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Coronavirus Pandemic

An outbreak of COVID-19 after a pilgrimage to Medjugorje due to Delta sub-lineages

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Abstract

Introduction: A COVID-19 outbreak occurred at the end of October 2021 among pilgrims returning from Medjugorje (Bosnia and Herzegovina).

Methodology: Whole genome sequencing (WGS) of SARS-CoV-2, epidemiological data, and phylogenetic analysis were used to reconstruct outbreak dynamics.

Results: The results suggest that only in one case, associated with the SARS-CoV-2 sub-lineage AY.9.2, it is possible to trace back the place of contagion to Medjugorje, while the other cases were likely to be acquired in the country of origin.

Conclusions: The combined use of phylogenetic data derived from WGS, and epidemiological data allowed us to study epidemic dynamics and to formulate a possible hypothesis on the place of exposure to SARS-CoV-2. The identification of different sub-lineages of the SARS-CoV-2 Delta variant also suggested that different chains of transmission contributed to the outbreak.

Key words: SARS-CoV-2; pilgrimage; Delta variant; phylogenetic analysis.

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Introduction

The continuous evolution of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has led to the emergence of several variants, and distinct lineages and sub-lineages [1-3]. According to the European Centre for Disease Prevention and Control (ECDC) [2] and the World Health Organisation (WHO) [3], variants such as Delta and Omicron are classified as variants of concern (VOCs).

The Delta variant was first identified in India in late 2020 [4]. This variant quickly spread worldwide, evolving with related sub-lineages AY.x [1], and since its introduction became predominant in numerous countries in slightly different time periods [5]. In Italy, the Delta variant appeared early in 2021 [5,6].

The sub-lineages AY.x were reported with different prevalences [7] until the emergence and spread of the Omicron variant, which represents, currently, the only variant responsible for Coronavirus disease 2019 (COVID-19) cases in Italy [8,9].

Mass gathering events, such as pilgrimages, were considered to be an important risk factor for SARS-CoV-2 transmission [10]. Here, we describe the whole genome sequencing and a comparative analysis of SARS-CoV-2 genomes which was performed using samples of COVID-19 cases identified after a pilgrimage. The genomic data were analyzed using phylogenetic approaches combined with clinical data to investigate the phylogenetic relationship among the cases and to track the source of the infection.

Methodology

Whole genome sequencing

Out of 152 pilgrims from Sardinia who had visited Medjugorje, 70 resulted positive for SARS-CoV-2 as confirmed by Real-Time PCR (attack rate 46%); 52 confirmed cases were symptomatic. Thirty-eight samples collected from pilgrims plus two secondary contacts were eligible for whole-genome sequencing.

The samples, collected and analyzed by the Microbiology and Virology Unit - COVID North Sardinia Reference Centre (Azienda Ospedaliera Universitaria, Sassari) - within the Italian SARS-CoV-2 genome surveillance framework, were then sequenced.

Sequencing libraries for each sample were prepared using the CleanPlex® FLEX SARS-CoV-2 Panel (Paragon Genomics, Fremont, CA, USA), pooled and sequenced for 150 bp of both ends on an Illumina sequencer with MiSeq Reagent Kit v2 (300 cycles) (MS-102-2002) (Illumina, San Diego, CA, USA). FASTQ files were generated from MiSeq Local Run Manager (Illumina, San Diego, CA, USA) and uploaded to I-Co-Gen Platform (https://irida.iss.it/irida21-aries/login - by ISS) for external quality check, variant call review, and determination of the consensus genome. The sequences are available in the GISAID database (https://gisaid.org/).

Phylogenetic analysis

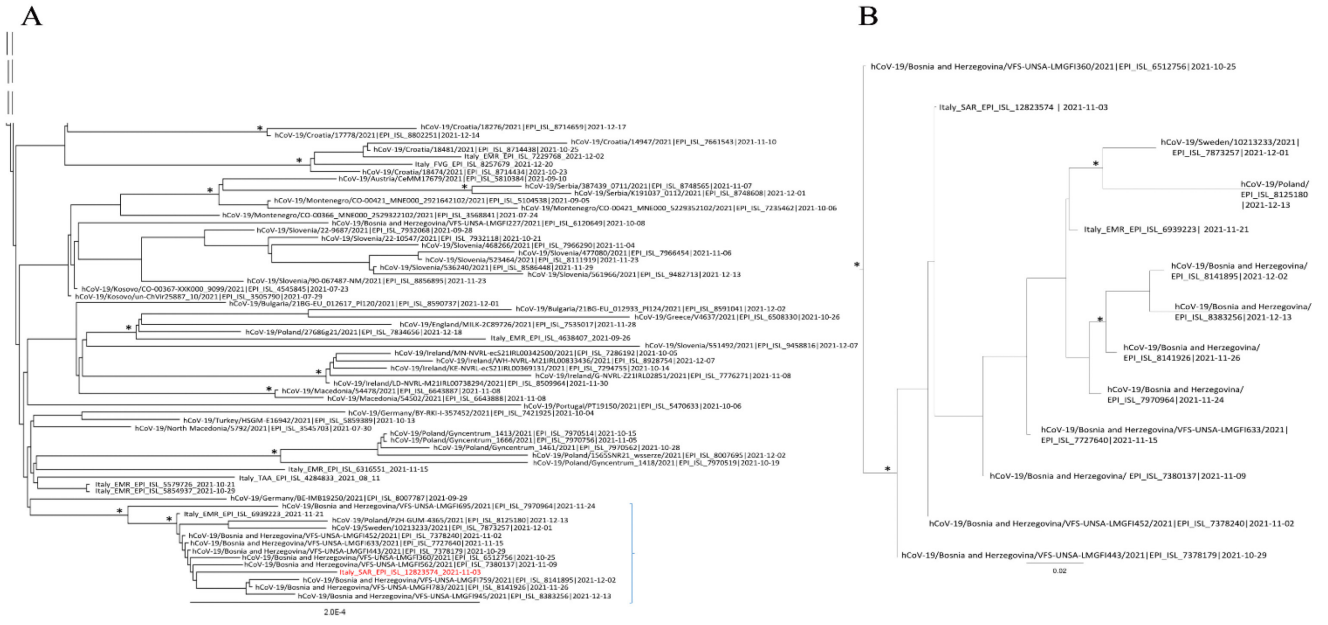
The phylogenetic analysis was performed at the ISS (Rome, Italy). Specifically, the sequence alignments were obtained using MAFFT v.7 [11] under the Galaxy platform [12], manually edited by Bioedit [13].

The lineage of the sequences was established using Pangolin Lineage assigner [14] and Pangolin v.3.1.20 (2022-02-02), subsequently verified by phylogenetic analysis.

A first dataset including 40 genomes together with 20 genomes belonging to sub-lineage AY.9.2 and 19 of sub-lineage AY.126 downloaded from GISAID [6], was used for the analysis (last access February 15, 2022). A second and third datasets were built for AY.9.2 and AY.126 sub-lineages, for a total of 467 and 558 genomes, respectively.

Maximum Likelihood (ML) phylogenetic trees and the best-fitting substitution models were estimated using the IQ-TREE [15]. Statistical support has been inferred by the SH-like aLRT and ultrafast bootstrap (1000 replicates). A specific cluster was further analyzed with a Bayesian method using Beast v.1.10.4.

Figure 1. A: A focus on the cluster including the AY.9.2 genome (EPI_ISL_12823574, highlighted in red) in the ML tree showed as a whole in Supplementary Figure 2. The tree was rooted with the midpoint method. Branch lengths were estimated with the best-fitting nucleotide substitution model according to a hierarchical likelihood ratio test. The scale bar at the bottom represents nucleotide substitutions per site. An asterisk (*) along a branch represents significant statistical support for the clusters subtending that branch (Ultrafast Bootstrap > 95% and aLRT > 80%). **B:** Bayesian maximum clade credibility tree including 13 SARS-CoV-2 AY.9.2 genomes highlighted with the curly bracket in panel a. The asterisk along the branches represents significant statistical support for the clade subtending that branch (posterior probability > 0.80). The Bayesian tree revealed sequences from Bosnia and Herzegovina as an outgroup of this cluster and related to the genome from our case (EPI_ISL_12823574).



The trees were visualized by Fig Tree software v.1.4.4, as previously reported [16].

Results

Seventy COVID-19 cases due to two SARS-CoV-2 Delta sub-lineages occurred at the end of October 2021. A total of 40 subjects (38 of whom were obtained from people who had participated in the pilgrimage thattook place between October 23 and October 27, 2021, and two from secondary contacts) were included in the molecular investigation. Twenty percent (8/40), were not vaccinated, while 5% (2/40) were immunized against COVID-19 only with one dose, and 75% (30/40) with two doses.

Based on the phylogenetic analysis, one genome out of 40 was classified as belonging to the AY.9.2 sub-lineage (Supplementary Figure 1). The ML tree of the second dataset (Figure 1 A and Supplementary Figure 2) showed AY.9.2 sequence (EPI_ISL_12823574) in a supported cluster with nine genomes from Bosnia and Herzegovina, one from Poland, one from Sweden, and one from Italy (Emilia Romagna region). Bayesian analysis suggested that the genomes from Bosnia and Herzegovina were an outgroup of this cluster and related to the genome from Sardinia (Accession ID: EPI_ISL_12823574; Figure 1 B). The dates of onset of symptoms and departure, combined to Bayesian analysis, suggest that this case might have become infected during the pilgrimage (departure from Sardinia on October 23, 2021; return to Sardinia on October 27, 2021).

The phylogenetic analysis showed that most of the genomes collected from the pilgrims (97.5%, 39/40) belonged to the AY.126 sub-lineage (Supplementary Figure 1). The maximum likelihood phylogenetic tree of the third dataset (Figure 2, Supplementary Figure 3) showed that the 39 AY.126 genomes were located in a supported clade that included other genomes from Sardinia and one from Tuscany region (EPI_ISL_7045301). By comparing the dates of onset of symptoms with the dates of travel (departure/arrival) (Figure 3), it can be assumed that approximately 25.6% (10/39) cases from the AY.126 line could have become infected before the pilgrimage.

Discussion

A large outbreak involving a high proportion of pilgrims returning to Sardinia from the sanctuary of Medjugorje (Bosnia and Herzegovina), occurred. The majority of the genomes investigated in this study belonged to the AY.126 sub-lineage, which is the alias of B.1.617.2.126 [1], only one sequence belonged to AY.9.2. Data obtained from GISAID [6] (last access May 10, 2022) showed that AY.126 and AY.9.2 sub-

Figure 2. Focus on the lower part of the tree from Supplementary Figure 3, which shows the clade including AY.126 SARS-CoV-2 genomes. The tree was rooted with the midpoint method. The 39 AY.126 genomes investigated in this study were highlighted in red. Branch lengths were estimated with the best-fitting nucleotide substitution model according to a hierarchical likelihood ratio test. The scale bar at the bottom represents nucleotide substitutions per site. An asterisk (*) along a branch represents significant statistical support for the clusters subtending that branch (Ultrafast Bootstrap > 95% and aLRT > 80%).

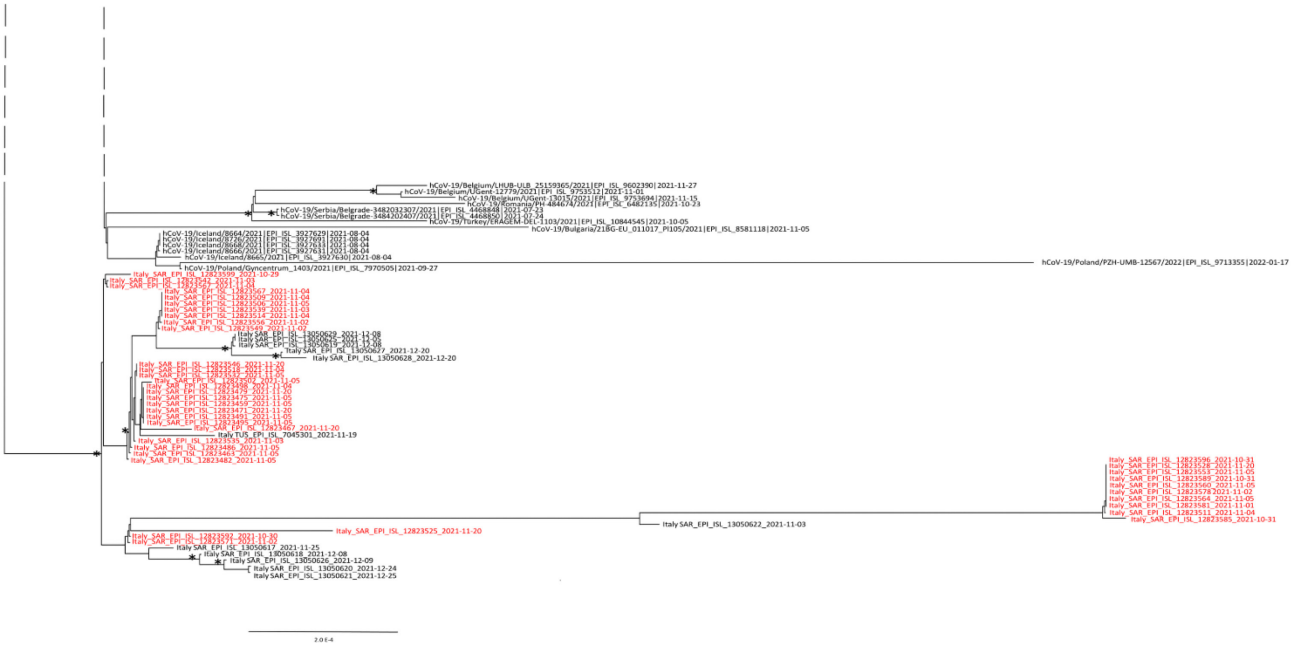
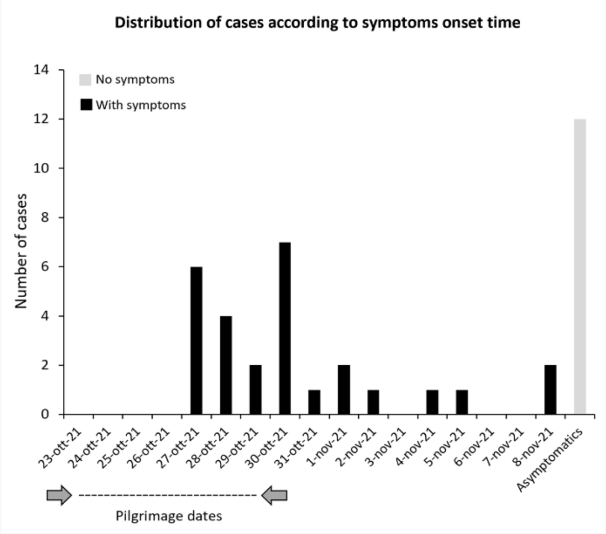


Figure 3. Distribution of cases according to timing of onset of COVID-19 symptoms and respect to pilgrimage dates.



lineages represented, respectively, 0.8% (99/13037) and 2% (259/13037) of the SARS-CoV-2 Italian genomes collected between October 1, 2021, to November 30, 2021, highlighting the low circulation of these delta sub-lineages in the country.

In this study, the analysis suggested that part of the patients was already infected before the departure. Other participants may have acquired the infection in Sardinia, in a period following the dates of the pilgrimage.

It was found that the patient infected by the sub-lineage AY.9.2 might have been infected during the pilgrimage (starting from October 23, 2021). This hypothesis is supported by the proximity of this genome, in the phylogenetic tree, to those from Bosnia and Herzegovina, which appeared as an outgroup of the cluster, although other sources of infection cannot be completely excluded.

SARS-CoV-2 transmission is not unusual during prolonged close contact with infected people, especially during mass gatherings, such as a pilgrimage [10].

A limitation of this analysis was due to the restricted number of genomes from Bosnia and Herzegovina and neighbouring countries in the dataset.

Conclusions

In conclusion, the combined use of phylogenetic data derived from WGS and epidemiological data allowed us to formulate an hypothesis on the place of exposure to SARS-CoV-2. The identification of different sub-lineages of the Delta variant also suggested that different chains of transmission might have contributed to the outbreak.

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We acknowledge the originating and submitting laboratories and Authors for the other Italian sequences shared via the GISAID Initiative, on which this research is based, reported in Supplementary Table 1.

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Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

Supplementary Table 1. Originating and Submitting laboratories and Authors for the other Italian sequences shared via the GISAID Initiative, on which this research is based.

Originating laboratories: A.S.L. BIELLA - OSPEDALE DEGLI INFERMI - SC LABORATORIO ANALISI (Ponderano, Italy) - A.S.L. CN1 - A.S.L. CUNEO 1 (Cuneo, Italy), A.S.L. NOVARA (Novara, Italy), A.S.L. TO4 (Chivasso, Italy), A.S.L. VERCELLI (Vercelli, Italy) - A.S.L. ALESSANDRIA (Alessandria, Italy) – A.S.L. VERBANO-CUSIO-OSSOLA - A.S.L. ASTI (Asti, Italy) - Submitting laboratory: Fondazione del Piemonte per l'Oncologia IRCCS, (Candiolo, Italy), Authors: Antonino Sottile, Silvia Brossa, Paola Marino, Giorgio Giardina.
Originating laboratories: SC (UCO) Igiene e Sanità Pubblica, ASUGI, Trieste, Via della Pietà, 2/2, 34129, Trieste, Italy - Submitting laboratory: ARGO Laboratorio Genomica ed Epigenomica, Trieste - Italy S.S. 14 km 163.5, 34149 Basovizza Area Science Park – Authors: Licastro D, Dal Monego S, Degasperi M, Marcello A, Segat L, Piscianz E, Basaglia G, De Rosa R, Fontana F, Barbone F, Pipan C, Busetti M, Forciniti G, Koncan R, D'Agaro P.
Originating Lab: ASST MONZA (Monza, Italy) – Submitting Laboratory: ASST MONZA (Monza, Italy) – Author: Sergio Maria Ivano Malandrin.
Originating lab: Laboratorio HUB -Azienda Ospedaliero Universitaria - AOU – Cagliari, Presidio Ospedaliero Duilio Casula- Blocco N – Submitting Lab: Laboratorio SPOKE Biologia Molecolare -Azienda Ospedaliero Universitaria - AOU – Cagliari, Presidio Ospedaliero S. Giovanni di Dio. Authors: Sara Fais, Valentina Medda, Alessandra Scano, Germano Orrù, Miriam Loddo, Riccardo Cappai, Ferdinando Coghe.
Originating lab: Azienda Sanitaria dell'Alto Adige - Laboratorio Aziendale di Microbiologia e Virologia (Bolzano, Italy) – Submitting Lab: Azienda Sanitaria dell'Alto Adige - Laboratorio Aziendale di Microbiologia e Virologia (Bolzano, Italy) - Authors: Irene Bianconi.
Originating lab: Center of Advanced Studies and Technology, Molecular Genetics Laboratory (Chieti, Italy) - Submitting Lab: Center of Advanced Studies and Technology, Molecular Genetics Laboratory (Chieti, Italy) - Authors: Anaclerio Federico, De Fabritiis Simone.
Originating lab: Laboratorio CQRC (Palermo, Italy) - Submitting Lab: CQRC_QUALITY CONTROL CHEMICAL BIOLOGICAL RISK_AOOR (Palermo, Italy) – Authors: Di Gaudio F., Contino F., Brunacci G.
Originating lab: UOC Microbiologia e Virologia, Azienda Ospedaliera Universitaria Senese, Siena, Italy, Policlinico Le Scotte (Siena, Italy) – Submitting lab: Dipartimento di Biotecnologie Mediche, University of Siena, Policlinico Le Scotte (Siena, Italy) - Authors: Maria Grazia Cusi, David Pinzauti, Claudia Gandolfo, Gabriele Anichini, Gianni Pozzi, Gianni Gori Savellini, Francesco Santoro.
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Originating lab: Istituto Zooprofilattico Sperimentale del Mezzogiorno (Portici-Napoli-Italy) – Submitting Lab: Telethon Institute of Genetics and Medicine (TIGEM) (Pozzuoli, Napoli, Italy) – Authors: Antonio Grimaldi Patrizia Annunziata Francesco Panariello Biancamaria Pierri Claudia Tiberio Teresa Giuliano Valentina Bouche Chiara Colantuono Maria Concetta Cuomo Denise Di Concilio Lucio Di Filippo Anna Manfredi Marcello Salvi Antonio Limone Luigi Atripaldi Pellegrino Cerino Andrea Ballabio Davide Cacchiarelli.
Originating laboratories: PO AVEZZANO - U.O.C. Laboratorio Analisi (Avezzano, Aquila), Laboratorio Analisi Osp. Città di Castello - Azienda USL Umbria1 (Città di Castello, Italy), Azienda USL Umbria 2 (Spoleto, Italy), Azienda Ospedaliera Terni (Terni, Italy), Università degli Studi di Perugia (Perugia, Italy), OSPEDALE CIVILE TERAMO - CENTRO TRASFUSIONALE (Teramo, Italy), OSPEDALE SAN SALVATORE - MEDICINA DI LABORATORIO (L'Aquila, Italy)
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Originating Lab: Azienda Ospedaliero - Universitaria di Modena Policlinico - Virologia e Microbiologia Molecolare – Universita di Parma Laboratorio di Igiene e Sanita Pubblica (Parma Italy) - Submitting Lab: Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Risk Analysis and Genomic Epidemiology Unit (Parma- Italy) – Authors: Marina Morganti, Ilaria Menozzi, Maria Eugenia Colucci, Licia Veronesi, Paola Affanni, Erika Scaltriti, Stefano Pongolini.
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Originating laboratories: AOUIVR (Verona, Italy) – AOPD (Padova (Italy) - AULSS 6 Euganea (Monselice PD, Italy) - AULSS 9 Scaligera – Villafranca (Villafranca di Verona, Italy) - AULSS 7 Pedemontana – Santorso (Santorso, Italy) - AULSS 9 Scaligera - San Bonifacio (San Bonifacio, Italy) - AULSS 9 Scaligera – Legnago (Legnano, Italy) - AULSS 5 Polesana (Rovigo, Italy) - AULSS 2 Marca Trevigiana (Treviso, Italy) - AULSS 3 Venezia - Submitting laboratory: Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy - Authors: Adelaide Milani, Alessia Schivo, Annalisa Salviato, Elisa Palumbo, Erika Giorgia Quaranta, Luca Tassoni, Angela Salomoni, Ambra Pastori, Edoardo Giussani, Alice Fusaro, Isabella Monne, Calogero Terregino, Antonia Ricci. UOSD Genetica e Citogenetica - Azienda ULSS 3 Serenissima; Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy) – Authors: Mosé Favarato, Elisa Squarcina, Laura Squarzon, Claudia Perini, Noemi Laganà, Laura Bevilacqua, Luca Sorino, Adelaide Milani, Alessia Schivo, Annalisa Salviato, Elisa Palumbo, Erika Giorgia Quaranta, Luca Tassoni, Angela Salomoni, Ambra Pastori, Edoardo Giussani, Alice Fusaro, Isabella Monne, Calogero Terregino, Antonia Ricci.
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Originating Lab: SOC Microbiologia e Virologia PO Pugliese Catanzaro - Microbiologia e Virologia PO Annunziata Cosenza – Submitting lab: SOC Microbiologia e Virologia - AO Pugliese-Ciaccio Authors: Pasquale Minchella.
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Denise Di Concilio, Lucio Di Filippo, Anna Manfredi, Marcello Salvi, Antonio Limone, Luigi Atripaldi, Pellegrino Cerino, Andrea Ballabio, Davide Cacchiarelli.
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Originating Lab: ASL BARI, Policlinico (Bari, Italy) – Submitting laboratory: University of Bari Biomedical Sciences and Human Oncology, Policlinico (Bari, Italy). Authos: Maria Chironna.
Originating Lab: Istituto Auxologico Italiano (Milano, Italy) - Submitting laboratory: Istituto Auxologico Italiano (Milano, Italy) – Authors: Erminio Torresani.
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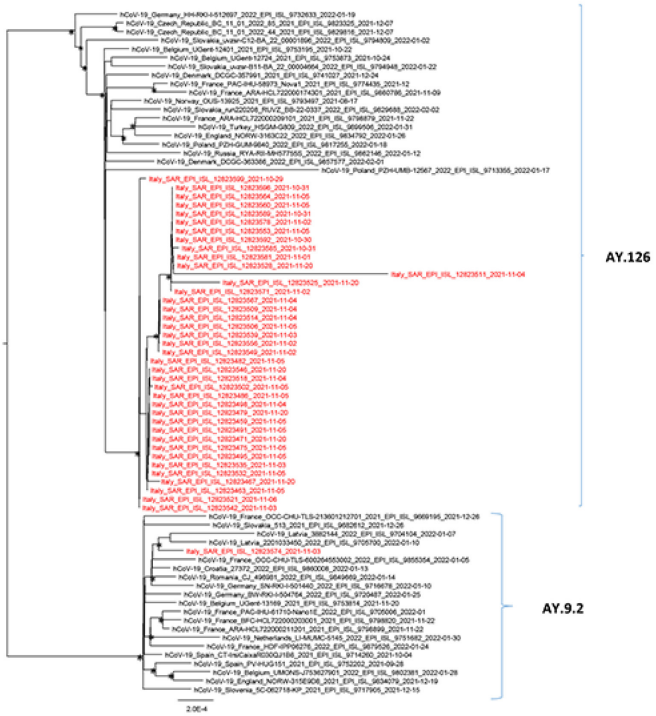
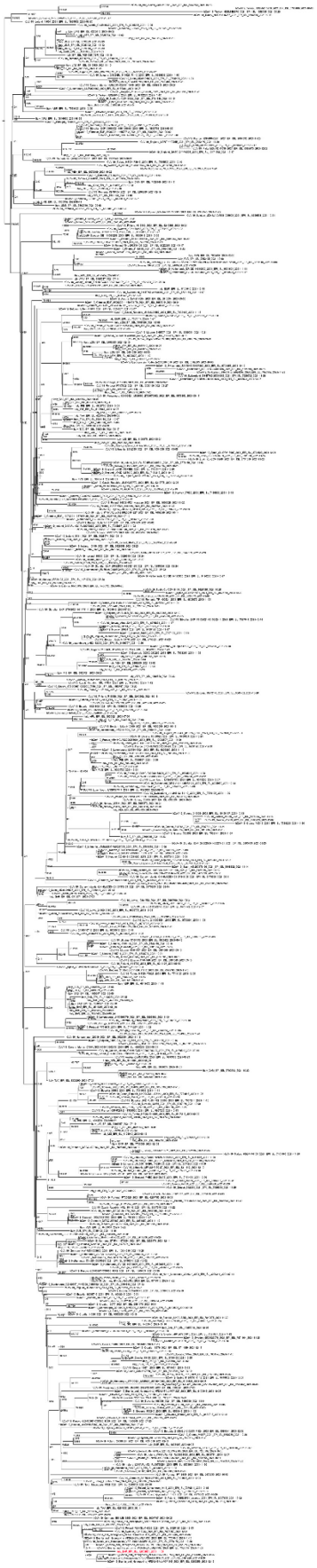


Supplementary Figure 1. Maximum likelihood phylogenetic tree of 40 SARS-CoV-2 genomes from cases (reported in red) together with 20 sub-lineage AY.9.2 and 19 sub-lineage AY.126 sequences downloaded from GISAID database (https://www.gisaid.org). The tree was rooted respect to the cluster AY.9.2. Branch lengths were estimated with the best-fitting nucleotide substitution model according to a hierarchical likelihood ratio test. The scale bar at the bottom represents nucleotide substitutions per site. An asterisk along a branch represents significant statistical support for the clusters subtending that branch (Ultrafast Bootstrap > 95% and aLRT > 80%). The main clades and clusters are highlighted.



Supplementary Figure 2. Maximum likelihood phylogenetic tree of 467 AY.9.2 SARS-CoV-2 genomes: 1) from our cases (EPI_ISL_12823574 - reported in red - collection date 3 November, 2021 and belonging to a different travel group respect to the other participants); 2) 114 Italian genomes downloaded from I-Co-Gen and also available in GISAID; 3) 352 foreign AY.9.2 genomes downloaded from GISAID database (9 from Austria, 1 from Andorra, 9 from Belgium, 10 from Bosnia and Herzegovina, 10 from Bulgaria, 10 from Canary Islands, 10 from Croatia, 10 from Czech Republic, 10 from Denmark, 8 from England, 10 from Estonia, 10 from Finland, 10 from France, 10 from Germany, 10 from Gibraltar, 10 from Greece, 1 from Hungary, 9 from Iceland, 10 from Ireland, 9 from Kosovo, 10 from Latvia, 2 from Liechtenstein, 10 from Lithuania, 10 from Luxembourg, 4 from Macedonia, 10 from Malta, 2 from Monaco, 6 from Montenegro, 10 from Netherlands, 10 from Norway, 10 from Poland, 1 from Monaco, 10 from Portugal, 10 from Romania, 1 from Russia, 3 from Serbia, 8 from Slovakia, 10 from Slovenia, 10 from Spain, 9 from Sweden, 10 from Switzerland, 10 from Turkey, 10 from Ukraine).
The tree was rooted with the midpoint method. Branch lengths were estimated with the best-fitting nucleotide substitution model according to a hierarchical likelihood ratio test. The scale bar at the bottom represents nucleotide substitutions per site. An asterisk along a branch represents significant statistical support for the clusters subtending that branch (Ultrafast Bootstrap > 95% and aLRT > 80%).

Supplementary Figure 3. Maximum likelihood phylogenetic tree of 558 AY.126 SARS-CoV-2 genomes: 1) 39 of which from our cases (reported in red); 2) 122 additional Italian genomes downloaded from the I-Co-Gen platform and available in GISAID; 3) 397 foreign genomes from GISAID (8 from Austria, 10 from Belgium, 23 from Bulgaria, 22 from Croatia, 9 Czech Republic, 10 Denmark, 6 England, 8 Estonia, 8 Finland, 11 France, 8 Germany, 2 Gibraltar, 8 Greece, 1 Hungary, 8 Iceland, 8 Ireland, 4 Kosovo, 8 Latvia, 8 Liechtenstein, 8 Lithuania, 8 Luxembourg, 1 Malta, 2 Moldova, 1 Montenegro, 8 Netherlands, 9 Norway, 10 Poland, 8 Portugal, 22 Romania, 8 Russia, 2 Scotland, 2 Serbia, 30 Slovakia, 34 Slovenia, 8 Spain, 8 Sweden, 8 Switzerland, 31 Turkey, 18 Ukraine, 1 Wales).
The tree was rooted with the midpoint method. Branch lengths were estimated with the best-fitting nucleotide substitution model according to a hierarchical likelihood ratio test. The scale bar at the bottom of the tree represents nucleotide substitutions per site. An asterisk along a branch represents significant statistical support for the clusters subtending that branch (Ultrafast Bootstrap > 95% and aLRT > 80%).