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Gene flow and Bayesian phylogeography of serogroup C meningococci circulating in Italy

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Abstract

Introduction. Hyperinvasive strains of *Neisseria meningitidis* serogroup C have caused outbreaks of severe disease in Italy. Here, we report the analysis of the migration patterns of C:P1.5-1,10-8:F3-6:ST-11(cc11) meningococcal strains from different Italian regions collected between 2012 and 2017.

Methods. *N. meningitidis* genomes were sequenced through the whole genome sequencing (WGS) method and were analyzed using the BIGSdb Genome Comparator tool. The phylogeography was performed using BEAST. The gene flows in Italy were tested by using MacClade.

Results. The C:P1.5-1,10-8:F3-6:ST-11(cc11) hyperinvasive meningococcal strain, for the data available at the time of the analysis, from UK reached at first Emilia Romagna region, and then, in 2012, was detected in the outbreak occurred in the port of Livorno. The *"Tuscany-outbreak strain"* was likely introduced in Italy between 2013 and 2014. Most of the observed gene flow events occurred from the Center to Northern part of Italy.

Discussion. The phylogeographic analysis allowed to track the dissemination of C:P1.5-1,10-8:F3-6:ST-11(cc11) strains in the country.

Key words

- public health
- surveillance
- invasive meningococcal disease
- phylogeography
- outbreak investigation

INTRODUCTION

Neisseria meningitidis is still cause of epidemic and outbreak with a high case fatality rate [1]. Molecular epidemiology has provided clear observational evidence for an association between specific bacterial genotypes (hyperinvasive lineages) and invasive meningococcal disease (IMD) [2]. In particular, serogroup C strains have been a common cause of IMD outbreaks in Europe, some of them belonging to hypervirulent clones.

Rapid and early detection of outbreaks is critical to guide prompt and appropriate public health interventions to control the outbreaks and prevent additional cases. Molecular typing has played an important role in outbreak investigations, identifying outbreak strains by assessing their genetic relatedness, and providing evidence to understand the chain transmission and linkage.

The rapid turnaround time and availability of automated whole genome sequencing (WGS) analysis is, nowadays, in the routine use for outbreak investigation and surveillance of IMD [3-5].

In a previous paper [6] we identified the pattern of dispersal of hyperinvasive strains of serogroup C meningococci in a large geographical area and the routes leading to their introduction in our Country.

The present study was then planned to decipher the sequence information of the complete genome of circulating meningococci of serogroup C isolated in Italy from 2012 to 2017. In particular, an extensive comparative genomic analysis was carried out for identifying the unique features and for phylogeography on strains belonging to C:P1.5-1,10-8:F3-6:ST-11(cc11) through the Italian regions.

METHODS

Setting, whole genome sequencing and phylogenetic dataset

In this study, 63 genomes of meningococci of serogroup C collected at the National Reference Laboratory (NRL), in the frame of the National IMD Surveillance System at the Italian Institute of Public Health (Istituto Superiore di Sanità, ISS), from January 1st 2012 to December 31st 2017 were analyzed. Sixthy-one of them belonged to C:P1.5-1,10-8:F3-6:ST-11(cc11) and two to C:P1.5-1,10-8:F3-6:ST-2780(cc11). The whole

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genome sequences were previously performed with the Illumina MiSeq platform (kit v3, 600 cycles), according to the procedure previously described [7]. The phylogenetic dataset, ranging from 2007 to 2017, comprised the 63 *N. meningitidis* genomes from Italy (collection dates ranging from 2012 to 2017), together with 70 C:P1.5-1,10-8:F3-6:ST-11(cc11) genomes from other countries (acting as reinforcement isolates for Bayesian analysis), and were compared using the BIGSdb Genome Comparator [8], through the gene-by-gene analysis. The genomes were analyzed by the core genome MLST (cgMLST), in the PubMLST Neisseria website [9], and the core genome has been generated [8]. The sampling administrative regions of the sixty-three Italian isolates were: Tuscany (n= 29), Lombardy (n=13), Liguria (n=2),Veneto (n=1), Lazio (n=2), Trentino (n=1), Emilia-Romagna (n=6), Piedmont (n=3), Apulia (n=3), Sardinia (n=1), Marche (n=1) and Basilicata (n=1).

Phylogenetic analysis

Core Genome Recombination analysis was performed with BratNextGen (BNG) [10] in the aligned core genome of the *N. meningitidis* dataset to obtain recombination-free input sequences for further phylogenetic analyses, as previously described [11-13]. Single-nucleotide polymorphisms (SNPs) were based on the core genome shared by all the isolates. SNPs were exported as variable sites using MEGA, removing SNP sites with ambiguities, missing data and gaps [14]. The SNPs introduced by putative recombination events were removed. The evaluation of the best fitting model of nucleotide substitution was performed with the JModeltest [15, 16]. The phylogenetic signal was investigated by using Treepuzzle, with the likelihood mapping analysis using 10,000 random quartets [17] to obtain a comprehensive picture of the phylogenetic quality and to estimate the amount of phylogenetic information.

Evolutionary rate estimate and phylogeography

In order to investigate the phylogeography, including evolutionary rate and dates estimates of the *N. meningitidis* dataset, a Bayesian Markov Chain Monte Carlo (MCMC) method implemented in the BEAST software [18], with the GTR model, previously estimated, was used. Alternative clock models (strict and relaxed clock with an uncorrelated log normal rate distribution) and demographic models (constant population size, exponential growth, non-parametric smooth skyride plot Gaussian Markov random field, GMRF, and non-parametric Bayesian skyline plot, BSP) were tested, on the filtered core genome SNPs alignment, accounting for invariant sites. The Bayes factor (BF, using marginal likelihoods) tests were used to compare the models and to estimate the best fitting, as previously described [19]; only values of 2ln BF>6 were considered significant. Chains were conducted until convergence was reached and assessed by calculating the effective sampling size (ESS) for each parameter. Only parameter estimates with ESS's of >200 were accepted. Uncertainty in the estimates was indicated by 95% highest posterior density (95% HPD) intervals. The continuous-time Markov chain (CTMC) process over discrete sampling locations in the BEAST software [18], with Bayesian stochastic search variable selection (BSSVS) model was used for the phylogeographic inference. The maximum clade credibility (MCC) tree (the tree with the largest product of posterior clade probabilities) was summarized with Tree-Annotator, after a 10% burn-in. The final tree was manipulated in FigTree [20] for display purpose. Statistical support for specific clades was assessed by the posterior probability (pp>0.90). The analysis and visualization of the different aspects of the phylogeographic diffusion was performed with Spread [21]. The temporal dynamics of the spatial *N. meningitidis* diffusion were provided by snapshots-maps of the dispersal pattern by Google Earth [22].

Gene flow and migration analysis

The Mac Clade version 4 program (Sinauer Associates, Sunderland, MA) was used to test gene out/in flow in Italy, among *N. meningitidis* infected subjects, using a modified version of the Slatkin and Maddison test [23]. A maximum likelihood (ML) tree was built by using the Phyml [24] with the GTR model and used as starting tree for this analysis. A one-character data matrix was obtained from the dataset by assigning to each taxon in the tree a one-letter code indicating its own sampling location, according to three different geographic groups: A: Northern Italy including the following regions: Lombardy, Liguria, Piedmont, Veneto, Trentino Alto Adige; B: Central Italy including Emilia-Romagna, Lazio, Marche, Tuscany (also including the isolates from Livorno's port); C: Southern Italy including Basilicata, Apulia and Sardinia. The putative origin of each ancestral sequence (i.e., internal node) in the tree was inferred by finding the most parsimonious reconstruction (MPR) of the ancestral character. The final tree length, that is the number of observed gene flow events in the genealogy, can easily be computed and compared to the tree-length distribution of 10,000 trees obtained by random joining-splitting (null distribution). Observed genealogies significantly shorter than random trees indicate the presence of subdivided populations with restricted gene flow. The gene flow among the different geographic groups (character states) was traced with the State changes and stasis tool through the Mac Clade software [23], which counts the number of changes in a tree for each pair wise character state. When multiple MPRs were present, the algorithm calculated the average migration count over all possible MPRs for each pair.

RESULTS

Evolutionary rate and Bayesian phylogeography.

After removing the SNPs introduced by recombination, the phylogenetic signal was assessed by likelihood mapping analysis which indicated <30 % of "noise", meaning sufficient phylogenetic signal. The Bayes factor tests indicated that the relaxed clock fitted the data significantly better than the strict molecular clock (relaxed *vs* strict 2ln BF=226,754 in favor of the relaxed). The BF comparisons showed that, under the

relaxed clock, the demographic BSP model fitted the data significantly better than the other models (BSP *vs* exponential 2ln BF=38.326, BSP *vs* constant 2 ln BF=68.558, BSP *vs* GMRF 2ln BF=732.512). Under the selected relaxed BSP model, the estimated mean value of the evolutionary rate was 3.1×10^{-6} substitutions per site per year (95% HPD, lower value, 2.4×10^{-6}–95% HPD upper value, 3.8×10^{-6}).

The phylogeographic analysis (*Figure 1*), showed that the root of the tree dated back to the year 2005 (95% HPD: 2002-2007) and most probably originated in UK (sp=0.95). Six cluster (I, II, III, IV, V, VII) and a main clade (VI), all statistically supported, were identified (tMRCA and most probable location reported in *Table 1*). The information regarding the isolates included in this study is available as *Supplementary Material Table 1S*.

The main clade (VI) included the majority of isolates (*Figure 1*), dated back to 2007 (95% HPD: 2006-2010) and showed two distinct introductions, with strains segregating in sub-clade VI a and VI b. The sub-clade VIa, which was dated back to 2008 (95% HPD: 2007-2011), included two isolates from the UK, two from Emilia-Romagna, and four isolates from the Livorno's port out-

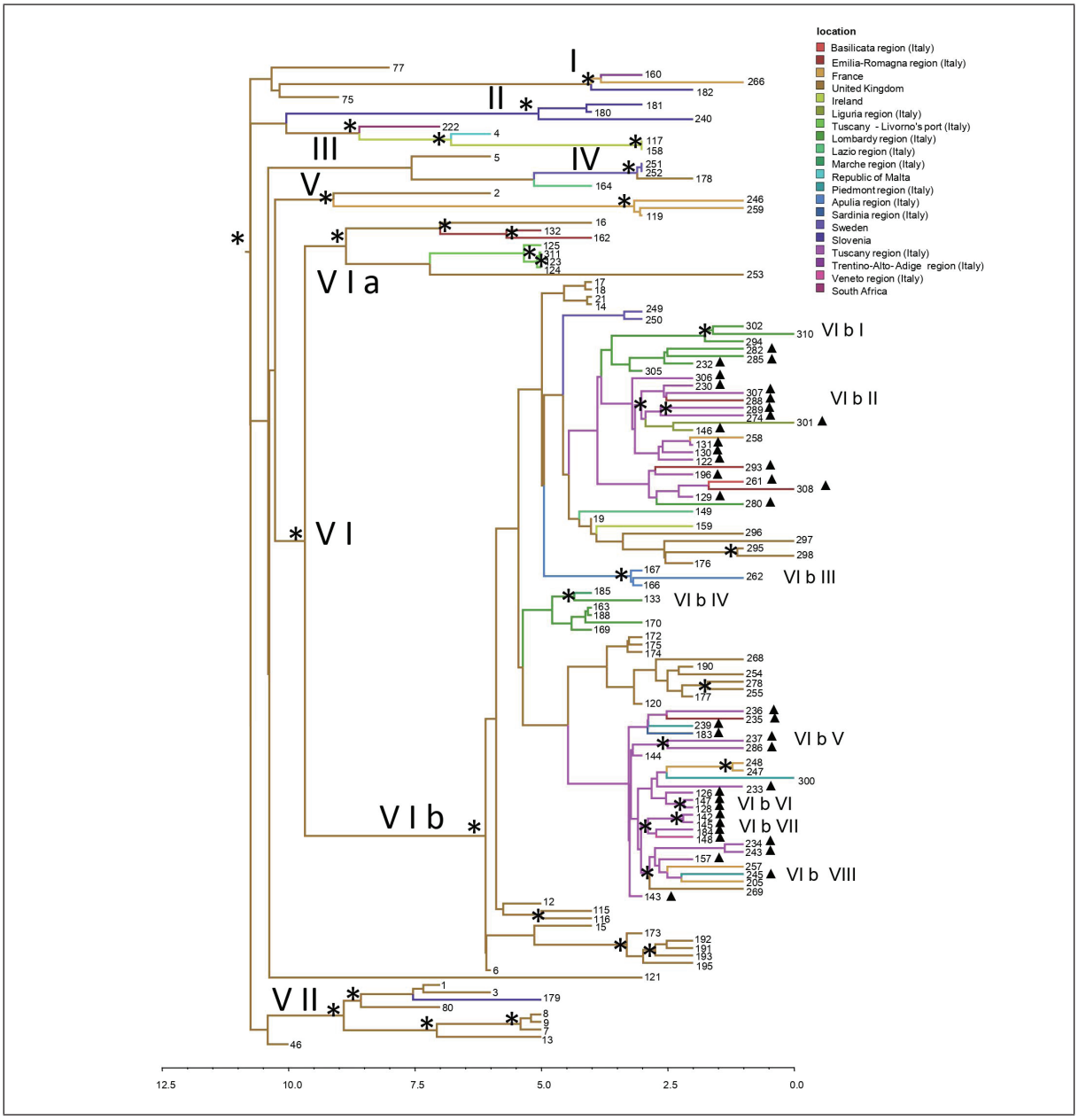

Figure 1

Bayesian phylogeographic tree of *N. meningitidis* strains belonging to the phylogenetic dataset. The scale axis below the tree showed the time (years) before the present. Main statistically supported clade and cluster were indicated. The * along the branch represents significant statistical support for the clade subtending that branch (posterior probability > 90%). Geographic locations were shown with different colors in the tree and reported as legend.

break. The four isolates from the port of Livorno ship outbreak occurring in 2012 were closely related to each other, as previously described [25], dated to 2011 (95% HPD: 2011-2012). The sub-clade VIb, dated to 2011 (95% HPD: 2009-2011), originated from UK (sp=0.99) and included European strains intermixed with Italian isolates from different regions (Tuscany, Piedmont, Veneto, Sardinia, Emilia-Romagna, Lombardy, Marche, Apulia, Lazio, Basilicata, Liguria) (*Figure 1*). Eight statistically supported internal clusters, dating from 2013 to 2015, were identified (VIb $_{I}$ - VIb $_{VIII}$, *Figure 1*, *Table 1*). All the isolates belonging to the "Tuscany-outbreak strain" were included inside VIb, (*Figure 1*, highlighted by a full triangle), some of them segregating in statistically supported clusters. The "Tuscany-outbreak strain" was likely introduced in Italy between 2013 and 2014. The two ST-2780 Italian meningococci (ID code 142 and 145) from Tuscany appeared closely related inside cluster VI b $_{VII}$, together with two Italian meningococci (ID code 184 and 148) collected respectively from Tuscany and Veneto regions in the same time period (year 2015).

Temporal dynamics of spatial diffusion

The spread of the hyperinvasive C:P1.5-1,10-8:F3-6:ST-11(cc11) meningococcal strains isolated in Italy over time, obtained from the location annotated MCC tree, indicated that starting from 2011 meningococci from UK reached at first Emilia Romagna region (*Supplementary Material: Figure 1S A, panel a*). By 2012, C:P1.5-1,10-8:F3-6:ST-11(cc11) meningococci from UK reached the port of Livorno causing a small outbreak, and subsequently, another migration occurred from UK to Lombardy (*Supplementary Material: Figure 1S A, panel b*). A spread from Lombardy to Marche and from UK to Lazio occurred by 2013 (*Supplementary Material: Figure 1S A*, *panel c*). Subsequently, waves of migration from UK to Tuscany (*Supplementary Material: Figure 1S A*, *panel d*), from Tuscany to Lombardy, and from UK to Apulia (*Supplementary Material: Figure 1S A*, *panel d*) were observed. By 2014 (*Supplementary Material: Figure 1S A*, *panel e*), flows from France to Trentino Alto Adige, from Tuscany to Liguria, and from Tuscany to Sardinia probably occurred. By the beginning of 2015, C:P1.5-1,10-8:F3-6:ST-11(cc11) meningococci probably continued to spread from Tuscany to Veneto, from Tuscany to Piedmont, from Tuscany to UK, from Tuscany to France (*Supplementary Material: Figure 1S A*, *panel f*). Between the end of 2015 and the beginning of 2016, a strain from Tuscany probably reached Basilicata. The most frequently invoked and supported links, involving Italian regions and foreign countries, were those between Slovenia and Sardinia, Slovenia and Marche, Slovenia and Piedmont, Slovenia and Basilicata, Sardinia and South Africa, Sardinia and Sweden, South Africa and Marche, Marche and Sweden, South Africa and Piedmont, South Africa and Basilicata, Piedmont and Sweden, Sweden and Basilicata (*Supplementary Material: Figure 1S B*). The most frequently invoked and supported links between Italian regions were, those between Apulia and Basilicata, Sardinia and Marche, Sardinia and Piedmont, Sardinia and Basilicata, Marche and Piedmont, Marche and Basilicata, Piedmont and Basilicata (*Supplementary Material: Figure 1S B*).

The gene flow analysis

The "null hypothesis" of panmixia (i.e., no population subdivision or complete intermixing of sequences from different geographic areas) was rejected by the

Table 1
Time to the most recent common ancestor for the internal nodes, 95% highest posterior density estimates, the most probable location and the state probability of the main clade and cluster

Clade and cluster	tMRCA (years)	95% HPD	Locality	State probability
I	2013	2008-2013	United Kingdom	0.51
II	2012	2011-2013	Slovenia	0.99
III	2008	2006-2009	United Kingdom	0.31
IV	2013	2012-2014	Sweden	0.54
V	2008	2006-2011	United Kingdom	0.95
VI	2007	2006-2010	United Kingdom	0.99
VI a	2008	2007-2011	United Kingdom	0.93
VI b	2011	2009-2011	United Kingdom	0.99
VI b I	2015	2014-2016	Italy: Lombardy	0.99
VI b II	2014	2013-2015	Italy: Tuscany	0.99
VI b III	2014	2013-2014	Italy: Apulia	0.99
VI b IV	2013	2012-2013	Italy: Lombardy	0.81
VI b V	2015	2014-2016	Italy: Tuscany	0.99
VI b VI	2015	2014-2015	Italy: Tuscany	0.99
VI b VII	2014	2014-2015	Italy: Tuscany	0.99
VI b VIII	2014	2014-2015	Italy: Tuscany	0.89
VII	2008	2006-2009	United Kingdom	0.99

tMRCA: time to the most recent common ancestor; HPD: highest posterior density.

randomization test, (P <0.0001) [23]. The gene flow analysis revealed that most of the observed gene flow events (54.5%) occurred from the Center to Northern Italy; a lower proportion of gene flow (18.2%) was identified from Northern to Center and from Center to Southern Italy (18.2%) (*Figure 2*). Only 9.1% of gene flow was observed from Northern to Southern Italy (*Figure 2*).

DISCUSSION

Neisseria meningitidis represents a public health issue globally [25-27] and a leading cause of morbidity and mortality in all age groups [1].

In Italy, where serogroup C is the second most common meningococcus serogroup [28], the hyperinvasive C:P1.5-1,10-8:F3-6:ST-11(cc11) strain caused several IMD sporadic cases, clusters, and small outbreaks in several regions, and a major outbreak in Tuscany in 2015-2017 [29]. In this study, a Bayesian MCMC approach was used to define the epidemiological history and phylogeographic analysis of C:P1.5-1,10-8:F3-6:ST-11(cc11) *N. meningitidis* isolates, circulating in different Italian regions, Moreover, the gene flow analysis was used to test the amount of gene out/in flow in Italy, among the different areas of the country.

The estimation of the rate of molecular evolution is critical for understanding a variety of evolutionary and epidemiological processes. In this study, we estimated a mean evolutionary rate of 3.1×10^{-6} substitutions per site per year (95% HPD: 2.4×10^{-6}–3.8×10^{-6}), which is comparable to those reported by Lamelas *et al.* 2014 [30] for *Neisseria meningitidis* serogroup A strains (3.1×10^{-6}; 95% HPD: 2.30×10^{-6}–3.85×10^{-6}). Values of the same order of magnitude were also reported in the literature [31] for *A. baumannii,* with a value of 3.15×10^{-6} (2.34×10^{-6} –4.44×10^{-6}), and for *Staphylococcus aureus,* which showed a mean rate of 2.43×10^{-6} (1.14×10^{-6}–3.98×10^{-6}) substitutions per site per year. The lowest estimates reported in the literature [31, 32] were those of *Klebsiella pneumoniae* and *Y. pestis*, with mean rates of 1.03×10^{-6} (95% HPD: 8.09×10^{-7} to 1.24×10^{-6}) and 1.57×10^{-8} (95% HPD: 1.03×10^{-8}–2.27×10^{-8}) substitutions per site per year, respectively.

The phylogeographical analysis suggested that C:P1.5-1,10-8:F3-6:ST-11(cc11) *N. meningitidis* strains, after accumulating by 2005 (95% HPD: 2002-2007) in UK, probably spread a few years later to the locations identified through the phylogeographic tree and diffusion analysis [33]. C:P1.5-1,10-8:F3-6:ST-11(cc11) meningococci most probably reached Emilia Romagna in 2011 (95% HPD: 2011-2012), and by 2012 was responsible of an outbreak occurred in the port of Livorno. The spatial diffusion suggested an intensification of the migration pathway since 2013 and during the following years towards several Italian regions. This finding is consistent with epidemiological data reporting an increase in the number of Men C IMD cases in Italy [29, 34, 35]. In the same time period, linkages and migration events were also identified between other foreign countries and Italian regions, together with exchanges involving only Italian regions. As recently described [33] the outbreak occurred in Tuscany was probably due to the introduction of the C:P1.5-1,10-8:F3-6:ST-11(cc11) strain between 2013 and 2014 through a dispersal pattern originated from UK in 2011 (95% HPD: 2009-2011). The analysis of the diffusion rates allowed

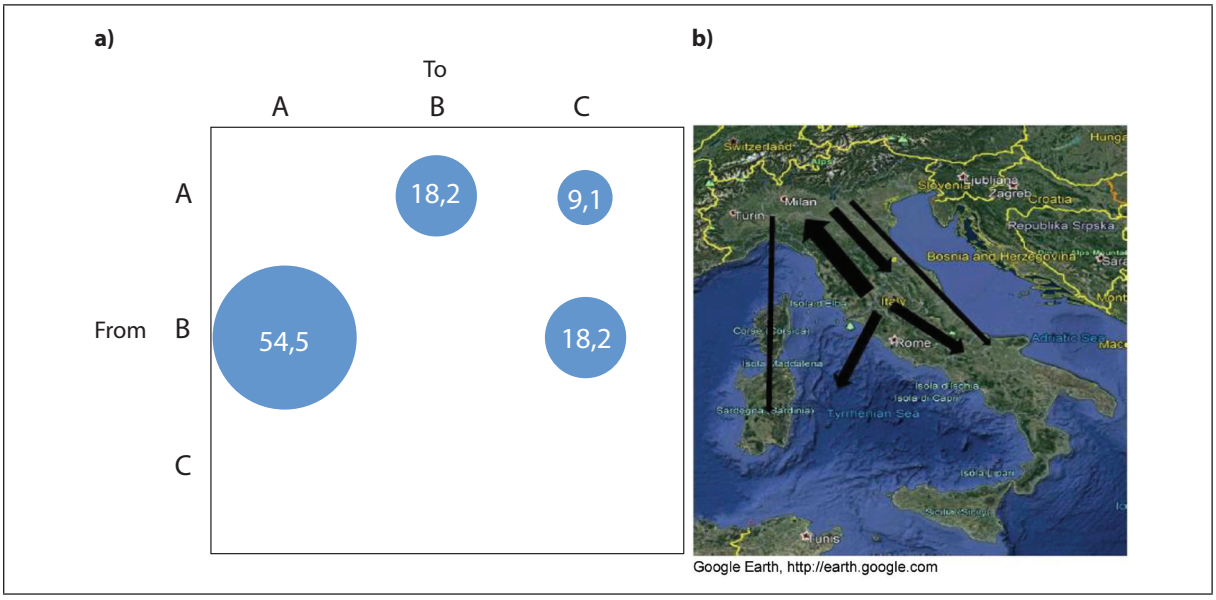

Figure 2

a) Maximum parsimony migration patterns of *N. meningitidis* strains to/from different areas of Italy. The bubblegram shows the frequency of gene flow (migrations) to/from different geographic areas, as the percentage of the total observed migrations estimated from the maximum likelihood tree. A, Northern Italy; B, Center- Italy; C, Southern Italy.

b) Projections and reconstructions of the *N. meningitidis* gene flows on a geographical map made available from Google Earth (http://earth.google.com; Google Earth Pro V.7.3.2.5491). The arrows indicate the gene flow to/from different areas of Italy (North, Center and South), the thickness of the arrows is proportional to the amount of gene flow (migrations obtained with Mac Clade software).

to highlight both frequently invoked rates acting as long distance connections and short distance linkages. Focusing only on the migrations identified by phylogeography and most probably occurred between Italian regions, most of the them regarded regions located in the Center and in the North of the country. Other migrations, probably involved the Center and the South of Italy. Few connections were identified between Northern and Southern regions. Linkages were also observed between regions located in the same geographic area. For example, Basilicata region resulted connected with Apulia located in Southern Italy, and with Sardinia. It is worth to mention how the phylogeographic analysis allowed to track the dissemination of the strains and to identify the highly connected locations. To this purpose, the migration patterns identified local and intercountries exchanges, probably explained by increased mobility of affected population, attendance to places of interconnection or crowded sites, close contacts with carriers or symptomatic individuals.

In this study, we also reported for the first time the gene flow analysis of C:P1.5-1,10-8:F3-6:ST-11(cc11) *N. meningitidis* isolates, which is able to test the migrations to/from different geographical areas. This analysis identified the Center of Italy as a major source of dissemination, with a 54.5% of gene flows to the North. Furthermore, as mentioned above, Central Italy showed a significant flow (18.2%) to the South, suggesting that C:P1.5-1,10-8:F3-6:ST-11(cc11) *N. meningitidis* mainly expanded from the Center to the North and, to a lesser extent to the South of the country. A low proportion of *N. meningitidis* gene flow from the North to the Center and to the South of Italy was also identified. Overall, these data, even though obtained by using a different methodology, confirm what previously observed using Bayesian phylogeography, which also showed a greater number of migrations between the Center and the North of Italy. Our data highlighting the Center of Italy as a major source of dissemination, can be probably explained by the increased number of meningococcal disease cases observed in Tuscany between 2015 and 2016 [30], together with an efficient surveillance system. Another possible assumption could be linked to the high prevalence of risk-behaviour that facilitate close contacts with infected individuals, promoting the spread of meningococci as previously described by Miglietta *et al*., 2018 [35]. Finally, our findings according to the dynamics of migration and phylogeography highlight the increase of IMD cases due to MenC in the Tuscany outbreak among adults [29, 34, 36] and the smaller increase of MenC incidence rates in other Italian regions in the same period, i.e. Emilia-Romagna, Lombardy, Trentino Alto Adige, Piedmont, Veneto [34, 36].

CONCLUSIONS

Invasive meningococcal disease due to hypervirulent serogroup C strain needs to be carefully monitored considering phylogeographic analysis of genomes in order to follow the spread in the country.

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Conflict of interest statement

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