Population-level genomics identifies the emergence and global spread of a human transmissible multidrug-resistant nontuberculous mycobacterium.

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ABSTRACT (117 words)

Lung infections with *Mycobacterium abscessus,* a species of multidrug resistant nontuberculous mycobacteria, are emerging as an important global threat to individuals with cystic fibrosis (CF) where they accelerate inflammatory lung damage leading to increased morbidity and mortality. Previously, *M. abscessus* was thought to be independently acquired by susceptible individuals from the environment. However, using whole genome analysis of a global collection of clinical isolates, we show that the majority of *M. abscessus* infections are acquired through transmission, potentially *via* fomites and aerosols, of recently emerged dominant circulating clones that have spread globally. We demonstrate that these clones are associated with worse clinical outcomes, show increased virulence in cell-based and mouse infection models, and thus represent an urgent international infection challenge.

MAIN TEXT

Nontuberculous mycobacteria (NTM; referring to mycobacterial species other than *M. tuberculosis* complex and *M. leprae*) are ubiquitous environmental organisms that can cause chronic pulmonary infections in susceptible individuals *[1, 2]*, particularly those with pre-existing inflammatory lung diseases such as cystic fibrosis (CF) *[3]*. The major NTM infecting CF individuals around the world is *Mycobacterium abscessus;* a rapidly growing, intrinsically multidrug-resistant species, which can be impossible to treat despite prolonged combination antibiotic therapy *[1, 3-5]*, leads to accelerated decline in lung function *[6,7]*, and remains a contraindication to lung transplantation in many centers *[3,8,9]*.

Until recently, NTM infections were thought to be independently acquired by individuals through exposure to soil or water *[10-12].* As expected, previous analyses from the 1990s and 2000s *[13-16]* showed that CF patients were infected with unique, genetically diverse strains of *M. abscessus,* presumably from environmental sources. We used whole genome sequencing at a single UK CF center and identified two clusters of patients (11 individuals in total) infected with identical or near-identical *M. abscessus* isolates, which social network analysis suggested were acquired within hospital *via* indirect transmission between patients *[17]*; a possibility further supported by genomic sequencing *[18]* of a separate *M. abscessus* outbreak in a Seattle CF center *[19]*.

Given the increasing incidence of *M. abscessus* infections in CF and non-CF populations reported globally *[3, 20, 21]*, we investigated whether cross-infection, rather than independent environmental acquisition, might be the major source of infection for this organism and therefore undertook population-level, multinational, whole genome sequencing of *M. abscessus* isolates from infected CF patients, correlating results with clinical metadata and phenotypic functional analysis of isolates.

We generated whole genome sequences for 1080 clinical isolates of *M. abscessus* from 517 patients, obtained from UK CF clinics and their associated regional reference laboratories, as well as CF Centres in the US (UNC Chapel Hill), the Republic of Ireland (Dublin), mainland Europe (Denmark, Sweden, The Netherlands), and Australia (Queensland). We identified 730 isolates as *M. a. abscessus*, 256 isolates as *M. a. massiliense*, 91 isolates as *M. a. bolletii,* with three isolates (from 3 different patients) containing more than one subspecies.

Phylogenetic analysis of these sequences (using one isolate per patient), supplemented by published genomes from US, France, Brazil, Malaysia, China, and South Korea (**Table S1**), was performed and analysed in the context of the geographical provenance of isolates (**Figure 1; Figure S1**). As done previously *[17]*, we obtained maximum likelihood phylogenetic trees demonstrating separation of *M. abscessus* into three clearly divergent subspecies (*M. a. abscessus, M. a. bolletii,* and *M. a. massiliense*), challenging recent reclassifications of *M. abscessus* into only two subspecies *[22]*.

Within each subspecies, we found multiple examples of deep branches (indicating large genetic differences) between isolates from different individuals, consistent with independent acquisition of unrelated environmental bacteria. However, we also identified multiple clades of near-identical isolates from geographically diverse locations (**Figure 1**), suggesting widespread transmission of circulating clones within the global CF patient community.

To investigate further the relatedness of isolates from different individuals, we analysed each subspecies phylogeny for the presence of high density phylogenetic clusters (see Supplementary Methods *[23]*). We identified multiple dense clusters of isolates, predominantly within the *M. a. abscessus* and *M. a. massiliense* subspecies (**Figure 2A**), indicating the presence of dominant circulating clones. We next excluded clusters found in only one CF centre from further analysis to remove related isolates that might have been acquired from a local environmental point source. We found that most patients (74%) were infected with clustered, rather than unclustered, isolates, principally from *M. a. abscessus* Cluster 1 and 2, and *M. a. massiliense* Cluster 1 (**Figure 2B**). The median branch lengths of almost all clusters found in two or more CF centers was less than 20 SNPs (range 1-175 SNPs), indicating a high frequency of identical or near identical isolates infecting geographically separate individuals.

To determine how much of the genetic relatedness found within clusters was attributable to recent transmission, we first examined the within-patient genetic diversity of *M. abscessus* isolates from single individuals. In keeping with our previously published results *[17]*, we found that 90% of same-patient isolates differed by less than 20 SNPs, while 99% of same-patient isolates differed by less than 38 SNPs (**Figure S2**). We therefore classified isolates from different individuals varying by less than 20 SNPs as indicating 'probable*',* and those varying by 20-38 SNPs as indicating 'possible'*,* recent transmission (whether direct or indirect). We thereby

identified multiple likely recent transmission chains in virtually all multi-site clusters of *M. abscessus* (**Figure 2B),** and across the majority of CF centers (**Figure S3**).

We next examined the global distribution of clustered isolates and found that, in all countries, the majority of patients were infected with clustered rather than unclustered isolates (**Figure 2C**; **Table S2**), suggesting frequent and widespread infection of patients with closely related isolates. Moreover, the three dominant circulating clones, *M. a. abscessus* Clusters 1 and 2, and *M. a. massiliense* Cluster 1, were all represented in the USA, European, and Australian collections of clinical isolates, indicating trans-continental dissemination of these clades.

We then compared the genetic differences between isolates (measured by pairwise SNP distance) as a function of geography. As expected from our previous detection of hospital-based transmission of *M. abscessus [17]*, average genetic distances were significantly shorter for *M. abscessus* isolates from the same CF center than those from different CF centers within the same country or from different countries (**Figure 2D**). However, we also detected numerous examples of identical or near-identical isolates infecting groups of patients in different CF centers and, indeed, across different countries (**Figure 2D**), indicating the recent global spread of *M. abscessus* clones throughout the international CF patient community.

We applied Bayesian analysis *[24]* to date the establishment and spread of dominant circulating clones (**Figure S4, S5**), focusing on *M. a. massiliense* Cluster 1, which includes isolates from both the Seattle *[19]* and Papworth *[17]* CF Center outbreaks, as well as isolates from CF centres across England (Birmingham, London, Leicester), Scotland (Lothian, Glasgow), Ireland (Dublin), Denmark (Copenhagen), Australia (Queensland), and the USA (Chapel Hill, NC) (**Figure 3A**). We estimate that the most recent common ancestor of isolates infecting patients from all these locations emerged around 1978 (95% CI: 1955-1995), clearly indicating recent global dissemination of this dominant circulating clone amongst individuals with CF (**Figure 3A**).

Furthermore we were able to resolve individual transmission events between patients infected with dominant circulating clones through two orthogonal approaches. Firstly, using high-depth genomic sequencing of colony sweeps, we were able to track changes in within-patient bacterial diversity in sputum cultures of a single individual over time. By linking the frequency of occurrence of minority variants in longitudinal samples, we were able to define the presence of particular subclones within infected individuals, assign their likely evolutionary development (involving the successive

acquisition of non-synonymous mutations in likely virulence genes; **Figure 3B**), monitor their relative frequencies over time, and demonstrate their transmission between patients (**Figure 3B**). Secondly, through longitudinal whole genome sequencing of isolates collected over time from individuals, we were able to find multiple examples of the complete nesting of one patient's sampled diversity within another's (**Figure S6**). Such paraphyletic relationships are strongly indicative of recent person-to-person transmission *[25]*.

We next examined potential mechanisms of transmission of *M. abscessus* between individuals (which our previous epidemiological analysis had suggested was indirect rather than *via* direct contact between patients *[17]*). We provide proof of concept for fomite spread of *M. abscessus* (detecting three separate transmission events associated with surface contamination of an inpatient room by an individual infected with a dominant circulating clone; **Figure S7**), and also for potential airborne transmission (by experimentally demonstrating the generation of long-lived, potentially infectious cough aerosols by an infected CF patient; **Figure S8**).

A potential explanation for the emergence of dominant clones of *M. abscessus* is that they are more efficient at infection and/or transmission. We therefore analysed clinical metadata to establish whether outcomes were different for patients infected with clustered rather than unclustered isolates. We correlated clinical outcomes with bacterial phylogeny and the presence of constitutive resistance to two key NTM antibiotics, amikacin and macrolides *[26, 27]*, acquired through point mutations in the 16S and 23S ribosomal RNA respectively (**Figure 4A**). We found no differences in the proportions of *M. abscessus*-positive individuals diagnosed with ATS-defined NTM pulmonary disease *[1]*, (namely the presence of two or more culture-positive sputum samples with NTM-associated symptoms and radiological changes), but did observe increased rates of chronic infection in individuals infected with clustered rather than unclustered isolates (**Figure 4B**). As anticipated for transmissible clones exposed to multiple rounds of antibiotic therapy, we also found high rates of constitutive amikacin and/or macrolide resistance in clustered isolates (**Figure 4B**). Of note, resistance to these two antibiotics did not necessarily result in a poor clinical outcomes (**Figure S9**), suggesting that additional bacterial factors might contribute to worse responses in patients infected with clustered isolates.

To explore differences in intrinsic virulence between clustered and unclustered *M. abscessus*, we subjected a panel of representative isolates (27 clustered and 17 unclustered *M. a. abscessus*; 25 clustered and 13 unclustered *M. a. massiliense*) to

a series of *in vitro* phenotypic assays. While we found no or only minor differences between groups in their colony morphotype, biofilm formation, ability to trigger cytokine release from macrophages (**Figure S10**) and their overall phenotypic profile (by multifactorial analysis; **Figure S11**), we detected significantly increased phagocytic uptake (**Figure 4C**) and intracellular survival in macrophages (**Figure 4D**) of clustered isolates of both *M. a. abscessus* and *M. a. massiliense* compared to unclustered controls, indicating clear differences in pathogenic potential. Moreover, infection of *SCID* mice revealed significantly greater bacterial burden (**Figure 4E**) and granulomatous inflammation (**Figure 4F**) following inoculation with clustered rather than unclustered isolates of *M. a. abscessus* and *M. a. massiliense*, confirming differences in virulence between these groups.

In summary, our results reveal that the majority of *M. abscessus* infections of individuals with CF worldwide are caused by genetically-clustered isolates, suggesting recent transmission, rather than independent acquisition of genetically-unrelated environmental organisms. Given the widespread implementation of individual and cohort segregation of patients in CF centres in Europe *[28]*, the USA *[29]*, and Australia *[30]* (which have led to falling levels of MRSA, *Burkholderia*, and transmissible *Pseudomonas* infections *[31-33]*), we believe that the likely mechanism of local spread of *M. abscessus* is via fomite spread or potentially through the generation of long-lived infectious aerosols (as identified for other CF pathogens *[34-36]*). Although further research is needed, both transmission routes are plausible given our findings (**Figures S7, S8**), and would be potentially enhanced by the intrinsic desiccation resistance of *M. abscessus*. Such indirect transmission, involving environmental contamination by patients, is supported by our previous social network analysis of a UK outbreak of *M. abscessus [17]* in CF patients, which revealed hospital-based cross-infection without direct person-to-person contact, and by the termination of a Seattle *M. abscessus* outbreak associated with the introduction of clinic room negative pressure ventilation and double room cleaning *[19]*. The long-distance spread of circulating clones is more difficult to explain. Importantly we found no evidence of CF patients or of equipment moving between CF centers in different countries indicating that the global spread of *M. abscessus* may be driven by alternative human, zoonotic or environmental vectors of transmission.

Our study illustrates the power of population-level genomics to uncover modes of transmission of emerging pathogens and has revealed the recent emergence of global dominant circulating clones of *M. abscessus* that have spread between continents. These clones are better able to survive within macrophages, cause more

virulent infection in mice, and are associated with worse clinical outcomes, suggesting that the establishment of transmission chains may have permitted multiple rounds of within-host genetic adaptation to allow *M. abscessus* to evolve from an environmental organism to a true lung pathogen.

Figure legends

Figure 1. Global phylogeny of clinical isolates of M. abscessus.

Maximum likelihood phylogenetic tree of clinical isolates of *M. abscessus* collected with relevant local and/or national Ethical Board approval from 517 patients (using one isolate per patient), obtained from UK CF clinics and their associated regional reference laboratories, CF Centres in the US (UNC Chapel Hill), the Republic of Ireland (Dublin), mainland Europe (Denmark, Sweden, The Netherlands), and Australia (Queensland), supplemented by published genomes from US, France, Brazil, Malaysia, China, and South Korea (listed in **Table S1**).

Figure 2. Transcontinental spread of dominant circulating clones.

(**A**). Hierarchical branch density analysis of phylogenetic trees for each subspecies of *M. abscessus* identifies multiple clusters of closely related isolates predominantly within the *M. a. abscessus* and *M. a. massiliense* subspecies (numbered, and spectrally coloured red to blue, from most densely clustered to least; black indicating no significant clustering). (**B**). Analysis of *M. abscessus* clusters found in two or more CF centers showing (*top*) numbers of patients infected with each cluster (*grey bars*) or unclustered isolates (*green)* and median branch length (SNPs) of different patients' isolates within each cluster (*blue circles*); (bottom) numbers of potential recent transmission events with < 20 SNPs (*red*) or 20 - 38 SNPs (*yellow*) difference between isolates from different patients. (**C**) Global distribution of clustered *M. abscessus* isolates showing *M. a. abscessus* Cluster 1 (*red*) and Cluster 2 (*green*), *M. a. massiliense* Cluster 1 (*blue*), other clustered isolates (grouped together for clarity; *white*) and unclustered isolates (*black*) with numbers of patients (*n*) sampled per location. (**D**) Genetic differences between isolates (measured by pairwise SNP distance) from different patients attending the same CF center, different CF centers within the same country, or CF centers in different countries (boxes indicate median and interquartile range; *p* values obtained from Mann Whitney Rank Sum tests). To exclude multiple highly distant comparisons, for each isolate only the smallest pairwise distance with an isolate from another patient is included. Colour coding indicates whether there were < 20 SNPs difference (*red*), 20-38 SNPs difference (*yellow*), or >38 SNPs difference (*grey*) between isolates from different patients.

Figure 3. Dating the emergence of dominant circulating clones.

(**A**). Dating the emergence of the *M. a. massiliense* Cluster 1 (responsible for the Papworth and Seattle CF center outbreaks), using Bayesian analysis, with geographical annotation of isolates within the cluster. (**B**). Predicted evolution of

subclones (identified through minority variant linkage; ***see Supplementary Methods [23]***) within a single patient with CF (Patient 2 from *Ref. 19*) chronically infected with the dominant circulating clone *Massiliense* Cluster 1 (representative of a total of 11 patients studied). (i) Analysis revealed successive acquisition of non-synonymous polymorphisms (NS) by the most common recent ancestral clone (MRCA; white) in potential virulence genes (UBiA, MAB_0173; Crp/Fnr, MAB_0416c; mmpS, MAB_0477; PhoR, MAB_0674) and then transmission of a single subclone to another patient from the same CF center (Patient 28 from *Ref. 19*). (ii) Frequency of each subclone within longitudinal sputum isolates analysed during the course of Patient 2's infection and the subsequent transmission of a subclone to Patient 28. We observed considerable heterogeneity in the detected repertoire of subclones within each sputum sample (vertical rectangles coloured to illustrate the proportion of detected subclones coded as for (i) in each sputum sample), reflecting either temporal fluctuations in dominant sub-lineages or variable sampling of geographical diversity of subclones within the lung (as previously described for *P. aeruginosa [37]*). Previously determined opportunities for hospital-based cross-infection between the two patients (using social network and epidemiologic analysis *[17]*), are shown in grey vertical bars.

Figure 4. Comparison of clinical outcomes and functional phenotyping of clustered and unclustered *M. abscessus* isolates.

(**A, B**). Relationship of phylogeny with clinical metadata. Phylogenetic tree of *M. abscessus* isolates (one isolate per patient) with dominant circulating clones *M. a. abscessus* 1 (*Absc 1*), *abscessus* 2 (*Absc 2*), and *M. a. massiliense* (*Mass 1*) highlighted (*grey*). For each isolate, clinical data (where available) was used to determine whether (*column 1*) the infected patient fulfilled the ATS/IDSA criteria for NTM pulmonary disease, namely the presence of two or more culture-positive sputum samples with NTM-associated symptoms and radiological changes *[1]* (yes: *blue;* no: *orange*); whether (*column 2*) isolates have acquired amikacin resistance (through 16S rRNA mutations; *red*), macrolide resistance (through 23S rRNA mutations; *yellow*), or both (*orange- B only*); and whether (*column 3*) patients culture converted (*green*) or remained chronically infected (*red*) with *M. abscessus.* (**C, D**). *In vitro* phenotyping of representative isolates of clustered (*blue*) and unclustered (*green*) *M. a. abscessus* and clustered (*red*) and unclustered (*yellow*) *M. a. massiliense* comparing phagocytosis by (C) and intracellular survival (normalised for uptake) within (D) differentiated THP1 cells. Data points represent averages of at

least three independent replicates. (**E, F**) Using SCID mice, infection with clustered *M. a. abscessus* (*blue*) and *M. a. massiliense* (*red*) led to (E) greater intracellular survival within (i) bone marrow-derived macrophages *in vitro* and (ii) higher bacterial burdens in lung and spleen after *in vivo* inoculation with 1 x 10^7 bacilli per animal with (F) worse granulomatous lung inflammation (*arrowheads*), than unclustered controls (*M. a. abscessus green; M. a. massiliense yellow*), *scale bar x 4*. CFU data is shown as mean ± *sem*; * $p < 0.05$; ** $p < 0.005$ (two-tailed unpaired Student's t-test).

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3-03 (f)/2012), for Scottish centres from NHS Scotland Multiple Board Caldicott Guardian Approval (NHS Tayside AR/SW), and locally for other centres. Aerosol studies were approved by The Children's Health Queensland HHS Human Research Ethics Committee HREC/14/QRCH/88 and TPCH Research Governance Office SSA/14/QPCH/202.

Supplementary Material

Materials and Methods
Supplementary References (*38-58*)
Figs. S1 to S12
Tables S1, S2

Figure 1

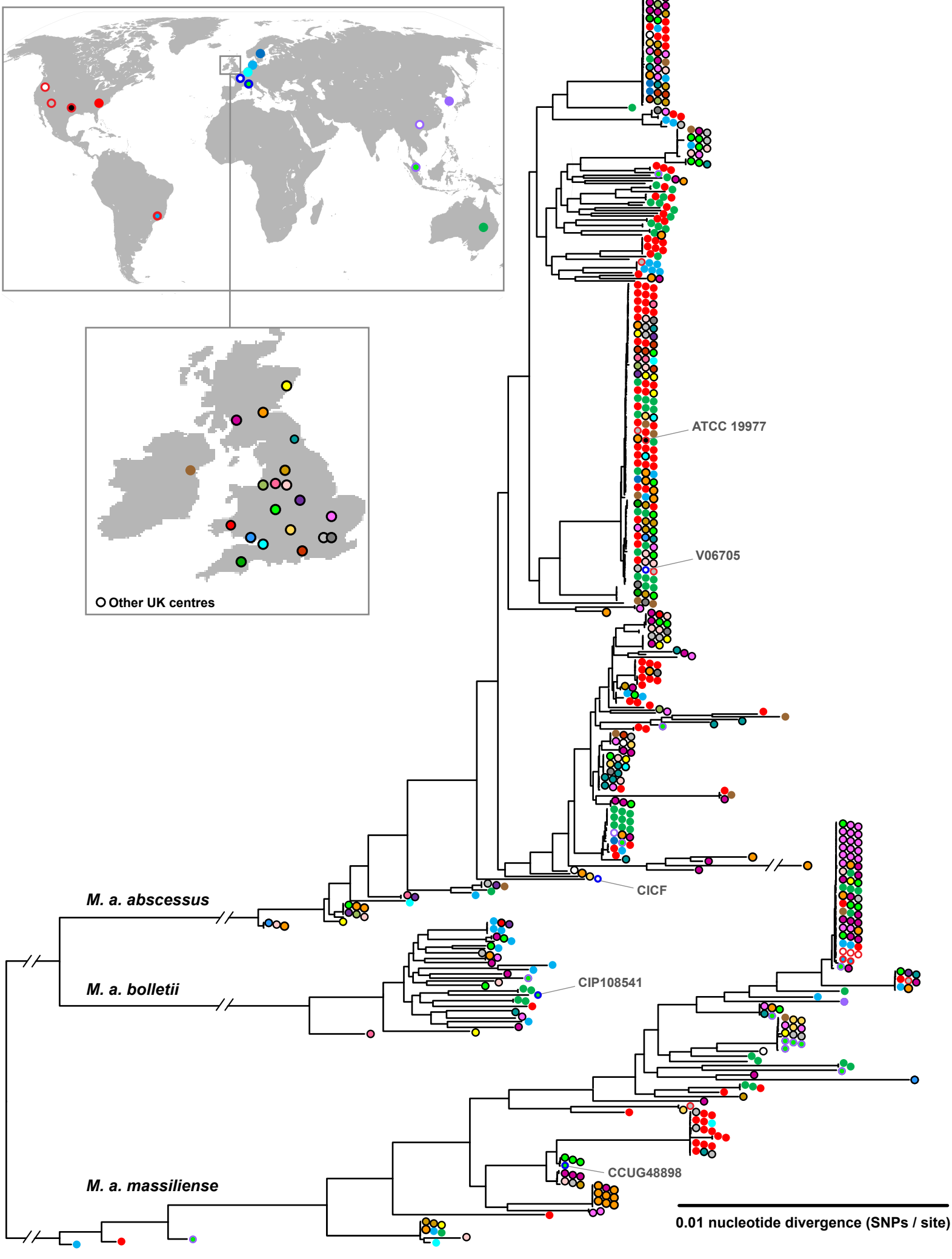

Other UK centres
ATCC 19977
V06705
CICF
M. a. abscessus
M. a. bolletii
CIP108541
CCUG48898
M. a. massiliense
0.01 nucleotide divergence (SNPs / site)

Figure 2

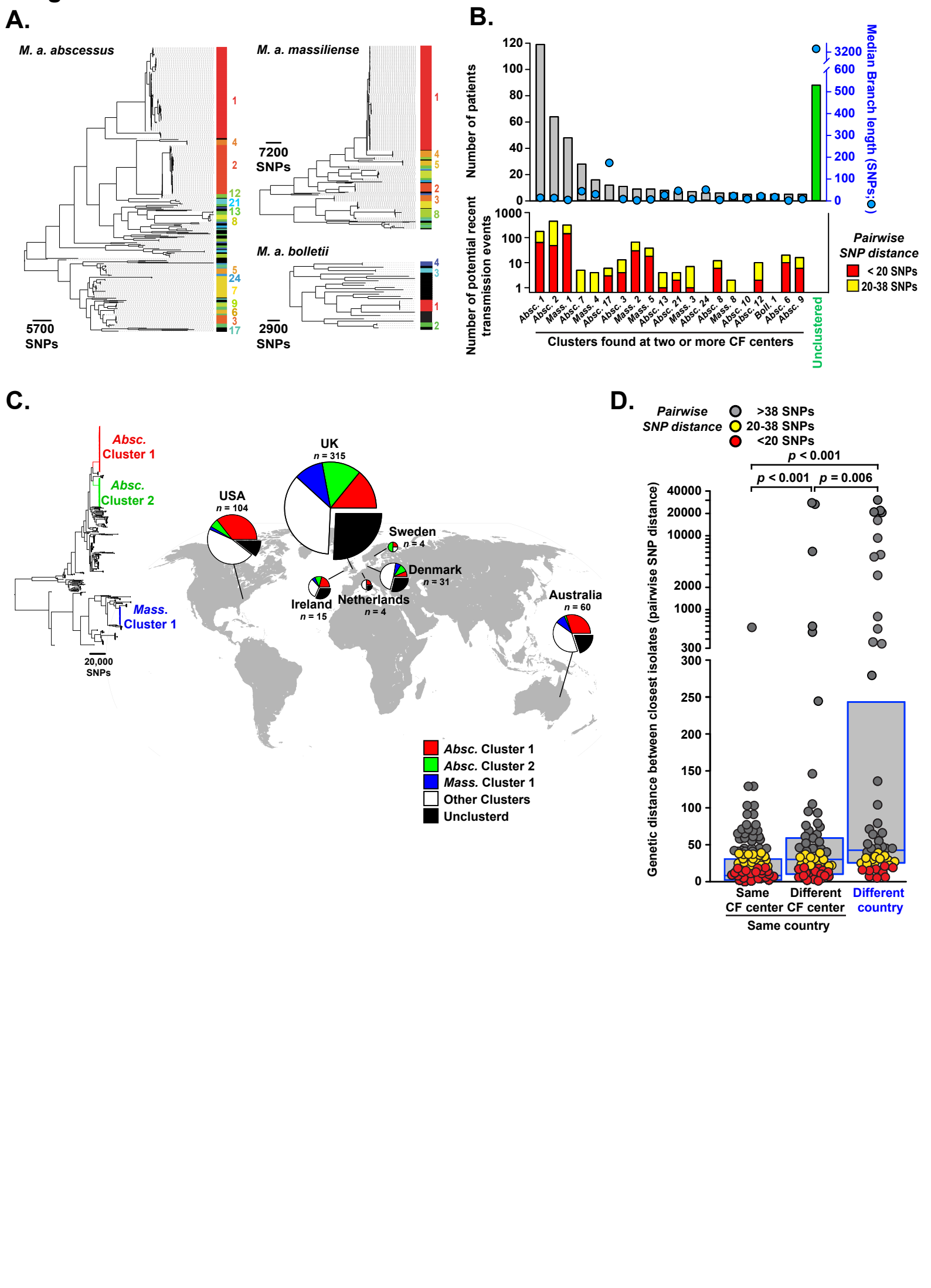

A.
M. a. abscessus
5700 SNPs
M. a. massiliense
7200 SNPs
M. a. bolletii
2900 SNPs
B.
Number of patients
Median Branch length (SNPs;●)
Number of potential recent transmission events
Pairwise SNP distance
< 20 SNPs
20-38 SNPs
Clusters found at two or more CF centers
Unclustered
C.
Absc. Cluster 1
Absc. Cluster 2
Mass. Cluster 1
20,000 SNPs
USA n = 104
UK n = 315
Sweden n = 4
Denmark n = 31
Ireland n = 15
Netherlands n = 4
Australia n = 60
Absc. Cluster 1
Absc. Cluster 2
Mass. Cluster 1
Other Clusters
Unclusterd
D.
Pairwise SNP distance
>38 SNPs
20-38 SNPs
<20 SNPs
p < 0.001
p < 0.001
p = 0.006
Genetic distance between closest isolates (pairwise SNP distance)
Same CF center
Different CF center
Different country
Same country

Figure 3

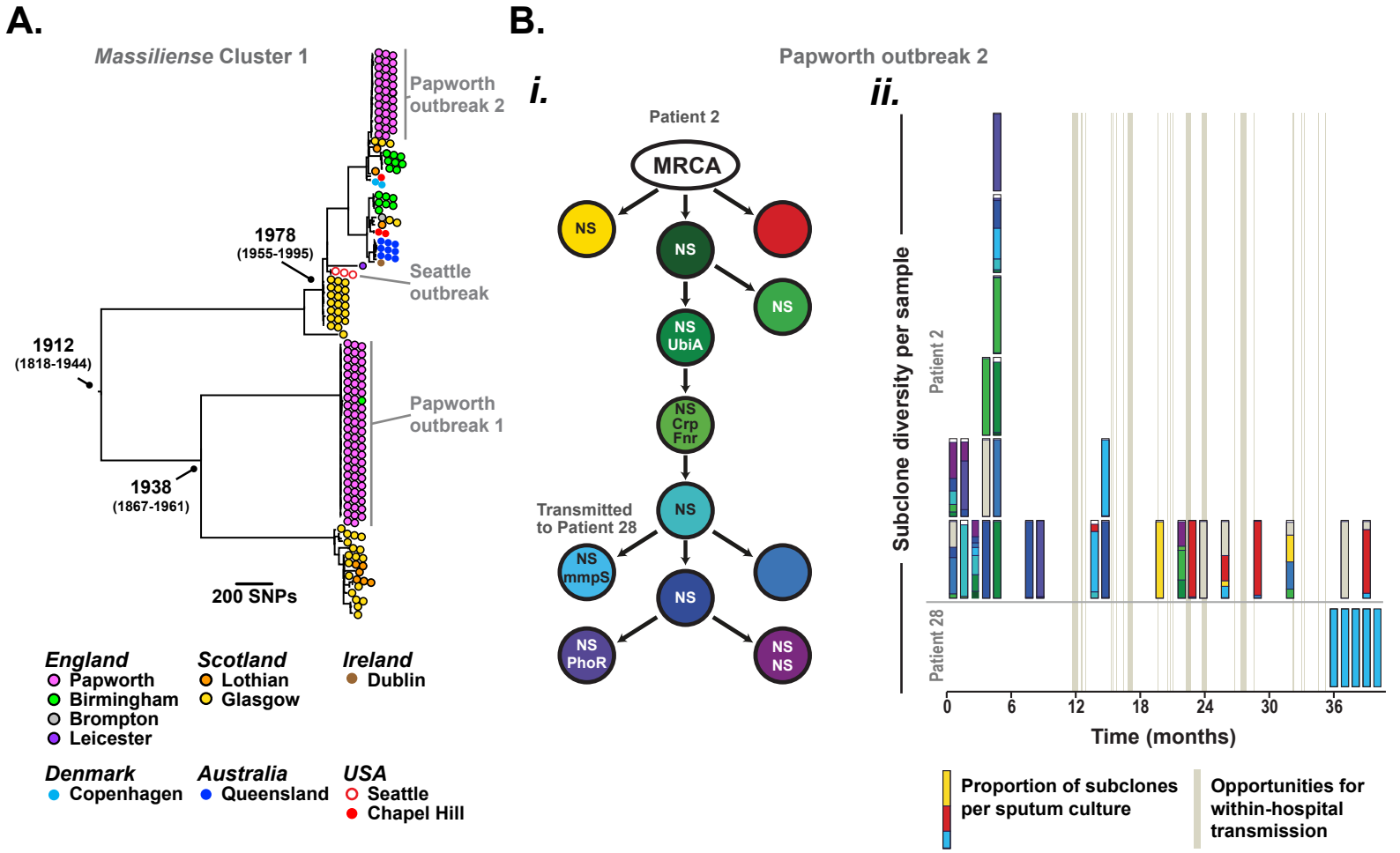
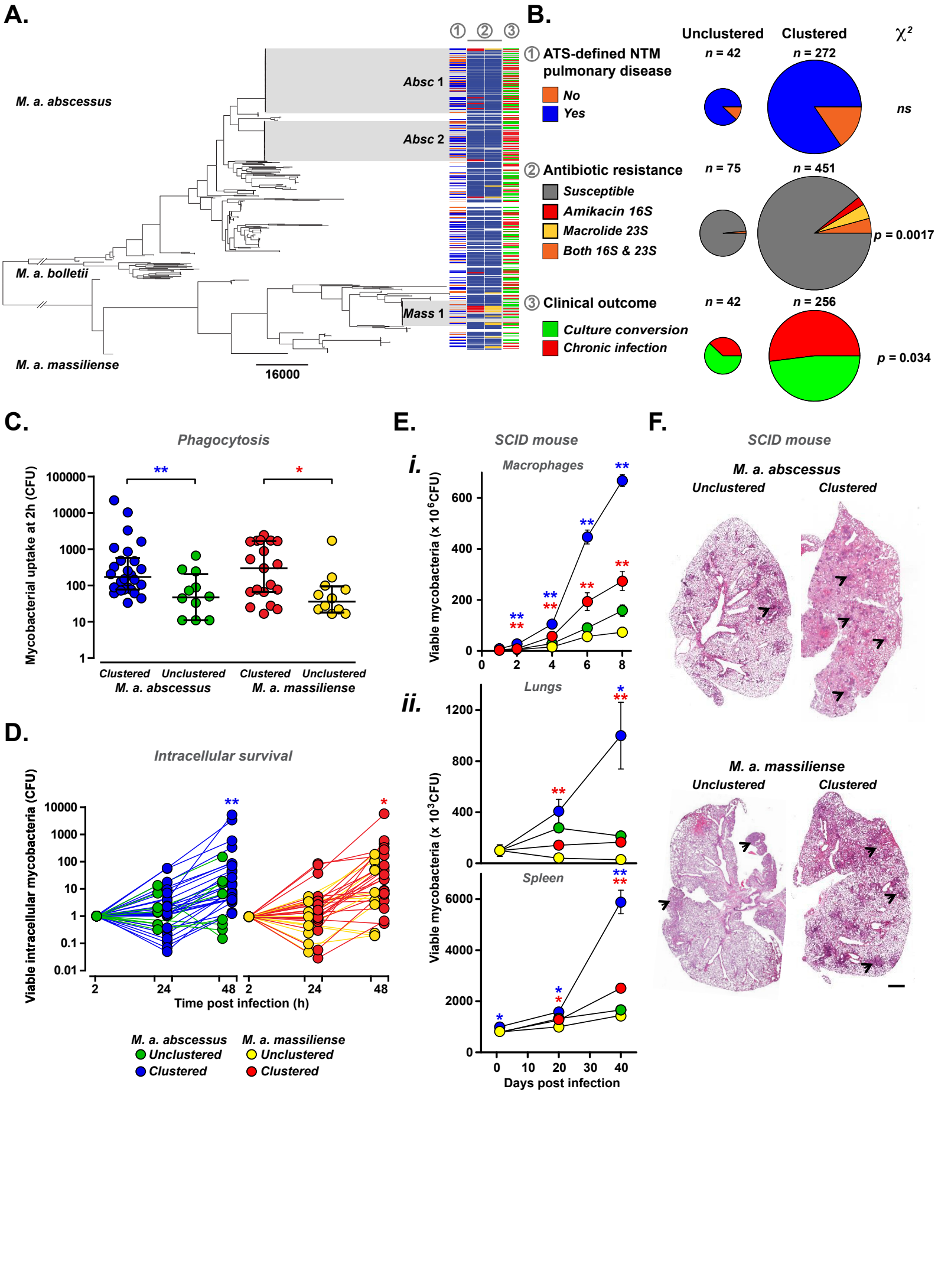

A.
Massiliense Cluster 1
Papworth outbreak 2
1978
(1955-1995)
Seattle outbreak
1912
(1818-1944)
Papworth outbreak 1
1938
(1867-1961)
200 SNPs
England
Papworth
Birmingham
Brompton
Leicester
Scotland
Lothian
Glasgow
Ireland
Dublin
Denmark
Copenhagen
Australia
Queensland
USA
Seattle
Chapel Hill
B.
i.
Patient 2
MRCA
NS
NS
NS
NS UbiA
NS Crp Fnr
NS
Transmitted to Patient 28
NS mmpS
NS
NS PhoR
NS NS
ii.
Papworth outbreak 2
Subclone diversity per sample
Patient 2
Patient 28
0
6
12
18
24
30
36
Time (months)
Proportion of subclones per sputum culture
Opportunities for within-hospital transmission

Figure 4


A.
M. a. abscessus
M. a. bolletii
M. a. massiliense
Absc 1
Absc 2
Mass 1
16000
B.
① ② ③
① ATS-defined NTM pulmonary disease
No
Yes
② Antibiotic resistance
Susceptible
Amikacin 16S
Macrolide 23S
Both 16S & 23S
③ Clinical outcome
Culture conversion
Chronic infection
Unclustered
Clustered
χ²
n = 42
n = 272
ns
n = 75
n = 451
p = 0.0017
n = 42
n = 256
p = 0.034
C.
Phagocytosis
Mycobacterial uptake at 2h (CFU)
Clustered
Unclustered
Clustered
Unclustered
M. a. abscessus
M. a. massiliense
D.
Intracellular survival
Viable intracellular mycobacteria (CFU)
Time post infection (h)
M. a. abscessus
Unclustered
Clustered
M. a. massiliense
Unclustered
Clustered
E.
SCID mouse
i.
Macrophages
Viable mycobacteria (x 10⁶CFU)
ii.
Lungs
Viable mycobacteria (x 10³CFU)
Spleen
Days post infection
F.
SCID mouse
M. a. abscessus
Unclustered
Clustered
M. a. massiliense
Unclustered
Clustered