5. Transmission of *Mycobacterium abscessus* within a cystic fibrosis clinic

Includes work published in:

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Statement of contribution

I carried out all bioinformatic analyses. DMG carried out collection of clinical data and sample preparation and DNA extraction. RAF, JP and SJP conceived the study. DMG, IR, TI and MR carried out epidemiological analysis.

5.1. Introduction

Although thought to be primarily an environmental bacterium, *M. abscessus* can also infect humans via wounds or as a respiratory pathogen. In particular, it is a significant cause for concern in cystic fibrosis patients, where infections are chronic and extremely difficult to treat due to its naturally high level of antibiotic resistance.

Worryingly, cases are thought to be on the rise, as observed in Taiwan, United States and Australia (Lai, Tan et al. 2010, Prevots, Shaw et al. 2010, Thomson 2010). The reason for this rise is not known, although possible explanations include: the increased use of intravenous antibiotics which by removing the resident microbiota may allow increased colonisation of Mycobacteria (Torrens, Dawkins *et al*. 1998); impairment of host anti-mycobacterial immunity through autophagy inhibition by chronic azithromycin therapy (Renna, Schaffner *et al*. 2011); patient-patient transmission; and increased surveillance.

Extensive person-to-person transmission of other cystic fibrosis pathogens, such as *Pseudomonas aeruginosa*, has long been demonstrated. For *M. abscessus* however, this was thought to be impossible or extremely rare; as previous small-scale studies had failed to find any evidence (Bange, Brown *et al*. 2001). Doubts over this assumption started to be raised however when an outbreak was reported at a cystic fibrosis clinic in Seattle in 2012, which involved five patients who all shared strains with an identical PFGE type (Aitken, Limaye *et al*. 2012).

In this study, 170 *M. abscessus* samples were sequenced from 31 patients at Papworth Adult Cystic Fibrosis Centre over four years. Prior to the initiation of this study only a single *M. abscessus* genome had been described, so a primary aim was to gain an overview of the population structure and diversity across the species. A secondary aim was to relate this population structure to the patients, and understand how their infections were acquired.

5.2. Methods

Since 2007, samples were collected from patients positive for *M. abscessus* and stored as frozen aliquots from MGIT samples. Following initial culture on solid media, sweeps of mycobacterial colonies were taken and sub-cultured (to remove contamination while maintaining genetic diversity). 170 pure cultures of *M. abscessus* were obtained from 31 patients (22 CF, 4 non-CF and 5 unknown), and DNA extractions were carried out. The DNA was subjected to 75bp paired-end sequencing on the Illumina HiSeq platform. Antibiotic sensitivity testing was performed on isolates before or shortly after starting anti-mycobacterial chemotherapy by serial broth microdilution and plates were read at 5 days.

For a species-wide analysis, the raw reads were mapped and SNPs called against the *M. abscessus* reference genome as described in Methods 8.2 and 8.3. In addition publically available data were analysed for isolates collected from Malaysia (M93 (Choo, Wong *et al*. 2012), M94 (Choo, Wong *et al*. 2012), M152 (Ngeow, Wong *et al*. 2012), M115 (Ngeow, Wong *et al*. 2012), M154 (Choo, Wong *et al*. 2012), M139 (Ngeow, Wee *et al*. 2012)), France (CCUG48898 (Tettelin, Sampaio *et al*. 2012), CIP108541 (Choi, Cho *et al*. 2012)), Birmingham (47J26 (Chan, Halachev *et al*. 2012)) and Brazil (GO-06 (Raiol, Ribeiro *et al*. 2012)). A high level of divergence was observed, so a separate analysis for each sub-species was carried out by generating two extra references to represent the *M. a. massiliense* and *M. a. bolletii* subspecies. These were produced by *de novo* assembly (using Velvet- see Methods 8.4) of the sequencing reads from appropriate samples, chosen on the basis of high coverage and central phylogenetic position. Mapping and tree construction as described in Methods 8.6 were then carried out separately for each of the subspecies. An in-house program (Croucher, Harris *et al*. 2011), was used to detect recombination and remove it from the dataset.

Bayesian inference was implemented in BEAST, a program used for Bayesian Markov chain Monte Carlo analysis of genetic sequences (Drummond and Rambaut 2007). BEAST was run on the dataset using both log-normal relaxed and strict clock models, for 100,000,000 states, excluding a 10% burn-in as described in Methods 8.7. The results quoted here were from runs with the best effective sample size (ESS), and

a log-normal relaxed clock. As substitution rate estimates in BEAST are dependent on a molecular clock signal being present, Path-O-Gen was used to informally assess the clock-likeness of the dataset using linear regression (See Methods 8.7). Monophyly was assessed by repeating the BEAST analyses above and reporting the "monophly statistic" for certain clades. This was carried out in triplicate, and the number of times a clade was reported as being as monophyletic was counted, with the burn-in excluded.

Epidemiological analysis was carried out by Dorothy Grogono and colleagues at Papworth Hospital. For this, all visits to the hospital were extracted from the patients' notes and cross-referenced. Public Health England (I Roddick and T Inns) carried out statistical analyses comparing clustered and non-clustered isolates and their association with specific ward visits.

5.3. Results

5.3.1. Species-wide overview

A maximum likelihood tree of all the samples in the dataset is shown in Figure 24. This revealed the presence of three major clades that represent the three main subspecies most often described as *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*. Although previously proposed to be separate species (Blauwendraat, Dixon *et al*. 2012), the whole genome phylogeny shows that the average nucleotide identity (ANI) between representatives is 99.1%; well above the 94% ANI previously associated with the species boundary (Konstantinidis and Tiedje 2007), suggesting they most likely represent subspecies. Of the 31 patients, 13 were infected with *M. a. abscessus*, 15 with *M. a. massiliense* and two with *M. a. bolletii*. One patient was co-infected with both *M. a. abscessus* and *M. a. massiliense* and was excluded from further analysis. The phylogeny was also supplemented with publically available CF and non-CF isolates from the UK, Brazil and Malaysia, which were distributed broadly across the tree, indicating that the Papworth sample collection is likely to be representative of the species.

5.3.2. Recombination

Manual observation of the whole genome alignment revealed sequences likely due to recombination between and within the sub-species. This could not be removed in an automated fashion due to the scale of the dataset, so variation due to recombination was maintained in Figure 24. For individual clusters recombination was detected and removed before analysis using a phylogenetic approach based on SNP density (Croucher, Harris *et al*. 2011). No recombination within patients was detected.

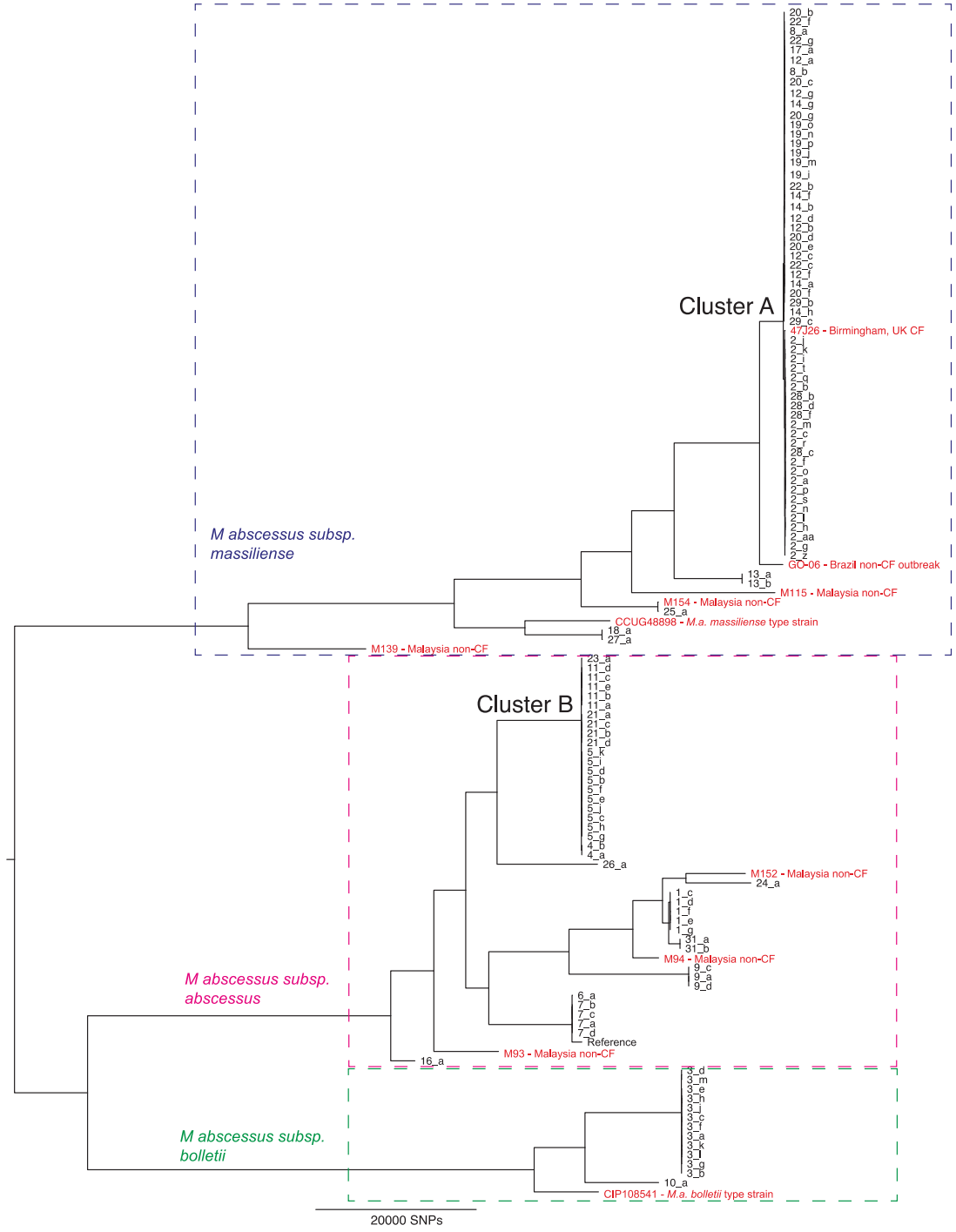

Figure 24 – Maximum likelihood phylogeny of all isolates in the collection (black) and publically available sequences (red). The three subspecies are indicated by the dashed boxes. The tree was built based on high quality SNPs called against the *M. a. abscessus* reference genome (labeled "Reference" in the tree). Possible transmission clusters A and B are indicated and shown in greater detail in Figure 25.

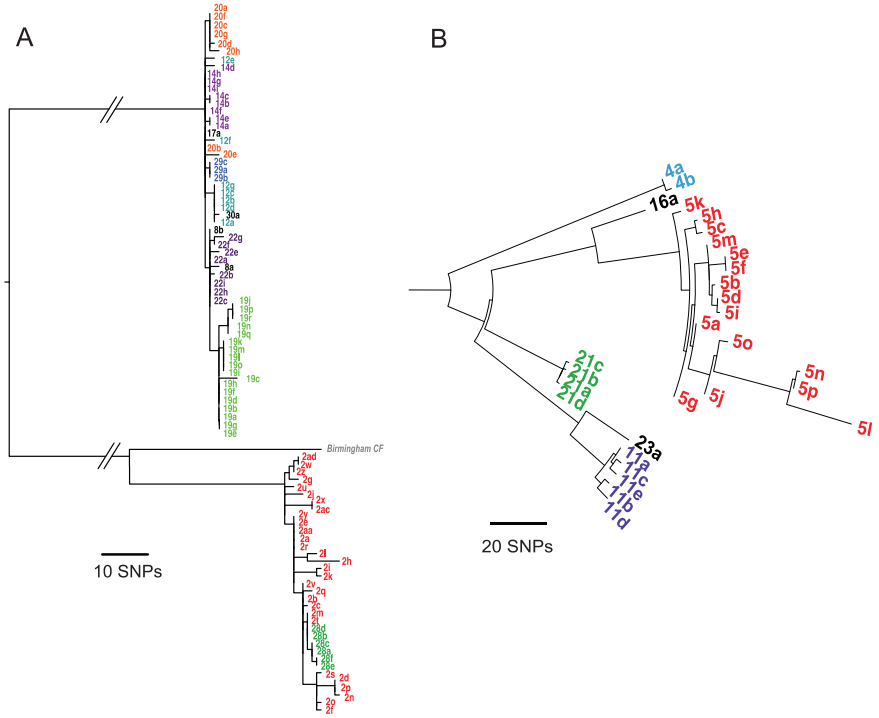

Figure 25: Maximum likelihood trees of cluster A and B as indicated in Figure 24. Trees were built based on SNPs called via mapping to a representative from each cluster. The bootstrap support for the sub-clusters in cluster A were 100%. Dashed lines represent branches shortened for illustration purposes.

5.3.3. Patient-level diversity

A detailed analysis was carried out for each subspecies, which showed that all patients had a clonal *M. abscessus* infection, as all their isolates were tightly clustered together on the tree. There were two clusters of multiple patient isolates (Cluster A and B: Figure 24) found in two of the subspecies, *M. a. massiliense* and *M. a. abscessus* respectively. Cluster A was comprised of two distinct sub-clusters separated by 185 SNPs, the first of which was composed of highly-related or identical isolates from nine different patients (Figure 25). The second of these was composed of two patients' isolates, with the diversity of one nested within the other: a pattern consistent with a directional patient-patient transmission event (Vandamme and Pybus 2013). The two SNPs (codon 42 in MAB_0477 and codon 233 in MAB_3748) conferring the monophyletic positioning of patient 28 within patient 2's isolates were found to be unique to these patients and of high quality. Furthermore the support for this reciprocal monophyletic topology was further supported by Bayesian analysis, discussed below in section 5.3.5.

In order to put the diversity observed within these clusters into context, the dataset was interrogated further by plotting the pairwise SNP distances (also known as Hamming distance (Pilcher, Wong *et al*. 2008)). This showed that whilst within patients most SNP distances are under 20 (red- Figure 26b), distances between patients mostly exceed 1,000 SNPs (not shown), which is consistent with independent acquisition from the environment. However, for the patients within *M. a. massiliense* cluster A (blue- Figure 26b), the inter-patient distances were very small, often the same or less than the number of SNPs seen within patients. There was also a second "mode" spanning 50 to 200 SNPs which represented the distance between the sub-clusters in *M. a. massiliense* cluster A in addition to the diversity within *M. a. abscessus* cluster B. Thus it appears that cluster B is "looser", with its inter-patient relationships more distant than observed for cluster A. However this diversity is still distinct from the diversity observed across the rest of the dataset as a whole (Figure 26).

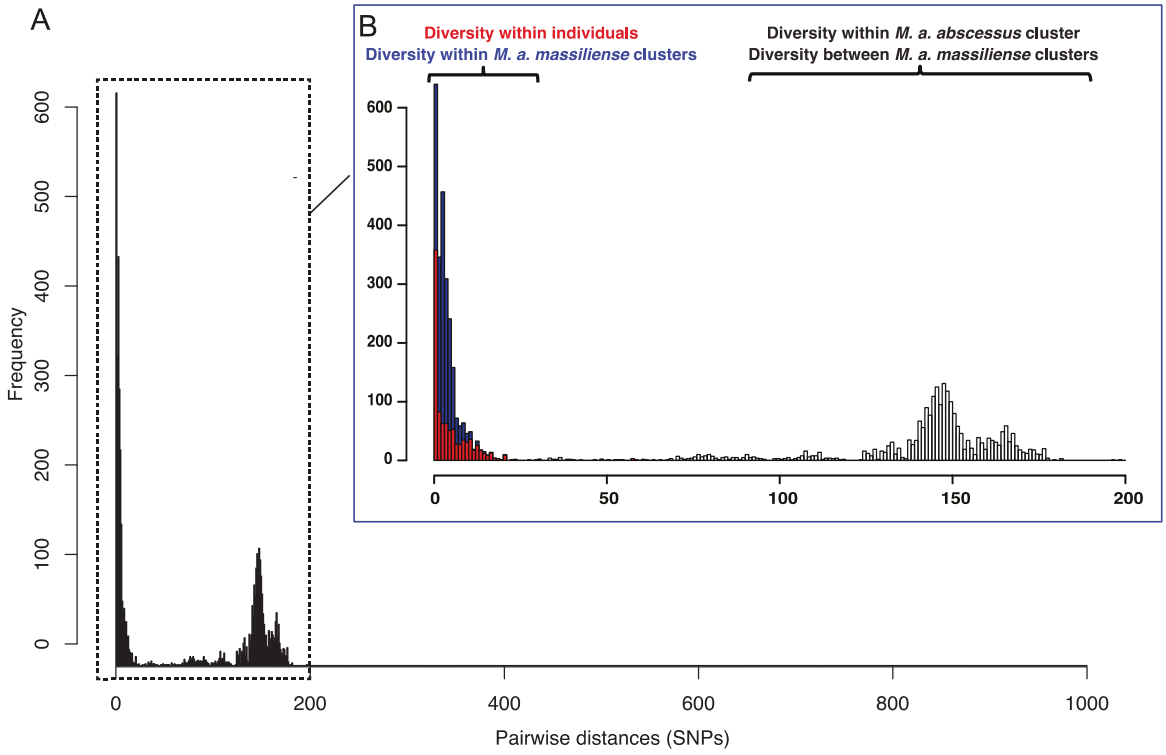

Figure 26: Histogram of pairwise SNP differences. A: all SNP differences under 1000. B: all SNP differences under 200 with subsets of data labeled. B shows two distinct "modes" existing in the data and A demonstrates how distinct these are from the rest of the dataset (all > 1000 SNPs).

5.3.4. Epidemiological investigation

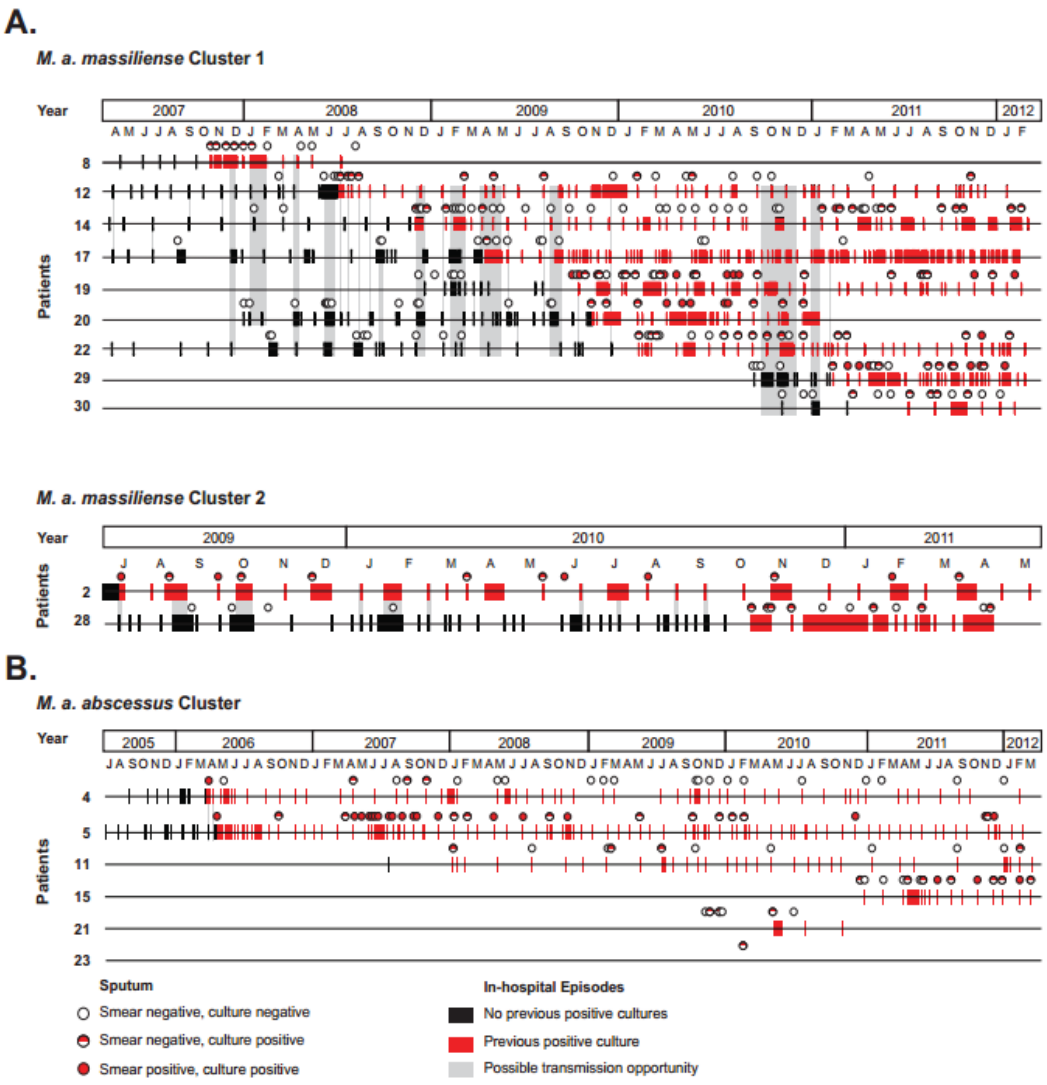

Figure 27 – Opportunities for transmission between patients. The timelines of individual patients within the *M. a. massiliense* sub-clusters 1 and 2 (A) and *M. a. abscessus* cluster B (B) are shown with short vertical lines denoting hospital visits to Papworth Hospital, and circles denoting sputum samples (culture negative white; smear negative culture positive half-red; smear positive red). Timelines become red following a positive sputum sample. Potential opportunities for transmission between patients (negative patient in hospital at the same time as a positive patient) are highlighted by grey vertical bars.

The tight relationships between different patients' isolates raised the possibility that either a point-source outbreak or patient-patient transmission was occurring. To investigate the former, Papworth hospital initiated extensive environmental sampling in June 2010 when the genomic analysis had revealed the possibility of an outbreak. The hospital water supply, which is chlorinated on site, was extensively sampled and

repeatedly found to be culture and PCR-negative for Mycobacteria. Showerheads, dishwashers and bronchoscopes were also shown to be free from NTM. In addition there was no geographical association between the patients' home water supply and genetic clustering.

To investigate the possibility of patient-patient transmission, Papworth hospital collected information from the patients' notes to determine when and where they attended the hospital. They could not find occasions, including social links, outside the hospital where direct patient-to-patient transmission might have occurred, but did, however, identify clear opportunities for transmission within the centre for all patients from the two *M. a. massiliense* sub-clusters (Figure 27). Except for the presumed index cases (patients 8 and 2 for sub-cluster 1 and 2 respectively), all previously uninfected patients were present at the centre at the same time as an infected individual on multiple occasions. In contrast, patients infected with the *M. a. abscessus* cluster B isolates had no clear opportunities for transmission within or outside the hospital, with the only overlap identified between patient 4 and 5 whose isolates are not monophyletic in the phylogeny (Figure 25).

This epidemiological analysis was extended further in order to determine whether patients with isolates belonging to the *M. a. massiliense* cluster had more opportunities for transmission than patients with non-clustered isolates. Individuals within *M. a. massiliense* sub-cluster 1 (n = 9) were compared to patients with unclustered *M. abscessus* isolates (n = 15). The incubation period of *M. abscessus* infections is unknown so it was assumed that patients might acquire infection any time during a 12-month period before their first positive sample and might transmit infection at any time from this point onwards. For every 100 days during periods of potential acquisition, clustered cases were significantly more exposed to hospital than unclustered cases (mean 10.8 days vs. 4.1 days; $p = 0.0126$), had greater exposure to the CF inpatient ward (mean 5.7 days vs 1.5 days; $p = 0.0133$) and were more likely to be in hospital at the same time as potentially infected individuals (4.18 days vs. 0.63 days; $p = 0.0053$).

5.3.5. Bayesian dating of possible patient transmission events

Next, dating techniques were used in order to estimated the ages of the two clusters, and whether they were consistent with the opportunities for transmission identified in the epidemiological analysis. Using BEAST (Drummond and Rambaut 2007) with a constant population size and lognormal relaxed clock, an estimated substitution rate of 1.8 (0.3-3.3 95% highest posterior density) was estimated for *M. a. abscessus* cluster B and 0.47 (0.2-0.8 95% HPD) SNPs per genome per year for *M. a. massiliense* cluster A. Estimates were also made for the ages of the most recent common ancestors (MRCAs) of linked patients found adjacent on the tree. In the case of both *M. a. massiliense* clusters, the estimates for the age of the inter-patient MRCAs overlapped with the opportunities for hospital transmission (Figure 28) For *M. a. abscessus* cluster B this was not the case as its inter-patient MRCA of patient 4 and 5 dated to several decades prior to either of the patients becoming positive (Figure 28).

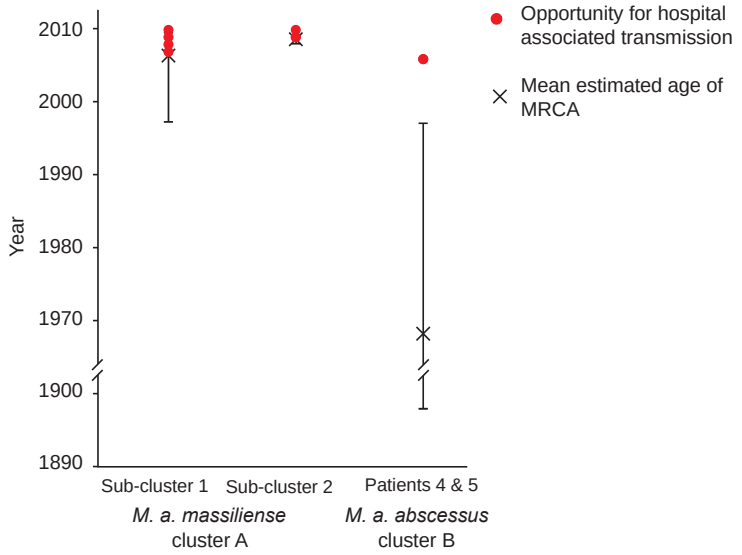

Figure 28 – Agreement between opportunities for patient-patient transmission and the estimated age of the corresponding most recent common ancestor (MRCA). Estimates from BEAST (Drummond and Rambaut 2007), of when the MRCA existed for isolates from different patients within *M. a. massiliense* clusters 1 and 2 and from the two patients within the *M. a. abscessus* cluster who had transmission opportunities

The substitution rates and the age of MRCAs presented here depend on the presence of a molecular clock in the dataset. In general a positive significant correlation between patient diversity and time was observed (see section 6.3.1), suggesting the

presence of a molecular clock. For individual clusters, Path-O-gen (Rambaut 2007) was used to plot root-tip distances against time to assess the presence of a strict clock-like signal. For the *M. a massiliense* sub-clusters, this test performed poorly, with a very weak positive signal in *M. a. massiliense* sub-cluster 1 (Figure 29a) and a complete absence in *M. a massiliense* sub-cluster 2 (Figure 29b). As *M. a. abscessus* cluster B has much deeper relationships between the patients, each patient (with more than 2 isolates) was plotted separately. In this case a positive molecular clock signal could be detected (Figure 30). The lack of a molecular clock observed in the *M. a. massiliense* data weakens the conclusions that can be drawn from the BEAST analysis. However it should be noted that the root-to-tip analysis is not a formal statistical test of a molecular clock, due to non-independence, so has its own limitations.

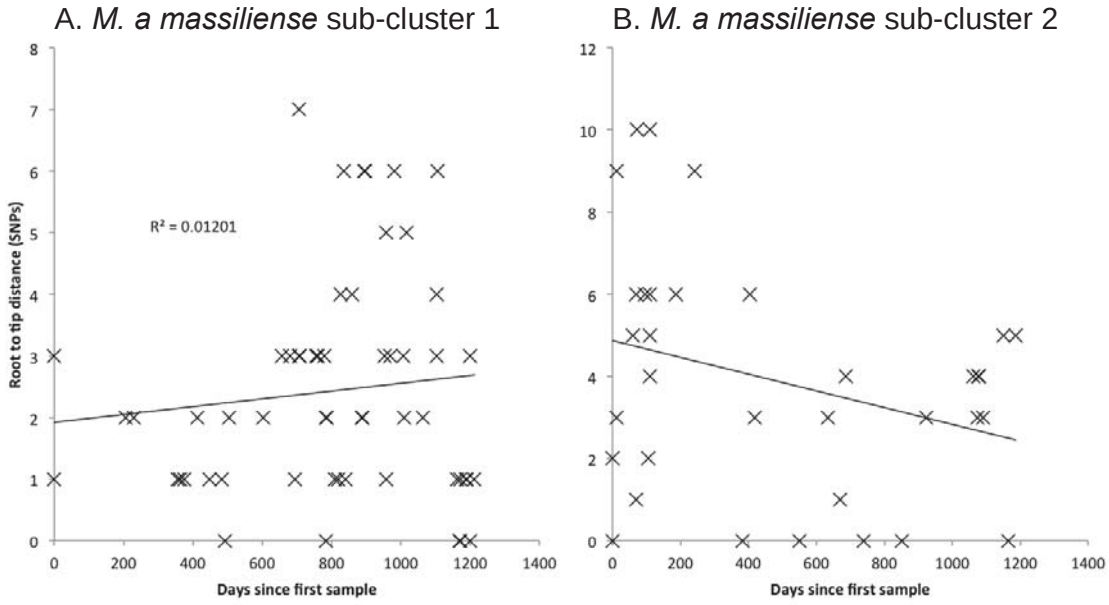

Figure 29 - Root to tip distances for *M. a. massiliense* sub-clusters. Rooted using Path-O-Gen (Rambaut 2007). No correlation coefficient is stated for sub-cluster 2 as the correlation is negative and shows a complete lack of evidence for a molecular clock.

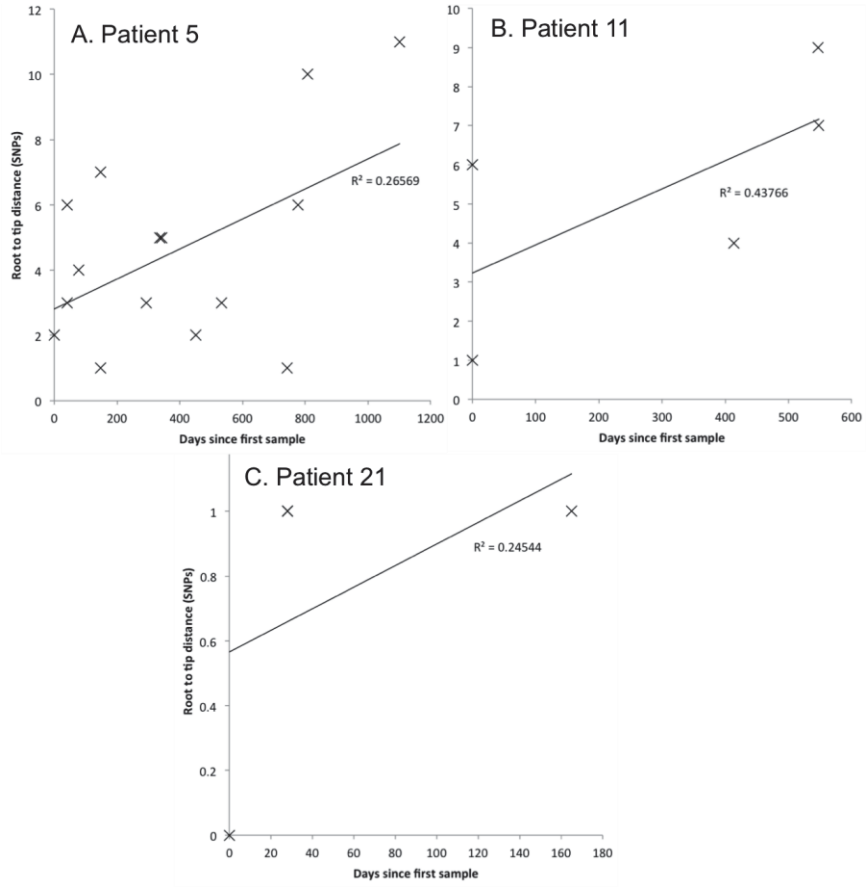

Figure 30 - Root to tip distances for individual patients within *M. a. abscessus* cluster. Rooted using nearest neighbor.

The reciprocal monophyly observed for patients 2 and 28 in *M. a. massiliense* sub-cluster 2 was tested further using BEAST. The "monophyly statistic", which determines for each state whether a certain group of taxa are monophyletic or not, was reported every 1000 states out of a total chain length of 100,000,000. This was carried out for isolates belonging to patient 28, isolates belonging to patient 2 and both patients combined. This confirmed that by far the most common scenario sampled is where patient 28 and patient 2 combined are monophyletic (Figure 31), and that patient 2 is never monophyletic, due to the nesting of patient 28 within it. This provides confidence to the observation of a nested topology involving these patients, a pattern strongly indicative of patient transmission.

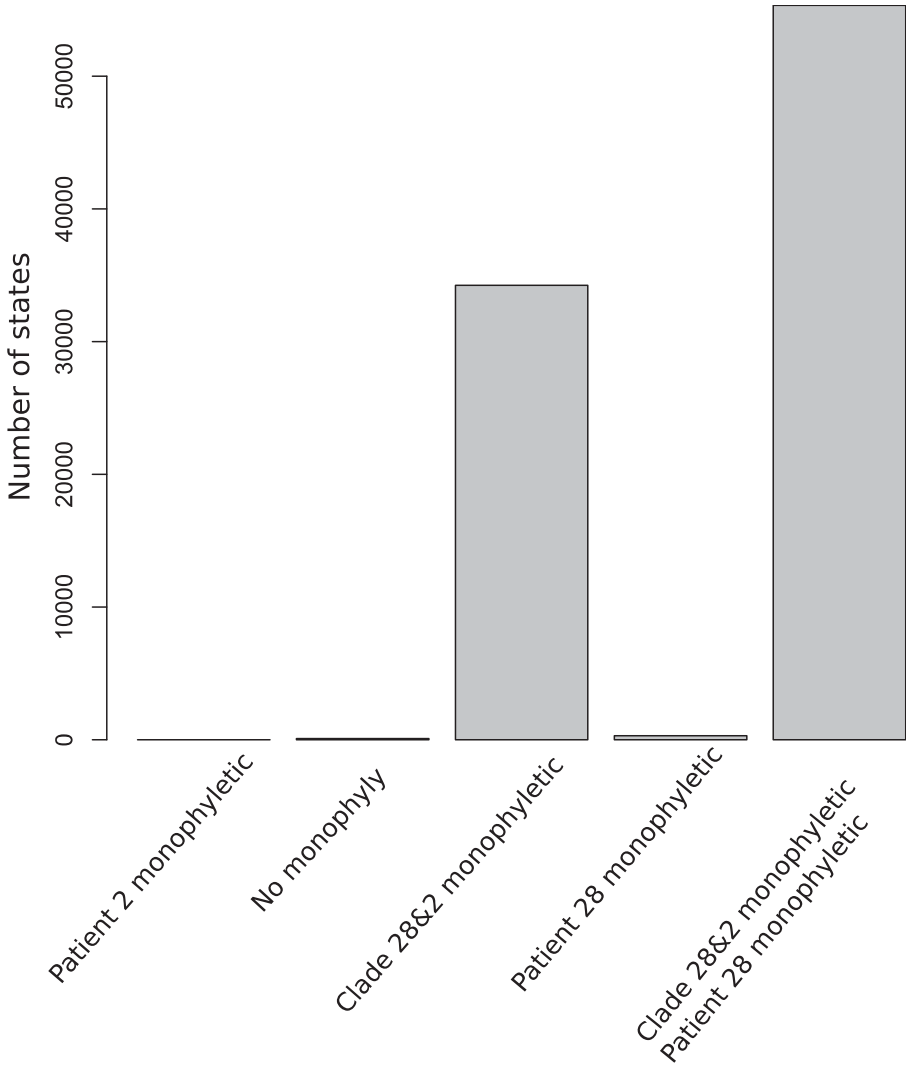

Figure 31 - Number of tree topologies where patients' isolates are monophyletic within *M. a. massiliense* sub-cluster 2. BEAST 1.7.5 was run on *M. a. massiliense* cluster A dataset described in the text with a chain length of 100 million states. The monophyly statistic was sampled every 1000 states, and a 10% burn-in was discarded. Patient 2, patient 28 and a clade comprising of both patient 2 28's isolates were tested. The number of times each scenario was counted is shown. Two additional runs on the same dataset showed highly similar results and are included in Appendix 9.5.

5.3.6. Antibiotic resistance patterns of *M. a. massiliense* cluster A

Amikacin and clarithromycin are commonly used to treat *M. abscessus* infections, and the molecular bases of acquired resistance to them are well characterised. Resistance to amikacin, an aminoglycoside, most often occurs through mutations in the 16s ribosomal RNA (Prammananan, Sander *et al*. 1998). Inducible clarithromycin (macrolide) resistance occurs through the up-regulation of the *erm(41)* gene (Nash, Brown-Elliott *et al*. 2009). Previously it was found that in *M. a. massiliense* strains, part of the *erm(41)* gene is deleted, rendering it inactive (Nash, Brown-Elliott *et al*.

2009). Instead constitutive resistance can evolve in *M. a. massiliense* through mutations in the 23s ribosomal RNA (Nash, Brown-Elliott *et al*. 2009). It was found that both *M. massiliense* cluster A sub-clusters were phenotypically and genotypically highly resistant to clarithromycin, but through independent mutations (Figure 32). This kind of acquired resistance is only thought to occur in patients upon exposure to macrolides (Wallace, Meier *et al*. 1996), and this assumption is supported by preliminary analysis of a global collection of *M. abscessus* isolates (manuscript in preparation), where all resistance conferring mutations were observed to occur at or close to the tips of the tree, and were not sustained in the population (Appendix 9.5, Figure 50). As three of the patients in sub-cluster 1 had not been exposed, this is perhaps evidence of human related transmission, assuming that resistance was acquired by a patient prior to sampling. Only sub cluster 1 was resistant to amikacin. However, the records of patient 8 (the presumed index case based on date of acquisition) revealed that they had acquired resistance during treatment before sampling was initiated. This suggests that resistance was acquired by this patient and then inherited by other patients through transmission.

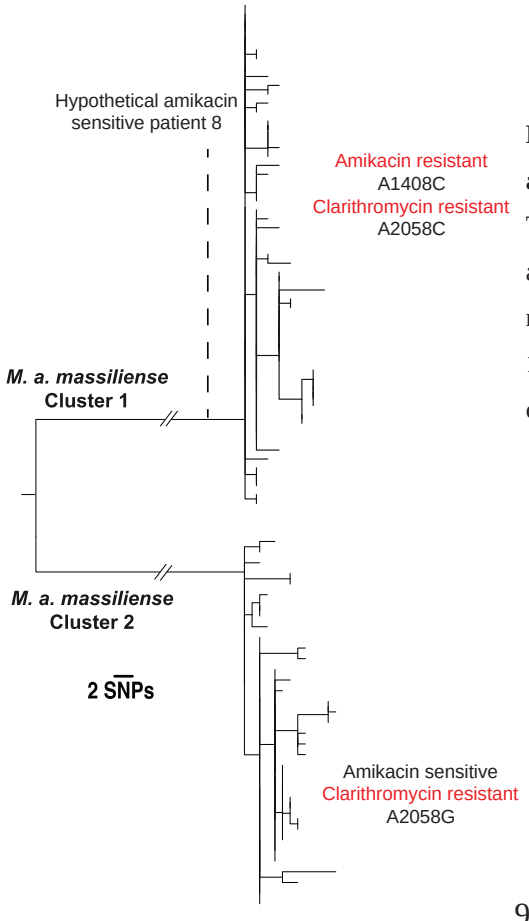

Figure 32 - Antibiotic resistance phenotype and genotypes of *M. a. massiliense* cluster 1. The dotted line indicates the hypothetical amikacin sensitive ancestor of cluster 1. When resistant, the causal mutation is shown in the 16S and 23S genes for amikacin and clarithromycin resistance respectively.

5.3.7. Evidence for dominant circulating clones

In contrast to the sub-clusters within cluster A, the possibility of patient-patient transmission in cluster B was unsupported by both its genetic diversity and epidemiology. However, the level of diversity observed (50-200 SNPs) is still lower than and distinct from the rest of the dataset, suggesting that this cluster is the result of a different underlying process. It's possible that this cluster represents a dominant circulating clone that may be the result of past transmission events amongst unsampled patients or a clone that is more prevalent in the general and/or cystic fibrosis population than would be expected by chance. In addition, the *M. a. massiliense* cluster A might also represent a dominant circulating clone, as the distance between the sub-clusters also falls within the 50-200 SNPs range. Of the published genomes, an isolate from a cystic fibrosis patient in Birmingham sits within cluster A between the two sub-clusters, and an isolate from a large outbreak in Brazil also appears to be ancestral but closely related to the cluster (Figure 24). Both of these sub-clusters are MLST type 23 and are found 40 times in the *M. abscessus* MLST database (total size 284, April 2014) maintained by the Pasteur Institute. The *M. a. abscessus* cluster B is MLST type 26 and is only found once on the database, from an isolate in France. Although the database is by no means extensive, it demonstrates that these putative dominant circulating clones have a further reach than Papworth or the UK, but that further sampling and characterization is required to determine their spread and nature.

5.4. Discussion

This analysis represents the first application of high-throughput genomics to *M. abscessus*, an important cystic fibrosis pathogen. The most significant finding of this work was that there was strong evidence for transmission between patients within a single cystic fibrosis clinic. NTMs are commonly cited as non-transmissible so cases aren't typically scrutinised for the possibility of cross-infection between patients. Therefore this work has an important clinical impact, and raises the possibility that other under-studied cystic fibrosis pathogens may also be able to transmit.

Identical or near-identical isolates of *M. abscessus* were observed in 11 patients in two sub-clusters, which was in contrast to the large genetic distances between isolates in the other patients. This could be due to either a point environmental source, or transmission between patients. Mycobacteria are notoriously difficult to culture from the environment (Falkinham 2002), and sampling occurred prospectively after the putative outbreak had been identified meaning that an environmental source cannot be completely ruled out. However there are several lines of evidence that suggest that a point source is unlikely. Firstly the point source would need to be maintained over a period of four years, and all of the patients would have needed to be exposed to this single source. The epidemiological analysis concluded that none of the clustered patients were associated with a single room or type of treatment. Secondly, the phylogenetic topology of one of the sub-clusters found in *M. a. massiliense* cluster A is highly consistent with directional transmission; where the diversity of the recipient (patient 28) is found to be nested within the diversity of the source case (patient 2), a pattern often observed for HIV transmission (Scaduto, Brown *et al*. 2010). This kind of pattern would be unlikely in a point-source scenario where a star shape, rather than a chain-like phylogeny would be expected (Ypma, Donker *et al*. 2013). Thirdly, the putative transmission clusters had acquired resistance, something that wouldn't be expected for an environmental bacterium, suggesting that there is at least some human element of the transmission chain. Therefore the most parsimonious explanation for these patterns is that transmission has occurred between these patients. This is counter to a previous study that found a complete absence of *M. abscessus* patient-patient transmission (Bange, Brown *et al*. 2001). However, the study was limited to a small number of patients (n=5) over a two year period, whereas this study encompasses a

much larger cohort (n=31) collected over four years, providing a greater opportunity to detect transmission.

Many of the patients involved in these transmission clusters were found to attend the hospital on the same day, perhaps providing an opportunity for cross-infection. However direct transmission between the patients is unlikely as strict infection control procedures are already in place at Papworth, preventing patients waiting in the same area or coming in direct contact within the hospital. Instead, indirect transmission is more likely, although in the absence of environmental evidence a specific mechanism can only be speculated upon. The ability of *M. abscessus* to withstand desiccation and other physical stresses and its resistance to many disinfectants (Wallace, Brown *et al*. 1998) may allow transmission *via* fomite contamination. Aerosol generation during physiotherapy and/or lung function testing could lead to the production and inhalation of airborne water droplets, from which NTM have been cultured in the environment (Wendt, George *et al*. 1980). Alternatively, it is possible that non-CF patients could be asymptomatic carriers, although there is currently no evidence to support this. It is also worth noting that transmission sometimes occurred between patients with persistently smear-negative (but culture positive) sputum, suggesting that the infectious dose may be low.

In addition to the patterns of diversity consistent with recent patient transmission, another distinct mode was detected that may represent the diversity seen within a dominant circulating clone. The existence of such clones is a well-recognised phenomenon of *P. aeruginosa*, where "epidemic" clones are found more often in cystic fibrosis patients than the environment and are thought to have a greater propensity to transmit (Wiehlmann, Wagner *et al*. 2007). Although there is still a possibility that *M. a. abscessus* or *M. a. bolletii* may be able to transmit, it is interesting that the only evidence of patient transmission was found within the *M. a. massiliense* dominant circulating clone (cluster A), raising the possibility that it may be particularly adapted for human spread. Subsequent to the work described here, several new whole genome analyses have been carried out on collections from outside the UK that strengthen the evidence for a dominant circulating clone. One study sequenced two strains from a large outbreak of *M. abscessus* of over 2,000 skin infections in Brazil, which are all assumed to be due to one PFGE-defined clone

(Leao, Viana-Niero *et al*. 2010). When comparing the Brazilian strains against the Papworth isolates presented here, they found they cluster closely with the *M. a. massiliense* cluster A (Davidson, Hasan *et al*. 2013). Surprisingly, although part of the same clone, they were relatively distant from the previously sequenced Brazillian strain GO-06, which suggests that the outbreak is likely to be polyclonal. One of these Brazilian strains (CRM-0019) has been shown to have a higher level of virulence in macrophages, than the type strain (Shang, Gibbs *et al*. 2011), which supports the idea that *M. a. massiliense* putative dominant circulating clone may be particularly adapted for human spread, although not necessarily through respiratory routes. Another recent study applying whole genome sequencing to the previously described outbreak in Seattle (Aitken, Limaye *et al*. 2012), also found that their strains clustered closely with, but were not identical to, the *M. a. massiliense* putative dominant circulating clone (Tettelin H, Davidson R.M *et al*. 2014). This again strengthens the hypothesis that these dominant circulating clones exist, and may be particularly clinically relevant. A larger and broader study will be required to determine their reach and nature.

The finding of frequent transmission amongst patients with cystic fibrosis raises several important questions about current infection control measures used in cystic fibrosis centres, as noted by commentators (Elborn 2013, O'Sullivan and Sassetti 2013). In response to these findings Papworth hospital has implemented new infection control measures including: continuous sputum screening for NTM in all patients; outpatient segregation of infected patients within a dedicated outpatient clinic with single use rooms; and use of negative pressure rooms for inpatient care. Follow up sequencing will be required to determine whether this has prevented further transmission.