

Genotyping of *Mycobacterium tuberculosis* cultured from elephants

Tuberculosis in Elephants: Science, Myth, and Beyond
APHIS Center for Animal Welfare
Kansas City, MO April 5 – 6 2011

**James Higgins, Mycobacteria and Brucella Section, National
Veterinary Services Laboratories
APHIS / USDA
Ames, IA**

'One Health': APHIS Veterinary Services 2015 initiative



Two major genotyping methods are currently used by mycobacteria laboratories world-wide:

- Spoligotyping ('medium' resolution)
- Variable number tandem repeat (VNTR) analysis also referred to as 'mycobacterial interspersed repetitive units' (MIRU), or 'multiple locus variable number tandem repeat analysis' (MLVA) ('high' resolution)

Spoligotyping ('spacer oligonucleotide typing')

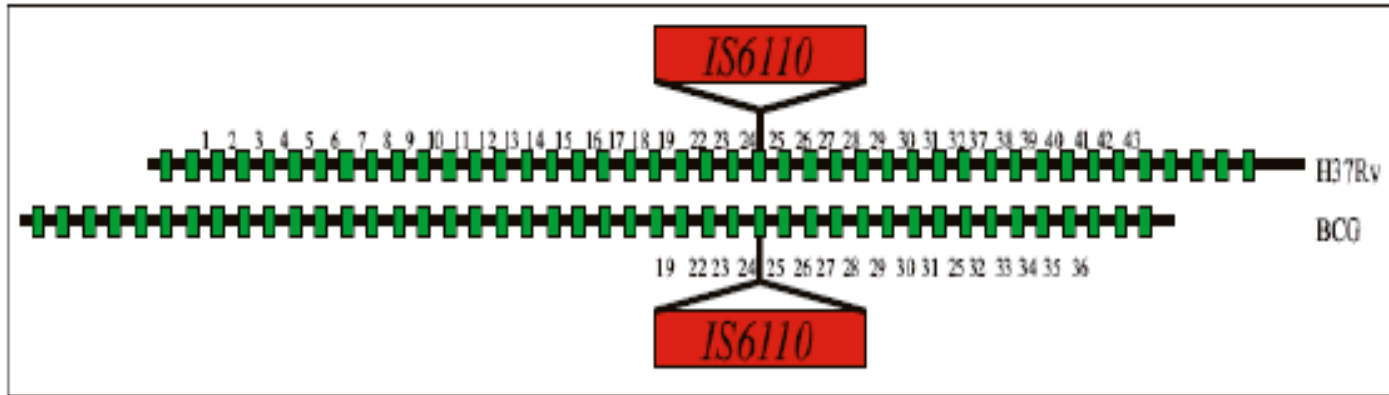


Figure 1: Structure of the DR locus in the genome of *M. tuberculosis* H37Rv and *M. bovis* BCG P3 strain. The green rectangle depicts 36 bp Direct Repeats(DR).

- Exploits the presence of a direct repeat (DR) region in the genome of *Mycobacterium tuberculosis* and *M. bovis*
- This region contains varying numbers of 36 base pair **direct repeats** and **intervening 'spacer' sequences**
- The mutation rate for a spoligotype locus is ~ 0.1 – 0.01 per year (Reyes & Tanaka 2010)
- Spoligotyping detects these **spacer sequences** via reverse line blot hybridization assay

Spoligotyping ('spacer oligonucleotide typing')

- PCR is used to amplify the DR region
- PCR products are applied to a membrane containing bound oligonucleotides representing the spacer sequences
- PCR products bound to the oligos on the membrane are visualized by a chemiluminescent reaction
- CDC contract labs now use Luminex® bead-based protocol (no longer membrane-based)

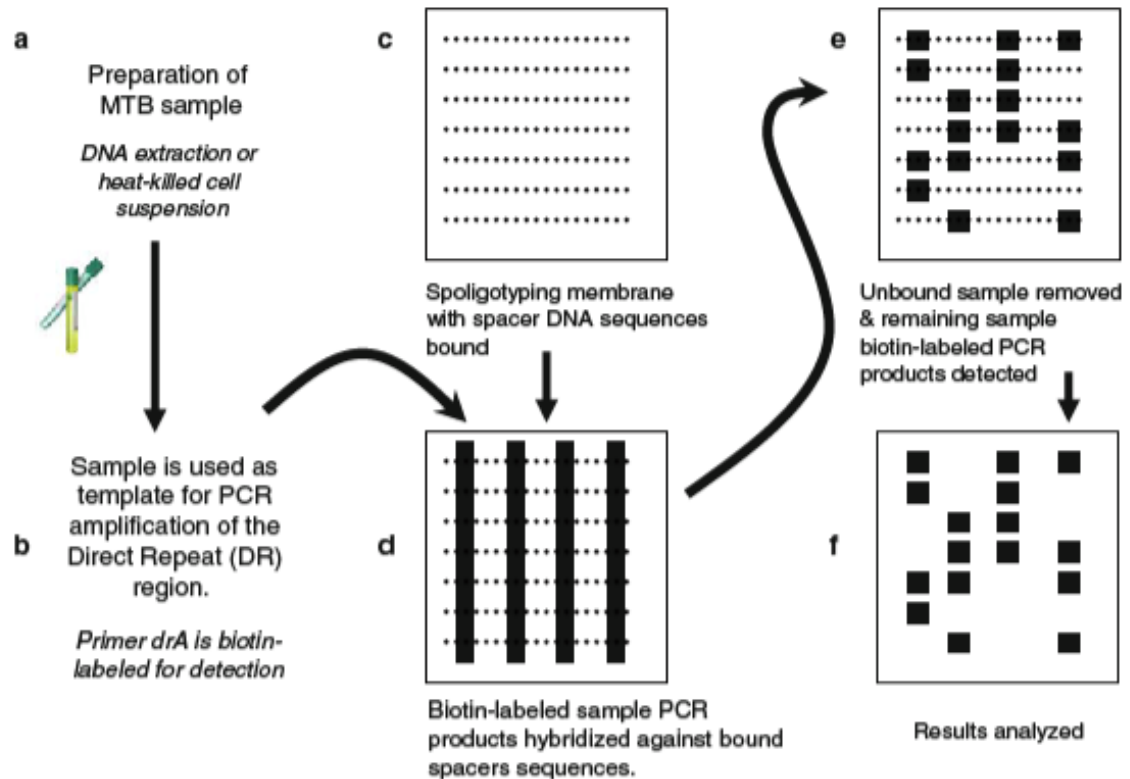


Fig. 1. Basis of the spoligotyping methodology. (a) A spoligotyping membrane. Dashed lines indicate the location of the bound polymorphic oligonucleotides, one corresponding to each of the 43 unique spacer sequences utilized in the assay. (b) The hybridization of the amplified samples (black bars) against each of the bound oligonucleotides. (c) The excess and nonspecifically bound sample is removed through a series of washes, and the remaining bound PCR products from the sample are detected. (d) A representation of the final results.

Spoligotyping ('spacer oligonucleotide typing')

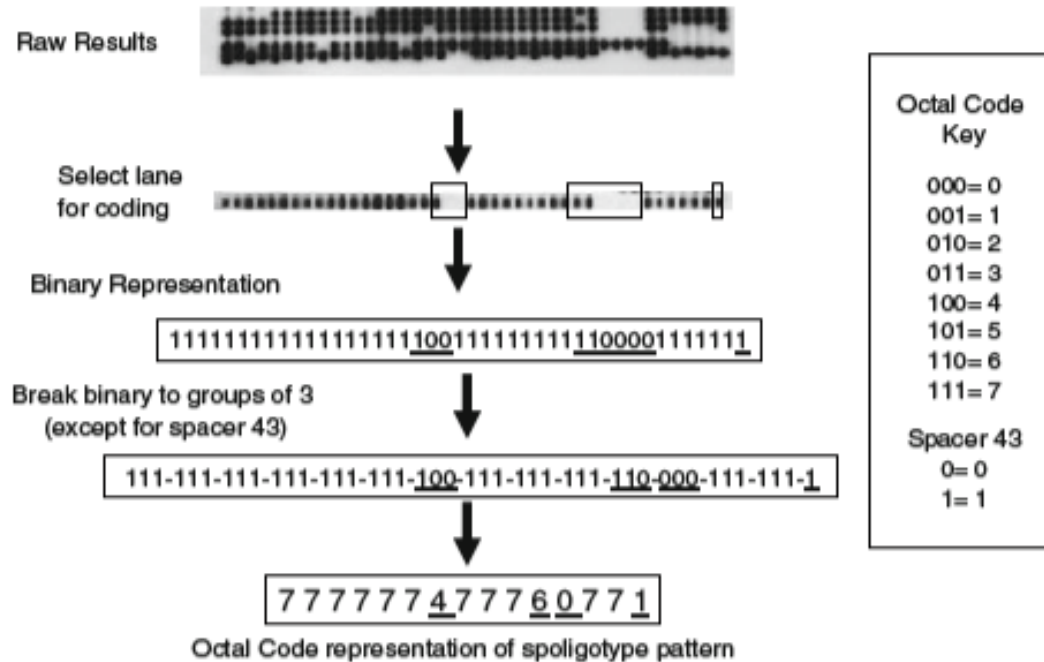


Fig. 2. Conversion of a raw spoligotyping results to octal code representation (5). The raw hybridization pattern is converted to a binary representation using 1's (indicating hybridization detected) and 0's (no hybridization detected). The binary string is separated into 14 groups of three, with spacer 43 remaining ungrouped. Each binary triplet is converted to the appropriate octal code designation (shown). The 15th digit of the octal is either 1 or 0 depending solely on the result for spacer 43.

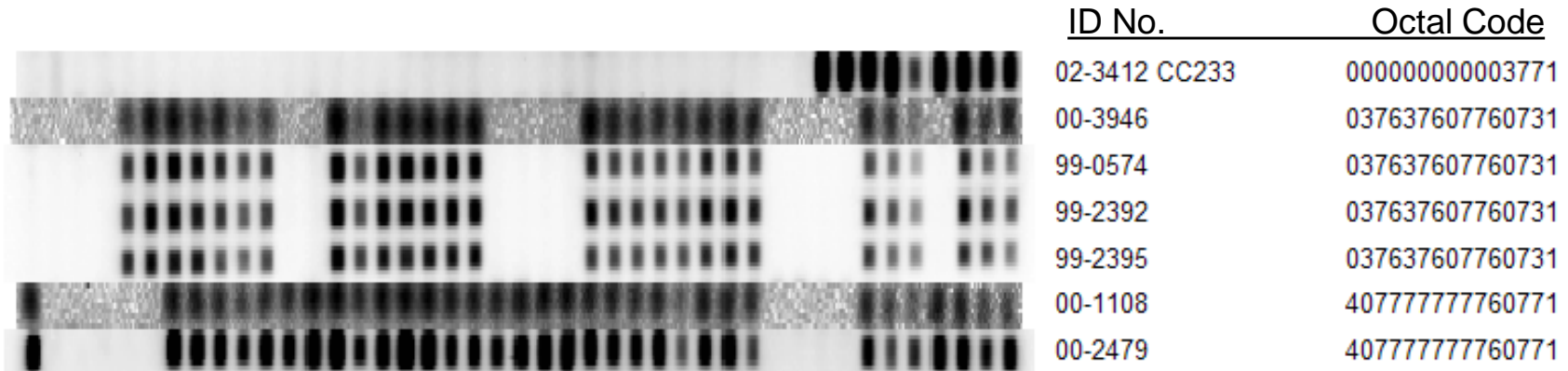
- Conversion of banding patterns to a **binary code** simplifies spoligo profile

Conversion of binary code to **octal code** simplifies still further and permits ready comparison via online databases

Safeguarding Animal Health

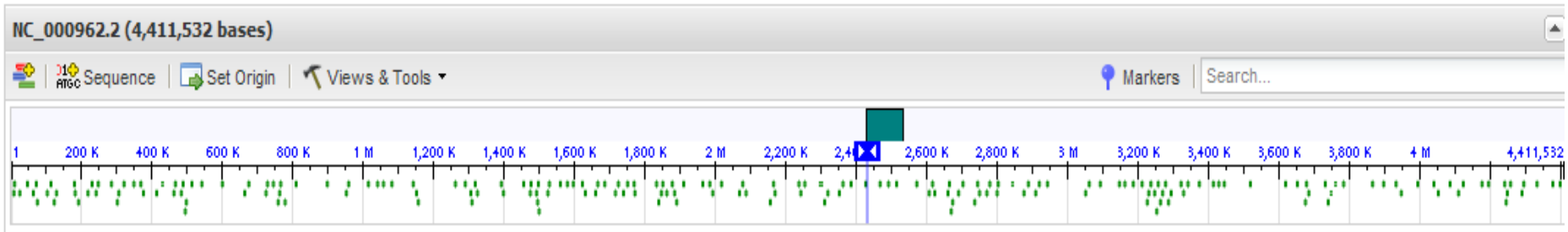
Spoligotyping ('spacer oligonucleotide typing')

Spoligotyping



Example of spoligotyping profiles generated for 7 *M. tuberculosis* isolates using the BioNumerics software package

Mycobacterial interspersed repetitive units (MIRU) & Variable Number Tandem Repeat (VNTR) analyses



- Exploits the presence of repeats regions in the genome of *M. tuberculosis*
- The genome contains large numbers of these repeats
- Mutation rate of a given VNTR / MIRU locus is $\sim 10^{-3}$ per year (Reyes and Tanaka 2010)
- Some of these repeats display variability *among* strains *within* a species
- Accordingly, variability in repeat numbers between strains can be a genotyping tool
- ‘simple’ and straightforward as opposed to spoligotyping

'close up' view of a VNTR / MIRU repeat region from the genome of *M. tuberculosis* strain H37Rv: locus 4052, known as 'QUB 26'

Locus 4052

>gi|57116681:4052801-4053708 *Mycobacterium tuberculosis* H37Rv, complete genome

Green highlighted sequences are forward and reverse primers

```
TCGCTGAAGTAAAAGAACGACCAGCCGAGCAGCACCACGACGGCGATCGCTGTTATGGTGCAGATCACGTTTTTCCGGTTCAT
CGATCATCGGCCGTGCCGGCCAGGTCCTTCCCGATACACGCAGCTGAAAGTCCAGGTTACCGCTCGTGCCGATCGCAAACCC
GGCGGAGCCGGGTGCAGCGGGTCGCCACCATCAGCCCCGTGGCGATCGCAAACCCCGCGCCTGGCGACAATGCGGCCCGCA
AAACGGGCCGAGGAGGAGCCAGGCAATCACCCAGAGCCGGGTGCAGCGGGTCGCCACCATCAGCCCCGTGGCGATCGCAA
ACCCCGCGCCTGGCGACAATGCGGCCCGCAAACGGGCCGAGGAGGAGCCAGGCAATCACCCAGAGCCGGGTGCAGCGGG
TCGCCACCATCAGCCCCGTGGCGATCGCAAACCCCGCGCCTGGCGACAATGCGGCCCGCAAACGGGCCGAGGAGGAGCCA
GGCAATCACCCAGAGCCGGGTGCAGCGGGTCGCCACCATCAGCCCCGTGGCGATCGCAAACCCCGCGCCTGGCGACAATGC
GGCCCGCAAACGGGCCGAGGAGGAGCCAGGCAATCACCCAGAGCCGGGTGCAGCGGGTCGCCACCATCAGCCCCGTGGC
GATCGCAAACCCCGCGCCTGGCGACAATGCGGCCCGCAAACGGGCCGAGGAGGAGCCAGGCAATCACCCAGAGCCGGGT
GCAGCGGGTGCAGCCTGGCTAGACCAACGACCGGTAGTTCACCGACGGCGTCGGAAAATCCGACAGCTGAGCGTTCGGGTCAA
ACACGCGGTGCACCGGACCTGATTTGGCTCGAATTGGTGCACCGAGGGTCGGGCACATCGCTCCGGTGCATGTGTCACT
GCACCGGGCG
```

Repeat region (111 bp long):

```
GAGCCGGGTGCAGCGGGTCGCCACCATCAGCCCCGTGGCGATCGCAAACCCCGCGCCTGGCGACA
ATGCGGCCCGCAAACGGGCCGAGGAGGAGCCAGGCAATCACCCCA
```

Copy number: 5.2

Historically, the mycobacterial research community has used nested sets of 12, 15, or 24 different loci for MIRU / VNTR analyses...
we at the MB Section at the NVSL use a set of 11 loci (highlighted in yellow) derived from the 'Master List' of 24

MIRU-VNTR Loci Set

12
15
24

Use MIRU-VNTR Loci Set: 24 ?

Composition of the different MIRU-VNTR sets:

Loci	Alias 1	Alias 2	24	15	12	Loci	Alias 1	Alias 2	24	15	12
154	MIRU02		X		X	2401	Mtub30		X	X	
424	Mtub04		X	X		2461	ETRB		X		
577	ETRC		X	X		2531	MIRU23		X		X
580	MIRU04	ETRD	X	X	X	2687	MIRU24		X		X
802	MIRU40		X	X	X	2996	MIRU26		X	X	X
960	MIRU10		X	X	X	3007	MIRU27	QUB5	X		X
1644	MIRU16		X	X	X	3171	Mtub34		X		
1955	Mtub21		X	X		3192	MIRU31	ETRE	X	X	X
2059	MIRU20		X		X	3690	Mtub39		X	X	
2163b	QUB11b		X	X		4052	QUB26		X	X	
2165	ETRA		X	X		4156	QUB4156		X	X	
2347	Mtub29		X			4348	MIRU39		X		X

MIRU / VNTR assay logistics

- Each strain / isolate of *M tuberculosis* is queried for the presence of 11 different MIRU / VNTR loci
- Use a multiplex PCR assay that covers each locus 2X – 3X
- PCR products are separated by capillary electrophoresis using an ABI 3500 sequencing instrument
- The PCR products sizes, and calculation of repeats per locus, are done using GeneMapper® software
- BioNumerics® software is used to compare VNTR profiles among cohorts / sets of *M. tuberculosis*



MIRU / VNTR assay data

VNTR

VNTR 0424	VNTR 0577	VNTR 1644	VNTR 1955	VNTR 2165	VNTR 2401	VNTR 2461	VNTR 2686	VNTR 2995	VNTR 3192	VNTR 4052	ID No.
1	4	3	2	2	2	2	1	5	3	5	99-0070a
1	4	3	2	2	2	2	1	5	3	5	99-0213a
2	4	2	2	2	2	2	1	5	3	5	97-2778
2	4	2	2	2	2	2	1	5	3	5	97-3059
2	4	2	2	2	2	2	1	5	3	5	97-3061

Example of VNTR profiles generated for 5 *M. tuberculosis* isolates using the BioNumerics software package

Genotyping of *M. tuberculosis* from elephants

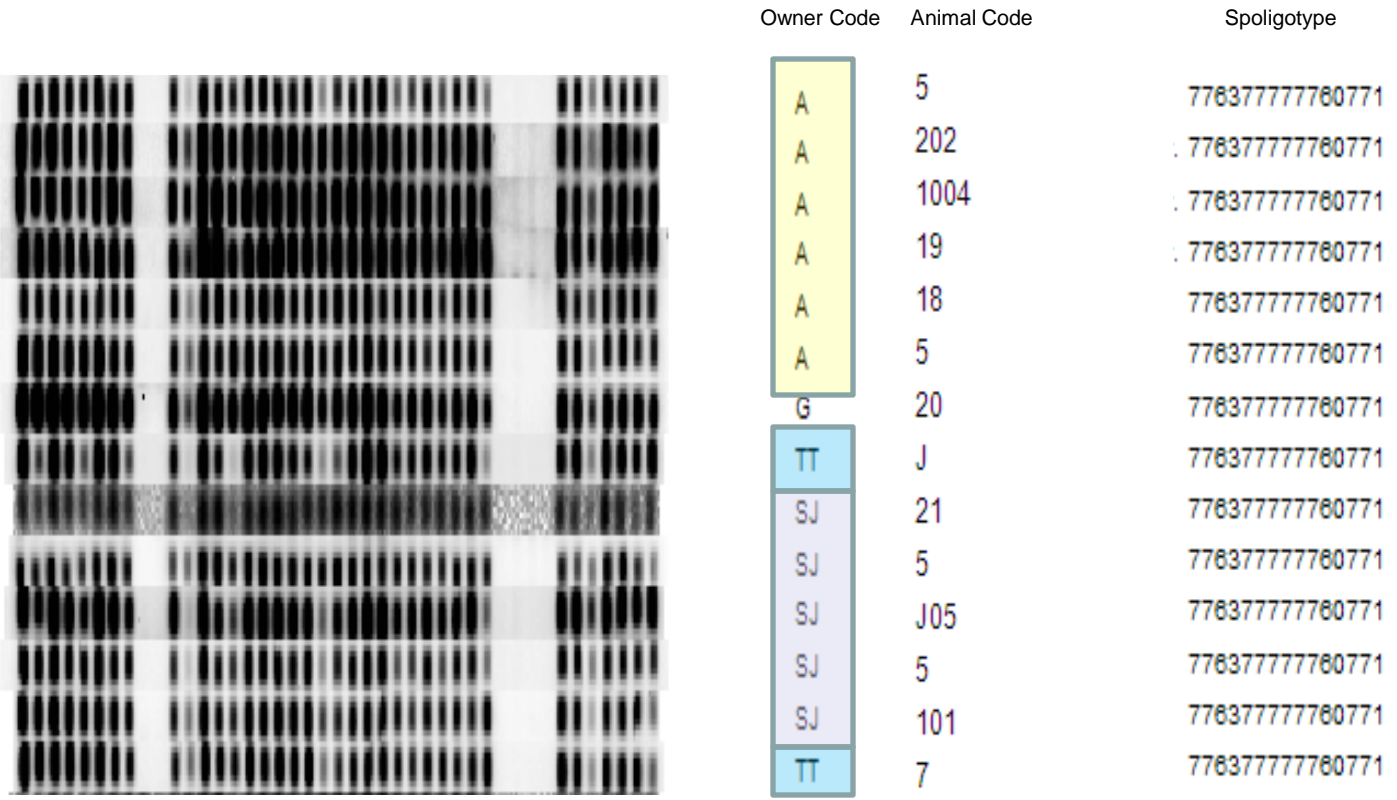
- As of March 2011, 48 entries from 33 different elephants
- Samples acquired from 1997 – present
- Trunk wash and tissue sample-derived isolates
- 11 states represented

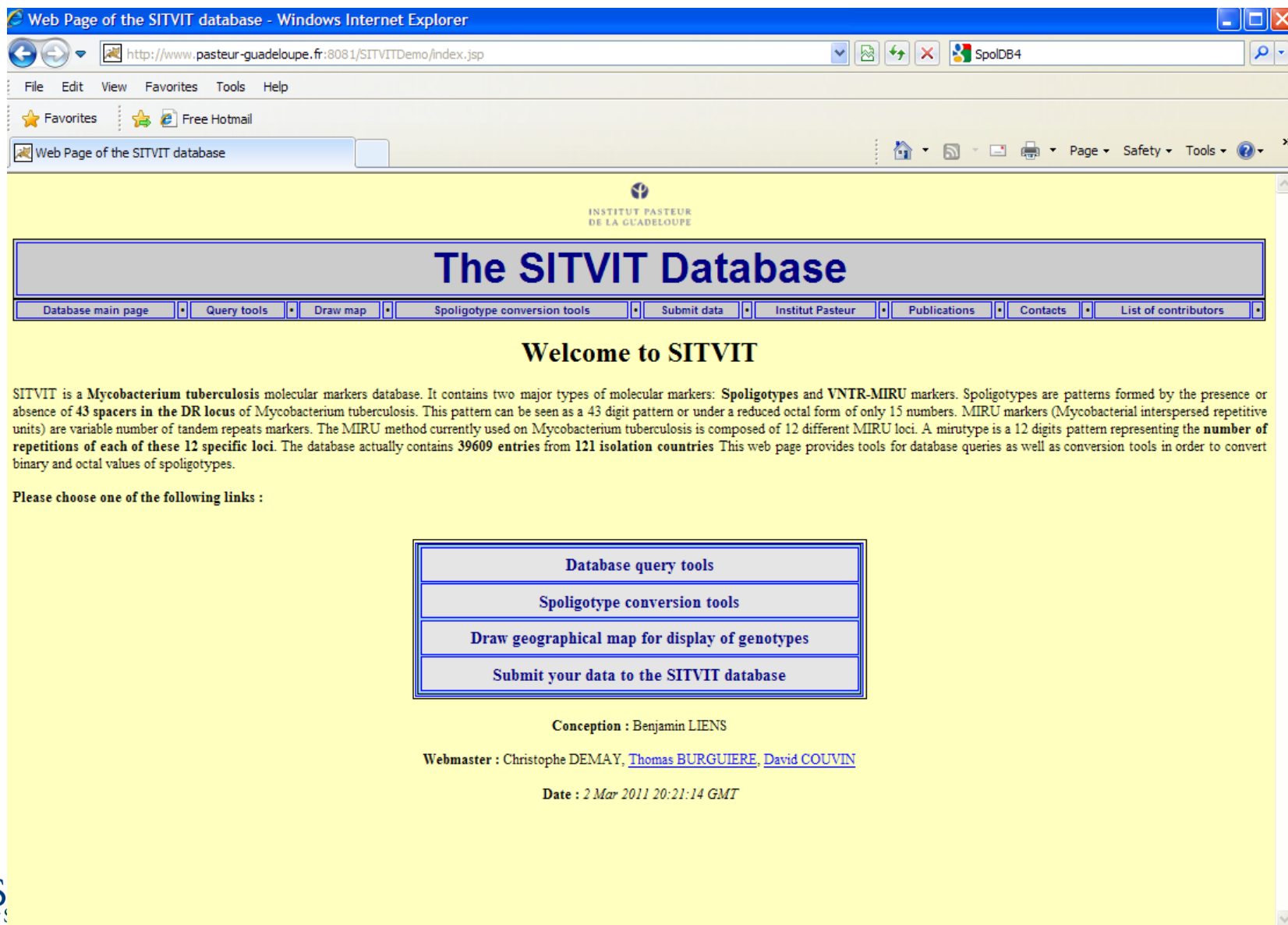
Spoligotype comparison for elephant *M. tuberculosis* isolates



Spoligotyping	TB No.	Owner Code	Animal ID Code	Spoligo octal code
	08-6843	G	1	000000007760771
	00-3946	I	1	037637607760731
	99-0574	FRFB	44	037637607760731
	99-2392	FRFB	44	037637607760731
	00-1108	FRFB	S57	40777777760771
	00-2479	FRFB	77	40777777760771
	00-2759	FRFB	77-2	40777777760771
	99-4445	FRFB	77 Jr #2	40777777760771
	99-4449	FRFB	S5	40777777760771
	00-3206	FRFB	6	47777777760731
	99-3867	RFRB	6	47777777760731
	00-4141	I	1	66407377777600
	08-6520	PZ	100227	77360377760771
	10-7449	BBR	2-2	776377057760771
	10-7450	BBR	2-2 2	776377057760771
	97-1413	A	5	77637777760771
	96-3895	A	202	77637777760771
	96-3927	A	1004	77637777760771
	02-6503	A	19	77637777760771
	95-1324	A	18	77637777760771
	97-0352	A	5	77637777760771
	08-5478	G	20	77637777760771
	10-3657	TT	J	77637777760771
	09-5638	SJ	21	77637777760771
	05-3549	SJ	5	77637777760771
	07-0665	SJ	J05	77637777760771
	05-1484	SJ	5	77637777760771
	11-0296	SJ	101	77637777760771
	11-2687	TT	7	77637777760771
	05-4400	GPZ	8	777774077560731
	10-8492	Sch	9	77777677760771
	98-1589	FRFB	10	77777607760771
	01-1905	GGC	11	77777777740371
	02-1430	GGC	12	77777777740371
	02-3412 CC218	ZGR	13	77777777740371
	98-2853	SER	17	77777777760771
	97-2778	C	16	77777777760771
	97-3059	D	15	77777777760771
	97-3061	D	14	77777777760771
	01-0082	H	808	77777777760771
	00-3703	H	808	77777777760771
	99-0070	FRFB	55 X6456	77777777760771
	99-0213	FRFB	55 65611	77777777760771
	07-7426	McG	808	77777777760771
	07-7120	HAM	202	77777777760771
	11-2528	SWAP	99	776377774020771

M. tuberculosis isolates from elephants with histories of sharing a Circus / Zoo / Sanctuary display similar spoligotypes





The screenshot shows a web browser window titled "Web Page of the SITVIT database - Windows Internet Explorer". The address bar shows the URL "http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp". The browser's menu bar includes "File", "Edit", "View", "Favorites", "Tools", and "Help". The address bar also shows "SpolDB4". The page content features the logo of "INSTITUT PASTEUR DE LA GUADELOUPE" at the top center. Below the logo is a large grey box with the title "The SITVIT Database" in blue text. Underneath the title is a navigation menu with the following items: "Database main page", "Query tools", "Draw map", "Spoligotype conversion tools", "Submit data", "Institut Pasteur", "Publications", "Contacts", and "List of contributors". The main heading is "Welcome to SITVIT". The text below explains that SITVIT is a *Mycobacterium tuberculosis* molecular markers database containing two major types of markers: Spoligotypes and VNTR-MIRU markers. It states that the database contains 39609 entries from 121 isolation countries. Below this text, it says "Please choose one of the following links :" followed by a vertical stack of four buttons: "Database query tools", "Spoligotype conversion tools", "Draw geographical map for display of genotypes", and "Submit your data to the SITVIT database". At the bottom, it lists "Conception : Benjamin LIENS", "Webmaster : Christophe DEMAY, [Thomas BURGUIERE](#), [David COUVIN](#)", and "Date : 2 Mar 2011 20:21:14 GMT".

Spoligotype information: the MIRU-VNTR_{plus} website for *M. tuberculosis*

MIRU-VNTR_{plus} - Home Page - Windows Internet Explorer

http://www.miru-vntrplus.org/MIRU/index.faces

File Edit View Favorites Tools Help

★ Favorites Free Hotmail

MIRU-VNTR_{plus} - Home Page

MIRU-VNTR_{plus}

Navigate Database Edit Options Tools Help

Home

Browse Database

Identification by Similarity Search

Nomenclature

Background Policy

Help

About us

Contact us

Imprint

Welcome to the MIRU-VNTR_{plus} web application!

Use the Home-button to return to this page at any time, or select "Home" from the Navigate-menu. Select one of the commands below to work with the database:

You have uploaded 2 user strains.

Enter a single user strain	Import multiple strains from file or clipboard
Remove all user strains	Browse user strains
Download user strains as MS Excel File	







Identify your strains

Identification by similarity search	Identification by a phylogenetic tree
Browse user strains and reference strains	Set Distance Measure

Analyze your strains, without using reference database

Map country of isolation	Calculate phylogenetic tree
Calculate minimum spanning tree	

Comparison with Reference strains: isolate No. 162337 CC217 (2002), owner 'ZGR',

Distance	ID	Species	Lineage	MLVA MtbC15-9	SpolDB4 ST	Spoligo pattern	RD Gagneux's Lineage
	162337	M. tuberculosis(u)	?-?		1		
0.0	3256/02	M. tuberculosis	Beijing 94-32		1		East Asian
0.0	3277/02	M. tuberculosis	Beijing 94-32		1		East Asian
0.0	3364/02	M. tuberculosis	Beijing 94-32		1		East Asian
0.0	3309/02	M. tuberculosis	Beijing 94-33		1		East Asian
0.0	4445/02	M. tuberculosis	Beijing 95-34		1		East Asian

Comparison with Reference strains: isolate No. 36240 (2000), owner 'FRFB',

Distance	ID	Species	Lineage	MLVA MtbC15-9	User Data 1	User Data 2	User Data 3	SpolDB4 ST	Spoligo pattern	RD Gagneux's Lineage
	36240			?-?				783		
0.0465	2173/99	M. tuberculosis	UgandaI	31-15				52		Euro-American
0.0465	2111/99	M. tuberculosis	UgandaI	32-15				52		Euro-American
0.0465	2263/99	M. tuberculosis	UgandaI	33-15				52		Euro-American
0.0698	2319/99	M. tuberculosis	UgandaII	26-13				135		Euro-American
0.0698	2307/99	M. tuberculosis	UgandaII	28-14				135		Euro-American

Spoligotype information: matching elephant strains with reference strains of *Mycobacterium tuberculosis* using national / international databases

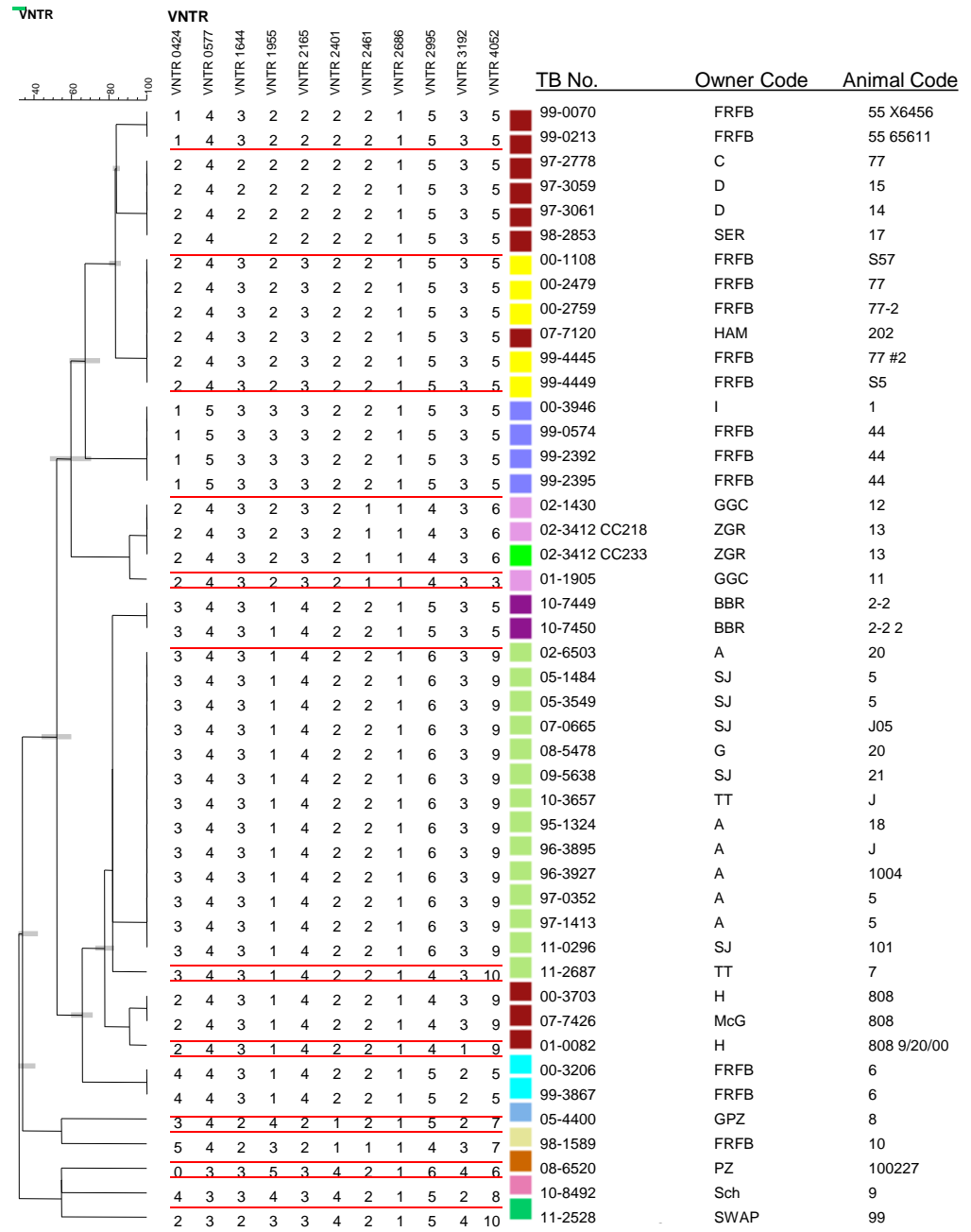
Accession Number	Octal code	CDC Spoligo database Family / Lineage	No. of isolates in CDC database sharing this spoligo	SITVIT / SpolDB4 Database shared-type number (SIT)*
162337	000000000003771	East Asian (Beijing)	7845	International Type 1
45767	037637607760731	EuroAmerican LAM	3	no match
15650	407777777760771	EuroAmerican	11	International Type 411
36240	477777777760731	EuroAmerican	4	International Type 783
560783	773603777760771	EuroAmerican	0	no match
10-029062	776377057760771	EuroAmerican S	0	no match
11-002174	776377774020771	Euro American both S and Haarlem signatures	0	International Type 383
6628	776377777760771	EuroAmerican S	557	International Type 34
365261	777774077560731	EuroAmerican	32	International Type 1232
10-038510	777776777760771	EuroAmerican X	2945	International Type 119
16164	777777607760771	EuroAmerican LAM	1947	International Type 42
89121	777777777740371	EuroAmerican	11	International Type 519
70967	777777777760771	EuroAmerican	4751	International Type 53
562603	000000007760771	Latin American - Mediterranean	183	International Type 4

Genotyping of *M. tuberculosis* from elephants: spoligotyping results

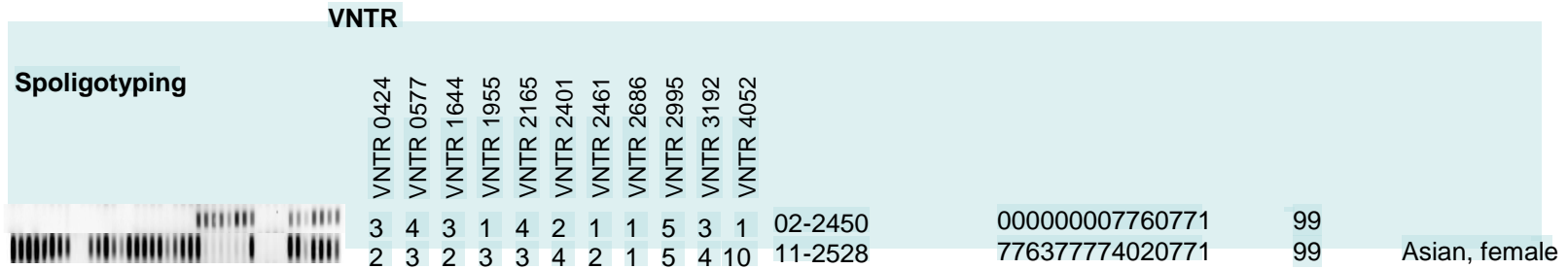
- Fourteen different spoligotypes represented
- Three spoligotypes have no match with International Type strains (?)
- 11 spoligotypes represented in CDC in-house reference database
- Spoligotypes are representative of common lineages (Beijing, EuroAmerican, LAM)

VNTR analysis: UPGMA dendrogram of profiles for selected elephant isolates

Color coding = shared spoligotype



Comparison with Reference strains: isolates No. 02-2450 and 11-002174 from elephant '99', owner 'SWAP'



Distance	ID	Species	Lineage	MLVA MtbC15-9	SpolDB4 ST	Spoligo pattern	RD Gagneux's Lineage
	02-2450	<i>M. tuberculosis</i> (m)		?-?	4		
0.0233	2224/99	<i>M. tuberculosis</i>	UgandaI	34-16	125		Euro-American
0.1395	12637/02	<i>M. tuberculosis</i>	Haarlem	90-31	2		Euro-American
0.1395	3103/03	<i>M. tuberculosis</i>	Haarlem	91-31	2		Euro-American

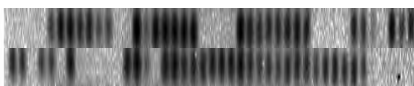
Distance	ID	Species	Lineage	MLVA MtbC15-9	SpolDB4 ST	Spoligo pattern	RD Gagneux's Lineage
	11-002174			?-?	383		
0.0465	3686/03	<i>M. tuberculosis</i>	Haarlem	92-15	47		Euro-American
0.0465	8750/03	<i>M. tuberculosis</i>	Haarlem	93-15	47		Euro-American
0.1163	4217/02	<i>M. tuberculosis</i>	Haarlem	84-28	1586		Euro-American
0.1163	6946/03	<i>M. tuberculosis</i>	Haarlem	84-28	1586		Euro-American
0.1163	8577/03	<i>M. tuberculosis</i>	URAL	163-15	262		Euro-American

Evidence of infection with two different strains of *M. tuberculosis* in ~ 8 years ?

One elephant ('1') from Southeastern region, 2000, remains the only elephant from which both *M. tuberculosis* and *M. bovis* were recovered

Spoligotyping

VNTR



	VNTR 0424	VNTR 0577	VNTR 1644	VNTR 1955	VNTR 2165	VNTR 2401	VNTR 2461	VNTR 2686	VNTR 2995	VNTR 3192	VNTR 4052	Acc No.	Owner Code	ID	Octal Code
1	5	3	3	3	2	2	1	5	3	5	00-3946	I	1	037637607760731	
2	5	2	3	5	4	5	2	5	3	4	00-4141	I	1	664073777777600	

The VNTR and spoligotype for this elephant's *M. bovis* isolate are similar / identical to five other isolates in the database...

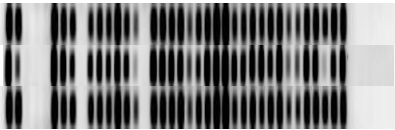


2	5	2	1	5	4	5	2	5	3	4	CA	Cattle
2	3	2	3	5	4	5	2	5	3	4	MX	Cattle
2	5	2	3	5	4	5	2	5	3	4		Elephant
2	5	2	3	5	4	5	2	5	3	4	TX	Cattle
2	5	2	3	5	4	5	2	5	3	4	TX	Cattle
2	5	2	3	5	4	5	2	5	3	4	TX	Cattle
2	5	2	3	5	4	5	2	5	3	4	NJ	Human
2	5	2	3	5	4	5	2	5	3	4	NL	Cattle - unk

One elephant ('000') from a Mid-Atlantic zoo, 2000, is the other entry in the NVSL database from which *M. bovis* was recovered

Spoligotyping

VNTR



VNTR 0424	VNTR 0577	VNTR 1644	VNTR 1955	VNTR 2165	VNTR 2401	VNTR 2461	VNTR 2686	VNTR 2995	VNTR 3192	VNTR 4052
2	5	4	3	2	4	2	2	5	3	5
2	5	4	3	5	4	2	2	5	3	5

TB No.	Epidemiology	Octal Code	Animal ID
00-5477	pos LN at necropsy	61677377777600	000 African Elep.
00-5478		61677377777600	000
00-5480	pos LN at necropsy	61677377777600	000

The spoligotype and VNTR profiles of this isolate of *M. bovis* remain unique (no match with >1550 isolates in the NVSL database)

Spoligotype	VNTR	Species	Epidemiology	Animal ID
2 4 3 3 5 4 2 2	2 4 3 3 5 4 2 2	Baboons		60277377777600
2 4 3 3 5 4 2 2	2 4 3 3 5 4 2 2	Baboons		
2 4 3 3 5 4 2 2	2 4 3 3 5 4 2 2	Baboons		60277377777600
2 5 4 3 2 4 2 2	2 5 4 3 2 4 2 2	Elephant	pos LN at necropsy	61677377777600
2 5 4 3 5 4 2 2	2 5 4 3 5 4 2 2	Elephant	pos LN at necropsy	61677377777600

Genotyping of *M. tuberculosis* from elephants: VNTR results

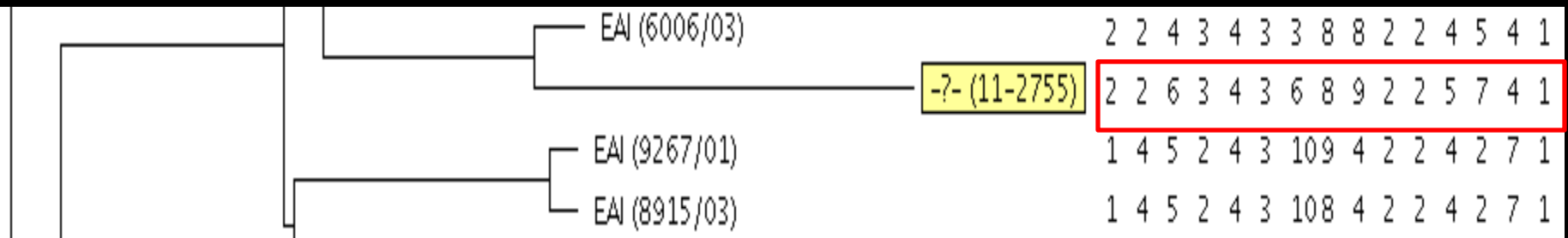
- 17 different VNTR profiles represented
- In general, partitioning by spoligo matched that of the VNTR profiles for isolates (i.e., good agreement with the two different genotyping methods)
- [In the process of generating ‘standardized’ VNTR 15 data for all elephant isolates]

M. tuberculosis isolate from a trunk wash from a 39 year-old Asian elephant from a zoo located in the Mid-West: March 22, 2011

Distance	ID	Species	Lineage	Country of Isolation	SpolDB4 ST	SpolDB4 Lineage	Spoligo pattern	RD Gagneux's Lineage
	11-02755	M. tuberculosis ^(m)		USA				
0.0233	6006/03	M. tuberculosis	EAI	Germany	138	EAI5		Indo-Oceanic
0.1163	11051/03	M. tuberculosis	EAI	Germany	236	EAI5		Indo-Oceanic
0.1395	7190/03	M. tuberculosis	EAI	Germany	591	EAI6_BGD1		Indo-Oceanic
0.1395	12778/03	M. tuberculosis	EAI	Germany	152	EAI5		Indo-Oceanic
0.1628	6538/03	M. tuberculosis	EAI	Germany	1628	EAI5		Indo-Oceanic

For spoligotype, the MIRU-VNTR^{plus} database has no match, 'nearest neighbors' are isolates from Germany, SpolDB4 shared type 138, lineage EAI5 (East African - Indian)

M. tuberculosis isolate from a trunk wash from a 39 year-old Asian elephant from a zoo located in the Mid-West: March 22, 2011



For VNTR-15, the MIRU-VNTR_{plus} database has no exact match for the elephant isolate profile..... again, nearest neighbor is lineage EAI5

Summary: Genotyping data

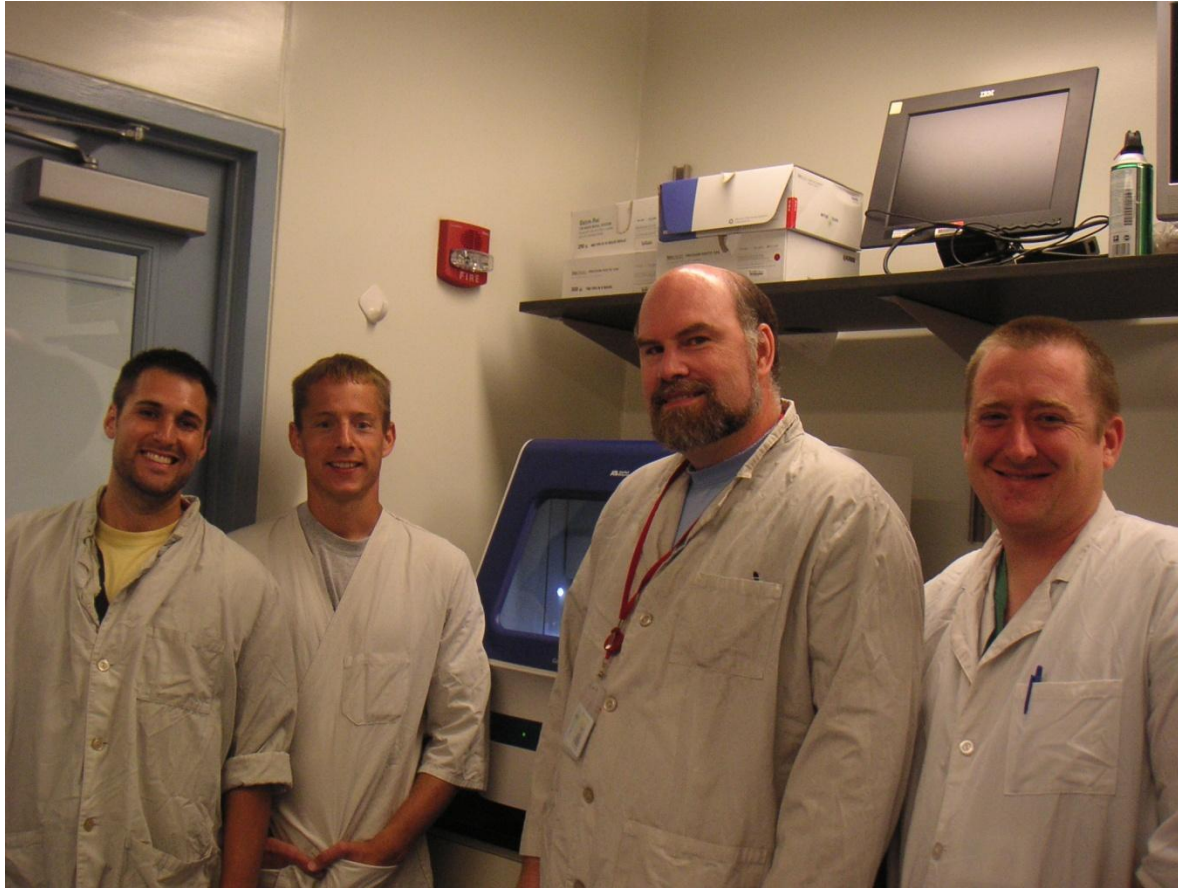
- Elephant isolates predominantly represented by EuroAmerican spoligo lineage of *M. tuberculosis*
- Successive isolates from the same elephant tend to have matching genotypes (recrudescence Vs newly acquired infections)
- Elephant '99' appears to have harbored two different strains of *M. tuberculosis* in 2002 and 2011
- Elephants with histories of shared exposures tend to harbor *M. tuberculosis* with similar genotypes
- Some *M. tuberculosis* strains recovered from elephants may represent rare or novel lineages

Future endeavors

- Subject all elephant isolates to analysis with ‘universal’ VNTR-15 set
- Use VNTR-15 data to further refine relationships with reference *M. tuberculosis* strains / lineages and support ‘One Health’ investigations into human disease risk
- Query elephant isolates for TbD1 element (‘modern’ Vs ‘ancestral’)
- Screen elephant isolates for antibiotic resistance
- In-depth sequencing of elephant isolates ?



Mycobacteria and Brucella Section specimen processing team (left to right): D. A. Hebeisen and H. Wilson (ISU students), A. Martindale, J. Case, J. Gerding, P. Dykema, and J. Fevold



Mycobacteria and Brucella Section molecular biology team: D. Farrell, T. Stuber, J. Higgins, P. Camp

Acknowledgements

- L. Cowan (CDC)
- D. Sofranko (APHIS)
- S. Robbe-Austerman (APHIS)
- D. Bravo (APHIS)
- R. Swanson (APHIS)
- B. Harris (APHIS)
- P. Brewer (Southwick's Zoo)
- B. Perri (NYC Dept Health)