) are unlikely to have occurred and therefore would not have biased the results.

Discussion

We discovered SNPs using high-density oligonucleotide microarray technology for genome-wide comparisons of a North American *Y. pestis* isolate collection. In the 34 isolates studied, 42 variable SNPs were identified, 18 of which were informative for delineating the geographical extent of *Y. pestis* clones in the western United States (Table 2). Results from the northern Colorado transect, which spanned sample locations from the Front Range mountains eastward to the Pawnee National Grasslands, suggested that plague epizootics in this region are localized events and that widespread plague activity is the result of several unique epizootic clones emerging simultaneously. The data do not support the notion of large-scale pandemics sweeping across the landscape. Perhaps the most compelling evidence of local maintenance of *Y. pestis* was that of our temporal comparison, in which unique SNPs supported the relationship between two Colorado isolates (CO991340, CO063310) collected from similar locations during epizootics that occurred 7 years apart (Table 3, Fig. 1). Existence of locally confined genotypes supports the idea that transmission and persistence of *Y. pestis* is facilitated by metapopulation turnover with localized epizootics.

Previous analyses have also demonstrated limited pathogen dispersal distances and subpopulation structure of *Y. pestis* during widespread epizootic activity (Girard et al. 2004, Snall et al. 2008, Gibbons et al. 2012). On the basis of the tree topology generated by the Colorado transect isolates, chain-reaction transmission of *Y. pestis* among many host species may not be necessary for widespread pathogen dispersal during epizootics, as previously suggested (Girard et al. 2004).

Several mechanisms of *Y. pestis* dispersal that would result in distances greater than prairie dog dispersal have been proposed. They include increased host abundance and contact with putative reservoir hosts (small rodents) (Snall et al. 2008), attraction of rodent-consuming predators that could carry infected fleas between *Cynomys* spp. colonies (Cully and Williams 2001, Salkeld et al. 2007), and interspecies transmission events facilitating *Y. pestis* translocation among dispersal-limited reservoir populations (Girard et al. 2004, George et al. 2013). Explanations of bacterial dispersal mechanisms have depended in the past on interpretation of rapidly mutating VNTR markers, suggesting that plague epizootics are initiated by precipitous spread of a single *Y. pestis* genotype across landscapes and subsequent genotypic differentiation within local, dispersal-limited reservoir populations (Girard et al. 2004). These mechanisms were no doubt important during the eastward expansion of *Y. pestis*, but our implementation of more slowly evolving SNPs suggests that multiple epidemic clones may have emerged independently (Fig. 1) and that longer-distance plague transmission is not

TABLE 2. SINGLE-NUCLEOTIDE POLYMORPHISM LOCATIONS AND MUTATION OUTCOMES RELATIVE TO THE CO92 GENOME

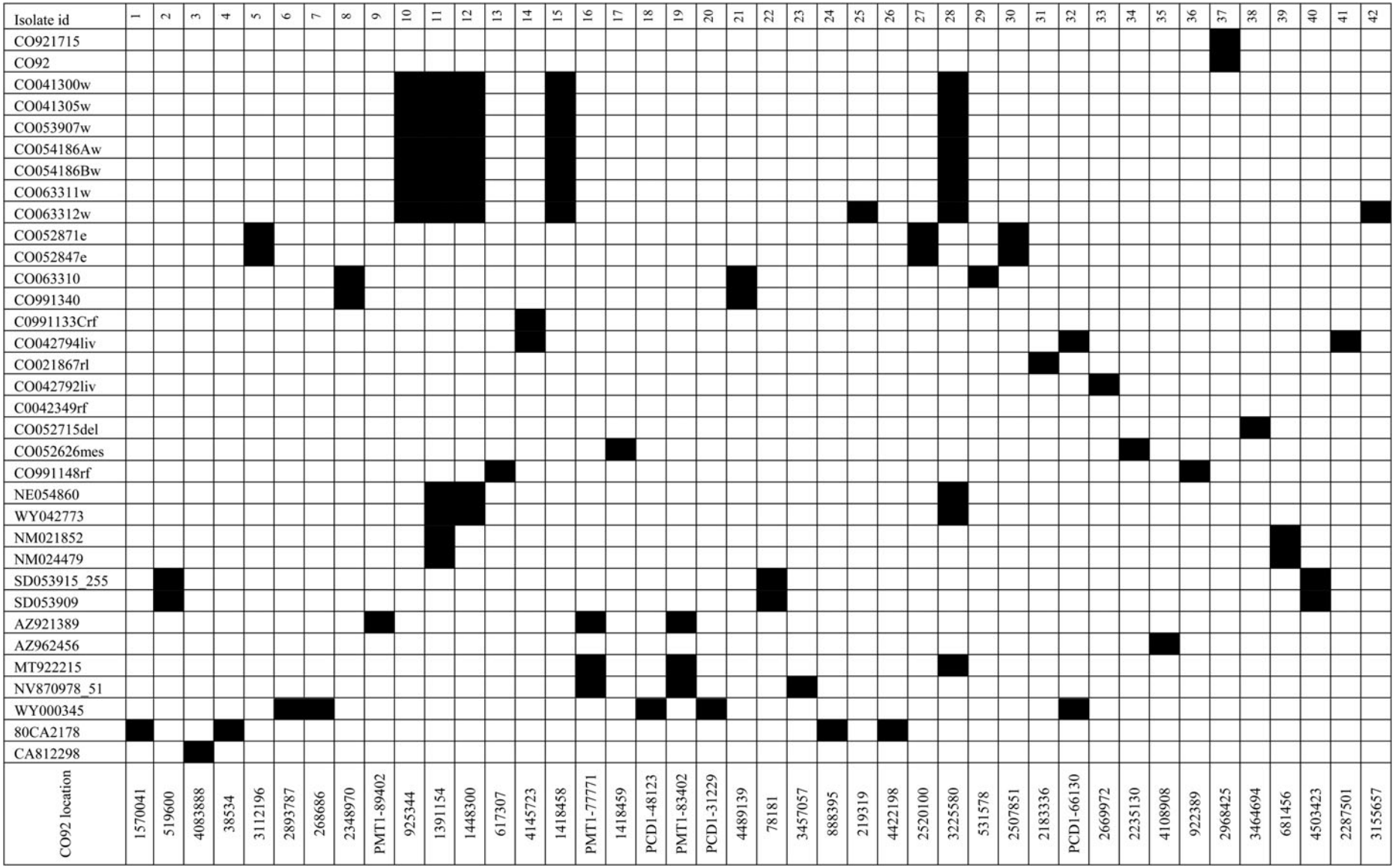
CO92 position	*CO92 gene*	*CO92*	*Anc*	*Der*	*Anc AA*	*Der AA*	*Anc codon*	*Der codon*	*Mutation*	*Feature related to sequence*
31229	YPCD1.42	C	C	A	T	K	aca	aaa	ns	Putative type III secretion protein
38534	YPO0028	G	G	A	V	M	gtg	atg	ns	Ribonuclease BN
48123	YPCD1.09c	G	G	T	M	I	atg	att	ns	Hypothetical protein YPCD1.09c
66130	YPCD1.91_92	A	A	T	N	Y	aac	tac	ns	PCD1
77771	YPMT1.78	T	T	C	D	D	gat	gac	s	PMT1_possible pseudogene
78181	YPO0067	G	T	T	A	S	gct	tct	ns	Protein-export protein
83402	YPMT1.83	A	A	T	N	I	aat	att	ns	caf1A, probable F1 capsule anchoring
89402	YPMT1.87	G	G	C	G	R	ggc	cgc	ns	PMT1_possible porphyrin biosynthetic protein
219319	YPO0203	T	T	A	D	E	gat	gaa	ns	Elongation factor Tu
268686	YPO0266	C	C	T	L	L	cta	tta	s	Putative type III secretion system ATP synthase
519600	YPO0486_87	C	C	A	Q	K	caa	aaa	ns	ig
531578	YPO0497_98	T	T	A	n/a	n/a	n/a	n/a	ig	ig
617307	YPO0572	G	G	A	STOP	STOP	tag	taa	s	Putative exported protein
681456	YPO0618	A	A	T	S	C	agt	tgt	ns	Putative exported protein
888395	YPO0809	C	C	A	N	K	aac	aaa	ns	General secretion pathway protein K
922389	YPO0842	T	T	G	V	G	gtc	ggc	ns	Sulfatase
925344	YPO0843_44	C	C	A	n/a	n/a	n/a	n/a	ig	ig
1391154	YPO1229_31	C	C	T	n/a	n/a	n/a	n/a	ig	ig
1418458	YPO1260	C	C	T	N	N	aac	aat	s	Putative membrane protein
1418459	YPO1260	C	C	A	Q	K	cag	aag	ns	Putative membrane protein
1448300	YPO1288_90	G	G	A	n/a	n/a	n/a	n/a	ig	ig
1570041	YPO1390	C	C	A	A	A	gcc	gca	s	3-Phosphoshikimate 1-carboxyvinyltransferase
2183336	YPO1925_26	C	C	T	n/a	n/a	n/a	n/a	ig	ig
2235130	YPO1966_67	G	G	T	n/a	n/a	n/a	n/a	ig	ig
2287501	YPO2015	T	T	A	L	Q	ctg	cag	ns	Putative lipoprotein
2348970	YPO2067_68	G	G	A	n/a	n/a	n/a	n/a	ig	Putative lipoprotein
2507851	YPO2232_34	T	T	C	n/a	n/a	n/a	n/a	ig	ig
2520100	YPO2243	C	C	T	H	Y	cat	tat	ns	Putative AraC-family transcriptional regulatory protein
2669972	YPO2377	T	T	C	I	T	atc	acc	ns	Putative membrane protein
2893787	YPO2573	G	G	T	T	T	acg	act	s	Putative membrane protein
2968425	YPO2641	G	G	A	Q	Q	cag	caa	s	Phage family integrase (partial)
3112196	YPO2777	C	C	A	H	N	cat	aat	ns	Histidine transport ATP-binding protein HisP
3155657	YPO2828	T	T	A	START	K	atg	aag	ns	Phosphoribosylaminoimidazole synthetase
3225580	YPO2886	C	C	T	T	I	act	att	ns	Putative autotransporter protein
3457057	YPO3098	G	G	T	L	F	ttg	ttt	ns	Probable glycosyltransferase
3464694	YPO3108	T	T	C	S	S	agt	agc	s	Putative glycosyltransferase (pseudogene)
4083888	YPO3663	G	G	T	G	C	ggt	tgt	ns	Probable zinc-binding dehydrogenase
4108908	YPO3678	G	G	A	S	N	agc	aac	ns	Insecticidal toxin complex
4145723	YPO3711	G	G	A	A	T	gct	act	ns	Maltoporin
4422198	YPO3937	G	G	A	Q	Q	cag	caa	s	Aerobic glycerol-3-phosphate dehydrogenase (partial)
4489139	YPO3984_85	C	C	A	n/a	n/a	n/a	n/a	ig	ig
4503423	YPO3995_96	T	T	C	n/a	n/a	n/a	n/a	ig	ig

SNP, single-nucleotide polymorphism; Anc, ancestral genotypes; Der, derived genotypes; s, synonymous SNPs that do not alter protein sequence; ns, nonsynonymous SNPs that alter protein sequence; ig, intergenic SNPs; n/a, not applicable.

necessary to explain the occurrence of widespread plague epizootics. Furthermore, our mountain isolates, which were subject to higher levels of geographic isolation, did not yield well-supported genetic relationships with each other or with plains isolates. Isolation by geographic barriers would speed differentiation between *Y. pestis* clones and prevent regular interspecies *Y. pestis* dispersal at large scales.

Phylogenetic relationships inferred among our samples are consistent with historic translocation across broad regional scales and provide clues to directional spread and

Table 3. Genomic Diversity in North American Collection



The positions of base changes are indicated according to location in the CO92 genome. Black squares indicate SNP presence. The base changes caused by each SNP mutation are shown in Table 2.
SNP, single-nucleotide polymorphism.

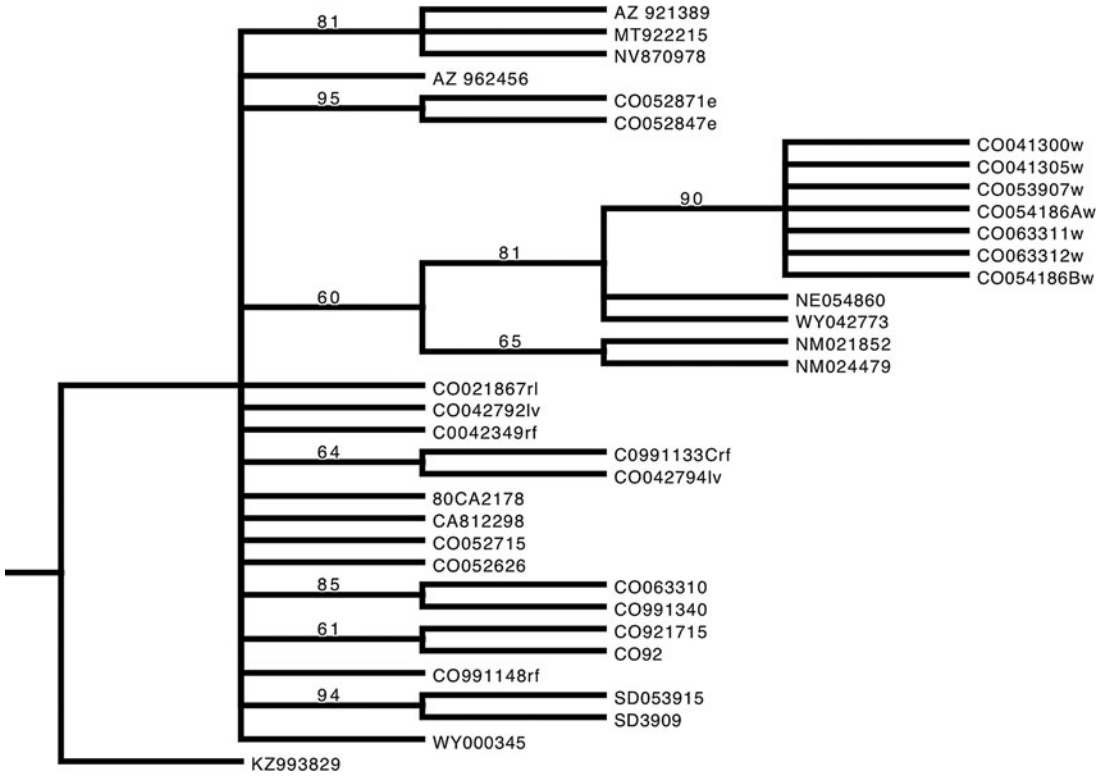


FIG. 3. Maximum parsimony tree of isolates used for SNP discovery. Isolate abbreviations are listed in Table 1 with the following modifications: Extensions del, mes, _51, and _255 were removed from CO052715, CO052626, NV870978, and SD053915, respectively, during phylogentic analysis. Jackknife support is indicated above branches.

establishment of *Y. pestis* in the United States (Enscore et al. 2002). Two SNPs were shared by isolates collected in 1987 from Nevada and in 1992 from Arizona and Montana. Whereas these isolates shared mutations, several singleton SNPs signify historic spread followed by local divergence (Table 3, Fig. 3). Furthermore, Nebraska and Wyoming isolates shared an ancestral SNP with the western Pawnee National Grasslands isolates, suggesting historic spread of a single clone, followed by local divergence. Additional sampling should reveal whether the singleton mutations mark clones that have spread regionally within mountain valleys.

Conclusion

The *Y. pestis* collection analyzed here suggested: (1) local-scale emergence of unique *Y. pestis* epizootic clones during widespread epizootic activity in Colorado, and that widespread interspecies chain reaction transmission events are not the sole mechanism of spread during epizootics; and (2) regional-level relationships among isolates, suggesting that *Y. pestis* diversifies at relatively local scales following long-range translocation events. Although this study would be strengthened by additional samples, the results strongly evoke a local maintenance mechanism for *Y. pestis* independent of interspecies and landscape-scale dispersal by rodent hosts or their predators. Future studies should focus on similar analyses using more samples clustered locally and more samples collected through time. A focus on potential local reservoirs, such as off-host fleas (Gage and Kosoy 2005), soil (Eisen et al. 2008), and nematodes (Tan and Darby 2004), should be included. Surveillance and public health recommendations could be improved by focusing more on climate models that may predict widespread epizootic activity based on ecological factors, rather than relying on prediction of directional spread.

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Author Disclosure Statement

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