Anthrax:

Evolutionary approaches for genetic-based investigative tools

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Hot or Not?

The 2001 anthrax letter attacks demonstrated that *Bacillus anthracis* spores can heavily contaminate a facility before anyone becomes aware of a problem. Ideally, any future anthrax attack would first show up as a positive test from routine air monitoring, not as a crisis days later when seriously ill patients start appearing in emergency rooms. Unfortunately, researchers haven't found it easy to develop an accurate anthrax test, particularly when dealing with complex environmental samples. One big problem is that *B. anthracis* is highly similar to common spore-forming bacteria such a *Bacillus cereus* and *Bacillus thuringiensis*. The specter of multiple false alarms and consequent public apathy gives serious cause for concern. **Easterday et al. (p. 731)** now provide some hope for developing specific and sensitive anthrax detection methods. In previous work, they showed that a single nucleotide change corresponding to a nonsense mutation in the *plcR* gene, though present in 89 different *B. anthracis* isolates, is absent in the bacteria's genetic near-neighbors. In this report, the researchers describe the validation of a real-time PCR-based mismatch amplification mutation assay for specific quantitative detection of *B. anthracis* DNA. The assay successfully amplifies as little as 25 fg *B. anthracis* DNA, even in the presence of air filter extracts containing a 20,000-fold excess of DNA differing in sequence only at the SNP position. The task of homeland defense initiatives remains enormous, but advances such as this should help front-line personnel determine more rapidly whether a sample contains a hot agent.

Unknown author

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Preface

As humans it seems we have always been developing ideas and methods to describe the world around us in a context we can understand. From fairy tales to religious texts it has been our attempt to describe causation and order or in simpler terms the 'how.' Through the development of Logic and Science the how is on its way to be answered. Where the end is we don't know, and we can only guess at the possible infinity. In man there is a continuum of thought stretching from these minds that first sparked the ideas that would lead to science as we know it today. These are the moral scientists who stood up for truth and would accept nothing less. Today we still have an obligation to produce honest work. We are responsible for our future.

None of this work would have been possible without the support of my friends and colleagues. I would first like to thank my colleague and friend Matt Van Ert whose enthusiasm, encouragement and guidance solidified who I am as a scientist. I would also like to thank my colleagues Zack Jay, Jana U'Ren, Tatum Simonson, Leo Kenefic and Shaylan Zanecki. I would also like to express my gratitude to Nils C. Stenseth for supporting me in compiling this thesis.

Most of the work presented here was done in Paul Keim's Genetic Laboratory at Northern Arizona University. The focus of his lab has enabled the development and accomplishment of these projects. This work was funded largely by the U.S. Departments of Energy and Homeland Security, National Institutes of Health, the U.S. Federal Bureau of Investigation, General Medical Sciences, the Cowden Endowment at Northern Arizona University, and the Defense Threat Reduction Agency.

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Introduction

While many researchers see increasing incongruity in the biological sciences due to increasing specialization others see opportunity through collaboration creating synergy. This synergy will be created when we as researchers are able to span the gaps between the different disciplines in biology. Indeed here at the Centre for Ecological and Evolutionary Synthesis at the University of Oslo, Norway we are already beginning to see efforts to bridge different disciplines within biology together. Where genetics and molecular biology had been disciplines unto their own, they are now being practiced in many fields within biology including population biology, ecology and paleontology. The genetics and now genomics revolution has been infiltrating all parts of biology.

This thesis is a compilation of works done on a specific bacterial pathogen, *Bacillus anthracis*, and even more specifically on the evolutionary genetics of this organism with respect to its geography. Here, through the introduction I will be explaining my own personal views of how biology is naturally structured and interconnected. Given the present state of research, how will the future of biology naturally evolve? Although we cannot see extremely far into the future we can see the next logical steps in this progression by examining the tools and methods we have today and given the rate of development, we can imagine what the near future holds in store for biology. It continues into the introduction of the chapters and the considerations, such as evolution, ecology (epidemiology), biological warfare and forensics that have bearing this work.

Causation- event and outcome

After working for nearly a decade in biology in a diverse set of fields within biology I began to see the natural connectivity between these fields. Not so much in the fact that they dealt with life but that they all dealt with evolution and hence studied change. Change is what everything in biology, and the universe for that matter, centers around. In biology, broadly speaking, we try to take a measurement of something physical and then after some duration we measure it again and that difference is its rate of change. In experimental biology we try to fix all the components that can change naturally by controlling the variability through consistency across replicated experiments. Once we are able to control the variability in a system we have a null to compare against, after we have added some effecter. Here we can directly measure the change from this null to the aftermath of the experiment. The final differences between the null/control experiment and experiment with variables are the measure of the net effect the variation has on the outcome.

This type of process is used throughout biology to first fix the components that we want to measure to create a null or negative control then introduce a change or let it occur naturally. We then use these data of change to predict what will occur in the future for similar circumstances or systems given previous rates of change by a known effecter. This is true across all levels in biology throughout Central Dogma up to Landscape Ecology. In practice we must understand causation, or correlation where causation cannot be teased out from a number of possible effectors, to begin to learn from that which we study.

Biology and the processes that occur within this study are ruled by the laws of physics. It is therefore quite helpful to understand some of these physical laws of the universe, especially causation. Causation is a central law of the Universe where real matter, which makes up the Universe, is involved. Nothing in the Universe is static; specifically everything in the Universe is made up of matter which has energy or velocity. All matter is moving and at this specific moment in time all matter is at a finite distance from all other matter. Many of these distances will change at the very next moment, yet this change is ruled by laws where matter with a velocity cannot skip space through time, but it must occur at the adjacent space in the next future segment of time.

Take for instance a ball being thrown from one person (A) to another person (B) in outer space. The ball will travel the distance (10 meters) from person A to person B in 10 seconds. When the ball is released by person A, a velocity of 1 meter per second is imparted unto the ball. Velocity, direction and speed, determines the path of the object, the path is made up of continuous time, over 10 seconds, and discrete space. Think of this space-time as a 3 dimensional object. To help illustrate this if you as an observer had a camera set-up with a 10 second exposure and took a picture of the path of the ball, in the picture you would see a 3-dimensional rod between person A and person B. This rod is really a 4-dimensional object (3 dimensions of the ball plus one dimension of time). We can then break up this 4 dimensional rod into discrete segments of time. If we break up the duration of the path into 1 second segments (10-1 second exposures), in sequential order they would make up the path of the ball. Let's number these segments 1 through 10 respectively from A to person B. In space (uniform gravity) the velocity of the object will be unchanged therefore the 10 space-time objects (10- 'ball through space' for 1 second) will look exactly the same except for the relative position of the objects between person A and B. Given these conditions of position and velocity (speed and direction) an earlier section will solely determine the shape of next 4-d section. This prediction is possible in this type environment because we know the velocity (cause) will affect the subsequent event. Here we have one attribute (velocity) of one object causing the next subsequent event in time, an effect.

This is causation in a most simple form and this is what we must first understand and identify in all sciences including biology. In biology to truly understand what we are studying we must first identify and understand the natural course of the biological elements without influence from extrinsic sources. Let's go back to the ball in space, since we have this understanding of how the object will travel with respect to space and time with no forces involved, other than inertia of the ball we can predict its path in both directions of time, future and past. Yet if the outcome is different from what we predict, for instance that the ball doesn't reach person B we know that there must be other forces acting upon the ball changing the result. If we bring this example from space to earth where person A and B are now playing catch in a vacuum we will see new effectors on the path and outcome of the object. From our null example in space we predict that the ball will leave person A's hand at time 0 and follow a direct path to person B's hand in exactly 10 seconds. On earth given no previous understanding of gravity we expect the same outcome. Yet when we see a different outcome, the ball colliding with the ground, we can conclude that being on earth (the only variation in conditions) has a direct measurable and consistent (in the sense that it is reproducible) effect on the ball.

We can then measure this difference from our null in outer space from the ball on earth to calculate the affect of gravity on the ball. After we know this variable and its effect on the change of our 1 meter per second velocity, we can now determine the path of the ball in both directions of time given any position in its path between person A and the ground.

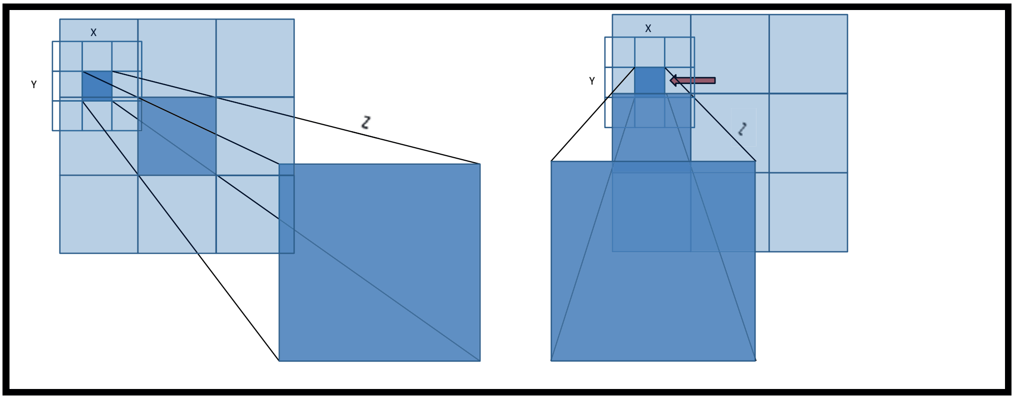


Figure 1: Simple diagram showing causation through space (compressed to 2-d: X, Y) and time Z.

To better illustrate causation there are two figures above (fig. 1). Within each of the two figures there are three sequential planes along the Z-axis (time) the further plane is the past, the nearest is the future. These X, Y planes represent a two-dimensional space which is some measurable quality or quantity. Along the Z- axis is time, time and sequence are not often actively thought about as we often take time for granted because it is a natural process that has bearing on everything we do.

At the beginning of the model on the left a force is applied to the dark blue square into the lower figure which creates change in velocity (directional), indicated by an arrow (fig. 1). We are able to recognize and measure this change because we are able to subtract that from the null control on the left side.

This very simple diagram showing causation is illustrative for nearly every process that belongs to the universe including biology, where everything has a sequential path through space and time. In static conditions, where there is a null effect from the surrounding environment, the path is straight through space over time. Yet where there is an effect from the surrounding environment the object's path is affected leading to a different than predicted (null) outcome.

Biology

The individual organism is something we can identify with because we are all individuals. The individual is the biological entity which interacts with the world (biotic and abiotic) proximate to it. Every organism looks the way it does for two reasons: 1) the heritable traits of the genome contained within and 2) the development of the organism that is driven by the interaction of the genome and the environment. If you look at biological history from the present to the beginning of life it divides into a history of converging lineages or paths going back through time. These can be broken into smaller pieces down to the life of individual organisms which are small segments of any given lineage, similar to the ball in space discussed earlier which starts at one point and ends at another occupying the 4-dimensions of space and time. The life of individual organisms can be further simplified into a series of events or effecters (like gravity although its effects are constant). Each event has an outcome that is predicated upon the natural laws in physics which bears directly on development, development on fitness and fitness on evolution. Evolution is the addition of these small physical events (feeding, reproduction, agility, etc.) where sometimes luck but ultimately fitness (these small differences in phenotype that make the difference) determines the outcome: perpetuation of life. Each event will have an outcome and every outcome influences the next event. This event and outcome is just causation which is a theme that runs through all disciplines in biology and it is the mechanism of evolution.

In biology there are often outcomes caused by culmination of small events, we see their subsequent effects on higher orders of complexity such as within Central Dogma. Central Dogma is useful to help understand biology and evolution. Central Dogma is structured in a way that the smaller things create and make-up the bigger things. This starts at the level of genes where the genes are responsible for the coding of proteins. These proteins are the machinery and building blocks of cells. Cells together (in multi-cellular organisms) create tissues which serve specific functions. These create organs and structure which serve as machinery (organs) for the organism. Although not typically talked about as being part of Central Dogma we can continue to extrapolate: the organism is a single member of a population, the population is part of an ecosystem and the ecosystem is part of the biome.

Now think of the above structure as a linear progression from genes to organism to population to ecosystem to biome. Everything biological is built by this process. It is difficult to identify all the factors contributing to or changing the expression of genes without removing the effecters. How do we study and learn the true effects of the external proximate environment on the outcome of phenotype; and how does this phenotype interact with the world around it?

The Null Organism

There are a couple ways to study the genotype-phenotype relationship, one way can be done in the natural environment (Gilbert 2004) which I will discuss later, the other is in a lab and until recently has been our only real option. An organism whose developmental needs are not limiting in a completely controlled environment is a good model to start with. To do this you'd need to strip away all the extrinsic factors that influence the development of an organism and not limit the necessary resources for growth and development. Our goal will be to see the true translation of genotype to phenotype. One of the best example of this, was work that was done in the MIR space station, even though this is not what they were intending to study directly (NASA 2006), it provides a very good example of how small influences shape the development of life.

On MIR research teams have been growing soybeans (*Glycine max*) in microgravity to develop processes for growing food using aeroponics to supply long distance space missions, such as a mission to Mars. As a biologist some of the small anecdotes they mentioned fascinated me. Growth rates of the soybeans (on earth) using aeroponics was much higher than those grown in soil (aeroponics is a growing system where roots are not planted but suspended by a trellis in the open air and misting system supplies water and nutrients directly to the roots). In addition to this, soybeans grown using aeroponics on MIR in the microgravity of space had an even faster growth rate than those grown aeroponically on earth. In space we have a plant close to a ‘null’ in terms of development. If we go from space to earth the effect of gravity is added to the outcome of the plant, which is seen in the reduced growth rate as there is now energy being spent to overcome gravity.

The more influences or constraints we put on the organism as it develops we can begin to measure the effects the proximate environment has on the phenotype. These induced phenotypes sould provide advantages to the organisms which express them (i.e. reduced predation as seen in Daphnia and carp, increased maturation rate like in the development of spade-footed toad tadpoles) with some cost or trade-off (i.e. slower speed/less efficient locomotion or smaller size at maturation).

The Null Plus

Since null organisms do not occur in natural settings they are a null plus the net effect of environmental influences. The expression of genes under the conditions of the specific proximate environment is responsible for the phenotype. This phenotype is the organism that interacts with the environment and other organisms around it. However small or insignificant these interactions with the environment seem to impact an organism, their sum can have great influences on that organism’s life. For instance the European map butterflys’, *Araschnia levana*, development of wing patterns is changed by differential expression of genes driven by climate (temperature). During the cooler spring the outcome is a more reticulated pattern and during the warmer summer a darker less reticulated pattern (Gilbert 2004).

If the interaction is an event which is ubiquitous across a habitat such as an unusual temperature or precipitation fluctuation the event can affect the development impacting overall fitness of a population having a ripple effect in the evolution or success of the lineage. If we had a series of warmer springs and began having summer morphs in the spring, how would this affect the success of: these individuals? the population? These are things we are not certain of because we do not understand the degree of phenotypic plasticity that is achievable determined by the influences of environment. Nor do we understand how ‘fit’ this phenotype will be to its environment, although we do have examples with *Daphnia* (Agrawal *et al* 1999) and carp, *Carassius carassius* (Brönmark *et al* 1994)

Biology’s Future

-*Yesterday*

When thinking of evolution, which elements play an applied role to diversifying species? More specifically which physical elements mechanically drive evolution and explain why differences within and among species exist? Evolution of life on earth can be thought of as similar to Newtonian Gravity with respect to its relativity. This description of relativity is the strength (gravity) of the relationship between two objects, with respect to size (mass) of and the distance between the two objects. Many of the physical mechanisms driving selection and evolution have a higher effect with higher relativity (proximity between two or more organisms in space and time and the strength of the relationship(s)). We see these types of relationships from the very small gene networks (Tong *et al* 2004) to the large ecological networks. Where and when an organism exists in space and time is its occurrence. Occurrence determines the context of the object or organism and its proximity to other real matter. This context is a compilation of physical factors, biotic and abiotic which make up the real earth we know.

This context is the measure, quantification and qualification of the physical environment, the data can be organized and related using space and time. Context and the change of context have a profound effect on the development, survival, adaptation and evolution of organisms. The variety of organisms and their genes within are directly linked to their occurrence. The genes and the influence of environment create the phenotype which is adapted to specific environments. This was a lesson learned by Darwin nearly one hundred and eighty years ago and became a foundation in evolutionary thought.

It was at first not apparent to the young researcher that part of what defined a species was its occurrence, as adaptation to these environments has guided the species' evolution. "I have not as yet noticed by far the most remarkable feature in the natural history of this archipelago; it is, that the different islands to a considerable extent are inhabited by a different set of beings. My attention was first called to this fact by the Vice-Governor, Mr. Lawson, declaring that the tortoises differed from the different islands, and that he could with certainty tell from which island any one was brought. I did not for some time pay sufficient attention to this statement, and I had already partially mingled together the collections from two of the islands (Darwin 1845)."

Due to Lawson's statement, Darwin realized that there was a link between the specific physical habitat and the types of organisms inhabiting it. The occurrence of a species depends on its ability to exploit the resources of the specific environment and this environment has shaped the evolution of the species. The physical environment is in many ways primarily responsible for the genes that exist with it, their expression into phenotypes and the evolutionary pressures which maintain stasis or force/allow change/drift.

-*Tomorrow*

As biologists we stand on the verge of whole genome sequencing becoming a tool that is available to all institutes and all budgets. Single-molecule whole-genome sequencing will dramatically lower costs in both the technology and the data analysis (Venter 2010). This capability will produce vast amounts of genetic data. How we manage, couple or integrate these data will not only directly impact the value of the parts but will dramatically impact the value as a whole.

When collecting organisms and sequencing their genomes we are describing these organisms in a most detailed way. However, as was mentioned earlier this genome or genotype does not necessarily correspond to a phenotype. There are many other factors that influence the expression of genes and their influence on the development and plasticity of the organism's traits. These factors are the components of its context of existence. This existence occupies a discrete dimension consisting of both real-world space and time. Along this flux of existence biotic and abiotic factors are literally helping shape the organism by extrinsic pressures. Intrinsic and extrinsic biotic and abiotic factors (diet, competition, climate, etc.) drive how an organism's genes are expressed and ultimately resulting into a phenotype. This phenotype interacts with the world around it and the fitness of this phenotype to the environment directly impacts the survivability of the organism, its genes and the perpetuation of its lineage. The genotype/phenotype and their applied fitness to their environment have been honed by the normalities of the environment and impacted severely by dramatic events that have occurred to the lineage. This is what truly worries many biologists about dramatic climate change. If a climatic event is too extreme for a key species or many species, will the overall system be able to cope and function without completely collapsing.

The genome harbored within an organism is truly rare as it exists once in discrete time and space. The continuation of any lineage must occur from one individual to the next, between the parent and the progeny. Here there must be a mechanical movement of genes from one organism to the next. Genes are passed and linked directly through time and space, creating an unbroken four-dimensional continuation between parent and offspring. This coupling between parent and progeny is one physical link in the chain or lineage which occupies space through time, it stretches from the present back to the origins of life. Along any lineage are the forces external to it, the proximate environment, which have guided its evolution and direction in real space through time.

Increasingly larger data sets are now and have been the trend in biology. Larger data sets over longer time series gives more insights into the natural variation that occurs within populations and help reveal how selection and environment shape the evolution and distribution of species. Increased data sets will also help shed light on atypical patterns of occurrence and variation. In the future, genomic and other possibly heritable data from an individual and data of its occurrence will naturally allude to the connections between each scale in central dogma from DNA to organism to population. Although from a different point of view. Currently RNA transcription libraries are created to determine how variations of one gene are being expressed under controlled or defined expression parameters. In the future we will be able to measure variations in the genome that account for these phenotypic differences. We can find these differences by subtracting likeness between to sets of genomic data, whether it is between two organisms or an organism and a population or two populations of a species etc., we can identify genetic differences responsible for phenotypic differences between groups. In fact this type of approach has been used to identify new pathogens specifically viruses that have become cryptic by embedding themselves within their host's genome (MacConaill *et al* 2008). In short large genetic data sets alone can be very powerful tools for everything from evolution to public health as seen in the 1000 human genomes project (Consortium 2010).

Yet there will be instances where genetic or other heritable data will not provide answers for observations in terms of phenotype. In lieu of these genetic differences the data would suggest external factors that are responsible for differences in phenotypes. Specifically data associated with the context of that organism. These data include all of the environmental data associated with the organism's habitat throughout its development. These factors can also be teased out by subtraction. Subtle differences within one habitat can have large impacts on the phenotype of organisms. Larger data sets combined with environmental data Central dogma with the impact of the environment that translates into a phenotype

Why these genes are the way they are and function the way they function is because of where they have originated. This is something that has largely been taken for granted or ignored by many geneticists and biologists. Yet, very recently ecologists and population biologists are beginning to use genetics as tools to explain phenotype. Even though unifying these disciplines is still a ways off, what will be next?

If we look at these trends in biology such as larger data sets, the incorporation of genetic data, genomics, proteomics over longer time series then linking these with phenotype data and add them to trends we see in our databases, computing power and internet networking; what should happen next? Our work as biologists at times seems quite abstract from the real world even though it is quite real. All life has its place on this earth and every time we collect an organism from its environment we should be collecting standardized information on its context also (Field *et al* 2008). Eventually from these genomes we will have genotype phenotype maps available that show the strength of association between the two (Thorisson *et al* 2009). Once we have this information we can organize these data on the web in space a 3-dimensional globe (Liolios *et al* 2008).

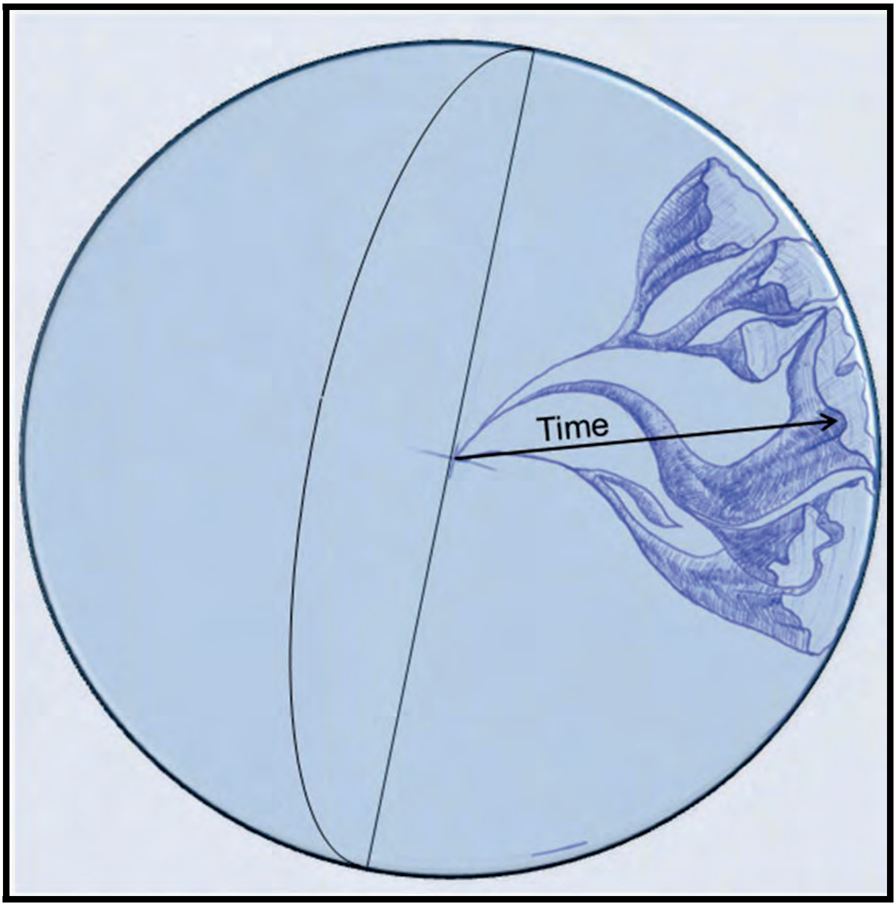


Figure 2: **Biology's future** interactive database to visualize genetic and environmental information with occurrence/migration patterns. The user will upload genome data along with MIGS (Field *et al* 2008) that will eventually include phenotype data (Houle *et al* 2010), long-term GPS data for animals capable of movement along with progeny data where available. The user can build phylogenies in space-time according to a specific gene or multiple genes, species, genera, phenotypic traits, etc. The user can also use the database to build 4-D models of ecological niches and run evolutionary simulations.

We can even go one step further and plot these data in time within the globe. To visualize this take the earth and cut it into equal halves (Fig

2). At its center will be time zero for life, some 3.5 billion years, at its crust will be the present. In order to fit everything into this globe the visual data would need to be compressed logarithmically as data moves from the 'crust' to its core. As we are able to collect more and more information on individuals such as movement and migration patterns using GPS tracking, we can incorporate these data to build a 4-dimensional existence of an individual. Collecting these types of data over multiple members within a population over generations we can build digital reconstructions of real world phylogenies. The user will also be able to build models changing environmental variables to see their impact on species or systems.

With the data accompanying these phylogenies we can begin to see how individuals and populations interact with the changing environment. Over longer periods of time we can begin to quantify and qualify properties of the environment that are selecting for the fit types from a population over time. We will be able to watch genes change with the environment or migrating to avoid environmental changes. We can see specific environmental ques that are responsible for phenotypes. The larger this database becomes the more powerful it will be to answer the finer peculiarities from the biological world.

For biology to answer bigger questions we need bigger tools the earth through time is the all encompassing (assuming they won't find life on Mars) context for life. It relates all life through space and time with respect to the abiotic environment. We can use a tool like this to study the life histories of organisms including pathogens and their hosts.

Pathogens

I have always found pathogens interesting, especially the semi-obligate pathogens because they seem to have one foot in each door: unable to walk away from their existence as a pathogen and also not able to make the complete jump to an organism that solely relies on its host.

Pathogens are really no different than other forms of life. They require extrinsic energy to live and to proliferate. Although they have specialized to parasitize other life to acquire their energy. Having an ability to transmit between hosts and exploit its resources is the simple reason for their existence. The evolution of pathogens and their virulence (ability to exploit the host) largely depends on the specific type of relationship between the pathogen and its host. These relationships have been classically divided into three groups: *obligate*, *opportunistic* and *accidental* pathogens. Although these classifications describe the necessity of the host for the pathogen to proliferate they do not always take into account the necessity of the host for the pathogen's persistence and the evolutionary pressures that persistence plays in virulence.

For instance two bacterial pathogens *Mycobacterium tuberculosis* and *Bacillus anthracis* would both be put into the 'obligate' pathogen category. Yet there is a big difference between the two in terms of disease pathogenesis and mortality. *M. tuberculosis* is transmitted directly from one host to the next and causes a chronic pulmonary infection. Whereas *B. anthracis* causes an acute and fatal infection. The evolutionary pressure on virulence (strategy of exploiting the host) lies in the transmission from one host to the next. For *M. tuberculosis* a chronic infection lengthens the time and increases the opportunity for transmission. For *B. anthracis* transmission only occurs after the death of the host, leaving no immediate selective pressure to lower virulence. This specific relationship between this pathogen and its host create the selective pressures which determine the morbidity caused by the pathogen to the host.

Managing infectious diseases that effect livestock, crops, wildlife and human health requires the ability to predict, detect, and effectively curtail naturally occurring infectious disease epidemics and epizootics. Central to this is the development of tools that allow us to monitor the environment and hosts for selected pathogens, as well as detect and track the progression of pathogens through an outbreak. Following the 2001 anthrax attacks in the United States, it became apparent that the same tools used in epidemiological and evolutionary studies of infectious disease are very relevant in law enforcement and intelligence applications.

Since biocrimes and bioterrorists attacks are typically by design covert, prevention is unlikely. Instead, a retrospective microbial forensic investigation of the event is a more probable outcome, followed by attribution and, if possible, apprehension. The microbial forensic investigation and attribution relies on methods and tools to precisely identify the attack strain that was released and the ability to link biological evidence among crime scenes, and ultimately to a source and a responsible person or party. Considering the paucity of physical characteristics available to uniquely identify and differentiate microbial evidence, genetic signatures are of central importance in any investigation.

Introduction of the Chapters

This dissertation describes the development of highly precise and sensitive molecular detection and typing tools for the pathogen *Bacillus anthracis*, and the application of these tools for forensic and epidemiological analyses. The chapters herein represent a top down approach to genetically describing *B. anthracis* isolates in the context of their world population using both Single Nucleotide Polymorphisms (SNPs) and Multiple Locus Variable Number of Tandem Repeat Analysis (MLVA)*,* although much of my contributions to chapters 4, 5 and 6 specifically focus on the application of SNP data. It begins at chapter 1 'Use of Single Nucleotide Polymorphisms in the plcR Gene for Specific Identification of *Bacillus anthracis* in which we define the species as compared to its nearest known genetic relatives. At this division there is a distinct phenotypic dimorphism caused by this nonsense mutation in the *plcR* gene. A single nonsense mutation in this gene shuts off the downstream genes that it is responsible to regulate. Shutting these genes off is a necessary trade-off to allow *B. anthracis* to sporulate after killing its host, which is a central step in the ecology and persistence of this pathogen. Using a ubiquitous and specific SNP allows fast and clear typing between typical *B. anthracis* strains and their nearest relatives.

Chapter 2 continues with the use of this same mutation 'Specific detection of *Bacillus anthracis* using a TaqMan® mismatch amplification mutation assay (taqMAMA)' which only allows the polymerase to extend off the specific nonsense mutation in *B. anthracis*. This allows the researcher or investigator to detect *B. anthracis* by specifically 'fishing' it out of a pool of genetic templates that may only differ by a single SNP without cross reactivity (false positives). Although not published this same type of assay, taqMAMA, was designed and used for some of the SNP markers in chapter 3 to specifically detect the Ames strain.

The use of this method was extremely important to forensic investigators during the Amerithrax investigation. Because the goal of this case was to identify a suspect then trial that person using evidence collected during the investigation. In order for the evidence to hold up in court much forethought was put into the analysis of materials collected, especially in instances where there was no *B. anthracis* that was culturable in environmental type samples. The risk of false positives was quite high using traditional PCR- based detection assays because of their ability to cross-react with the DNA found in close relatives of *B. anthracis* in the *Bacillus cereus* group, a very common environmental bacterial group. This method allowed very sensitive detection of B. anthracis even the presence of near neighbors and extracts that could inhibit or create false positives using PCR.

Where this method really differs from most real-time PCR assays used for detecting pathogens is that it uses a small yet significant marker that has true biological significance for this pathogen (Easterday *et al*

2005, Mignot *et al* 2001). In contrast up to this point many markers were either developed by using genes that were assumed to be unique or by BLAST at a time when the genetic databases were quite small, to identify unique regions in the pathogen of interest. After designing makers in these unique regions they were often tested against a variety of lab type strains within the species of interest and among other species.

Chapter 3 'Strain-specific single-nucleotide polymorphism assays for the *Bacillus anthracis* Ames strain' demonstrates the ability to rapidly detect a specific strain of *B. anthracis*, in this case Ames, the classic laboratory and infamous 2001 letter attack strain, by using SNPs and the dramatic advantages this approach allows. Any one of these 6 SNPs are quite specific to Ames. In fact 5 of the 6 SNPs can differentiate Ames from the known diversity of this pathogen including its closest genetic relatives isolated from neighboring counties in Texas, USA. Most importantly this chapter illustrates the dramatic advantages this approach allows. This is similar to chapter 1 in the sense that the SNPs define specific lineages, although they differ from one another as the plcR SNP is most basal in *B. anthracis* phylogeny, whereas the Ames SNPs are much more derived in recent evolutionary history. This approach that utilizes SNPs can be used to define species, clades or isolates is continued in the following chapter.

This method as well as many of these others were developed from the combination of demand and curiosity. There was a specific need for these types of tools to aid in the Amerithrax investigation. Typically it would take days to DNA fingerprint a hundred samples to forensic standards. In contrast with this method that combined smarter markers with faster scoring methods an investigator could now identify process thousands samples to identify the presence of the Ames strain to forensic standards in a single day.

Chapter 4 is the most complete description of the world population of *B. anthracis* in the literature to date. 'Global Genetic Population Structure of *Bacillus anthracis*' is a description of genetic groups and types as defined by SNPs and MLVA. The geographic distribution of many of these groups has revealed trends of occurrence for anthrax. This in turn has led to further more focused investigations into the dispersal of *B. anthracis* such as chapter 6, as well as Kenefic et al's research into the origins of anthrax in North America (Kenefic *et al* 2009). Despite being a highly monomorphic species, the evolutionary history of *B. anthracis* proves to be interesting as it is a good example to understand the evolution of pathogens routinely going through population bottlenecks (Handel *et al* 2008) and how they spread and evolve in the absence of horizontal gene transfer and genetic recombination which is common in many other pathogens, *Burkholderia spp.* and *Bartonella spp.*

Many of these *B. anthracis* strains were provided by Dr. Hugh-Jones who has spent a life time amassing this collection.

Chapters 5 and 6 are more focused reviews of anthrax in two Asian countries, Kazakhstan and China respectively. Kazakhstan is a unique data set, and in fact is the first genetic description of naturally occurring *B. anthracis* strains from any part of the former Soviet Union: 'Historical Distribution and Molecular Diversity of *Bacillus anthracis* in Kazakhstan,' Chapter 6, '*Bacillus anthracis* in China and its relationship to worldwide lineages' describes interesting trends of diversity within China and the relationship between some Chinese strains with North American strains. These trends were first recognized by M. Van Ert during the preparation of chapters 3 and 4.

Both Kazakhstan and China have problems with anthrax killing livestock and humans. In these countries where often much of a family's wealth is invested into their livestock, the untimely death of these animals can have huge impacts on the family. It is then decided whether to destroy the carcass and suffer the loss or try to salvage some of the wealth by butchering and selling the meat. Occasionally the animal dies from an infectious disease sometimes it is anthrax. This contaminated meat is then sold, becoming a public health problem. DNA fingerprinting tools are helpful to investigate outbreaks. Building baseline data such as in chapters 5 and 6 give investigators tools to find the source of infection and route of transmission.

Prior to these chapters, I present background on *B. anthracis* since understanding the challenges associated with molecular forensics and epidemiology of this pathogen requires a discussion of; 1) the role of *B. anthracis* as a bioweapon, 2) the ecology and evolution of the pathogen, 3) the identification of genomic variation and genetic markers between *B. anthracis* and genetic near-neighbors and within the species; and 4) the forensic considerations when leveraging assays and global genetic data for forensic applications. First, however, it is of benefit to examine the significance of *B. anthracis* as a bioweapon.

***B. anthracis* as a Biological Warfare Agent**

The communicability of disease has been known by humans for centuries and this knowledge has been leveraged to disseminate diseases creating morbidity, mortality and fear. Some of the first accounts of the use of biological weapons date back to 400 BC, when Assyrian archers used a blood/manure mixture on their arrows to promote wound infection. Even more notably, at the beginning of the Black Death in 1344, plague victims' bodies were catapulted into the besieged city of Caffa by the Tartars in an attempt to spread the Plague, caused by the bacterium *Yersinia pestis*. Eventually the besieged Genoese fled back to Italy bringing with them this disease and starting the Medieval Plague in Europe (Handysides 2009). In more recent history, after germ theory became fact and a working discipline, this type of warfare has been increasingly researched and refined.

In the 20th and 21st centuries, considerable state-sponsored research and funding has gone into selecting effective organisms for biological warfare and a diversity of bacterial and viral agents have been weaponized, including; *B. anthracis* (Anthrax), *Brucella spp.* (Brucellosis), *Fransicella tularensis* (Tularemia) and variola major (smallpox)

(Kortepeter *et al* 1999). Among the bacterial biothreat agents, *B. anthracis* represents a particularly attractive choice as a bioweapon for a number of reasons. The ease of cultivation and high virulence of *B. anthracis* likely contributes to its attractiveness as a weapon. However, the ability of the bacteria to form highly stable, environmentally resistant, infectious spores is a central reason for its weaponization by many countries during the 20th century including the Soviet Union, the U.S.A., Great Britain and Japan (Handysides 2009). The pathogen has gained further notoriety in recent history as a weapon of biological terrorism in Japan in 1993 (Kortepeter *et al* 1999) and the U.S.A. in the 2001 letter attacks (Inglesby *et al* 2002). It was the latter attack that spawned one of the largest and most expensive criminal investigations in U. S. history, and illustrated the real-world efficacy of *B. anthracis* as an agent of bioterror.

Not surprisingly, the fields of biosecurity and bioforensics grew immensely following the 2001 letter attacks as governments started pouring huge sums of money into the development of tools for pathogen detection and monitoring (Bohannon 2003). To focus regulatory and research efforts, a select group of disease agents that were thought to represent the greatest threat to the public were identified. These select agents were identified based on several criteria, including availability, ease of weaponizing, morbidity/mortality and persistence in the environment. In 2002 these select agents were divided into categories by the U.S. Center for Disease Control and Prevention (CDC) A, B and C, in the order of perceived threat; *B. anthracis* was classified as an A category pathogen at the top of the list.

Ecology and Evolution of *B. anthracis*

B. anthracis belongs to the *Bacillus cereus* group, which consists of three genetically and phenotypically similar species; *Bacillus cereus*, *Bacillus thuringiensis* and *B. anthracis*. The group is alike with all being gram positive, soil emanating, and spore-forming bacteria. Pathogenic members are found in all three species; *B. cereus* toxins are known to cause food poisoning (Granum *et al* 1997), *B. thuringiensis* is a known insect pathogen and *B. anthracis,* the causative agent of anthrax, is a mammalian disease that primarily infects herbivores. Despite the differences in pathogenesis, the core chromosome of the three species shows a high degree of genetic similarity (Helgason *et al* 2000) and among these three 'species' is likely a continuum of organisms found in the environment that span these gaps between defined species. Indeed environmental isolates have been described that genotype with one species and share a phenotype with another. For instance, an environmental isolate was found that is genetically and phenotypically more like *B. cereus*, yet was capable of producing anthrax-like pneumonia using many of the same virulence factors (Hoffmaster *et al* 2004). The existence of these previously unknown near neighbors present unique problems and complicate the design of genetic-based species detection assays.

B. anthracis is generally considered an obligate pathogen since evidence of common soil propagation remains scarce (Hugh-Jones *et al* 2009). As a result, understanding its transmission dynamics is critical for understanding its evolution. Anthrax has three clinical manifestations: cutaneous, caused by infection through a break in the epidermis; pulmonary, inhalation of spores into the lungs; and gastrointestinal caused by ingestion of spores. It is the latter, gastrointestinal route which is typical of anthrax transmission in wildlife. In this case, herbivores ingest spores which, aided by internal abrasions, are phagocytized by macrophages in the mucosa and transferred to lymph nodes where the spore germinates into a vegetative cell and a subsequent systemic infection proliferates. [In gastrointestinal anthrax, if the spore is not taken in by this process it will not germinate and will be passed through the feces (Hugh-Jones 2010).] Following infection, spores germinate and undergo rapid proliferation killing the host; sporulation begins, as decay, aided by scavengers reintroduces the pathogen back into the soil.

Importantly, the transmission cycle of *B. anthracis* slows the genome's evolution relative to other pathogens as there is a brief period of infection and replication, through which mutations can occur, is followed by long periods of dormancy, potentially for decades (Graham-Smith 1941)during which time genetic mutations are paused. This 'stop' for long periods (years) and 'go' for short periods (days) greatly reduces the number of generations from its first emergence as a pathogen to the present. Here the number of generations is relatively low compared to other bacteria that exhibit continual growth and replication. For instance *Escherichia coli* is estimated to undergo 300 generations per year (Guttman *et al* 1994), whereas *B. anthracis* is estimated at a magnitude less with only 20 to 40 generations per year . The small number of generations greatly reduces the number of genetic mutations among members within the population.

Although mutation is likely the primary diversifying force in *B. anthracis*, selection, drift and recombination may all potentially affect allelic distributions in *B. anthracis* (Keim et al 2004). For example, the manifestations of the disease likely exert a distinct evolutionary selective pressure on the virulence of this pathogen. In the anthrax cycle, spores persist in the soil until they are ingested, inhaled or come into contact (through skin lesions) with a host and cause their respected pathology as described above. In lieu of an unknown alternate path in the transmission cycle or long-term chronic infection (which there is no evidence for in the literature), failure to cause mortality from any form of infection becomes a dead end for the pathogen. Specifically strains that infect and are unable to cause mortality of the host will not be selected for and will be literally aberuncated from the population. In all the manifestations of the disease the core mechanism of transmission to a host is through the soil, this transmission step to the soil is only accomplished by killing the host.

Necessity of mortality of the host in the *B. anthracis* transmission cycle creates evolutionary pressure to maintain or increase virulence and as long as there is not a trade-off in the transmission (spore) phase (Moran 2002). The selective mechanism behind maintenance or increase in virulence is sheer numbers. If mutations arise creating faster division in a certain subpopulation within a host, those strains in greater numbers should eventually dominate the population through many generations (Levin *et al* 1994). Although *B. anthracis* may already be quite optimized to this habitat within the host which if true may act as a constraint on an already optimized genome and its expression allowing little divergence from this fit genotype/phenotype, preserving the genetic homogeneity of this species.

As a result of the transmission cycle, and potentially other processes, there exists very little molecular variation among globally, geographically widespread *B. anthracis* isolates. It is because of the low levels of intra-species genetic diversity that *B. anthracis* is generally considered a 'recently emerged pathogen'; although the ecology of the pathogen, and the stochastic nature of the spore phase, complicates molecular clock determinations (see chapter 4). The monomorphic nature of the *B. anthracis* genome and its extremely close genetic relationship with its environmentally common near-neighbors complicates efforts to develop molecular tools for its precise identification. However, use of genomic and evolutionary analyses was used to develop species and strain specific assays for *B. anthracis*.

Genomics of *B. anthracis* and Genetic Markers

The use of new genetic tools for pathogen work, in many ways, greatly surpasses the traditional 'gold standards' of classical microbiology. Frequently prior to 2001 and to some extent now, *B. anthracis* identification (now confirmation) is accomplished through classical microbiological methods; using techniques to isolate and phenotype the microbe biochemically and morphologically. This process is laborious, time consuming and potentially yields inconclusive results; it also suffers from a limited number of diagnostics. In the case of forensic science, human genetic data have been used extensively to attribute crimes to perpetrators (Pena *et al* 1993). However these types of data had not been used to their potential in microbial forensics prior the 2001 attacks and the co-occurring genomics revolution, when there was a push to use genetics to identify and subtype pathogens, here *B. anthracis*, in forensic and clinical settings (Popovic *et al* 2003, Swaminathan *et al* 2001).

To meet the needs of forensic and epidemiological investigations of anthrax outbreaks, new genetic markers and tools needed to be developed for more definitive and precise identification. Having these types of tools in place in the event of an intentional release can produce key data for investigators. For example, in the circumstance of a release the speed and throughput of identifying a pathogen and mobilizing the appropriate response could have a significant effect on number of deaths within a population. In criminal justice the use of these tools give investigators the ability to rapidly include and exclude biological material and, by association, suspects.

Prior to the anthrax attacks, certain Polymerase Chain Reaction (PCR) – based methods were already available for the identification of *B. anthracis* as well as resolving genetic groups within the species (Keim *et al* 2000, Qi *et al* 2001). However the existing research methods suffered from limits in through-put and strain discrimination, limiting the application to the epidemiological and forensic investigation. The 2001 anthrax letters forced a change in the development and use of these systems from research applications to investigative applications and is the focus of this thesis: developing high-throughput, trace-level detection of pathogens used in biocrimes; smart tools and markers to detect specific pathogens and specific strains, in this case for the pathogen *B. anthracis*.

Although mutations are rare within *B. anthracis*, examination of genomic sequences permitted the identification of Variable Number of Tandem Repeat loci. These were some of the first polymorphisms found between isolates of *B. anthracis* and were the first tools to give insight into the genetic and geographic history of the pathogen (Keim *et al* 2000). Afterwards, more exhaustive, comparative genomic surveys, allowed for the discovery of Single Nucleotide Polymorphisms (SNPs) for the identification of the species, as well as clonal groups or even a specific strains within the species.

To effectively find SNPs a phylogeny was built using a 15 marker Multi-Locus Variable Number of Tandem Repeat Analysis (MLVA) system. This method was applied to DNA 'fingerprint' over one thousand geographically diverse isolates of *B. anthracis*. An evolutionary hypothesis was constructed with Unweighted Pair Group Method with Arithmetic Mean (UPGMA), using these markers (chapter 4). From this phylogeny a total of five genetically diverse isolates were selected for whole genome sequencing which includes the Ames strain. A comparison between these genomes revealed around 3500 SNPs among these strains. These SNPs were then screened against a diverse set of 27 isolates that were representative of *B. anthracis* phylogeny. SNPs were then mapped on a phylogeny (Pearson *et al* 2004) and SNPs that defined major clonal lineages were identified. Twelve SNPs were used as binary markers to define subgroups within the species (figure 1) and real-time assays were designed to these markers to screen a large population of 1000+ globally diverse isolates.

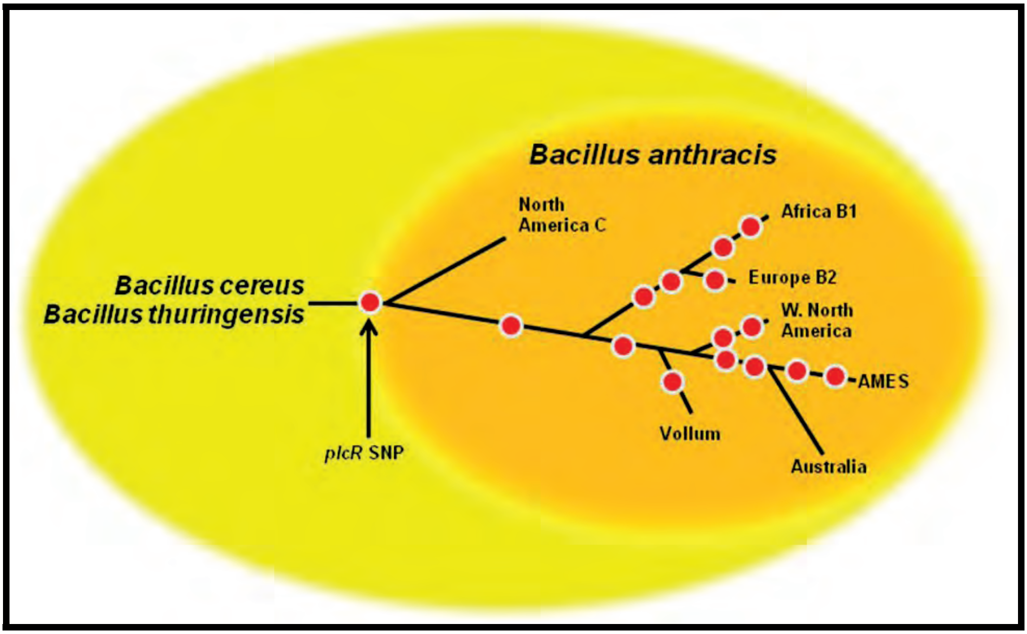


Figure 1 Representation of the location of Canonical SNPs on a simple *B. anthracis* phylogeny based upon seven diverse strains. The *plcR* SNP (nonsense mutation) occurs between *B. anthracis* species and other members of the *B. cereus* group. Also, two Ames strain specific SNPs are shown at the Ames terminus.

Genetic tools, assays and databases for forensics

In contrast to a natural outbreak, where the public health sector responds to and manages the outbreak, the bioterrorist attack of 2001 required the participation of both public health and law enforcement agencies. The amount of work in biodefense that needed to be accomplished to have information gathering systems in place was quite large, especially for the detection and fingerprinting *B. anthracis* and other pathogens. Hence a symbiosis has been formed between law enforcement and the public health sector and joint efforts between the two disciplines to structure research into developing new and more efficient tools is underway (Goodman *et al* 2003).

The 2001 anthrax letter attacks exposed the gaps in forensic capabilities for the specific detection and identification of *B. anthracis* and other pathogens. Furthermore there was no established standard method or protocol for strain identification and the need for precise, sensitive, high-throughput tools for the identification of *B. anthracis* strains became apparent. In the 2001 Amerithrax case, the strain of interest was Ames, and the tools to specifically identify this strain for the purposes of including and excluding evidence required development and validation. Major challenges in examining evidence arise from inherent limitations of the PCR method. Even so the benefits of using PCR outweigh the limitations of this method. Although other PCR based methods are used for the identification of genetic markers (Van Ert *et al* 2004), here for the detection of SNPs we specifically used real-time PCR.

Some of these limitations are intrinsic to the method itself including inhibition of PCR due to environmental contaminates, such as humic acids (Tebbe *et al* 1993) and the limit of detection which is the lowest copy number of a given template yielding a positive result (figure 2). Similarly, yet not inherent to PCR, are the problems which may arise where sequences which have a similar composition cross-react with the primers and probes of the assay used. Given the right conditions this can yield false positives and may occur in the negative controls if a combination of cycling conditions, chemistry and the design of the oligos allow.

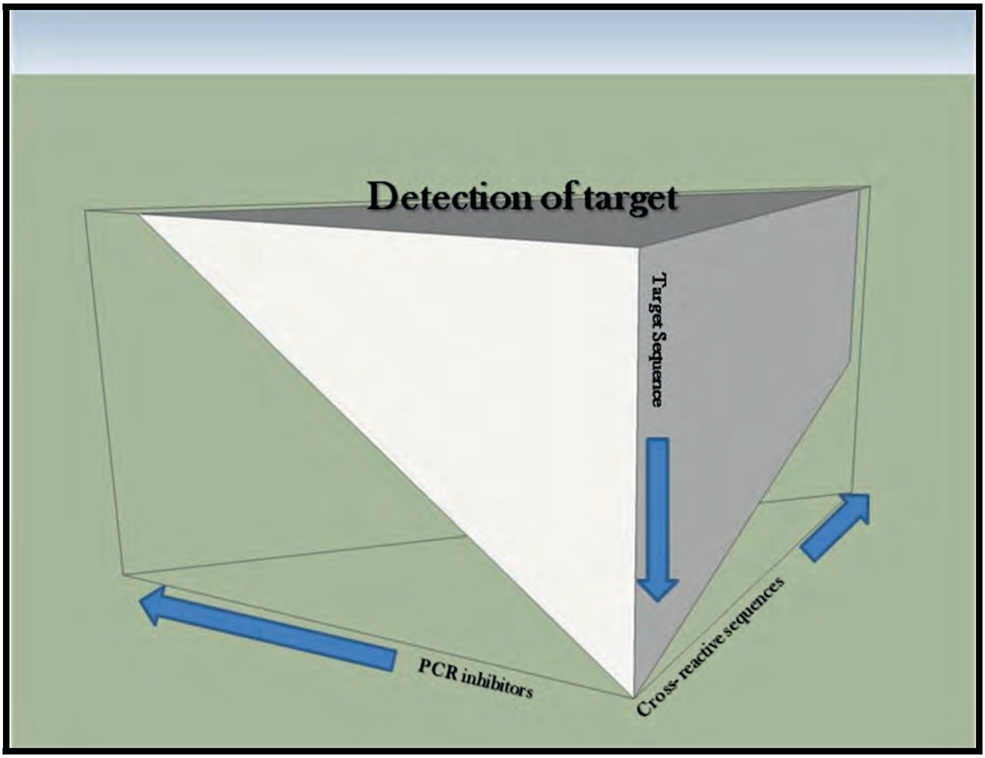


Figure 2 Representation of the parameters of detection for a pathogen signature in an environmental sample. As the amount of target DNA sequence decreases, the larger the impact that PCR inhibitors have on any particular assay to the threshold of causing false negatives. The cross-reactive sequences also can cause false results. As the amount of cross-reactive sequence is increased within a sample the murkier the results may become, to the point of creating false positives or negatives.

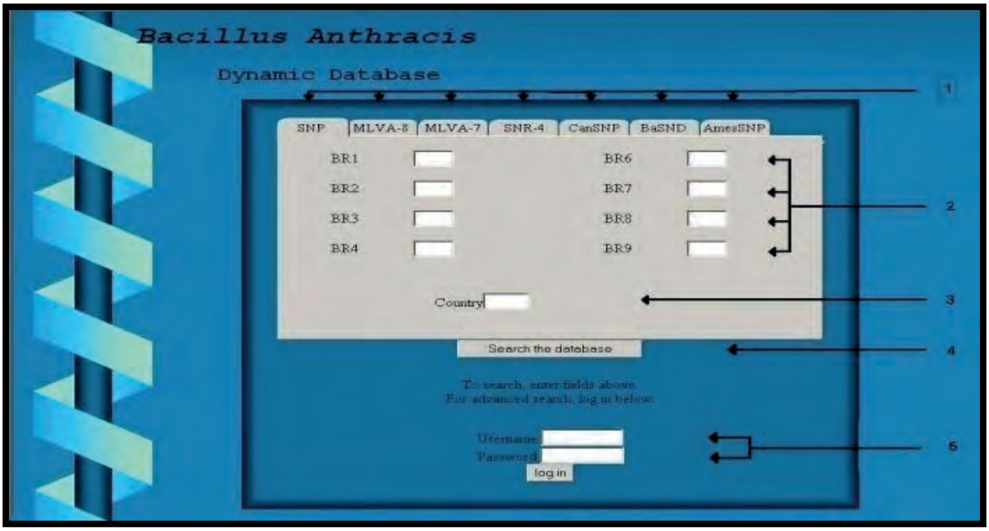
Although academic laboratories are known for pioneering research in molecular biology, many researchers have developed ritual habits that are based on taught and or learned procedures from incorrect interpretation of data due to a lack of appropriate controls. In practice many researchers will throw out data after 40 cycles of this process, real-time PCR, because the validity is in question due to the de novo fabrication of PCR products which allow for the binding and cleavage of probes creating a fluorescent signal in controls (personal correspondents). This is the case when one or more parameters are not optimized to prevent these false positives and where appropriate controls were not run creating inaccurate conclusions or ambiguous results. In the case of the research herein tightly controlled experiments were run with exhaustive controls to nullify this notion.

When samples are taken as evidence for forensic analyses the quality cannot always be controlled for. There is the possibility for a range of quality when it comes to samples taken as evidence. Obviously the first step is to attempt to culture *B. anthracis* when isolated and grown creates a situation where the resource is not finite. Yet culturing the organism is often not possible. In these instances confirmation for the presence of the organism relies on genetic methods. Here the danger of cross-reactive sequences in any sample is always possible and can be likely.

Genetic/Geographic Databases

One of the most important tools in epidemiology and criminal justice are databases. Databases can be used to quickly query data once it is gathered. The likeness of the match, if not perfect, enables investigators to focus and structure their investigation to more likely sources while excluding or lowering the priority of less like matches. Genetic, spatial and temporal data allow epidemiologists and investigators to attribute a particular case to a likely source.

For instance in Hong Kong 2003 Cheung et al. documents a fatal case of anthrax in a boy. Anthrax in Hong Kong is an extremely rare disease, with only three cases in the last 20 years (Cheung *et al* 2005). Their ability to closely genetically match the isolate to other isolates from Guangxi an adjacent province enabled the epidemiologists to attribute the bacterium’s presence to probable contamination of a food product.



To search, enter fields above.
For advanced search, log in below.

Figure 3 A screenshot from the '*Bacillus anthracis* Dynamic Database.' The database allows the user to enter any combination of genetic data [1, 2] (SNPs, MLVA, and single nucleotide repeats) and spatial data [3] and query these data [4]. The information within the database is secured with registered usernames and passwords [6]; each database user is given a specific level of access.

These types of investigations became possible for the following reasons. The first was amassing a large enough collection of isolates to represent the genetic diversity in a global (spatial) context. The second was developing genetic tools that create a fine enough resolution of the isolates which allows discrimination of similar isolates from adjacent locations. These data then need to be collected into a database. The database should be intuitive and easy to query (figure 3). This allows the user to query specific data and have specific clear data returned. In turn it helps guide investigators to a probable source of the pathogen. Despite having a database some of the biggest challenges in these types of investigations are actually capturing these data. The following chapters present the data and the methods to access these data, even from some of the most challenging samples.

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Chapter 1

Use of Single Nucleotide Polymorphisms in the plcR Gene for Specific Identification of *Bacillus anthracis.*

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Chapter 2

Specific detection of *Bacillus anthracis* using a TaqMan® mismatch amplification mutation assay.

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Specific detection of *Bacillus anthracis* using a TaqMan® mismatch amplification mutation assay

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Single nucleotide polymorphisms (SNPs) are increasingly recognized as important diagnostic markers for the detection and differentiation of Bacillus anthracis. *The use of SNP markers for identifying* B. anthracis *DNA in environmental samples containing genetically similar bacteria requires the ability to amplify and detect DNA with single nucleotide specificity. We designed a TaqMan® mismatch amplification mutation assay (TaqMAMA) around a SNP in the* plcR *gene of* B. anthracis. *The assay permits specific, low-level detection (25 fg DNA) of this* B. anthracis*-specific SNP, even in the presence of environmental DNA extracts containing a 20,000-fold excess of the alternate allele. We anticipate that the ability to selectively amplify and detect low copy number DNAs with single nucleotide specificity will represent a valuable tool in the arena of biodefense and microbial forensics.*

INTRODUCTION

The 2001 anthrax letter attacks resulted in five fatalities, cost several billion dollars to the U.S. economy, and illustrated the practical efficacy of this pathogen (*Bacillus anthracis*) as a bioterrorism agent. Routine monitoring and genetic testing of environmental samples for *B. anthracis* nucleic acids is critical for early detection and response in the event of a bioterrorist attack and for subsequent forensic investigations. Yet, molecular detection of *B. anthracis* in the environment is particularly challenging, since samples may contain complex mixtures of DNA signatures, including genetically similar innocuous organisms. In particular, the genetic similarity of *B. anthracis* to other common spore-forming soil bacteria, such as *Bacillus cereus* and *Bacillus thuringiensis*, has presented challenges to identifying *B. anthracis*-specific chromosomal targets (1).

Single nucleotide polymorphisms (SNPs) are valuable markers for the detection and characterization of *B. anthracis* (1–5). For instance, Hurtle et al. (1) demonstrated that a SNP in the *gyrA* gene was a possible *B. anthracis*-specific chromosomal marker. A TaqMan® minor groove-binding assay designed around the SNP sensitively detected 43 *B. anthracis* strains and did not cross-react with 36 *B. cereus* and 13 *B. thuringiensis* strains. In another example, a single nucleotide nonsense mutation has been identified in the *plcR* gene of *B. anthracis* that is absent in panels of *B. thuringiensis* and *B. cereus* isolates (5–7). Recently, we demonstrated that this nonsense mutation in the *plcR* gene was ubiquitous in 89 globally and genetically diverse *B. anthracis* isolates and is absent from *B. cereus* and *B. thuringiensis* isolates that are known to be genetic near-neighbors of *B. anthracis* (7). Given this, the single nucleotide change in the *plcR* gene represents a promising target for the specific detection of *B. anthracis*.

From a biodefense and bioforensic perspective, using SNP markers for biological threat agent identification should require clear detection of the targeted SNP sequence in the presence of a background of DNA containing the alternate allele. A recent advance in the area of SNP-specific detection was the combination of the fluorogenic 5′ nuclease PCR (TaqMan) and the mismatch amplification mutation assay (MAMA) (8,9). Developed by Glaab and Skopek (9), this novel approach, termed TaqMAMA, utilizes the MAMA to exploit mismatched bases between the PCR primers and the targeted SNP template to support allele-specific amplification, while TaqMan fluorescence allows monitoring of amplification in a real-time, quantitative manner. A powerful attribute of this technique is that it allows for the amplification of low-copy templates with a single nucleotide change in 1000-fold excess of wild-type DNA (9). Here we demonstrate the use of TaqMAMA to selectively amplify and detect *B. anthracis*-specific DNA in the presence of complex mixtures of DNA signatures, including signatures from genetically similar near-neighbors.

MATERIALS AND METHODS

TaqMAMA Primer and Probe Design

Sequences flanking the *plcR* nonsense mutation were obtained either from GenBank® or from sequencing efforts in our laboratory (7) (Figure 1) and were aligned using DNASTAR SeqMan™ II software (DNASTAR, Madison, WI, USA). To promote *B. anthracis* specific amplification, the MAMA primer was designed so that the ultimate 3′ base was complementary to the *plcR* nonsense mutation. The penultimate 3′ base was designed to mismatch with the shared sequence between *B. anthracis* and the near-neighbors sequences (9). The TaqMan minor groove-binding probe and the reverse primer were designed using Primer Express® software (Applied Biosystems, Foster City, CA, USA) to exploit additional nucleotide differences between *B. anthracis* and near-neighbor sequences that were proximal to the nonsense mutation (Figure 1).

TaqMAMA PCR Protocol

In all experiments, TaqMAMA PCR was conducted in 10-µL reactions that contained 600 nM both forward (5′-GAGTTTGATGTGAAGGTGAGACATAATC-3′) and reverse (5′-TTTGCATGACAAAGCGCTAA-3′) primers (see Figure 1 for sequences), 250 nM probe (5′-6FAM-TACTTGGACAATCAA-MGB-3′), 1× Platinum®

qPCR SuperMix-UDG (5 mM $MgCl_2$; Invitrogen, Carlsbad, CA, USA), and 1 µL template. Thermal cycling was performed on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) with the following conditions: 50°C for 2 min, 95°C for 2 min, and 50 cycles of 95°C for 15 s, 60°C for 1 min.

Specificity Tests

To initially test the specificity of the assay, 10 pg DNA from the *B. anthracis* Ames strain and 29 genetic near-neighbors (see Figure 1 for strains and sequence information) were used as templates in the assay. We also performed PCR on genomic DNA from *B. cereus* strain 3A and *B. thuringiensis* strain HD1011 at 10 pg, 100 pg, 500 pg, and 1 ng input. We performed 32 replicates of each DNA input level for each strain, with the exception of HD1011 at 500 pg input, which we tested at 64 replicates (288 reactions total).

Sensitivity Tests

Sensitivity was determined by analyzing serial 10-fold dilutions of genomic DNA from the *B. anthracis* Ames strain ranging from 250 pg to 25 fg. To determine assay sensitivity in nontarget DNA backgrounds, we performed analysis on the same *B. anthracis* template ranges in the presence of 50 or 500 pg DNA from the *B. thuringiensis* strain HD1011, air filter DNA extracts from a Biodefense monitoring program, and a combination of air filter DNA extracts and HD1011 DNA (50 or 500 pg).

RESULTS AND DISCUSSION

The TaqMAMA *plcR* assay is designed to amplify this gene fragment in *B. anthracis* but not in closely related isolates of *B. cereus* and *B. thuringiensis* (Figure 1). We found that the assay amplified 10 pg *B. anthracis* DNA but did not amplify any of the 10 pg near-neighbor DNA templates after 50 thermal cycles (Figure 2A). In contrast, the same samples analyzed using a traditional single probe TaqMan minor groove-binding assay (probe recognizes *B. anthracis* SNP allele) showed low-level cross-reactivity with near-neighbors in sequence group 3 [see Figure 1, mean cycle threshold (C_t) = 34.27]. To evaluate if the TaqMAMA would cross-react with higher levels of near-neighbor DNA templates that differed by only the *plcR* nonsense mutation, we performed extensive PCR analysis of genomic DNA from *B. thuringiensis* HD1011 and *B. cereus* 3A. We tested 64 replicates of these near-neighbor templates at 10 pg, 100 pg, 500 pg, and 1 ng per PCR. The assay did not amplify any of the near-neighbor templates at 10 or100 pg. However, 2 of the 96 replicates at 500 pg input (*B. cereus* 3A) and one at 1.0 ng (*B. cereus* 3A) exhibited weak amplification after 40 amplification cycles (mean C_t = 42.9, C_t range = 41.6–45.0).

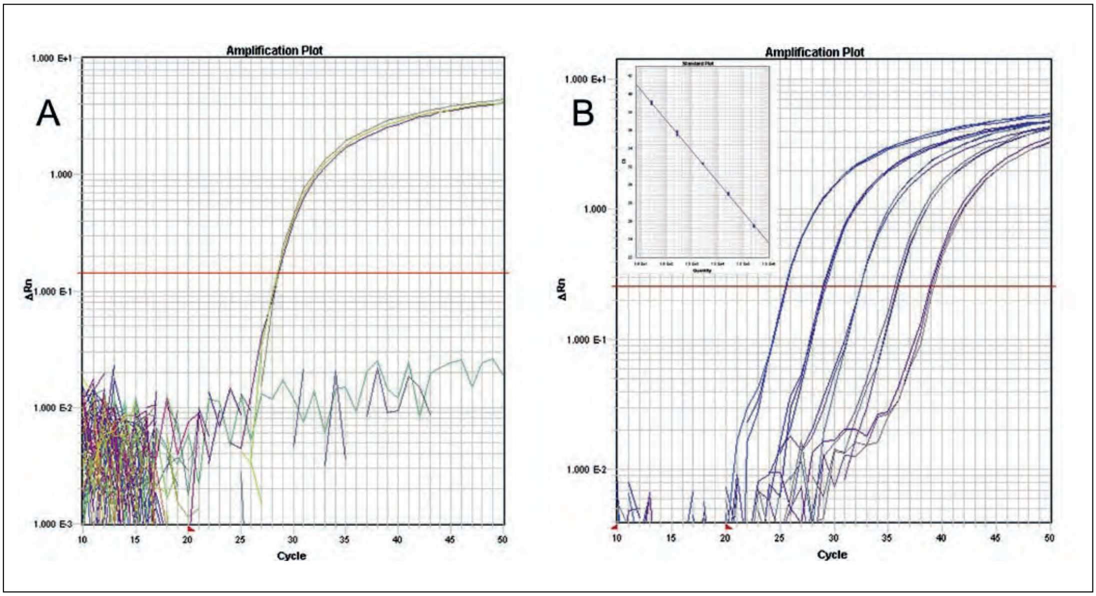
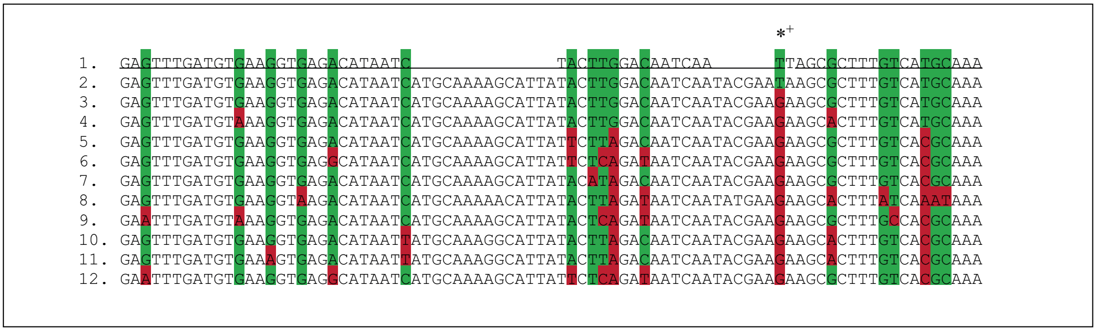


Figure 2. Real-time plots indicating the specificity and sensitivity of the *plcR* TaqMan mismatch amplification mutation assay (TaqMAMA). (A) Results of triplicate analysis of 10 pg DNA from *Bacillus anthracis* and 29 near-neighbors. The 29 near-neighbors include the 17 listed in Figure 1 and the following isolates: *Bacillus cereus* strains ATCC 4342, ATCC 14579, F3-27, F3350/87, S2-4; and *Bacillus thuringiensis* strains HD1015, HD681, HD288, HD526, HD974, HD30, and HD50. *B. anthracis* DNA amplified at an average cycle threshold (C_t) of 28.46, whereas the near-neighbor templates did not exhibit amplification. Additional information on strains is presented in Hill et al. (11). (B) Results of TaqMAMA PCR analysis of 10-fold serial dilutions of *B. anthracis* DNA (triplicate samples illustrated). The average C_t values (triplicate analysis) in molecular grade water were as follows: 250 pg, 25.5; 25 pg, 29.0; 2.5 pg, 32.4; 250 fg, 35.7; and 25 fg, 39.0. Insert in the upper left-hand corner is the standard curve plot; R^2 = 0.9995, slope = -3.376.



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                                                                        *+
1.  GAGTTTGATGTGAAGGTGAGACATAATC              TACTTGGACAATCAA      TTAGCGCTTTGTCATGCAAA
2.  GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATACTTGGACAATCAATACGAATAAGCGCTTTGTCATGCAAA
3.  GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATACTTGGACAATCAATACGAAGAAGCGCTTTGTCATGCAAA
4.  GAGTTTGATGTAAAGGTGAGACATAATCATGCAAAAGCATTATACTTGGACAATCAATACGAAGAAGCACTTTGTCATGCAAA
5.  GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATTCTTAGACAATCAATACGAAGAAGCGCTTTGTCACGCAAA
6.  GAGTTTGATGTGAAGGTGAGGCATAATCATGCAAAAGCATTATTCTCAGATAATCAATACGAAGAAGCGCTTTGTCACGCAAA
7.  GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATACATAGACAATCAATACGAAGAAGCGCTTTGTCACGCAAA
8.  GAGTTTGATGTGAAGGTAAGACATAATCATGCAAAAACATTATACTTAGATAATCAATATGAAGAAGCACTTTATCAAATAAA
9.  GAATTTGATGTAAAGGTGAGACATAATCATGCAAAAGCATTATACTCAGATAATCAATACGAAGAAGCGCTTTGCCACGCAAA
10. GAGTTTGATGTGAAGGTGAGACATAATTATGCAAAGGCATTATACTTAGACAATCAATACGAAGAAGCACTTTGTCACGCAAA
11. GAGTTTGATGTGAAAGTGAGACATAATTATGCAAAGGCATTATACTTAGACAATCAATACGAAGAAGCACTTTGTCACGCAAA
12. GAATTTGATGTGAAGGTGAGGCATAATCATGCAAAAGCATTATTCTCAGATAATCAATACGAAGAAGCGCTTTGTCACGCAAA
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Figure 1. Sequence alignment of *plcR* gene fragments from *Bacillus anthracis* and genetic near-neighbors. The numbered lines indicate the sequences of: (1) primer and probe locations; (2) *B. anthracis*; (3) *Bacillus cereus* 3A, S2-8, and *Bacillus thuringiensis* HD1011; (4) *B. cereus* D5; (5) *B. cereus* AH527; (6) *B. cereus* D17, *B. thuringiensis* HD571, HD44; (7) *B. cereus* F3502/72, R6; (8) *B. cereus* F2-1; (9) *B. cereus* R4, ATCC 33018, *B. thuringiensis* HD1012; (10) *B. thuringiensis* 97-27; (11) *Bacillus* spp. TET 2b-3; and (12) *B. thuringiensis* HD682. Additional information on strains is presented in Hill et al. (11). Colored shading indicates polymorphic sites that are targeted in this assay. Red shading indicates nucleotide differences between near-neighbors and *B. anthracis*. *, *plcR* nonsense mutation; $^+$, penultimate 3′ mismatch.

Although these results indicate exceptional specificity of the assay, the rare low-level cross-reactivity with *B. cereus* 3A at 1.0 ng and 500 pg indicates that a weak false positive result could occur in certain instances. In our estimation, this scenario is unlikely to occur during routine environmental monitoring, in that it requires fairly high concentrations of a template that differs by only a single nucleotide. *B. cereus* 3A and *B. thuringiensis* HD1011 isolates were selected for specificity testing, since they shared identical sequence in the

Table 1. Average Cycle Threshold Values for Sensitivity Test[a]

***Bacillus anthracis* Template**	**Experiment[b]**	**Average C_t (C_t - C_t of control sample)**			
		Control Sample (no background)	**HD1011 DNA**	**Air Filter Extract[c]**	**Air Filter Extract and HD1011 DNA**
250 pg	Exp1	24.9	24.4 (-0.5)	26.2 (+1.3)	26.4 (+1.5)
	2	25.7	26.9 (+1.2)	26.8 (+1.1)	27.5 (+2.2)
25 pg	Exp1	28.2	28.2 (0.0)	29.5 (+1.3)	28.9 (+0.7)
	2	28.8	30.0 (+1.2)	29.8 (+1.0)	31.6 (+2.8)
2.5 pg	Exp1	31.0	31.4 (+0.4)	32.8 (+1.8)	33.6 (+2.6)
	2	31.9	32.3 (+0.4)	32.6 (+0.7)	34.1 (+2.2)
250 fg	Exp1	34.6	35.0 (+0.4)	36.4 (+2.2)	35.8 (+1.2)
	2	34.2	35.4 (+1.2)	35.4 (+1.2)	35.3 (+1.1)
25 fg[d]	Exp1	40.4[e]	40.5[f] (+0.1)	40.7[h] (+0.3)	41.1[f] (+0.7)
	2	40.6[f]	41.7[g] (+1.1)	42.3[h] (+1.7)	40.9[f] (+0.3)

[a] Cycle threshold (C_t) values are an average of duplicate reactions unless otherwise noted.
[b] Experiment: Exp1 HD1011 DNA background = 50 pg/PCR; Exp2 HD1011 DNA background = 500 pg/PCR.
[c] 1.0 µL air filter DNA extract spiked into PCR.
[d] All 25 fg samples were replicated 10 times, the C_ts from samples that amplified were averaged.
[e] Of the 10 replicates, 7 amplified.
[f] Of the 10 replicates, 9 amplified.
[g] Of the 10 replicates, 10 amplified.
[h] Of the 10 replicates, 8 amplified.

plcR gene fragment with *B. anthracis*, with the exception of the single nonsense mutation. In our sequence analysis of the *plcR* gene fragment of near-neighbors, the majority of isolates (26 of 29) had additional mutations in the sequence fragment that is targeted by the assay. We have not observed the *plcR* assay to cross-react with these isolates at 1 ng input levels (data not shown). Therefore, analyzing 500 pg to 1 ng genomic DNA from *B. cereus* 3A and *B. thuringiensis* HD1011, which we estimate corresponds to approximately 100,000–200,000 genome equivalents, represents a rare worse case scenario. Despite this, we suggest that weak positive results in the TaqMAMA could be rapidly investigated by using a dual-probe system (7) that detects the alternate SNP state. This control would differentiate between a very infrequent misamplification of a near-neighbor or the legitimate detection of very low levels of the real target. Additional experiments are underway to investigate factors affecting assay specificity.

The TaqMAMA exhibited quantitative detection of the *B. anthracis* template over a dynamic range (Figure 2B) and permitted sensitive detection in PCRs containing different backgrounds. We compared the sensitivity of the TaqMAMA in PCRs containing: (*i*) no background; (*ii*) 50 or 500 pg of *B. thuringenesis* HD1011 genomic DNA; (*iii*) air filter DNA extracts; and (*iv*) a combination 50 or 500 pg of *B. thuringenesis* HD1011 genomic DNA and air filter extracts (Table 1). We used HD1011 genomic DNA in these experiments since we did not observe any cross-reactivity at any concentration (see above) in these templates despite extensive PCR analysis. As illustrated in Table 1, the average C_t values were similar among treatments, although we did consistently measure a slight increase in C_ts in samples containing 500 pg HD1011 background and/or air filter extracts, suggesting weak inhibition of the reaction. This inhibition was not alleviated by the addition of 400 ng/µL bovine serum albumin (BSA) to the PCR (Reference 10 and data not shown). Interestingly, this apparent inhibition did not impact the threshold of detection of the assay, which permitted routine detection of the *B. anthracis*-specific SNP at template levels as low as 25 fg, even in the presence of air filter extracts containing a 20,000-fold excess of DNA containing the alternate allele (Table 1). It is important to note that the air filter extracts did not cross-react with the assay or impact the specificity of the assay as illustrated by the lack of amplification in air filter extracts and air filter extracts spiked with 100 or 500 pg HD1011 template (data not shown).

This *B. anthracis* detection assay represents a significant advance in rapid and specific detection of *B. anthracis* for several reasons. First, the assay is based upon a well-studied chromosomal marker that is present in *B. anthracis* but absent in genetic near-neighbors. The presence of the *plcR* nonsense mutation in globally and genetically diverse *B. anthracis* lineages (7) limits the likelihood of false negative results, whereas the absence of the mutation in *B. cereus* and *B. thuringiensis* strains (5,7) and the specificity of the reaction decreases the probability of false positive results. Second, the sensitivity and specificity of the assay permits low-level detection of the targeted SNP, even in the presence of environmental DNA extracts containing 20,000-fold excess of the alternate allele. Third, the assay is amenable to high-throughput real-time PCR platforms that are currently the mainstay of homeland defense initiatives, such as Biowatch.

In conclusion, the use of the TaqMAMA method to detect the *plcR* mutation in *B. anthracis* permits the specific, low-level detection of this biological threat agent in samples containing environmental extracts and/or genetic near-neighbor DNA. We anticipate that this ability to selectively amplify and detect low copy number biothreat agent DNAs with single nucleotide specificity will represent a valuable tool in the arena of biodefense and bioforensics.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Chapter 3

Strain-specific single-nucleotide polymorphism assays for the *Bacillus anthracis* Ames strain.

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Chapter 4

Global Genetic Population Structure of *Bacillus anthracis*.

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Global Genetic Population Structure of *Bacillus anthracis*

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Anthrax, caused by the bacterium *Bacillus anthracis*, is a disease of historical and current importance that is found throughout the world. The basis of its historical transmission is anecdotal and its true global population structure has remained largely cryptic. Seven diverse *B. anthracis* strains were whole-genome sequenced to identify rare single nucleotide polymorphisms (SNPs), followed by phylogenetic reconstruction of these characters onto an evolutionary model. This analysis identified SNPs that define the major clonal lineages within the species. These SNPs, in concert with 15 variable number tandem repeat (VNTR) markers, were used to subtype a collection of 1,033 *B. anthracis* isolates from 42 countries to create an extensive genotype data set. These analyses subdivided the isolates into three previously recognized major lineages (A, B, and C), with further subdivision into 12 clonal sub-lineages or sub-groups and, finally, 221 unique MLVA15 genotypes. This rare genomic variation was used to document the evolutionary progression of *B. anthracis* and to establish global patterns of diversity. Isolates in the A lineage are widely dispersed globally, whereas the B and C lineages occur on more restricted spatial scales. Molecular clock models based upon genome-wide synonymous substitutions indicate there was a massive radiation of the A lineage that occurred in the mid-Holocene (3,064–6,127 ybp). On more recent temporal scales, the global population structure of *B. anthracis* reflects colonial-era importation of specific genotypes from the Old World into the New World, as well as the repeated industrial importation of diverse genotypes into developed countries via spore-contaminated animal products. These findings indicate humans have played an important role in the evolution of anthrax by increasing the proliferation and dispersal of this now global disease. Finally, the value of global genotypic analysis for investigating bioterrorist-mediated outbreaks of anthrax is demonstrated.

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INTRODUCTION

Anthrax, caused by the bacterium *Bacillus anthracis*, is a disease with a natural transmission cycle involving wildlife, livestock, and, occasionally, humans. Recently *B. anthracis* received notoriety for its use as an agent of bioterrorism in the 2001 letter attacks in the United States [1], and an unsuccessful aerosol attack in Japan in 1993 [2]. Prior to its use as a bioterrorism agent, *B. anthracis* was developed as a biological weapon by the governments of several countries, including the United States, the United Kingdom, and the former Soviet Union [3]. Despite the emphasis on its role as an agent of bioterrorism or biological warfare, anthrax has been and continues to be an important global disease of wildlife and livestock. Global dispersal of spores via commodities has been prevalent, such that there are currently endemic anthrax foci on all continents except Antarctica (http://www.vetmed.lsu.edu/whocc/). In the environment, *B. anthracis* primarily exists as a dormant, highly stable spore, which is central to the ecology, evolution, and contemporary weaponization of this pathogen. During the spore phase, which may persist for decades, evolution is static or at least greatly reduced in rate, which limits the amount of genetic diversity found among isolates of this species.

In the past the genetic homogeneity of *B. anthracis* severely compromised efforts to reconstruct its evolutionary history. Two molecular approaches, multiple locus variable number tandem repeat analysis (MLVA) and whole genome single nucleotide polymorphism (SNP) discovery and analysis, have greatly enhanced the identification of genetic markers that help to establish the phylogenetic relationships among *B. anthracis* isolates [4,5]. For example, Keim *et al.* [4] used eight variable number tandem repeat (VNTR) markers to examine a worldwide collection of over 400 *B. anthracis* isolates and described two major clonal lineages (A and B) and 89 unique MLVA8 genotypes. This

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same VNTR typing scheme also has been used to examine the diversity of *B. anthracis* in France, [6] Poland, [7], Italy [8], and countries in southern [9] and northern Africa [10]. This process has now been expanded to 15 marker-loci, MLVA15 [11].

Although individual SNPs have limited resolving power relative to MLVA, researchers have used phylogenetic approaches to identify SNPs that efficiently partition bacterial strains into genetic groups consistent with their recognized population structure [3,11,12]. Recent whole genome sequencing efforts discovered approximately 3,500 SNPs among five strains of *B. anthracis* [5,13] (J. Ravel, unpublished). Pearson *et al.* [5] mapped nearly 1,000 of these SNPs across 27 diverse isolates and proposed an extremely robust and conserved phylogenetic model for *Bacillus anthracis*. The conserved distribution of SNPs within the *B. anthracis* phylogenetic tree was reflected in the observation that only a single character conflict (homoplasy) was detected from >25,000 data points. These results indicated that that a select number of SNPs representative of specific branches and nodes in the *B. anthracis* SNP-derived tree would be sufficient to accurately determine the current phylogenetic position of any *B. anthracis* isolate. A working hypothesis was formulated [3] where a small number of canonical SNPs (canSNPs) located at key phylogenetic junctions along the *B. anthracis* SNP tree could replace a tedious genome-wide SNP analysis. This strategy is analogous to the TagSNP concept that has been suggested by the International HapMap Consortium for the human genome[14] that "only a minority of sites need to be examined" to fully capture the genotype information in various conserved regions throughout the genome. CanSNPs in *B. anthracis* represent an extreme example of the TagSNP concept where a single SNP can represent the entire genome of an isolate.

In this study, the canSNP hypothesis for *Bacillus anthracis* was tested against a diverse global collection containing >1,000 isolates. An initial set of 12 canSNPs representing different points in the evolutionary history of *Bacillus anthracis* were queried against DNA preparations from this entire collection. These experiments demonstrate that all of the *B. anthracis* isolates can be placed into one of 12 conserved groups or lineages. The slowly evolving canSNP data set was then coupled to the more rapidly evolving MLVA15 marker set to greatly enhance the resolution beyond the original 89 *B. anthracis* genotypes [4]. The analysis of slowly evolving canSNPs allowed the definition of major clonal lineages in *B. anthracis*, whereas the more rapidly evolving MLVA15 markers elucidated younger population-level structure in the species. We also utilized molecular clock models, based upon simple assumptions and exhaustive whole genome synonymous SNP surveys of representative strains, to estimate the age of major events in the evolution of *B. anthracis*. Collectively, our phylogenetic and molecular clock analyses, as well as information on isolate frequencies and global geographic distribution, facilitate the most comprehensive description to date of the global diversity and historical transmission patterns of this pathogen.

RESULTS

Canonical SNP analysis

CanSNP analysis subdivided all of the *B. anthracis* isolates into three previously recognized major lineages (A, B and C), with further subdivisions into one of 12 distinct sub-lineages (Figure 1, stars) or sub-groups (circles). Seven completed whole genome sequences (C.USA.A1055, KrugerB, CNEVA.9066, Ames, Aus-

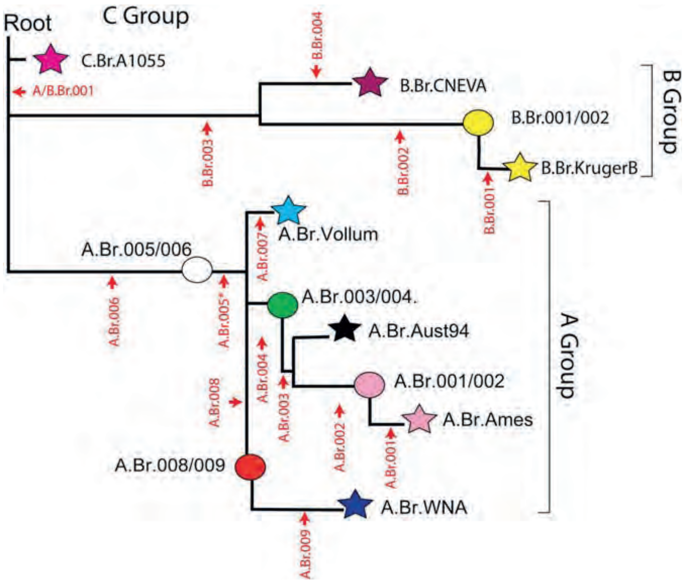

Figure 1. The relationship between canSNPs, sub-lineages and/or sub-groups: The stars in this dendrogram represent specific lineages that are defined by one of the seven sequenced genomes of B. anthracis. The circles represent branch points along the lineages that contain specific subgroups of isolates. These sub-groups are named after the canSNPs that flank these positions. Indicated in red are the positions and names for each of the canSNPs (also see Table 1).
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tralia94, Vollum, Western North America, see Table 1) defined endpoints (stars) that describe 7 distinct sub-lineages within the canonical SNP tree. These seven strains were picked to represent previously recognized diversity within *B. anthracis* [4,5]. In addition to the 7 lineages the canSNP analysis identified 5 sub-groups labelled as positions along the branches in the canSNP tree. The positions of each of the canSNPs are illustrated in Figure 1 and the canSNP genotype for each of the 7 sub-lineages and the 5 sub-groups is shown in Table 1. It is important to note that all of the 1,033 isolates in this *B. anthracis* collection fell into one of these 12 subdivisions and that the specific sequenced lineage isolates are only representative of a cluster of related isolates within that lineage.

MLVA15 analysis

UPGMA cluster analysis of the MLVA15 data alone clearly identifies the three major genetic lineages (A, B, and C; Figure 2). The longer B and C branch lengths (Figure 1) are underestimated in this analysis (Figure 2) due to mutational saturation of the rapidly evolving MLVA markers. This dataset also increased the number of unique *B. anthracis* MLVA genotypes from 89 (MLVA8, [3]) to 221 owing to both a larger subset of isolates and the expanded resolving power of the MLVA15 marker set (Figure 2, Tables S1 and S2). The MLVA15 tree (Figure 2) illustrates that the majority of isolates are located in shallow branches within the A lineage whereas the B and C lineages have rarer genotypes and fewer isolates. The MLVA15 dataset indicates that 89.6% (198) MLVA genotypes are from the A branch, 10% (22 MLVA genotypes) are from the B branch, and only 0.4% (1 MLVA genotype) are from the C branch.

Geographical distribution of clonal sub-lineages and sub-groups

Figures 2 and 3A graphically depict the distribution of the 1,033 isolates into the 12 canSNP sub-lineages and/or sub-groups (Column N in Fig. 2 and 3A) and also indicates the number of unique MLVA15 genotypes that were found in each of the 12 canSNP groupings (Figure 3A, column G; also see Table S1 in the Supplemental Section). The canSNP sub-lineages and sub-groups in Figure 3A also were assigned unique color codes to assist in establishing correlations between these 12 canSNP groupings and the geographic origins of each isolate. These data are presented in Figure 3B as color-coded pie charts for various geographic regions. Each pie chart illustrates the proportion of each canSNP grouping and the total number of isolates that originated from a particular geographic region. North America, Europe, China and parts of Africa are very well represented in these studies, whereas South America and Australia have reasonable representation. Countries from the Middle East and the former Soviet Union are under-represented. These sample biases are important considerations but do not appear to mitigate major genetic and geographic trends in this data set.

There are distinct differences in the global distributions of the major *B. anthracis* clonal lineages (A, B, and C). The A lineage isolates are widely distributed and are found in all countries included in this study. In contrast, the geographic distributions of the B and C lineage isolates are restricted, for example, the B lineage is primarily found in South Africa [B.Br.Kruger B sub-lineage and B.Br.001/002 sub-group [9] and portions of Europe [B.CNEVA-9006 sub-lineage; [4,6,7] with geographical differentiation at the sub-group level. Examples of these sub-lineages are rarely found outside of these regions.

Table 1. CANONICAL SNPs.

Lineage/Group	Type Strain.	Sequence	A.Br.001	A.Br.002	A.Br.003	A.Br.004	A.Br.006	A.Br.007	A.Br.008	A.Br.009	B.Br.001	B.Br.002	B.Br.003	B.Br.004	A/B.Br.001
C.Br.A1055	C.A1055	C.USA.A1055	T	G	A	T	C	T	T	A	T	G	G	T	**G**
B.Br.KrugerB	B1.A0442	KrugerB	T	G	A	T	C	T	T	A	**C**	T	A	T	A
B.Br.001/002	B1.A0102		T	G	A	T	C	T	T	A	**T**	**T**	A	T	A
B.Br.CNEVA	B2.A0402	CNEVA.9066	T	G	A	T	C	T	T	A	T	G	A	**C**	A
A.Br.Ames	A2.A0462	Ames	**C**	A	G	C	A	T	T	A	T	G	G	T	A
A.Br.001/002	A2.A0034		**T**	**A**	G	C	A	T	T	A	T	G	G	T	A
A.Br.Aust94	A1.A0039	Australia94	T	**G**	**G**	C	A	T	T	A	T	G	G	T	A
A.Br.003/004	A2.A0489		T	G	**A**	**C**	A	T	T	A	T	G	G	T	A
A.Br.Vollum	A1.A0488	Vollum	T	G	A	T	A	**C**	T	A	T	G	G	T	A
A.Br.005/006	A1.A0158		T	G	A	**T**	**A**	**T**	T	A	T	G	G	T	A
A.Br.008/009	A1.A0293		T	G	A	T	A	T	**G**	**A**	T	G	G	T	A
A.Br.WNA	A1.A0193	W. N. America	T	G	A	T	A	T	G	**G**	T	G	G	T	A

CanSNPs and profiles for the lineages/groups: This table lists each of the 12 lineages and groups and indicates the canonical SNPs that help to define each of the sub-lineages and sub-groups (canSNPs that define a particular sub-lineage or sub-group are indicated in yellow). Each lineage is named after the whole genome sequence that is positioned as an end point in a branch created by a comparison of that particular genome sequence to 6 other genomes (stars in Figures 1 and 3). As endpoints all but one of the lineages are defined by a single canSNP (see profiles in yellow for B.Br.Kruger , B.Br.CNEVA, A.Br.Vollum, A.Br.Ames and A.Br.WNA. Although Aust94 is an endpoint the canSNPs that define this lineage were developed before the draft sequence and as a result two canSNPs A.Br.002 and A.Br.003 define the branch point where this isolate is located. Similarly, the groups are positions that define branch points [5,35] along the different lineages (Circles in Fig. 1 and 3). They carry the group name designations corresponding to the canSNPs that flank these positions and are indicated in blue in this table (e.g. A.Br.001/002). Note that the sub-group need at least two canSNPs (one SNP on either side of the node) to assign a correct sub-group. Sub-group A.Br.005/006 requires three canSNPs to assign an exact genotype because a canSNP for A.Br.005 has not yet been tested. The whole genome sequences for Bacillus anthracis strains A0155, Ames Ancestor, CNEVA-9066, Kruger B, Vollum, Western North America (WNA) and Australia 94 can be found in the NCBI microbial genome website at http://www.ncbi.nlm.nih.gov

doi:10.1371/journal.pone.0000461.t001

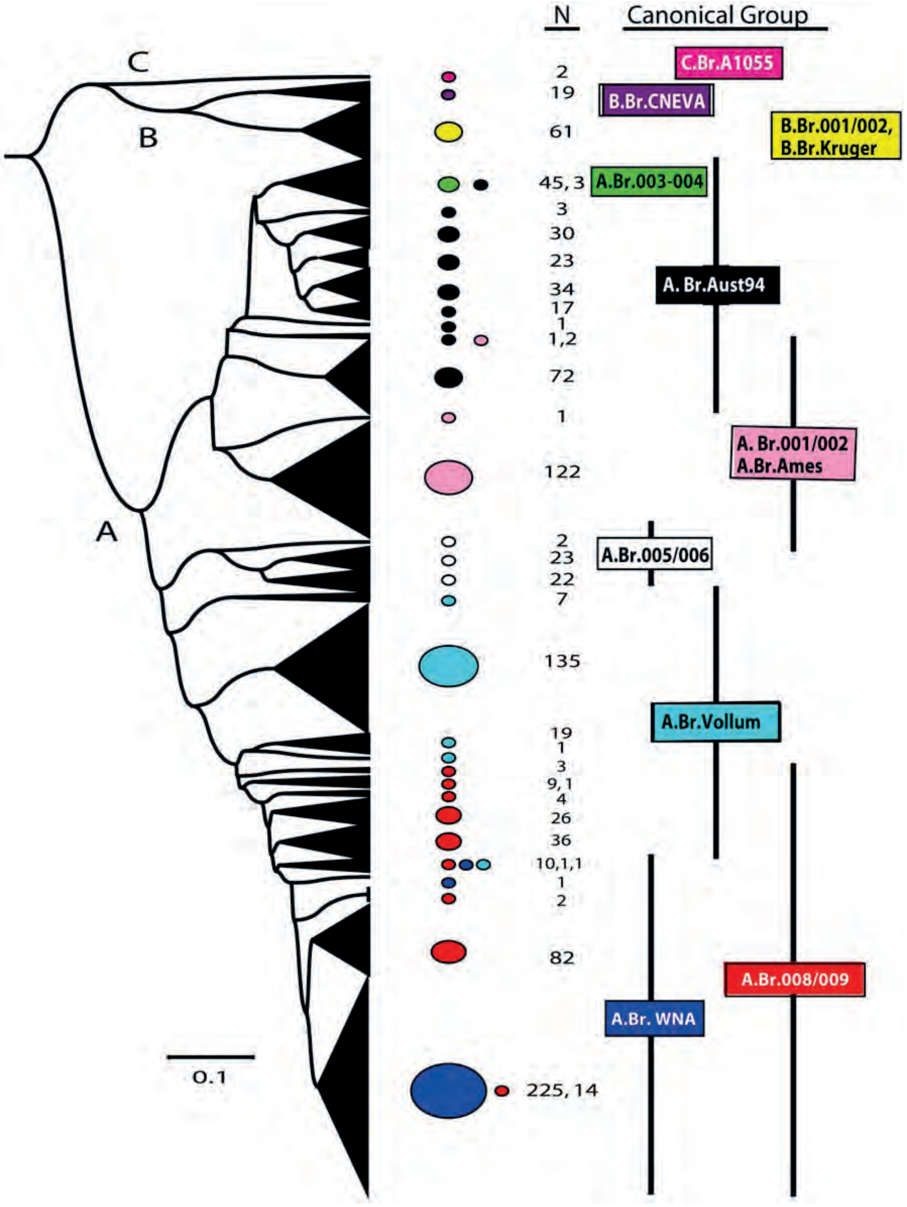

Figure 2. UPGMA dendrogram of VNTR data from worldwide B. anthracis isolates: Fifteen VNTR loci and UPGMA cluster analysis were used to establish genetic relationships among the 1,033 B. anthracis isolates. In this UPGMA dendrogram, which was created using MEGA software [39], groups of genetically similar isolates are collapsed into black triangles that are sized in proportion to the number of isolates in that particular lineage. VNTR loci mutate at faster rates than SNPs and, hence, provide greater resolution for terminal branches. Longer branches, such as the B and C lineages, have length underestimation in this analysis due to mutational saturation. The scale bar indicates genetic distance. Also illustrated on this figure is the distribution of the canonical SNP groups relative to the MLVA phylogeny (right columns). The number of isolates (N) associated with each canSNP group is shown in the second column. The correlation between the phylogenetic clusters identified by the canSNP and MLVA analysis with regards to the world wide geographic distribution of these clusters can be seen in Figure 3.
doi:10.1371/journal.pone.0000461.g002

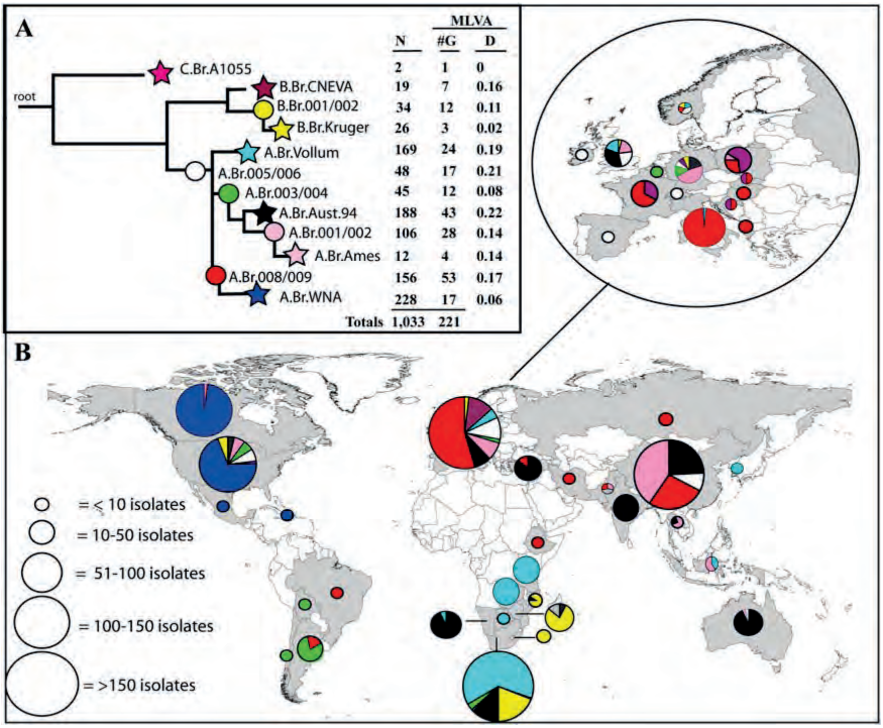

Figure 3. Worldwide distribution of B. anthracis clonal lineages:Phylogenetic and geographic relationships among 1,033 B. anthracis isolates. (A) Population structure based upon analysis of data from 12 canSNP (Protocol S1). The numbers of isolates (N) and associated MLVA genotypes (G) within each sub-lineage are indicated as well as the average Hamming distance (D) as estimated from VNTR data. The major lineages (A, B, C) are labelled, as are the derived sub-lineages (1–12), which are also color-coded. (B) Frequency and geographic distribution of the B. anthracis sub-lineages. The colors represented in the pie charts correspond to the sub-lineage color designations in panel A.
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Although A branch genotypes appear to be scattered throughout the world, there are distinct subgroup geographic compositions for many regions. The dominant genotypes in Southern Africa, for example, belong to the A.Br.Vollum sub-lineage, whereas in Europe isolates from A.Br.008/009 sub-group are dominant. Although central Asia is poorly represented in our collection, the genetic diversity in Eurasia appears to change along a longitudinal axis. Isolate collections from the west (Europe) are dominated by A.Br.008/009 sub-group isolates, and collections from western and south-central Asia (Turkey, India) and western China are dominated by genotypes belonging to A.Br.Aust94 sub-Lineage, (regional data not shown). Further into central and eastern China the genotypes are dominated by isolates belonging to the A.Br001/002 sub-group and A.Br.Ames sub-lineage (regional data not shown). Distinctive genotype compositions are also observed in the western hemisphere, which is dominated by unique clonal lineages that are not observed in the eastern hemisphere. Within the Americas, North and South America contain different genetic groups of *B. anthracis*: North American genotypes belong mainly to the A.Br.WNA sub-lineage and South American genotypes belong mainly to A.Br.003/004 sub-group.

A striking feature of isolate collections from the Americas is that the dominant clonal groups are rarely observed outside of these regions. These collections also exhibit low genetic diversity even when analyzed using high-resolution MLVA markers (Figure 2). For instance, in South America isolates from the A.Br.003/004 sub-group (mean within-group VNTR distance = 0.08; Fig. 3A) comprise more than 80% of the total isolates from this region yet are rarely observed elsewhere in the world. A similar trend is observed in the more extensive isolate collection from North America, which is dominated (70%) by a single group (sub-lineage A.Br.WNA; mean within-group VNTR distance = 0.06; Fig. 3A) that is not observed outside of North America. In contrast, the dominant sub-lineages in Europe, Asia, and Africa exhibit greater within-group genetic distances [Europe = A.Br.008/009 sub-group, mean within-group genetic distance = 0.17; South Asia (India, Turkey) = A.Br.Aust94 sub-lineage, mean within-group genetic distance = 0.22; East Asia (China) = A.Br.001/002 sub-group, mean within-group genetic distance = 0.14; Southern Africa = A.Br.Vollum sub-lineage, mean within-group genetic distance = 0.19]. In more industrialized regions, such as Western Europe and the United States, we observe dominant clonal lineages but also the co-occurrence of greatly differing genetic types. Important "donor" regions can be identified and differentiated from "recipient" regions based upon their strain diversity and the positions of these strains in phylogenetic models.

Molecular Clock Estimates

Our models, based upon simple assumptions and whole genome synonymous SNP surveys, allowed us to generate age estimates for the major events in the evolutionary history of *B. anthracis* (Methods). The divergence of the rare C branch isolates from the lineage containing the A and B branches appears to have occurred approximately 12,857 to 25,714 ybp. The more recent divergence of the A and B branch from a common ancestor occurred approximately 8,746 to 17,493 ybp. On a more recent time scale, we estimate that the primary A-radiation in *B. anthracis*, which is clearly evident in Figures 2 and 3A, occurred approximately 3,277 to 6,555 ybp, or in the mid-Holocene (Table 2).

DISCUSSION

B. anthracis is thought to have diverged from a *B. cereus* ancestor by the evolutionary acquisition of two virulence plasmids (pX01 and pX02) and several important chromosomal mutations, such as the nonsense mutation in *plcR* [15–18]. Subsequent evolution within this pathogen is evidenced by differences in the global distribution and abundance of isolates from the major clonal lineages (A, B, and C). In *B. anthracis*, the more common genotypes and the majority of isolates are located in shallow branches within the A lineage (Figures 2, 3A); whereas the B and C lineages are associated with rarer genotypes and fewer isolates. If isolate abundance is used as a fitness estimator, with rare genotypes considered less fit than common types, genotypes from the C branch and, to a lesser extent, the B branch appear to have very low fitness relative to the A branch genotypes (Figures 2, 3). Indeed, the C branch has significantly slower evolutionary rates than the A branch ([5]; Figure 2), suggestive of fewer infective cycles in nature.

The A branch of *B. anthracis* has experienced a recent and massive radiation (Figures 2 and 3A) that was clearly a very important event in the evolution of anthrax. Evidence for this event includes the great success of the A branch and its clonal derivatives, the involvement of A genotypes in most of the recent anthrax outbreaks around the world, and short phylogenetic branch lengths within this group. This last point is best illustrated in the dendrogram generated from the MLVA data alone (Figure 2), which capitalizes upon the rapid evolution of VNTR loci to depict the recently-derived radiative lineages within the A branch. The domination of A branch genotypes on a global scale is indicative of great reproductive success (hence, fitness) and considerable long-distance dispersal (Figure 3B). In the absence of the A-lineage expansion, anthrax likely would be a highly restricted and rare disease.

There are several possible explanations for the differences in global distribution and abundance observed among the major lineages of *B. anthracis*. One explanation is adaptive genetic differences that affect survival and propagation in either the environment or hosts. A comparison of A vs. B isolates from Kruger National Park, South Africa indicated that A strains were adapted to more diverse environments than B strains, which were restricted to more narrow environmental conditions [9]. This trend is also reflected on a global scale, where the B and C types may be successful locally or regionally but, unlike the A strains, are not a dominant presence worldwide. The limited abundance and geographic distribution of these rarer lineages may arise from fitness costs associated with niche specialization [9,19].

In addition to possible adaptive differences among lineages, stochastic processes such as human-mediated dispersal may explain the greater success of particular genetic groups. The global genetic population structure of *B. anthracis* suggests human activities have played a role in the proliferation and dispersal of this now global disease and we see evidence for these human impacts on several time scales. For example, models based upon simple assumptions and whole genome synonymous SNP surveys suggest the primary A-radiation in *B. anthracis* occurred approximately 3,277 to 6,555 ybp, or in the mid-Holocene (Table 2). These age estimates coincide with periods of increased human activities in animal domestication and domesticate population expansion [20–24]. Although the importance of the development of human civilization and animal domestication in the natural history of anthrax has been recognized [20,21], our study presents genetic evidence that it dramatically influenced the global population structure of *B. anthracis*.

As an important disease of livestock, it seems logical that major evolutionary events in anthrax, such as the A radiation, coincide with human developments in agriculture, animal domestication, and Old World trade routes. Animal husbandry and farming

Table 2. Molecular clock estimates of separation times among *B. anthracis* sub-lineages.

Compared lineages^{a}	Major groupings	Total synonymous sites^{b}	Observed sSNPs	sSNP substitution frequency	1 death per year model (ybp±2 STD)^{c}	0.5 death per year model (ybp±2 STD)^{c}
^{d}Vollum /^{e}Ames	A vs. A	899,987	153	1.7E-04	3,801±123	7,603±174
^{d}Vollum /^{f}WNA	A vs. A	899,957	129	1.4E-04	3,205±113	6,411±160
^{e}Ames/^{f}WNA	A vs. A	902,239	114	1.3E-04	2,825±106	5,651±150
Average among A branch divergence times =					**3,277±114**	**6,555±162**
^{g}CNEVA/^{e}Ames	B vs. A	901,936	322	3.6E-04	7,983±179	15,966±253
^{h}KrugerB vs ^{e}Ames	B vs. A	902,983	384	4.3E-04	9,509±195	19,019±276
Average B branch/A branch divergence times =					**8,746±187**	**17,493±264**
^{g}CNEVA/^{h}KrugerB	B vs. B	901,935	188	2.1E-04	**4,661±137**	**9,322±193**
^{i}C.A1055/ ^{g}CNEVA	C vs. B	901,783	484	5.4E-04	**12,002±219**	**24,003±310**
^{i}C.A1055/^{e}Ames	C vs. A	901,791	553	6.1E-04	**13,713±234**	**27,425±331**

a Sub-lineages according to Fig. 1, bTotal Syn Sites = The total sites for synonymous substitutions were determined separately for each pair-wise comparison. c The model for sSNP substitution rate is particularly sensitive to number of death cycles per year. Therefore, two possible scenarios (1 and 0.5 deaths per year) were modelled (see supporting methods on the PNAS website for more details). STD = The standard deviation for observed sSNPs, calculated as the square root of the time estimate. Thus, 2 STD represents ~95% confidence interval based upon fluctuation in this parameter of the model. d Sequence from the Vollum strain, The Institute for Genome Research (TIGR). e Sequence from the 'Ames Ancestor' strain, GenBank Reference Sequence NC 007530. f Sequence from the Western North America USA 6153, TIGR.g Sequence from the CNEVA-9066, TIGR. h Sequence from the Kruger B strain, TIGR. I Sequence from A1055, TIGR

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practices, which forced animals into confined areas, are likely to have increased *B. anthracis* infection and evolutionary rates, which would rapidly increase genotypic diversification. Similarly, the population expansion of large mammal domesticates from the centers of domestication in Eurasia and North Africa would function to disperse *B. anthracis* genotypes. Molecular clock models suggest that African and Eurasian cattle populations expanded 9,000 ybp and 5,000 ybp, respectively [24]; a time period that roughly corresponds to the A lineage radiation (3,277 to 6,555 ybp) and the divergence of the two major B branches from a common ancestor (4,661–9,322 ybp).

Independent domestication and domesticate expansion events may provide an explanation for the different assortments of A and B lineages on these two continents. For example, the two major B lineages are spatially separated, one is found in southern Africa (B.Br.001/002 sub-group and B.Br.KrugerB sub-lineage) and the other (B.Br.CNEVA-9066 sub-lineage) is found in portions of Europe, suggesting that after diverging from a common ancestor, these two groups experienced independent evolutionary histories. The divergence of the B.Br.CNEVA and B.Br.Kruger sub-lineages are similar in molecular clock estimates to the A radiation and, again, could represent human influences on this pathogen. Taken together, human-mediated events in the mid-Holocene provide plausible explanations for both the dramatic events in *B. anthracis* evolution observed during this time period and the diversity among and within clonal lineages on the African and Eurasian landmasses.

The dispersal of *B. anthracis* to the western hemisphere was probably via intercontinental transport of animal products during European colonization [25,26]. Evidence for this includes isolate collections from the western hemisphere that are dominated by clonal groups that are rarely observed outside of these regions and exhibit low genetic diversity when analyzed using high-resolution markers. These patterns are consistent with single, relatively recent introductions followed by widespread dispersal, ecological establishment, and local differentiation. The close derived genetic relationship between the North American sub-lineage A.Br.WNA and the dominant European sub-group A.Br.008/009 is consistent with an introduction to North America from Europe, possibly via French or Spanish colonization [25,26].

More recent human activities in commerce and industrialization also appear to have impacted the global population structure of *B. anthracis*. For instance, in addition to a single dominant genetic type, North America also contains a cosmopolitan assortment of rarer *B. anthracis* genotypes that are likely a consequence of international industrial trade (*e.g.,* wool, skins, bone meal, shaving brushes). A similar phenomenon is observed in other industrialized regions, such as Western Europe, where we observe the co-occurrence of greatly differing genetic types. The dispersal of these genotypes to industrialized regions has been tied to the trade of spore-infected items [25,27]. For instance, in the United Kingdom, the presence of minor genetic types that are dominant in portions of southern and eastern Asia (sub-lineages A.Br.Aust94, A.Br.001/002, A.Br.Ames; Figure 3) is consistent with reports tracing anthrax infections to imported animal products from these regions during the 19th and early 20th century [28–31]. Certainly, the highly-stable *B. anthracis* spore plays an important role in the importation of diverse genotypes into industrialized countries via transport and trade of contaminated commodities across large distances.

Trade also seems the likely source of *B. anthracis* in Australia. It has been hypothesized that anthrax was first introduced to Australia in 1847 via contaminated bone meal-based fertilizer shipped from India. Following this initial introduction at Sydney, the disease is thought to have spread along stock routes to the interior of the country [32]. Our genetic data provide some support for this hypothesis. All ten of the isolates we examined from India were assigned to sub-lineage A.Br.Aust94, which also appears as the dominant sub-lineage in Australia. It must be noted that the preponderance of isolates from A.Br.Aust94 lineage in Australia stems in part from a collection that is dominated by isolates from a single anthrax outbreak. Our genetic data, in fact, indicates separate introductions into Australia of isolates that belong to the A.Br.005/006 and A.Br.001/002 sub-groups; sub-groups that are more commonly found in Southern Africa and Eastern Asia, respectively.

B. anthracis has been developed as a biological weapon by several nations and terrorists groups and this has greatly increased the value of genotyping analysis for applications that attempt to differentiate between natural and bioterrorist-mediated outbreaks of anthrax. This is illustrated in the identification of the Ames strain as the source for the weaponized material from the 2001 anthrax letter attacks in the USA [1,3,13]. We found that the Ames strain genotype, which was originally obtained from a dead cow in Texas in 1981, is unique in this isolate collection and, hence, apparently rare in nature. North America is well represented in this study with 273 isolates spanning 44 MLVA genotypes (A.Br.WNA plus isolates from other sub-lineages, Figure 3B). However, the Ames genotype was present only once (although genetically similar isolates to the Ames strain were also identified in Texas, USA). The rarity of the Ames genotype in nature, coupled with its widespread use as a laboratory strain, makes it unlikely that the source material utilized in the 2001 bioterrorist attack was acquired directly from nature. These findings further highlight the importance of large genetic-geographic databases for distinguishing between intentional and environment-acquired infections caused by organisms that are both potential biological weapons and widespread in the environment [8,33,34].

In summary, our analyses of both canSNP and MLVA data provide a description of the global diversity and historical transmission patterns of *B. anthracis*. Our data suggest that although *B. anthracis* is a naturally occurring pathogen, human activities have dramatically influenced its current distribution and occurrence. We observe the effects of human activities at three levels: 1) the massive radiation of the A-branch in the mid-Holocene, 2) the more recent colonial-era importation of specific *B. anthracis* genotypes from the Old World into the New World, which lead to their ecological establishment, and 3) the repeated industrial importation of rare diverse genotypes into developed countries through animal products (e.g. wool, hides, and bone meal). The genetic population structure of *B. anthracis* is indicative of these long distance transmission events and illustrates its ability to become ecologically established in new locations. Fortunately, natural outbreaks of anthrax can be managed effectively through vaccination and public health efforts. However, due to actual and potential nefarious use of the pathogen, anthrax will likely remain of great social and scientific importance.

MATERIALS AND METHODS

Nomenclature

The tree in Figure 1 is based upon an analysis of $>$1,000 SNPs discovered amongst seven complete or draft genomes of *B. anthracis*, which yielded a branched phylogeny containing seven lineages corresponding to the sequenced "discovery" genomes [5]. In a strictly clonal species, like *B. anthracis*, these genomes will be situated at the end of each branch. These terminal lineages are depicted as stars Figure 1 and each of these lineages is named after the sequenced isolate (e.g. Ames, KrugerB, Vollum, etc.). The canSNPs are named after one of the three main clades (e.g. A, B, or C) followed by a three digit number (A.Br.001, A.Br.002, A.Br.003). Where possible, we have tried to be systematic in naming the canSNPs. For example, the first canSNP in the A branch was proximal to the Ames genome sequence (or the lineage terminus) and is named A.Br.001 (red labels in Figure 1). The second canSNP position defines a position between canSNP A.Br.001 and the circled position called A.Br.001/002. Such a systematic naming scheme for canSNPs may be compromised by future studies that define additional lineages and branches (*i.e.* the order of the canSNPs from the terminus will be inconsistent with their names). Hence, this should only be considered an arbitrary numbering system, but it will function effectively as new phylgenetic discoveries are made. The circles in the dendrogram represent branches or branch points defined by flanking characters (canSNPs). The branch points and the ends of lineages (the circles and stars in Fig 1) encompass all 1,033 *B. anthracis* isolates (ranging from 2 isolates in the C lineage (C.Br.A1055) to 228 isolates in the Western North American lineage (A.Br.WNA). Branch points also have been defined and named by their flanking canSNPs (*e.g.* B.Br.001/002). The near total absence of homoplasy (character conflicts in the tree), coupled with character discovery bias, has caused "branch collapse" in this clonally propagating pathogen [5,35]. A collapsed branch is still defined by its flanking canSNP characters.

B. anthracis isolates

We examined a global collection of 1,033 *B. anthracis* isolates. Table S3 contains information on the numbers and distribution of strains used in this study. These isolates were obtained from known anthrax cases, environmental sources, or other materials associated with the disease. Our isolate collection is biased toward anthrax outbreaks that occurred in the last several decades and towards countries actively engaged in the international exchange of scientific material. It is important to note that all of the isolates analyzed in this study were shown to possess the *plcR* inactivating mutation as detected by the PCR assay described in Easterday *et al.* [16]. This nonsense mutation is considered essential for maintenance of virulence plasmids and represents a definitive character of *B. anthracis* [16,36].

DNA isolation

A 1.0 µl inoculating loop was used to transfer *B. anthracis* colony material into 200 µl of Brain-Heart Infusion broth (Hardy Diagnostics, Santa Maria, CA) within the wells of a sterile, untreated polycarbonate 96-well culture plate (Costar Corning Inc., Acton, MA). The plate was then covered with an adhesive plastic film, placed in a secondary containment device, and incubated overnight at 37°C without shaking. Following incubation, 10.0 µl of broth was transferred to a Microseal^{TM} Polypropylene Microplate (MJ Research, Waltham, MA). The samples were then flash-frozen in 96-well cold block (−80°C) for 15 s and then immediately thawed in a 96-well heat block (96°C) for 15 s. This freeze-thaw cycle was repeated two additional times. The cell lysates were then transferred into a 96-well GV 0.2 µM Durapore Multiscreen Plate (Millipore, Billarica, MA) containing 100 µl of TE (10 mM Tris-HCl [pH 8.0], 1.0 mM EDTA) per well. Cellular debris and spores were removed from the 96-well filter plate by vacuum filtration using a MultiScreen Separations System Manifold (Millipore, Bedford, MA). The filtrate was collected into a 96-well plate and used to support PCR for downstream SNP and MLVA genotyping. The sterility of each sample was confirmed by plating 1.0 µl of each filtrate onto a TSA II 5% Sheep Blood prepared media plate (Becton Dickinson and Company, Cockeysville, MD) and incubating at 37°C for 48 hr.

Genetic Markers

Two types of genetic markers were used to analyze the *B. anthracis* collection: canonical single nucleotide polymorphisms (canSNPs) and variable number tandem repeats (VNTRs). We used data from the Pearson *et al.* [5] and unpublished genomic sequence data (Ravel *et al.*, unpublished data) to identify canSNPs that can be used for identifying a particular phylogenetic point in the evolutionary history of *B. anthracis*. In total, 2 *B. anthracis* specific SNPs and 12 canSNPs to analyze DNA from the 1,033 *B. anthracis* isolates. CanSNP alleles were determined using TaqMan^{TM} -Minor Groove Binding (MGB) allelic discrimination assays. TaqMan^{TM} MGB probes and primers for the canSNPs were designed using ABI Primer Express software and guidelines, with the exception that allele-specific probe lengths were manually adjusted to match melting temperatures [37]. The genomic location for each of the canSNPs can be found in Table S4 while the probe and primer sequences for each are listed in Table S5. Each 10.0 µl reaction contained 1× ABI Universal Master Mix, 250 nM of each probe, and 600 nM each of forward and reverse primers and 1.0 µl of approximately 350 pg/µl template DNA. For all assays, thermal cycling parameters were 50° C for 2 min., 95° C for 10 min., followed by 40–50 cycles of 95° C for 15 sec and 60° C for 1 min. Endpoint fluorescent data were measured on the ABI 7900.

DNA from the isolates was also analyzed using 15 VNTR loci; eight of these VNTRs are described by Keim *et al.* [4] MLVA8 and the additional 7 markers are described by Zinser [38]. These markers were compiled together into a multiple-locus VNTR analysis (MLVA15) subtyping system (see Protocol S1, Table S6 for details on the markers and methods).

Phylogenetic analyses

Two basic approaches were used to analyze genetic relationships among the 1,033 *B. anthracis* isolates. First, canSNP and VNTR data were used in a hierarchical approach to analyze phylogenetic relationships: data from the slowly evolving canSNPs loci were used to categorize the isolates into clonal lineages and followed by the use of data from the 15 rapidly-evolving VNTR loci to measure diversity and determine the number of genotypes within each of these clonal categories. This system allowed us to effectively analyze both older phylogenetic relationships and younger population-level structure [3]. Second, we used UPGMA cluster analyses of the MLVA15 data alone to illustrate the global population genetic structure in an unbiased manner. All phylogenetic analyses were conducted using MEGA3 software [39].

Geographic distribution of clonal lineages

To examine genetic-geographic patterns in *B. anthracis*, we mapped the worldwide distribution of the clonal lineages that were identified by the analysis of the canSNP data.

Age Estimates

To estimate the age of several events in the evolutionary history of *B. anthracis*, we performed whole genome synonymous SNP comparisons of strains that represent major clonal lineages. We utilized the following equation to estimate the time since pairs of strains last shared a common ancestor:

$$Age = \frac{sSNPs}{[MR \times sSites \times generations \times 2]},$$

where *sSNPs* is the total number of synonymous SNPs between two strains as determined by whole-genome comparisons, *MR* is the per site synonymous mutation rate in *B. anthracis* (5.2×10^{-10} mutations/generation; [40], *sSites* is the number of synonymous sites in common between the two strains, and *generations* is the estimated number of generations undergone by a given lineage in each year (estimated as 43 per transmission cycle). The number of generations per year is based upon an ungulate transmission model and the number of infection/death cycles per year (see detailed descriptions below). The age estimates are particularly sensitive to the number of infection/death cycles per year. As such, we calculated the estimates using both 1 (43 generations/year) and 0.5 (21.5 generations/year) infection/death cycles per year (Table 2).

Details of the Age Estimates

The use of sSNPs for the substitution rate restricts these estimates to nearly neutral evolutionary characters. While all SNPs are relatively infrequent among *B. anthracis* isolates, the use of whole genome analysis has identified many sSNPs (Table 1) and resulted in highly robust estimates of relationships among isolates [5]. sSNP occurrence between two strains is modeled well by the Poisson probability distribution. The relative large number of observations makes the error in this estimate small. When the expected number is high, the Poisson become fairly symmetrical with a standard deviation equal to the square root of the expected number. Thus, two standard deviations from the maxima are very close to the 95% confidence interval.

The mutations rates for single nucleotide changes have been reported in *B. anthracis* based upon selection for antibiotic resistance (Rif) and are very similar to the rates observed for other well-studied bacteria (2). In this case, Vogler et al. [40] estimated the rate using the Luria-Delbruck fluctuation test and then partitioned the phenotypic mutation rate (1.55E-09 mutants per generation) to different nucleotide positions in the *rpoB* gene by sequencing this gene in the mutants. Hence, we have a per site mutation rate (5.2E-10 mutations per generation) instead of merely a phenotypic rate.

While Drake [41,42] has argued for a universal substitution rate for microbial genomes, the extremely episodic nature of anthrax transmission makes this hard to justify among the clonal lineage of *B. anthracis*. Indeed, this is clearly the most sensitive aspect of the substitution rate model with certain parameters highly influential in the final estimates.

Ungulate transmission model

The number of *Bacillus anthracis* generations (G) in a single infected ungulate was determined using the following equation:

$$G=[\log_2(t\div i)],$$

where t = terminal number of *B. anthracis* organisms in a 100 kg ungulate (100 kg × d), i = initial number of *B. anthracis* organisms in the ungulate as obtained from an environmental source (10 organisms), and d = terminal density of *B. anthracis* organisms per unit body weight in a mammal ($10^{8.8}$ organisms per kg) [43]. Based on these parameters, it was estimated that $G=43.1$, which was rounded to 43. The model is not particularly sensitive to this particular parameter. Changing the size of the animal and, hence, the final *B. anthracis* population size is mitigated by the $\log_2$ transformation. The number of generations is altered only by 3.3 for every 10-fold increase in population size. This has a minimal affect upon the final number of generations.

Infection/death cycles per year

Estimating the number of infection/death cycles per year is difficult for anthrax. While hundreds or even thousands of individual animals might die in a single outbreak, it is unlikely that these multiple victims are sequential infection/death cycles. Rather, these clusters are likely to be from a single source, or due to environmental induction of the outbreak. For this reason, we believe that the average annual number of death/infection cycles will be one or less, even in the most endemic regions. *B. anthracis* spores are known to survive long periods of time; though very long-term spore survival is unlike to be important in the overall evolutionary rates as the viability does drop with time. In this study, we are primarily interested in the most highly fit branch of *B. anthracis* (A). Its worldwide distribution and fitness argues for a higher rate of transmission, probably close to one infection/death cycle per year. Because this is one of the most sensitive parameters in the model, we have modeled the molecular clock estimates using both 1 and 0.5 deaths per year. These values translate into 43 or 21.5 generations per year when combined with the population size estimates from a typical host (see above).

SUPPORTING INFORMATION

Table S1 The 221 MLVA Genotypes and Associated Can SNPs. The 221 genotypes (1–221, Column A) are organized according to their Keim Genetics Lab ID Designation ("A" number - Column B), prior designations when available ("K" numbers - Column C), their original MLVA8 GenoTyping designation ("GT" numbers: 1–89 - Column D) from Keim et al., (2000) and the alternative strain designations and original source codes for each isolate (Column E). This is followed by the isolate's canSNP lineage/group (Column F, also see Fig 1), two B. anthracis specific SNPs (Columns G and H), the13 canSNP scores (Columns I–U) and the 15 marker MLVA profile for that isolate (Columns W–AK). The first two SNPs (Column G and H) are Bacillus anthracis specific SNPs originally identified in the plcR and gyrA loci and are not part of the canSNP profile. There are 221 unique MLVA genotypes listed in this table.

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Table S2 The MLVA Sizing Code. The VNTR alleles for each MLVA marker in Supplemental Table S1 are letter coded according to size to allow these data sets to be utilized by various tree drawing programs. Apparent MLVA fragment sizes vary from instrument to instrument and even with various size standards. Allele codes provide a common designation in the face of this variation. Table S2 provides a code that describes the fragment sizes for these alleles based on analysis on an ABI 3100 Genetic Analyzer (see Protocol S3), a custom made LIZ®-labeled internal size standard (5), and subsequent analysis using Genotyper. The numeral 1 appears as a code when a fragment failed to amplify; eg., an isolate lacking the pXO1 plasmid would not be able to amplify the pXO1.1AAT VNTR marker.

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Table S3 Geographical Composition of B. anthracis isolates used in this study

Found at: doi:10.1371/journal.pone.0000461.s003 (0.06 MB DOC)

Table S4 CanSNPs Description and Chromosomal Location

Found at: doi:10.1371/journal.pone.0000461.s004 (0.03 MB DOC)

Table S5 Canonical SNP Primers/Probes used in molecular typing of B. anthracis

Found at: doi:10.1371/journal.pone.0000461.s005 (0.03 MB DOC)

Table S6 15 VNTR loci in the B. anthracis 15 VNTR MLVA system.
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Author Contributions

Conceived and designed the experiments: PK TP Mv DW WE LH TS JU SK KS LK. Performed the experiments: Mv WE LH SZ TS JU SK SR GZ LK RL. Analyzed the data: JR PK TP Mv DW WE LH. Contributed reagents/materials/analysis tools: CF JR TP Mv DW WE LH JF PC KS BW MH. Wrote the paper: PK Mv DW RO.

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Chapter 5

Historical Distribution and Molecular Diversity of *Bacillus anthracis* in Kazakhstan.

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Historical Distribution and Molecular Diversity of *Bacillus anthracis*, Kazakhstan

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To map the distribution of anthrax outbreaks and strain subtypes in Kazakhstan during 1937–2005, we combined geographic information system technology and genetic analysis by using archived cultures and data. Biochemical and genetic tests confirmed the identity of 93 archived cultures in the Kazakhstan National Culture Collection as *Bacillus anthracis*. Multilocus variable number tandem repeat analysis genotyping identified 12 genotypes. Cluster analysis comparing these genotypes with previously published genotypes indicated that most (n = 78) isolates belonged to the previously described A1.a genetic cluster, 6 isolates belonged to the A3.b cluster, and 2 belonged to the A4 cluster. Two genotypes in the collection appeared to represent novel genetic sublineages; 1 of these isolates was from Krygystan. Our data provide a description of the historical, geographic, and genetic diversity of *B. anthracis* in this Central Asian region.

Anthrax is a globally widespread disease of livestock and wildlife that occasionally infects humans. According to official estimates, the number of human anthrax cases worldwide ranges from 2,000 to 20,000 annually (*1*). *Bacillus anthracis*, the etiologic agent of anthrax, persists in the environment as a dormant, highly stable spore. The prolonged periods of dormancy during the spore phase slows evolution of this species, resulting in high levels of interstrain genetic homogeneity, which complicates efforts to subtype the pathogen. The availability of whole-genome nucleotide sequences of *B. anthracis* for single-nucleotide polymorphism (SNP) elucidation and the discovery of polymorphic markers such as variable number tandem repeat (VNTR) sequences (*2*,*3*) have enabled identification of unique subtypes within this species. Keim et al. (*4*) used 8 VNTRs to describe 89 unique genotypes in a global collection of over 400 *B. anthracis* isolates. Later studies used VNTRs to examine *B. anthracis* diversity in different global regions, including France (*5*)*,* Italy (*6*)*,* Poland (*7*)*,* Chad (*8*), and South Africa (*9*). More recently, SNPs that define major clonal lineages in *B. anthracis* have been identified and applied to describe global and regional patterns of *B. anthracis* diversity (*10*).

In the central Asian republic of Kazakhstan, anthrax is enzootic and still represents a human public health concern. A recent publication examined risk factors associated with 73 human anthrax cases in Kazakhstan over a 2-year period (*11*) and concluded that most cases were cutaneous and had resulted from the handling of infected livestock and contaminated animal products. Gastrointestinal anthrax in Kazakhstan has also been reported but is less common. Despite the widespread nature of the disease in this region, the historical incidence, distribution, and genetic diversity of

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B. anthracis in central Asia, and Kazakhstan in particular, has remained cryptic.

We mapped the historical distribution of anthrax in Kazakhstan over a 68-year period. Archived cultures from a subset of these outbreaks collected from 10 oblasts (provinces) over a 53-year period were analyzed by using genetic and biochemical tests. Multilocus variable number tandem repeat analysis (MLVA) and canonical single nucleotide polymorphism genotyping (*10*) of this collection enabled us to examine strain dynamics among and within these outbreaks and to understand the diversity of *B. anthracis* isolates from Kazakhstan on a local, regional, and global scale.

Materials and Methods

Mapping Historical Anthrax Outbreaks

To map the historical distribution of anthrax outbreaks and *B. anthracis* strain types across Kazakhstan, we constructed a geographic information system (GIS) database within ArcGIS 9.1 (www.esri.com). This database used archival data collected through the antiplague stations established by the Union of Soviet Socialist Republics. This system of stations remains in place under the current government, and Kazakhstan maintains a multiagency reporting protocol to update, document, and respond to the distribution of outbreaks. These data are archived at the Kazakhstan Scientific Center for Quarantine and Zoonotic Diseases. Outbreaks and strain locations were geolocated to the nearest village by using GIS data layers produced by the Kazakh Institute of Geography. Historical outbreaks were mapped for 1937 through 2005. To illustrate differences in the distributions of outbreaks in cattle and sheep, the 2 most affected livestock species, a kernel density estimation was performed by using the Spatial Analyst Extension in ArcGIS. We mapped outputs by using the standard deviation of density values to illustrate areas of greatest outbreak concentration by species (*12*).

Isolation of *B. anthracis*

Samples collected from anthrax outbreaks in Kazakhstan (with the exception of 2 isolates from the Kyrgyzstan border region) and cultures spanning a 53-year period were archived in the Kazakhstan National *B. anthracis* Collection. Most isolates were from human patients, some from blood or organs of ruminants (mainly sheep and cows), and a few from soil or other inanimate objects contaminated by contact with blood or tissues of infected animals. Archived cultures were confirmed as *B. anthracis* on the basis of colony morphologic appearance; absence of hemolysis and catalase, lipase, phosphatase and protease activity; and susceptibility to *B. anthracis*–specific γ phage.

DNA Preparation

B. anthracis cultures from the Kazakhstan National Collection were grown on Hottinger blood agar. A colony from each sample was harvested from the agar plates and dispersed in Tris-EDTA buffer for DNA extraction. A QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) was used to extract genomic and plasmid DNA by using the manufacturer's protocol. A total of 1.0 mL of DNA was collected from each of the isolates in the collection.

MLVA Genotyping

Eight VNTR (MLVA-8) markers were amplified by PCR by using primer pairs *vrrA*-f1 and *vrrA*-r1, *vrrB*$_1$-f1 and *vrrB*$_1$-r1, *vrrB*$_2$-f1 and *vrrB*$_2$-r1, *vrrC*$_1$-f1 and *vrrC*$_1$-r1, *vrrC*$_2$-f1 and *vrrC*$_2$-r1, CG3-f1 and CG3-r1, pXO1-AAT-f3 and pXO1-AAT-r3, and pXO2-AT-f1 and pXO2-AT-r1 (*4*). One microliter containing ≈1 ng of template DNA was added to each PCR.

Electrophoresis of amplified products was performed on an ABI 310 genetic analyzer (Applied Biosystems, Inc., Foster City, CA, USA). Data were analyzed by using GeneMapper software V4.0 (Applied Biosystems, Inc.). To ensure comparability and accuracy of raw VNTR scores from the strains from Kazakhstan with the genotypes published by Keim et al. 2000 (*4*), we performed electrophoresis on amplified fragments from 4 control DNAs (A0462-Ames, A0488-Vollum; A0071-Western North America and A0402; and French B2) in parallel with the isolates from Kazakhstan. In addition, DNA molecular size reference markers (Applied Biosystems, Inc) were included in each sample to accurately size the 8 VNTR fragments. Raw VNTR sizes were normalized to the sizes reported by Keim et al., 2000 (*4*) for genotypic comparisons.

Unweighted Pair Group Method with Arithmetic Mean Cluster Analysis of Genotypes

Unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of VNTR data from 92 confirmed *B. anthracis* isolates and the diverse 89 genotypes described by Keim et al. 2000 (*4*) were used to establish genetic relationships. Distance matrices were generated in PAUP 4.0 (Sinauer Associates, Inc., Sunderland, MA, USA) and imported into MEGA 3.1 (*13*) for tree-building purposes.

Spatial Patterns of Genetic Relationships

The strain database was constructed from museum records and contemporary epidemiologic investigations. This database was synchronized with the bacterial culture collection to geolocate the culture by using the GIS. To map strain diversity, we categorized culture collection locations by strain identifications based on the MLVA genotyping results.

SNP Typing of *B. anthracis* Isolates

Representative cultures from each Kazakh MLVA genotype plus the STI vaccine strain from Russia were genotyped by using previously described canonical SNPs discovered by whole-genome sequencing (*10,14*). SNPs were interrogated by using the Roche Light Cycler II real-time PCR instrument (Roche Diagnostics, Indianapolis, IN, USA). Allelic discrimination assays initially developed on the ABI 7900 real-time platform (*10*) were adapted for use on the Light Cycler II. The assay amplifies a fragment of DNA sequence containing the SNP site. Two probes complementing the 2 potential SNP states were used as real time markers. Each probe had a distinct fluorescent label; i.e., probe 1 was labeled with 6-carboxyfluorescein, and the alternate probe was labeled with VIC (Applied Biosystems, Inc.). The probe complementary to the sequence in the sample amplicon will hybridize over the SNP and surrounding sequence during the amplification process to generate a signal. It is possible for the incorrect probe to generate some signal but not enough to be confused as a positive reaction. The Light Cycler II discriminated which probe was the complementary sequence on the basis of the differential intensity of the reaction. Controls for each run included template DNA with both SNP states of interest.

Results

Historical Incidence and Geographic Distribution of Anthrax in Kazakhstan

A total of 1,037 human outbreaks were reported, representing 1,765 human cases. The outbreaks occurred in 665 locations; 198 of those locations reported repeat outbreaks throughout the study period (Figure 1; Table 1). Additional review of historical data at the Kazakhstan Scientific Center for Quarantine and Zoonotic Diseases identified 3,947 outbreak events reported for animal species and were entered into GIS. The outbreaks occurred over 1,790 locations; 805 of those reported repeated outbreaks.

Table 1. Outcomes for 1,765 human patients in mapped anthrax outbreak areas, Kazakhstan, 1937–2005

Status	Number
Recovered	1,541
Deceased	75
Lost contact	17
No data/unknown	132

Cattle and sheep were the primary livestock species affected during the study period; fewer outbreaks occurred among swine, and rarer, sporadic outbreaks occurred on mink farms and among foxes, and camels (Table 2). Cattle outbreaks were most common in northern Kazakhstan; several outbreaks occurred in the southernmost oblasts bordering Uzbekistan and Kyrgyzstan (Figure 2, panel A). Sheep outbreaks were prominent throughout eastern and southern Kazakhstan (Figure 2, panel B). The largest cattle outbreak (n = 174 cattle) in the dataset occurred in 1957 in the northernmost region of the Karaganda oblast in north central Kazakhstan. The largest sheep outbreak affected 851 sheep and occurred in the southern oblast of Zhambyl in 1971.

Biochemical Tests

All cultures except 1 (isolate no. 49) were biochemically and morphologically consistent for *B. anthracis*; 3 cultures (isolate nos. 65, 76, and 77) were consistent with *B. anthracis* but did not exhibit capsule formation. With the exception of culture no. 49, isolates were nonhemolytic; nonmotile; phosphatase and lecithinase negative; protease, oxidase, and catalase positive; and, with 3 exceptions, formed a capsule.

MLVA Genotyping

Of the 92 *B. anthracis* isolates, 88 isolates yielded complete data for the 8 marker MLVA; 3 isolates were missing the pX02 marker (isolate nos. 65, 76, and 77), and 1 was missing the pX01 plasmid marker (isolate no. 7). After we coded the raw VNTR fragment sizes, the Kazakh *B. anthracis* genotypes were analyzed by using PAUP 4.0

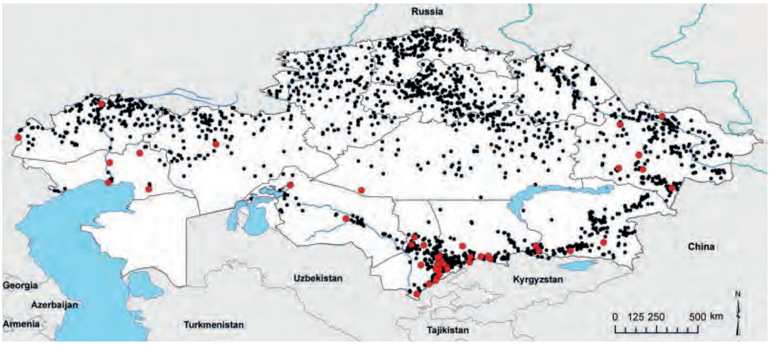

Figure 1. Anthrax outbreaks in Kazakhstan, 1937–2005. Each dot represents an outbreak; red dots indicate that cultures were isolated and analyzed from these outbreaks.

Table 2. Anthrax outbreaks, number of animal deaths per outbreak by species affected, and miscellaneous anthrax-positive samples, Kazakhstan, 1937–2005

Animal species	No. outbreaks/ samples	Deaths/ outbreak*	Total no. deaths
Sheep	1,735	0–851	16,080
Cattle	1,678	0–84	3825
Equine	304	0–28	634
Swine	192	0–78	832
Camel	5	1–2	7
Mink	3	28–37	95
Goat	1	1	1
Fox	1	1	1
Dog	2	1	2
Arctic fox	2	5	6
Unidentified	6	–	15
Miscellaneous anthrax-positive samples†			
Soil samples	17	–	–
Wool	1	–	–

*0 indicates animals that recovered from infection.
†*Bacillus anthracis* spores were recovered, but there were no infections.

and MEGA 3.1 phylogenetic software programs. UPGMA cluster analysis of the Kazakh isolates with complete MLVA-8 data (*4*) identified 12 unique MLVA subtypes.

UPGMA cluster analysis of the 12 Kazak MLVA genotypes (G_{kz}) with the diverse 89 genotypes reported by Keim et al. (*4*) showed that most isolates (n = 78) belonged to the previously described A1.a genetic cluster; 6 isolates belonged to the A3.b cluster; and 2 isolates belonged to the A4 cluster. More than half of the A1.a isolates belong to previously described genotypes (38/74; excluding samples with missing pX01, pX02 data), including the previously described MLVA genotypes 3 (n = 15), 6 (n = 2) and 13 (n = 21). Most of the novel genotypes reported from the Kazakhstan National collection represent slight variants of previously described genotypes that can be explained by the insertion or deletion of ≥1 tandem repeats at a particular locus, usually in pX01 or pX02 (Table 3). However, 2 of the genotypes from Kazakhstan (G_{kz}-9 and -11) appear to represent new sublineages on the basis of newly described allele combinations and distance-based clustering with the diverse 89 genotypes. In addition, the pX01 allele sizing at position 138 appears novel (G_{kz}-5); we have not seen this size reported in previous MLVA-8 studies (Table 3).

Geographic Distribution of MLVA Genotypes

The geographic distribution of MLVA types in Kazakhstan indicated that A1.a genotypes were widely distributed (Figure 3). For example, the most common Kazakh genotype (G_{kz}-1; n = 21) clusters on the Georgia–Kazakhstan border and on the southern border near Kyrgyzstan and Uzbekistan. The A1.a G_{kz}-4 (n = 17) is also widely dispersed across Kazakhstan; cases have occurred in the western, southern, and eastern regions and into Kyrgyzstan. Specific genotypes within the Kazakh A1.a group appear to exhibit geographic clustering, reflecting temporally linked outbreaks.

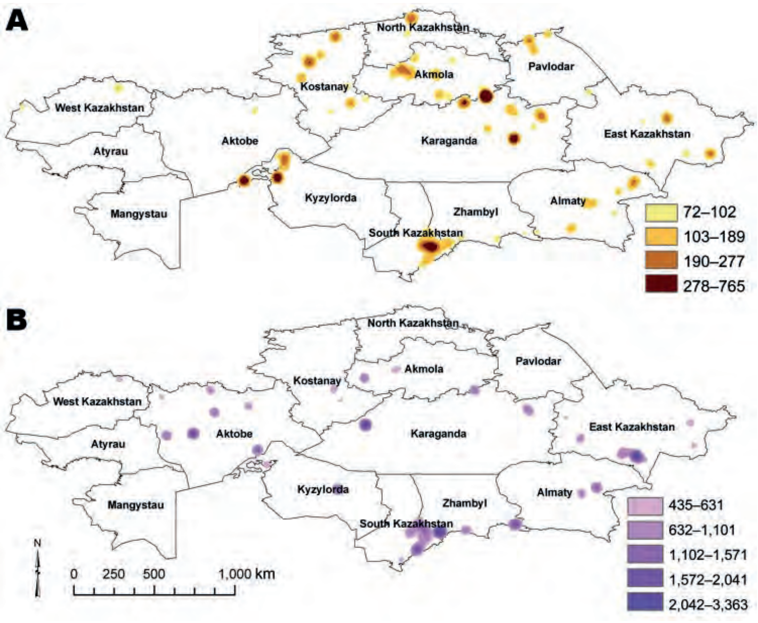

Figure 2. Kernel density estimates of anthrax outbreaks in cattle (A) and sheep (B), Kazakhstan, 1937–2005. Color shading represents SD values relative to density values from the kernel density estimate analysis for each species.

Table 3. Variable number tandem repeat sizes for *Bacillus anthracis* isolates, Kazakhstan*

Kazakhstan genotype no.	MLVA group†	MLVA genotype	*vrrA*	*vrrB1*	*vrrB2*	*vrrC1*	*vrrC2*	*CG-3*	*pX01*	*pX02*
1	A1.a	Gt-13 (*4*)	313	229	162	613	604	153	132	137
2	A1.a	Novel	313	229	162	613	604	153	135	137
3	A1.a	Novel	313	229	162	613	604	153	129	139
4	A1.a	Novel	313	229	162	613	604	153	129	137
5	A1.a	Novel	313	229	162	613	604	153	138	137
6	A1.a	Gt-6 (*4*)	301	229	162	613	604	153	126	137
7	A1.a	Gt-3 (*4*)	313	229	162	613	604	153	126	137
8	A1.a	Novel	313	229	162	613	604	153	132	139
9	Novel	Novel	325	229	162	613	604	158	132	137
10	A4	Novel	313	229	162	538	604	158	126	137
11	Novel	Novel	313	229	162	583	532	153	129	141
12	A3b	Novel	313	229	162	583	532	158	126	139

*Raw allele sizes were determined by electrophoresis on the ABI 310 (Applied Biosystems, Inc., Foster City, CA, USA); sizes were compared to control variable number tandem repeats and corrected to the sizes reported by Keim et al. (*4*).

†MLVA, multilocus variable number tandem repeat. MLVA group determined by unweighted pair group method arithmetic mean clustering with the diverse 89 genotypes described by Keim et al. (*4*).

The KZ genotypes 9–12 (G_{kz} -9–12) also appear to be more geographically confined, although this apparent confinement is likely a reflection of sample size. For example, isolates with G_{kz}-12 (n = 6; Figure 3) are exclusively found in the border region of the East Kazakhstan oblast, whereas the group 9 isolates (n = 5) are found in the Shymkent oblast in the south-central portion of the country. MLVA G_{kz}-11 (n = 1), which appears to represent a previously unreported genetic lineage, was isolated just south of Kazakhstan in Kyrgyzstan.

SNP Typing

Representative cultures from each of the Kazakh MLVA genotypes plus the Russian STI vaccine were SNP genotyped by using allelic discrimination probes and the Light Cycler II instrument. The SNP results were compared (Table 4) with the SNP profiles of Van Ert et al. (*10*), allowing assignment of the isolates to 1 of 12 sublineages. As with MLVA typing, all isolates tested with SNPs had genotypes characteristic of the A branches.

Representatives of MVLA genotypes 1–9 were assigned to A.Br.008/009, KZ genotype 10 to the A.Br.Vollum subgroup, and genotype 11 and 12 to the A.Br.Ames subgroup. The SNP data indicated that all representative A1.a Kazakh isolates belonged to the European branch of this group. The assignment of MLVA G_{kz}-10 to the A.Br. Vollum group is consistent with *B. anthracis* found globally in areas such as Pakistan and western China (*10*). Likewise, the assignment of Kazakh MLVA genotypes 11 and 12 to the A.Br.Ames genotype is consistent with the presence of this lineage in China (*10*).

Discussion

The historical occurrence and geographic distribution of anthrax outbreaks in Kazakhstan suggest anthrax foci are heavily concentrated in the southern region and broadly distributed across the northern portions of the country but are less common in the central regions. This may reflect regional differences in soil composition, availability of water and livestock and even case reporting. For example, the central region of Kazakhstan is dominated by desert, which likely has poor soils for long-term spore survival, whereas in the southern, northern, and eastern oblasts, the soils are more alkaline with higher organic matter and likely support spore survival (*15–17*). From a temporal perspective, outbreaks (or outbreak reports) have decreased in severity (number of animals infected), frequency (number of reported outbreaks), and have been associated with smaller geographic areas affected. However, the spatial distribution of the disease appeared to be relatively stable in the northern and southern Kazakh oblasts during the study period.

From a genetic perspective, *B. anthracis* in Kazakhstan was dominated by isolates clustering in the MLVA A1.a group, which is consistent with reports of the A1.a group being widely distributed globally (*4,5,6*). The widespread occurrence and apparent ecologic establishment of these VNTR genotypes in Kazakhstan supports the hypothesis that the A1.a group represents a very fit strain complex (*6*). Of the 8 A1.a genotypes in Kazakhstan, 5 were novel (G_{kz}-2, -3, -4, -5, and -8) and exhibited a previously undescribed pX01 allele (G_{kz}-5), which is not unexpected considering that this region has been underrepresented in prior MLVA-8 *B. anthracis* studies (*4–8*).

SNP typing of representative isolates from the A1.a Kazakh MLVA genotypes assigns these isolates to the A.Br.008/009 SNP lineage, which is widely distributed throughout Europe and has been reported in western China (*10,18*). Notably, the SNP data differentiate the Kazakh genotypes from the related North American genotypes, which are not effectively differentiated by MLVA alone. Since the representative Kazakh isolates in this SNP study were cultured from outbreaks spanning a 50-year

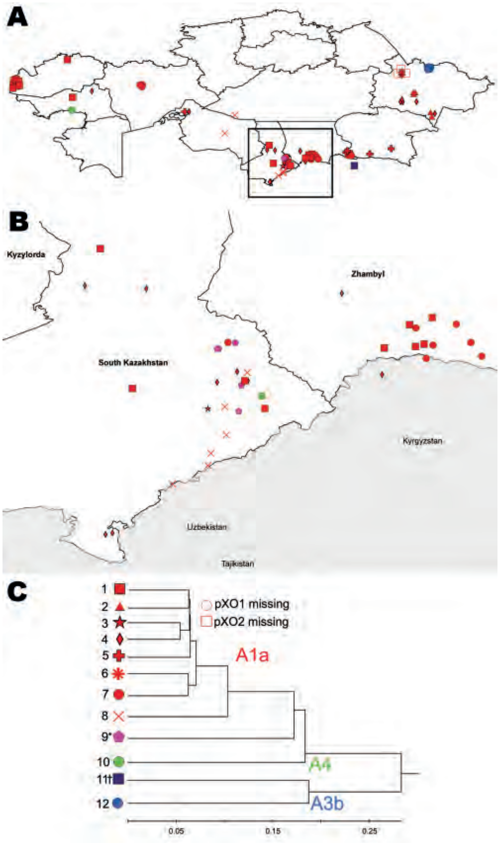

Figure 3. Geographic distribution of genotypes of *Bacillus anthracis* strains in Kazakhstan (A), with a closer view of outbreaks within eastern and southern Kazakhstan (B). Different genotypes are represented by different shapes and color coding reflecting major genetic affiliations (C). * and † indicate novel subgroups. Scale bar indicates genetic difference.

period (1952–2002), our data not only expand the understanding of the geographic range of this Eurasian lineage (A.Br.008/009) but also provide insights into its historical incidence and persistence in the country. Because of sampling limitations, the extent to which this dominant lineage is represented in the northern sections of Kazakhstan, and further into Russia, is unknown. However, in a recent study *B. anthracis* DNA from persons affected by the Sverdlovsk accident was assigned to the A.Br.008/009 SNP subgroup (*19*). Our data and the report that the Sverdlovsk strain was initially isolated in the 1950s in Kirov, Russia (*19*), underscores the need to genotype additional samples in northern Kazakhstan oblasts and Russia to measure the northern range of this apparently highly successful lineage.

The assignment of Kazakh isolates to the A3.b and A4 MLVA clades and the A.Br.Ames and A.Br.Vollum SNP groups is not surprising considering these MLVA and SNP types are also found in Middle Eastern countries, such as Pakistan and China (*10*). As first reported by Van Ert et al. (*10*), and later detailed by Simonson et al. (*18*), the A.Br.001/002 is common in China, whereas the closely related A.Br.Ames SNP lineage is more restricted geographically. The finding that the Kazakh isolates from the eastern border were assigned to A.Br.Ames SNP group is notable considering that the A.Br.Ames isolates that can be geolocated are found exclusively in Inner Mongolia. These genotypic similarities may reflect historical trade and nomadic routes linking those regions.

The absence of B lineage genotypes in Kazakhstan, as indicated by both MLVA and SNP data, is consistent with the lack of these genotypes in China, including the western province of Xinjiang (*10*,*18*), and supports the hypothesis that these lineages are restricted to narrow environmental conditions and, therefore, are more restricted in their global distribution (*9*). On a more local level, our MLVA data permit strain-level analysis of samples isolated during outbreaks. In several instances we were able to link strains collected from human anthrax patients to the infection source. For example, we identified the same strain in 10 cultures collected from an outbreak in western Kazakhstan that occurred from July–August 2005. The samples included cultures isolated from livestock, contaminated meat, human victims, and contaminated soil. The MLVA data linked the cultures and provided a mechanism for retrospective epidemiologic trace-back.

Sampling biases and limitations are important considerations in any study. For example, the distribution of cultures available for this study does not represent a balanced sampling of the entire country. There is an ongoing effort in Kazakhstan to expand the culture collection and to include a wider geographic sampling of the country, including the northern oblasts, which is underrepresented in the current culture collection but has a long historical record of anthrax. It would be worthwhile to revisit livestock burial sites and to isolate and analyze cultures from this region. In addition, the application of more comprehensive genetic analysis of Kazakh isolates would provide greater insight into the uniqueness of *B. anthracis* diversity in this region. For example, although canonical SNPs provide a powerful tool for assigning isolates into major clonal lineages, their resolution is limited by the use of relatively few representative SNPs and the diversity of the genomes used in the initial discovery process.

In summary, our work describes the historical incidence, distribution, and biochemical and genetic diversity

Table 4. *Bacillus anthracis* SNPs, Kazakhstan*

Isolate	KZ MLVA genotype	SNP group	SNPs											
			A branch								B branch			
			001	002	003	004	006	007	008	009	001	002	003	004
KZ 6	1	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 60	2	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 52	3	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 3	4	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 44	4	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 1	5	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 74	6	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 25	7	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 55	7	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 8	8	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 13	9	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 11	10	A.Br.Vollum	T	G	A	T	A	C	T	A	T	G	G	T
KZ 42	11	A.Br.Ames	C	A	G	C	A	T	T	A	T	G	G	T
KZ 66	12	A.Br.Ames	C	A	G	C	A	T	T	A	T	G	G	T
KZ ST1	NA	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T

*SNP, single nucleotide polymorphism; KZ, Kazakhstan; MLVA, multilocus variable number tandem repeats; NA, not applicable. SNP changes are shaded. SNP groups as described in Van Ert et al. (*10*).

of *B. anthracis* isolates in the central Asian republic of Kazakhstan. Our discovery of novel genotypes in this region contributes to the understanding of the global diversity of the pathogen and emphasizes the need for future studies in this geographic region. In addition, this study provides useful baseline data for future epidemiologic studies in Kazakhstan and for guiding future disease control programs

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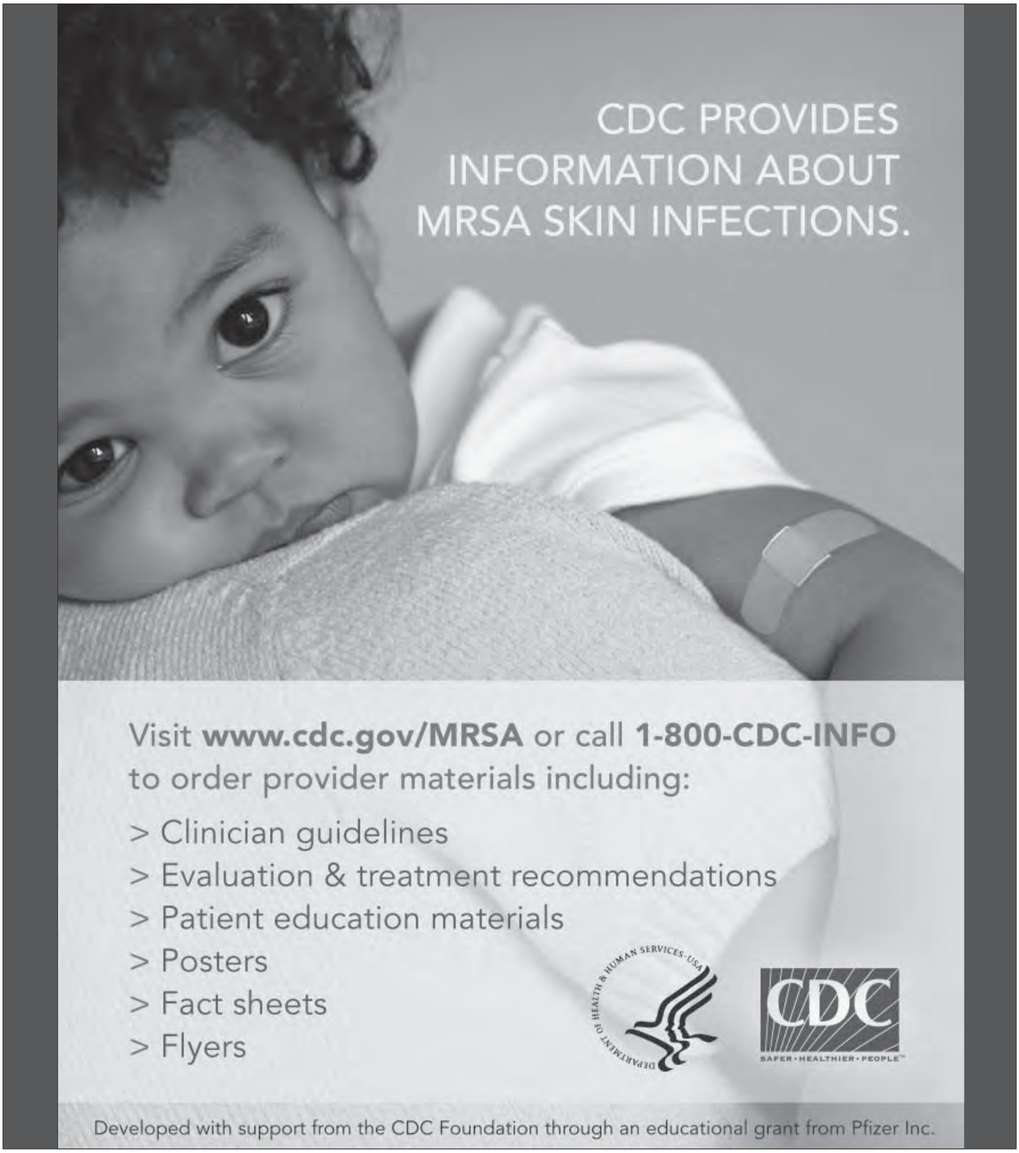

Chapter 6

Bacillus anthracis in China and its relationship to worldwide Lineages

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Bacillus anthracis in China and its relationship to worldwide lineages

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Abstract

Background: The global pattern of distribution of 1033 *B. anthracis* isolates has previously been defined by a set of 12 conserved canonical single nucleotide polymorphisms (canSNP). These studies reinforced the presence of three major lineages and 12 sub-lineages and sub-groups of this anthrax-causing pathogen. Isolates that form the A lineage (unlike the B and C lineages) have become widely dispersed throughout the world and form the basis for the geographical disposition of "modern" anthrax. An archival collection of 191 different *B. anthracis* isolates from China provides a glimpse into the possible role of Chinese trade and commerce in the spread of certain sub-lineages of this pathogen. Canonical single nucleotide polymorphism (canSNP) and multiple locus VNTR analysis (MLVA) typing has been used to examine this archival collection of isolates.

Results: The canSNP study indicates that there are 5 different sub-lineages/sub-groups in China out of 12 previously described world-wide canSNP genotypes. Three of these canSNP genotypes were only found in the western-most province of China, Xinjiang. These genotypes were A.Br.008/009, a sub-group that is spread across most of Europe and Asia; A.Br.Aust 94, a sub-lineage that is present in Europe and India, and A.Br.Vollum, a lineage that is also present in Europe. The remaining two canSNP genotypes are spread across the whole of China and belong to sub-group A.Br.001/002 and the A.Br.Ames sub-lineage, two closely related genotypes. MLVA typing adds resolution to the isolates in each canSNP genotype and diversity indices for the A.Br.008/009 and A.Br.001/002 sub-groups suggest that these represent older and established clades in China.

Conclusion: *B. anthracis* isolates were recovered from three canSNP sub-groups (A.Br.008/009, A.Br.Aust94, and A.Br.Vollum) in the western most portion of the large Chinese province of Xinjiang. The city of Kashi in this province appears to have served as a crossroads for not only trade but the movement of diseases such as anthrax along the ancient "silk road". Phylogenetic inference also suggests that the A.Br.Ames sub-lineage, first identified in the original Ames strain isolated from Jim Hogg County, TX, is descended from the A.Br.001/002 sub-group that has a major presence in most of China. These results suggest a genetic discontinuity between the younger Ames sub-lineage in Texas and the large Western North American sub-lineage spread across central Canada and the Dakotas.

Background

Ancient Chinese medical books suggest that an anthrax-like disease has been present in China for more than 5,000 years and that by 500–600 A.D. the epidemiology and symptoms of anthrax had been described [1]. A 1995 report from China described the results of an anthrax surveillance and control project in 10 provinces in China between 1990–1994 [2]. Stations in these 10 provinces (Sichuan, Tibet, Inner Mongolia, Xinjiang, Qinghai, Gansu, Guangxi, Guihou, Yunnan and Hunan) reported 72 outbreaks and 8,988 human cases of anthrax. These results, which are indicative of a long history and significant levels of contamination in these specific areas, are the reason for concern by the Chinese Institute of Epidemiology and Microbiology [2].

The population structure of *Bacillus anthracis* has only recently begun to be resolved with specific geographical patterns spread across areas mostly inhabited by man and his animals. Higher genetic resolution within *B. anthracis* has resulted from two molecular typing approaches: An ongoing comparative, single nucleotide polymorphism (SNP) analysis of diverse isolates that describes a conserved, clonally derived basal tree, [3] and a multiple locus variable number tandem repeat analysis (MLVA) system that provides improved resolution among individual isolates [4-7]. This process for molecular typing has now been applied to the study of isolates from China.

An archival collection of 191 *B. anthracis* isolates from China [collection dates from 1947–1983, except isolates A0034 (1993) and A0038 (1997)] was obtained and used in this study (see Methods and Additional file 1). This collection contained an unusual subset of 122 *B. anthracis* isolates recovered from soil, including 107 isolates collected between 1981/1982 in Xinjiang province. This province is located in the western most tip of China and was one of the 10 regions surveyed in the study conducted from 1990–1994. The remaining isolates originated from many regions across the whole of China. This report focuses on the molecular genotyping of these 191 isolates. Our goal was to determine the nature and distribution of genotypes found in China and to establish phylogenetic relationships between these isolates and those found elsewhere in the world.

Canonical SNP analysis

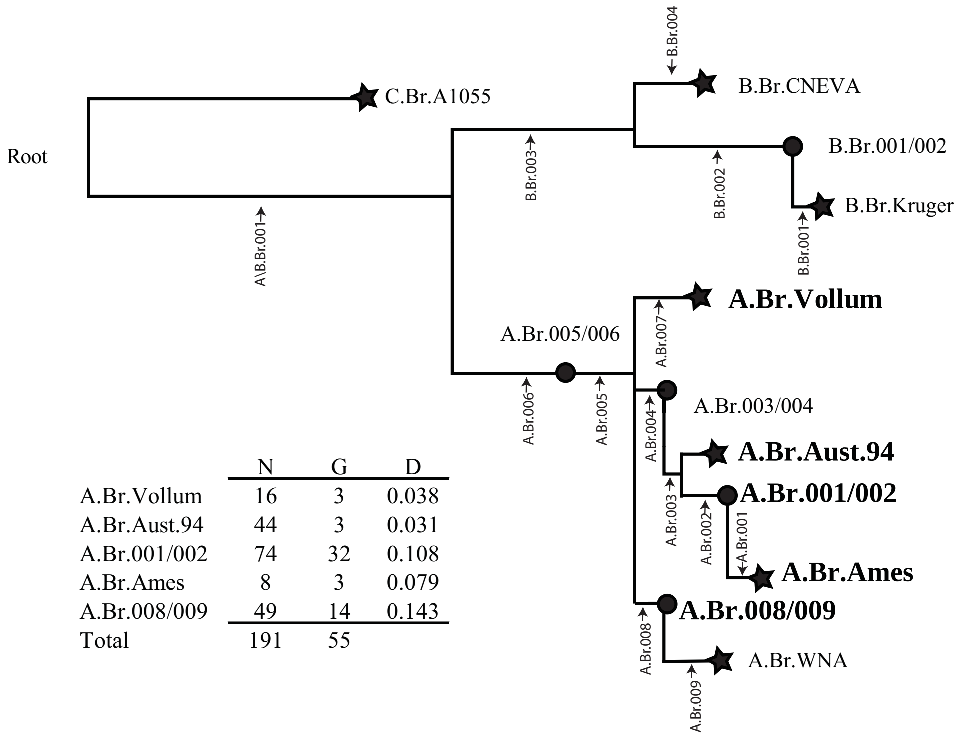
The original comparative analysis of 5 *B. anthracis* whole genome sequences examined the status of ~1,000 single nucleotide polymorphisms (SNPs) in 26 diverse isolates [3]. This study revealed an extremely conserved phylogenetic tree with only one homoplastic character in ~26,000 measurements. These results prompted the hypothesis that a few strategically placed "canonical SNPs" could replace the 1,000 assays and still describe an accurate SNP based tree. This idea was confirmed in a study using 13 canonical SNPs (canSNP) to examine 1,000 world-wide isolates of *B. anthracis* [5]. Figure 1 illustrates this original canSNP tree and is used here to define important nomenclature and terminology.

The basic tree is now defined by 7 sequenced genomes that form 7 sub-branches or sub-lineages ending in "stars" in Figure 1. Each of these sub-lineages is designated by the nomenclature from the whole genome sequence site in Genbank, e.g. A.Br Ames, A.Br.WNA (for western North America), and A.Br.Vollum. The relative position of each canSNP is indicated by vertical script and a small arrow and is arbitrarily defined, e.g., as A.Br.001 where A refers to the major subgroup and 001 is the first canSNP (see the A.Br.Ames sub-lineage in Figure 1, also [5]). In this case the derived A.Br.001 SNP defines all isolates that are on the same branch as the sequenced Ames strain. In addition to these 7 sub-lineages the analysis of 26 diverse isolates uncovered 5 nodes or sub-groups along the branches of this tree. Four of these nodes are in the major A Branch and one is in the B Branch (see "circles" in Figure 1). These nodes are defined by the two canSNPs on either side of the node position, e.g. A.Br.001/002 or A.Br.008/009. All of the initial 1,000 isolates in the Van Ert study [5] were placed into one and only one of these 12 sub-lineages or sub-groups.

Results

CanSNP analysis of isolates from China

The 191 *B. anthracis* isolates from China were distributed into only five of these 12 canSNP sub-lineages/sub-groups described by Van Ert et al. [5]. These canSNP groups were A.Br.Vollum, A.Br.Aust.94, A.Br.001/002, A.Br.Ames, and A.Br.008/009 (Figures 1 and 2). Four of the sub-lineages/



	N	G	D
A.Br.Vollum	16	3	0.038
A.Br.Aust.94	44	3	0.031
A.Br.001/002	74	32	0.108
A.Br.Ames	8	3	0.079
A.Br.008/009	49	14	0.143
Total	191	55	

Figure 1

The twelve canSNP subgroups and sub-lineages of *B. anthracis*. Determined by the analysis of 14 canSNP sites described by Van Ert et al[5]. The five canSNP groups represented in China are indicated in larger and bold fonts in this Neighbor Joining Tree. The number of isolates (N), genotypes (G), and Nei's Diversity Index [8] within groups (D) are illustrated in the table in the lower left. Neighbor-joining trees based upon additional MLVA genotypes within each of these 5 canSNP groups are illustrated in Figures 3 and 5.

sub-groups (A.Br.Vollum, A.Br.Aust.94, A.Br.008/009 and A.Br.001/002) were found in the western province of China, Xinjiang (Figure 2). But only isolates from A.Br.001/002 sub-group and the close relative A.Br.Ames sub-lineage were found scattered throughout the other regions of China from east to west. These findings clearly suggest 4 or 5 separate introductions of *B. anthracis* into or out of China, with 3 possibly involving the routes defined as the Silk Road.

The A.Br.008/009 sub-group is a cluster that predominates throughout Europe, the Middle East and China. Xinjiang province had 49 of the worldwide total of 156 A.Br.008/009 isolates (Table insert in Figure 1 and [5]). This province also had 44 of 188 worldwide isolates of the A.Br.Aust94 isolates. This is a sub-group that is also well represented in neighboring Turkey and India. A smaller subset of the A.Br.Vollum sub-lineage (also found in Europe and Africa) accounts for 16 Xinjiang samples out of a worldwide set that totals 48 isolates (Table insert in Figure 1).

The remainder of China is dominated by the A.Br.001/002 subgroup. Chinese isolates represent 74 of the 106 isolates from our worldwide collection of A.Br.001/002 sub-group isolates (Figure 1 and [5]). Only 9 of these isolates are from Xinjiang province to the west. Similarly there are 8 isolates out of 19 worldwide isolates in the A.Br.Ames sub-lineage in the main parts of China.

MLVA Analysis of A.Br.008/009, A.Br.Aust94 and A.Br.Vollum

CanSNP typing of these isolates has already indicated that there were 49 total Chinese isolates from the A.Br.008/009 subgroup, 44 from the A.Br.Aust94 sub-lineage and 15 from the A.Br.Vollum (Figure 1). Additional sub-typing using 15 MLVA markers indicates that there were only 3

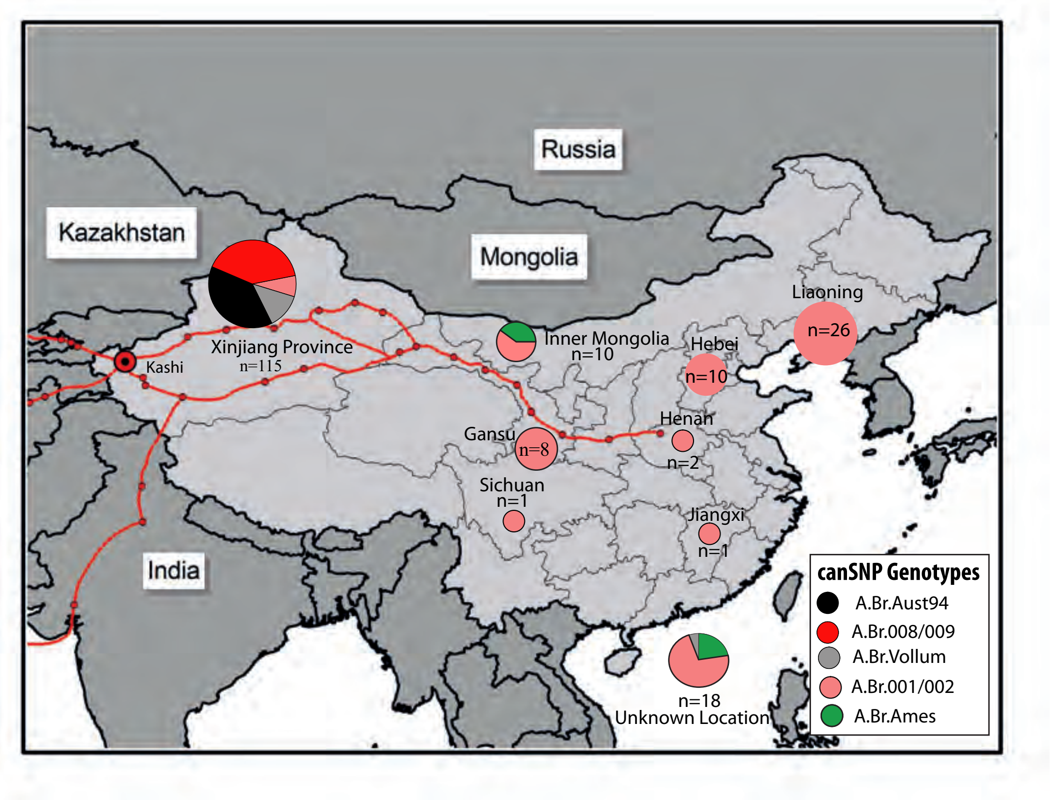


Figure 2

Geographical distribution of *B. anthracis* isolates in China. This distribution is based on 12 canSNP genotypes described in Figure 1 and the analysis of 191 isolates from China; also see [5]. The red routes include the western city of Kashi in Xinjiang Province, the main crossroads into China and around the Taklimakan Desert leading into the eastern Chinese provinces.

MLVA genotypes within both the A.Br.Vollum (Nei Diversity Index = 0.038 [8]) and A.Br.Aust94 (Nei's Diversity Index = 0.031) sub-lineages but 14 MLVA genotypes within A.Br.008/009 (Nei's Diversity Index = 0.143, Figures 1, 3a, 3b, and 3c). These results suggest repeated infections and outbreaks for each of these sub-groups of *B. anthracis*. The identification of 14 genotypes for the A.Br.008/009 subgroups is an indication of a combination of possibly repeated introductions and infections and a significantly longer history for this particular clade in this region.

Branch collapse and ongoing SNP analysis

One of the more remarkable findings from the whole genome SNP analysis of 5 diverse isolates by Pearson et al. [3] was a nearly total lack of homoplastic SNP markers in a query of the status of nearly 1,000 SNP positions in 26 diverse isolates. This finding uncovered a phenomenon called "branch collapse" that resulted in a tree that had no branching except for those created by 7 sequenced reference genomes. The remaining 26 isolates were then either part of one of these seven "sub-lineages" or part of 5 non-branching nodes ("sub-groups") on one of the 7 branches. While the canSNP tree is highly accurate in the typing of 1033 isolates, it lacks resolution because it reflects the results of only 13 of nearly 1,000 SNPs.

Improved resolution between two points was demonstrated by an extensive analysis of the Ames specific branch [9,10] when the status of 29 SNPs that define this branch were determined for the original 12 Ames-like isolates. These analyses have a direct bearing on the isolates from China that are either Ames-like or part of the A.Br.001/002 sub-group (Fig. 1 and 4). The extended analysis of the SNPs on the Ames branch indicate that

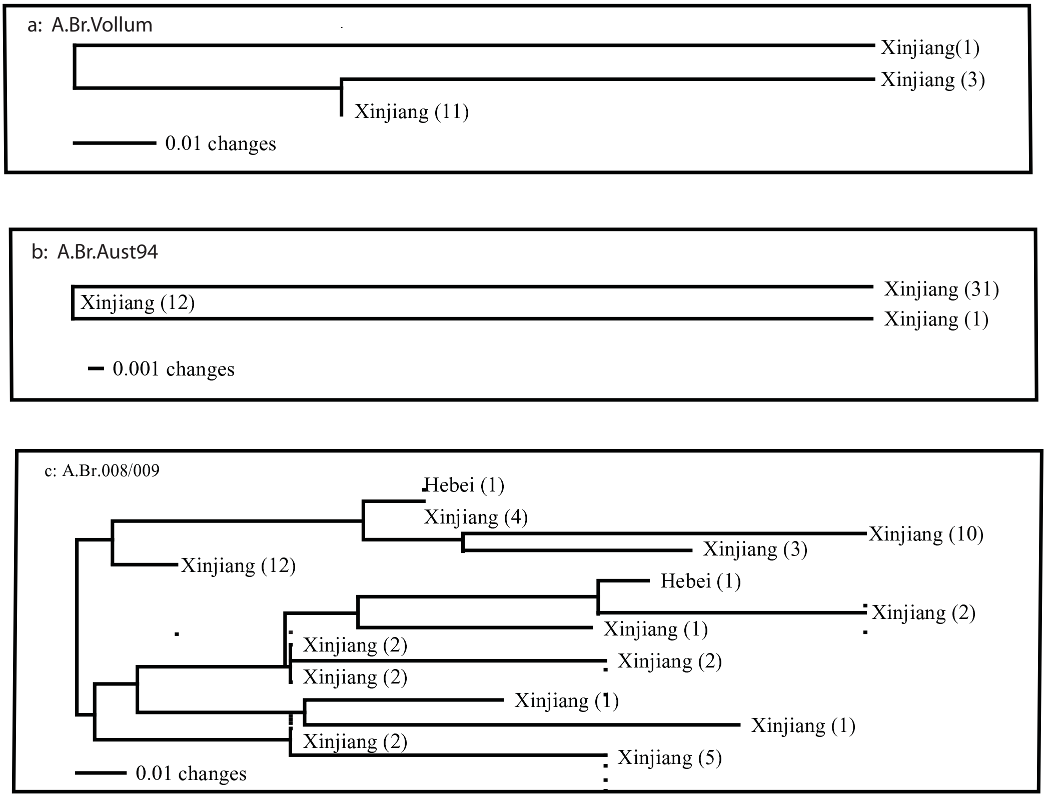


Figure 3
MLVA15 Analysis of Chinese isolates belonging to the A.Br.Vollum, A.BrAust94 and A.Br.008/009 canSNP sub-lineges/sub-groups. Representatives of these three sub-groups were only found in isolates recovered in Xinjiang Province, or in unknown locations within China (n = 2). All of these isolates were recovered from soil samples in this province.

there are 74 Chinese isolates in the A.Br.001/002 subgroup and 8 additional Chinese isolates (see the table insert in Figure 1) that form three new nodes or collapsed branch points between A.Br.001/002 and the Ames isolate (Figure 4). In addition, there is a fourth node closest to the Ames strain that contains 10 Ames-like isolates from Texas, one goat and 4 bovine isolates [9] shown in Figure 4 and an additional 5 Ames-like isolates from the CDC (Brachman collection, see Methods and Materials). The precise location for the recovery of these latter isolates is unknown except that they originated in Texas. These 19 isolates (8 Chinese, 10 Texas) and the Ames strain represent a highly resolved, SNP based A.Br.Ames sub-lineage. These results indicate that the original Ames strain and a subset of 10 Texas isolates are decendents of a rare lineage that is otherwise only found in China.

MLVA: A.Br.001/002

The 15 marker MLVA analysis (MLVA15) of the 74 isolates belonging to the A.Br.001/002 sub-group yielded 32 different genotypes (Nei Diversity Index = 0.108, Figures 1, 5a). This high diversity index is an indication that this sub-group, spread throughout the whole of China (Figure 2), is another sub-group of *B. anthracis* with a long and extensive evolutionary presence in China.

Discussion

Human anthrax has been an old and continuous problem in many rural regions in China where as much as six percent of environmental samples have been found to be contaminated with *B. anthracis* [2,2]. An archival collection of 191 *B. anthracis* isolates was obtained from China and canonical SNP typing indicated that only 5 of the 12 world-

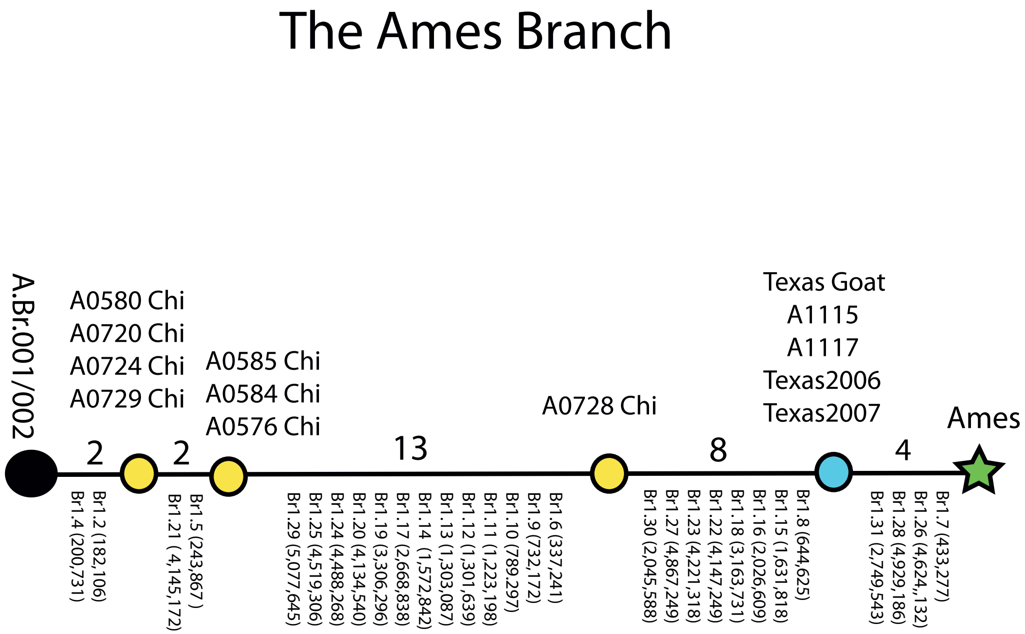


Figure 4
The Ames branch of *B. anthracis*. This figure shows the relationship between the Ames strain and its closest relatives in a worldwide collection [5]. Twenty-nine of 31 original [5] SNPs are defined by their positions in the Ames genome (NC_003997) and their positions along the Ames branch. Ames has the derived state for all 29 SNPs and the 4 SNPs between Ames and the Texas Goat are specific for the Ames strain alone [5]. A0728 was isolated in China in 1957 but the specific location/source of this isolate is unknown.

wide sub-lineages/sub-groups of this pathogen were represented in this collection. One striking feature of the distribution of these *B. anthracis* isolates within this country was the discovery that three of the five canSNPs sub-lineages/groups (A.Br008/009, A.BrAust.94, and A.Br.Vollum) are predominantly found in the western most Chinese province of Xinjiang. The previous observation [5] that these three sub-lineages/sub-groups are prominent genotypes in India, Pakistan, Turkey and most of Europe suggest a likely transmission pattern for anthrax along the ancient trade route known as the Silk Road [11] that extended from Europe, the Middle East, portions of Asia and into Xinjiang province and the whole of China, Figure 2.

More specifically, 107 isolates were recovered from "soil samples" between 1981–1982 from unspecified sites relatively close to the city of Kashi in this province. Kashi (also Kashgar, Kaxgar, Kxkr) was a major "oasis" crossroads city along the ancient Silk Road and dates back more than 2,000 years [11]. Consistent with the idea that the life cycle of *B. anthracis* can be maintained by viable spores in previously contaminated areas, the later 1990–1994 surveillance project in China described three regions in Xinjiang Province where severe anthrax outbreaks had previously occurred [2]. Two of these towns, Zepu and Atushi, are located approximately 144 and 33 kilometers respectively from the city of Kashi. In the 1990–1994 study, Zepu recorded 24 villages with 202 human infections and Atushi recorded 4 villages with 81 human infections.

Despite a clear correlation between canSNP genotypes from the A radiation and the spectrum of isolates found across the Trans-Eurasian continents, there is one set of genotypes in Europe that are clearly missing in China. These are representatives from the B branch that appear to be prevalent in several European states including at least 27 B2 isolates from France and isolates identified in both the B2 and B1 branches from Croatia, Germany, Poland, Italy, Norway and Slovakia [5,6,12]. It is not obvious why

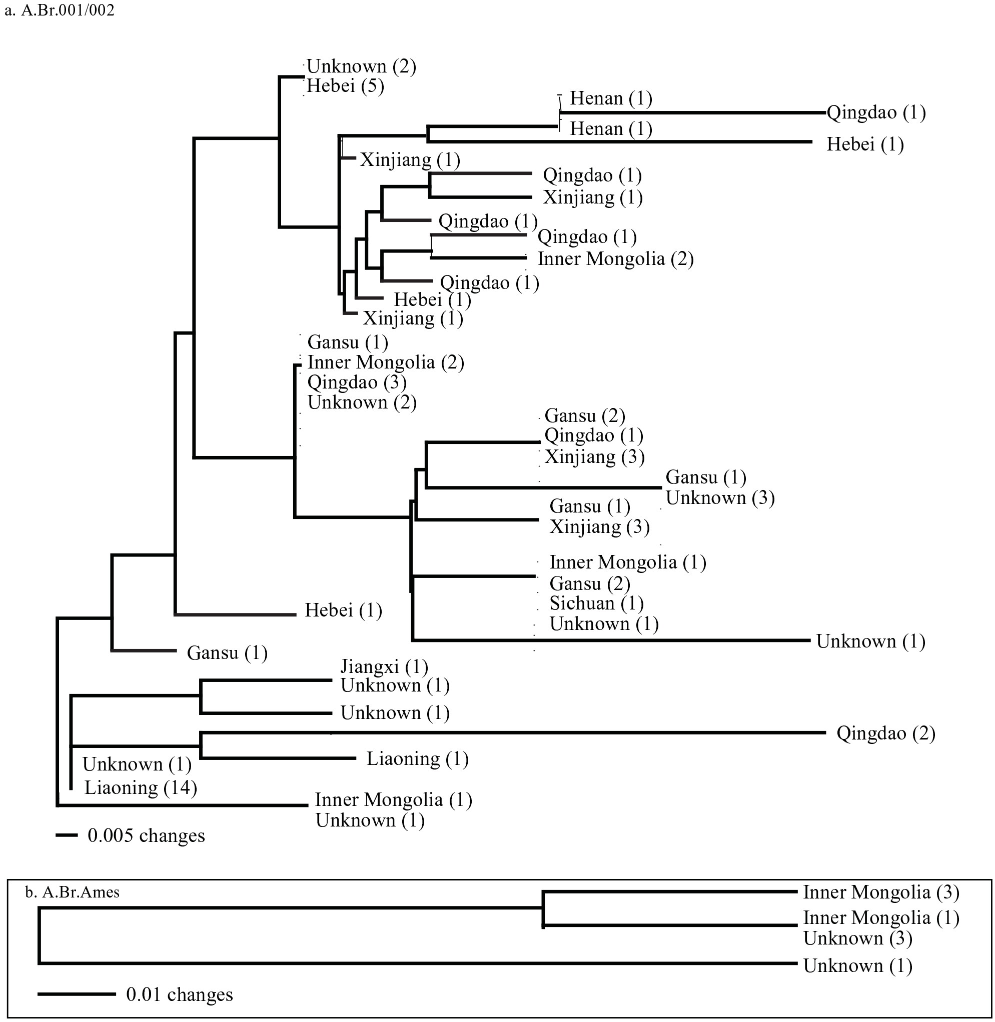


Figure 5

MLVA 15 Analysis of A.Br.001/002 and A.Br.Ames sub-group and sub-lineage respectively. The A.Br.001/002 sub-group has a relatively large diversity index (See Figure 2) and suggests that this sub-group has a long history in China with repeated outbreaks and eventual spread throughout much of the country.

examples of the B branch are limited mostly to Africa, this region of Europe and a small location in California, USA. Aside from sampling issues the B branch does not appear to have participated in the world-wide, dynamic radiation that has characterized the A branch [5].

Additional analyses with the rapidly evolving MLVA markers suggest that establishment in China of two of these sub-groups/sub-lineages, A.Br.Aust94 and A.Br.Vollum, resulted from relatively recent events (Figure 3a and 3b). In both of these instances, a sizeable number of isolates (44 and 15, respectively) are clustered into only three different MLVA15 genotypes (Nei's Diversity Indices = 0.031 and 0.038 respectively, Figure 2). Although these results may reflect a certain sampling bias, the MLVA comparison to other worldwide isolates from this branch indicates that the A.Br.Aust94 sub-lineage in China is most closely related to isolates recovered from the large 1997 outbreak in Victoria, Australia (data not shown). The precise origin and time-scale for this exchange is not certain but relatively recent exchanges between the Far East and Australia appear to have originated from India [13], which could represent a common ancestor or an intermediate step in the transmission route.

By direct contrast the MLVA analysis of 49 isolates belonging to the A.Br.008/009 sub-group revealed a more complex pattern with 14 different MLVA15 genotypes (Nei Diversity Index = 0.143, Figures 1 and 3c). This is a remarkable finding because it indicates that a variety of MLVA genotypes are persisting in the different soils from which the A.Br.008/009 isolates were recovered. These results are an indication that A.Br.008/009, a major subgroup in Europe and Asia [5], has had an extensive history in China. It is difficult to determine the precise origins of the A.Br.008/009 subgroup (e.g. China versus Europe) at this point because rapidly evolving MLVA markers are subject to homoplasy and potentially inaccurate phylogenetic reconstructions. These issues can eventually be resolved using additional whole genome sequencing and phylogenetic inference to more accurately predict the origins of the A.Br.008/009 sub-group.

The Ames sub-lineage appears to have descended from the A.Br.001/002 sub-group, a sub-group that has 106 isolates in our worldwide collection [5]. Seventy-four of these accessions were isolated from outbreaks in China and the remaining 32 isolates were recovered in the UK, other parts of Europe, North America and other parts of Asia. The large number of MLVA15 genotypes (n = 32) among the 74 Chinese isolates and a wide distribution throughout the country indicates that the A.Br.001/002 sub-group is a major part of the *B. anthracis* population structure in this region (Figure 5a). This sub-group also appears to be basal to the Ames sub-lineage, indicating that 8 isolates from China and 11 isolates from Texas may share common ancestors that originated in China (Figure 5b and [10]).

How then did the Ames lineage come to Texas and why is this lineage not found in Europe? This is still not known and subject to considerable speculation. By several accounts, it is believed that anthrax was introduced into the Gulf Coast states (Louisiana and Texas) by early settlers from Europe. Stein [14,15] indicates that the first recorded episodes of anthrax in livestock in Louisiana occurred in 1835, 1851 and 1884; and in Texas in 1860 and 1880. By 1916, when a first national survey was conducted to obtain nation-wide information on the incidence of anthrax, Texas already had 41 counties reporting infections. A composite of outbreaks compiled after the 4th National Survey by the U.S. Department of Agriculture between 1916–1944 (Figure 6) indicates three major outbreak pockets: one in California, one in the Dakotas/ Nebraska and the third along the coastal regions of Texas and Louisiana [15].

An important feature of the outbreaks in Texas is that the "modern" outbreaks have occurred repeatedly in many of the same counties depicted in this historical map (Figure 6 and USDA Report: Epizootiology and Ecology of Anthrax: http://www.aphis.usda.gov/vs/ceah/cei/taf/emerginganimalhealthissues_files/anthrax.pdf). A culture-confirmed study between 1974–2000 indicated that 179 isolates were spread across 39 Texas counties (counties outlined in yellow) that are in general agreement with the dispersal patterns observed in the early national surveys depicted in Figure 6. The one significant difference is a shift from the historical outbreaks in the coastal regions to counties more central and southwesterly in "modern" times. Similarly, culture-confirmed isolates from a 2001 outbreak in Val Verde, Edwards, Real, Kinney and Uvalde counties in southwest Texas are similar to outbreaks in 2006 and 2007 when 4 Ames-like isolates were recovered from Real, Kinney, and Uvalde county [9].

It appears that *B. anthracis* was introduced into the Gulf Coast, probably by early European settlers or traders through New Orleans and/or Galveston during the early to mid 1800s. The disease became established along the coastal regions and then became endemic to the regions of Texas where cattle and other susceptible animals are currently farmed. Are these *B. anthracis*, Ames-like genotypes from the Big Bend region (Real, Kinney, Uvalde counties) of Texas representative of the ancestral isolates brought to the Gulf Coast? Van Ert et al. [5] used synonymous SNP surveys to estimate the divergence times between the major groups of *B. anthracis* and these estimates suggest that the Western North American and the Ames lineages shared common ancestors between 2,825 and 5,651 years ago. Extrapolating to the much shorter SNP distances between the most recent Chinese isolate (A0728) and the recent Texas isolates on the Ames sub-lineage would approximate that these two shared a common ancestor between 145 to 290 years ago. These estimates would be consistent with the hypothesis that an Ames-like isolate was introduced into the Galveston and/ or New Orleans area in the early to middle 1800s.

This relatively recent expansion is in direct contrast to analyses of the Western North American (WNA) sub-lineage that appears to have an ancient and significantly longer evolutionary presence in North America; this group stretches from the central regions of Canada and into North and South Dakota (Figure 6; [16]). Phylogenetic reconstruction of > 250 Western North American isolates indicates that the more ancestral isolates of this sub-lineage are found in the upper reaches of central Canada and portrays a migration pattern where the youngest

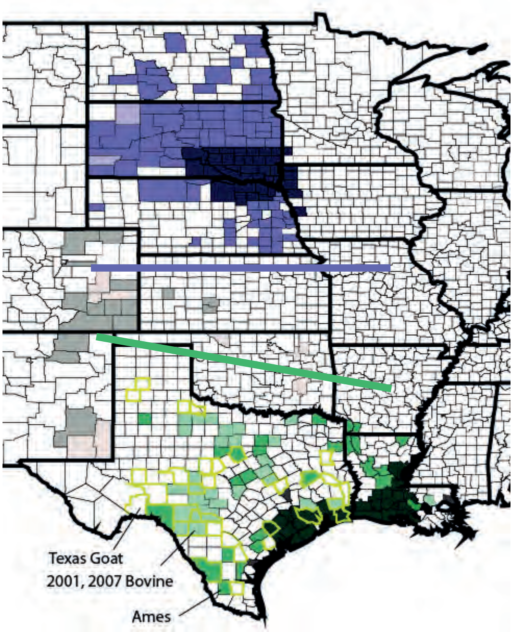


Figure 6
Historical Anthrax Incidences between 1915–1944 in Texas/Louisiana and The Dakotas/Nebraska/Iowa. Adapted from Stein (1945, [15]). Darker colors represent severe outbreaks and the lighter colors represent sporadic outbreaks. The blue and green colors were used to illustrate that two distinct genotypes (Western North America (WNA) and the Ames sub-lineage) have been indentified in "modern" isolates from these two regions. The counties bordered in yellow in Texas indicate counties where documented incidents of anthrax have occurred between 1974 and 2000. The numbers 1–4 indicate the counties in which the original Ames strain, 2 bovine samples and a goat sample have been analyzed by current genotyping methods as belonging to the Ames sub-lineage. The molecular analysis of more than 200 isolates from North and South Dakota indicates a pre-dominance of the sub-lineage WNA in this region. The gray colors indicate moderate to sparse outbreaks in the states adjoining the Dakotas and Texas.

isolates are found in cattle outbreaks in North/South Dakota and Nebraska. Kenefic, Pearson et al. [16] suggest that the ancestral isolates may have entered the North American continent via the Beringian straights 13,000 years ago.

A recent ecological niche model suggests that natural anthrax outbreaks are "concentrated in a narrow corridor from southwest Texas northward into the Dakotas and Minnesota" [17]. This model indicates that conditions like vegetation, precipitation and altitude along this corridor are suited for maintaining naturally occurring anthrax outbreaks in livestock and wildlife. Although historical records provide evidence that validate this model, there is a molecular and genotyping anomaly: there does not appear to be a direct epidemiological link between the "younger" Ames-like cluster and the Western North American lineage. Despite nearly 100 years of monitoring since the first national outbreak tabulations [15], there is still a clear physical division between the Ames-like isolates to the south and the Western North American lineage to the north (Figure 6). This gap is not obvious until the spatial patterns are examined in hindsight of the genetic discontinuity. These observations probably reflect the awareness and controls that were being observed for anthrax outbreaks as the US entered the 20th century.

Limited sample analysis of isolates from the Texas/Louisiana coastline prevents any conclusions about the overall dominance of the Ames sub-lineage in this area and we also cannot exclude the possibility that there are other sub-groups/sub-lineages that might have been imported and even become transiently established along the Texas/Louisiana Gulf region during this same time frame.

Conclusion

Despite containing only 5 of the initial 12 canSNP genotypes used to define a collection of world-wide isolates [5], the analysis of 191 Chinese *B. anthracis* isolates reveals an interesting impact on global distribution. The major diversity in these isolates is concentrated in the western province of Xinjiang and especially the city of Kashi, the hub of the Silk Road around the Taklimakan Desert into and out of China. These results reinforce the idea that this Silk Road region was central to the spread of anthrax between the trans-Eurasian continents.

In addition to the three distinct sub-groups found in the western Xinjiang province, the central and eastern regions of China are dominated by a different, highly diverse, can-SNP sub-group, A.Br.001/002. This sub-group is a major presence in relationship to our world-wide collection since 70% of all the isolates and most of the diversity for this sub-group were in this Chinese collection. These results suggest that the A.Br.001/002 cluster may have originated in China. Finally, the Ames and Ames-like strains in Texas are descended from common ancestors in Inner Mongolia in China as an extension of this sub-group. It is curious that this lineage would become established in Texas, and perhaps Louisiana, and not in Europe. This leaves behind a missing historical gap within the phylogeography of the Ames lineage.

Methods

B. anthracis *isolates*

The 191 *B. anthracis* isolates from China used in this study were previously isolated from a variety of sources and provinces in China (see Additional file 1). One hundred and fifteen isolates were from Xinjiang Province in western China including 107 isolates from soil samples. The remainder of the isolates were recovered from the following provinces with the number of isolates in parenthesis: Hebei (10), Gansu (8), Henan (2), Inner Mongolia (10), Jiangxi (1), Liaoning (26), Sichuan (1) and 18 isolates where the province of origin was not known. In addition to the 107 soil samples from Xinjiang Province isolates were obtained from the following sources: soil (15 additional), air (4), bovine (3), buffalo (1) fur (2), human (25), laboratory (1), marmot (1), sheep (3), swine (3) and unknown sources (26). In addition to the Chinese isolates there are 6 isolates that were used to describe Figure 4[9,10] and an additional 5 isolates that were obtained from the CDC as part of the "Brachman Collection" (CDC ID # 34064, 34279, 402, 482, 490). All 11 of these isolates belong to the Ames sub-lineage and all were isolated in Texas between 1959–2007. This analysis also includes the original Ames strain that was isolated in 1981 from bovine in Jim Hogg County.

All isolates were initially genotyped for a *B. anthracis* species-specific *plcR* nonsense mutation that has been suggested as being necessary for stabilization of the virulence plasmids [18]. This single nucleotide polymorphism appears to be diagnostic for *B. anthracis* [19]. In this study the ancestral state for this marker was used to root the *B. anthracis* SNP tree to the older and more diverse *B. cereus/ B. thuringiensis* tree. DNA was isolated from each of the 191 isolates as previously described [5].

CanSNP Genotyping

TaqMan™ -Minor Groove Binding (MGB) allelic discrimination assays were designed for each of 13 canSNPs and have been described in great detail by Van Ert et al. [5]. The genomic positions for each canSNP and the primer sequences and probes for each site can be found in Supplemental Tables 4 and 5 in the Van Ert et al. [5].

MLVA Genotyping

Multiple Locus Variable Number Tandem Repeat (VNTR) Analysis (MLVA) was used to determine the overall diversity of the isolates within each sub-group and sub-lineage. The first 8 marker set used in this analysis were initially described by Keim et al., [4] and a second set of 7 additional markers were described by Zinser [20]. This 15 marker, high-resolution, MLVA system is described in detail by Van Ert et al. [5] with the genomic positions and primer sets for these assays described in Supplemental Tables 2 and 6 of this reference.

Phylogenetic Inference

The genetic relationships among the Chinese isolates were established using a hierarchical approach where the slowly evolving, highly conserved, canSNP markers were first used to place each isolate into its appropriate clonal lineage. The 15 more rapidly evolving, VNTR loci, were then used to measure the genetic diversity and to determine the number of specific genotypes within each of these clonal lineages. Neighbor joining phylogenetic trees were constructed for both the canSNP and MLVA datasets using PAUP (Phylogenetic Analysis Using Parsimony) [21]; and the MEGA 3 software package [22] was used to calculate average within group distances for each of the five canSNP sub-groups/sub-lineages.

Authors' contributions

TSS: Conception, acquisition and analysis of data, interpretation of data, drafting of manuscript, approved final draft. RTO: Analysis and interpretation of data, drafting of manuscript, approved final draft, BW: Acquisition and interpretation of isolate data, approved final draft, RE: Acquisition and interpretation of DNA signature data, approved final draft, LYH: Acquisition and interpretation of DNA signature data, approved final draft, JMUR: Acquisition and interpretation of DNA signaturedata, approved final draft, MD: Acquisition and interpretation of DNA signature data, approved final draft, SRZ: Acquisition and interpretation of DNA signature data, approved final draft, LJK: Provide insight for relationship between worldwide and Chinese isolates, approved final draft, JB: Acquisition and interpretation of data, approved final draft, JMS: Acquisition and interpretation of data, approved final draft, TP: Input on phylogenetic analysis of datasets, draft manuscript, approved final draft, DMW: Provide insight into geographical relationships between worldwide isolates, draft manuscript, approved final draft, AH: Provide data and genotyping information for new Texas isolates belonging to Ames sub-lineage, approved final draft, JR: Initial sequencing, assembly and analysis of genomes, approved final draft.

PK: Responsible for concepts, vision and direction for the entire project, draft manuscript, approved final draft.

Additional material

Additional file 1

List and description of isolates including the canSNP and MLVA Genotypes for each isolate*. This table contains: The Keim Laboratory ID # for each isolate, the year of isolation, the source, the canSNP ID, and the originating province. This information is followed by the Keim Genetics Laboratory 15 MLVA genotypes for each isolate, see supplemental material from Van Ert et al., [5].*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-9-71-S1.doc]

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