

# Circulation of Enterovirus D68 during Period of Increased Influenza-Like Illness, Maryland, USA, 2021

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We report enterovirus D68 circulation in Maryland, USA, during September–October 2021, which was associated with a spike in influenza-like illness. The characterized enterovirus D68 genomes clustered within the B3 subclade that circulated in 2018 in Europe and the United States.

In early July 2021, the United States began to relax COVID-19 infection control measures. As the number of COVID-19 cases began to fall, cases of influenza-like illness (Appendix Table 1, <https://wwwnc.cdc.gov/EID/article/28/7/21-2603-App1.pdf>) continued to be seen in the Johns Hopkins Hospital system (Baltimore, MD, USA) through October 2021 (Appendix Figure 1). Enterovirus/rhinovirus were detectable throughout the pandemic (1,2), but their positivity markedly increased to reach 20.7% (of all samples tested for enterovirus/rhinovirus) in October 2021, surpassing all other respiratory viruses (Appendix Figure 2) (2).

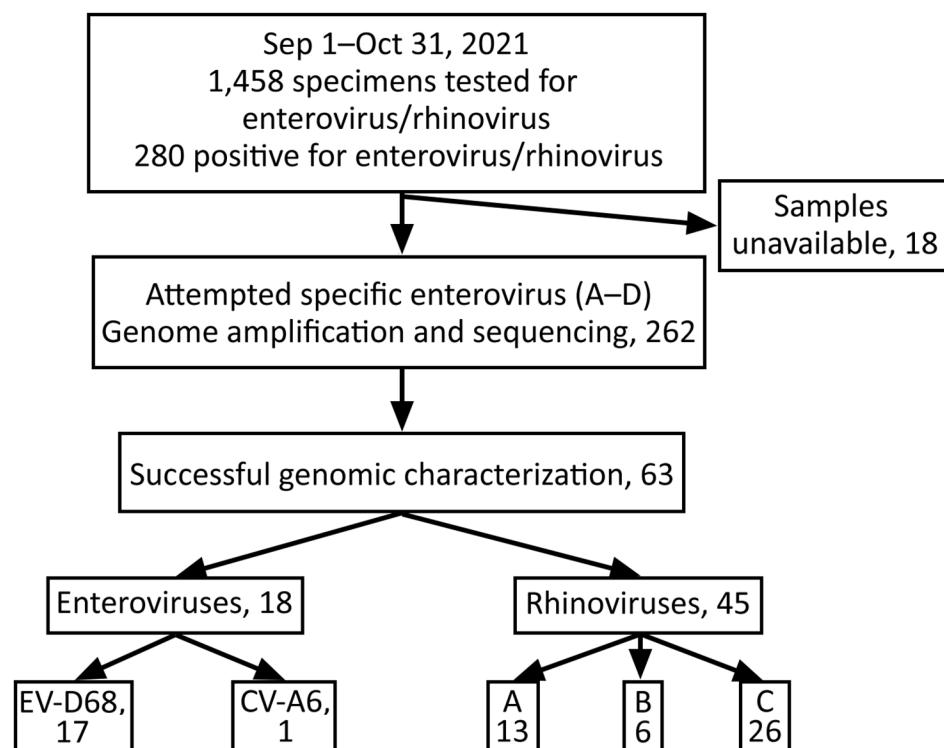
Enterovirus-D68 (EV-D68) was associated with a large outbreak of respiratory disease in children in North America in 2014 and was subsequently linked to the occurrence of acute flaccid myelitis (AFM) (3). After the 2014 outbreak, active surveillance of EV-D68 was implemented in many countries in Asia, Europe, Africa, and the Americas. Data obtained through surveillance during 2014–2018 suggested a biennial circulation cycle in Europe and North America (4,5). However, despite this expected biennial pattern, EV-D68 detection in 2020 was lower than anticipated, and limited cases were detected in the United States (6). This change in the circulation of EV-D68 in 2020 might have been

secondary to the widespread mitigation measures for COVID-19. Of note, a recent study from 8 countries in Europe reported a rapid increase in EV-D68 infections during July 31–October 14, 2021, which coincided with a period of relaxed COVID-19 mitigation measures (7).

For this study, we collected samples positive for enterovirus/rhinovirus after the standard-of-care diagnosis at the Johns Hopkins Medical Microbiology Laboratory during September–October 2021 (Figure; Appendix). Research was conducted under Johns Hopkins Institutional Review Board protocol IRB00221396 with a waiver of consent. Remnant nasopharyngeal clinical specimens from patients that tested positive for enterovirus/rhinovirus during September–October 2021 were retrieved for the study. Genomes were made publicly available in GenBank (accession nos. OL826825–36).

We employed an optimized typing approach by using Nanopore next-generation sequencing (NGS) to characterize the enterovirus types (September–October 2021) associated with the increase in influenza-like illness. In brief, we used primers specific for enterovirus species A–D to amplify a ≈4,500-nt fragment that covers the whole P1 region (about half of the genome) (8) and then performed sequencing (Appendix). Of 280 enterovirus/rhinovirus-positive samples, we collected 262 for genotyping (Figure). We detected enterovirus in 28.6% of the 63 successfully sequenced samples (18/63); 94.4% (17/18) were EV-D68 and 5.6% (1/18) were coxsackievirus A6 (CV-A6). Even though the primers used for amplification were specific for enteroviruses, rhinoviruses were characterized in 45 of the 63 samples; those rhinoviruses consisted primarily of species C (26/45), followed by A (13/45) and B (6/45).

The whole cohort of patients positive for enterovirus/rhinovirus during September–October 2021 ranged in age from <1 year to >90 years; mean age was 16.7 years and median age 5 years. The male:female ratio was 1:1. On the other hand, the median age of EV-D68–positive patients was 2 years, and the male:female ratio was 1:3 (Appendix Table 2). EV-D68 was detected in 15/168 (8.9%) pediatric specimens positive for enterovirus/rhinovirus during the study time frame. Symptoms or signs of viral or respiratory illness were reported in all pediatric patients with EV-D68 (N = 15) (Appendix Table 2), and 5 patients (33.3%) were admitted and required supplemental oxygen, admission to the intensive care unit, or both. No neurologic complications including AFM



**Figure.** Flowchart of patients and specimens in study of circulation of EV-D68 during period of increased influenza-like illness, Maryland, USA, 2021. CV-A6, coxsackievirus A6; EV-D68, enterovirus D68.

were observed (Appendix Table 2). Of note, no AFM cases were diagnosed at Johns Hopkins Hospital during the study time frame. Most cases of enterovirus were detected in residents of the city of Baltimore (11/17). A total of 12 EV-D68 sequences, subclade B3, had a complete 5' half of the genome (3000–4200 bp). EV-D68 genomes clustered with strains detected in 2019 from several countries in Europe (Appendix Figure 3).

We report a predominance of EV-D68 among the circulating enteroviruses during the same period in which enterovirus/rhinovirus positivity increased in this hospital system (2). The predominance of EV-D68 in our study (27% of total enterovirus/rhinovirus-typed genomes) was higher than the 0.4% and 3.6% observed in 2019 and 2020 in the United States (6) and comparable to the 24.3% reported before the COVID-19 pandemic in 2018 (6).

The EV-D68 strains detected belong to the B3 subclade, which had not been reported from the United States since 2018 (6) but was detected in Europe in 2019 (9). The strains we detected form a distinct cluster within the B3 subclade that circulated in 2018 in Europe and the United States but seem very close to those characterized in Europe in 2019. Nevertheless, it was reported that strains circulating in Europe in 2019 are common ancestors of strains detected in the United States in 2018

(9). That report might explain why the strains we identified are more closely related to subclade B3 from the United States than to those from Europe in 2018.

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## Genomic Evidence of In-Flight SARS-CoV-2 Transmission, India to Australia, April 2021

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Epidemiologic and genomic investigation of SARS-CoV-2 infections associated with 2 repatriation flights from Australia to India in April 2021 indicated that 4 passengers transmitted SARS-CoV-2 to  $\geq 11$  other passengers. Results suggest transmission despite mandatory mask use and predeparture testing. For subsequent flights, predeparture quarantine and expanded predeparture testing were implemented.

During the first epidemic wave of SARS-CoV-2, Australia closed its borders; during March 28, 2020–November 1, 2021, international arriving passengers were required to undergo mandatory supervised quarantine (1). This initial response contributed to the end of the first pandemic wave in June 2020 and resulted in periods of COVID-19 control throughout the country (2).

Beginning October 23, 2020, a quarantine facility in Darwin, Northern Territory, Australia, received persons who arrived via government-assisted repatriation flights. On April 15 and 17, 2021, two repatriation flights (flights 1 and 2) carrying pas-

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## Appendix

### Patients' Clinical Data

Demographic and clinical data were collected through bulk query from the Johns Hopkins Hospital electronic medical record system. Influenza-like illness encounters were defined based on the following criteria: all emergency department (ED) visits (regardless of age across the entire system) where: (1) patients presented with a chief report of upper respiratory infection (URI), pneumonia, influenza, flu-like symptoms, cough, or fever and sore throat (2) ED diagnosis of B97.89 (other viral agents as the cause of diseases classified elsewhere). Diagnosis codes are detailed in Appendix Table 1. Clinical data presented in Appendix Table 2 were collected by detailed manual chart reviews from the electronic patients' records.

### Specimens and Diagnostic Testing

The Johns Hopkins Microbiology laboratory serves a wide geographic area that includes Baltimore, Virginia, and Washington, DC. Testing for enterovirus/rhinovirus is performed as a part of multiplex respiratory viral panels using the GenMark ePlex RP1 and RP2. These panels do not differentiate between enteroviruses and rhinoviruses (Figure, <https://wwwnc.cdc.gov/EID/article/28/7/21-2603-F1.htm>) and detect Adenovirus, HCoV-229E, HCoV-HKU1, HCoV-NL63, HCoV-OC43, human Metapneumovirus (HMPV), enterovirus/rhinovirus, influenza A/A H1/A H1–2009/A H3, influenza B, parainfluenza (HPIV)1–4, RSV A, RSV B, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae* in addition to SARS-COV-2 (in RP2 only) (1,2).

## Genomic Sequencing for Genotyping

RNA was extracted from 300µL of clinical specimens using the Chemagic 360 system (Perkin Elmer) according to the manufacturer's specifications. RNA was eluted with 60µl nuclease-free water and stored at -80°C until use. cDNA synthesis has been performed using RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific) following the manufacturer's protocol. The amplification of the 5' half of the genome (~4500 nt) was performed as previously described (3). PCR products were barcoded using the Native barcoding genomic DNA kit (EXP-NBD196) according to the manufacturer's instructions and sequenced using R9.4.1 flowcells on a GridION (Oxford Nanopore Technologies). For the FASTQ files analysis, low-quality reads were filtered, and adapters trimmed with Trimmomatic. Denovo assembly was performed using metaSPAdes. Identification of the enterovirus types was carried out with the RIVM genotyping tool (<http://www.rivm.nl/mpf/enterovirus/typingtool/>). Sequence alignment and phylogeny were performed using MEGA 7.0. The robustness of the ML tree was assessed by bootstrap analyses of 1,000 replicates and the Fermon strain, collected in 1962 was used to root the tree. The evolutionary distances were derived using the Tamura 3 parameter method. BLAST analysis showed >99% homology with strains detected in Europe in 2019 and with a bootstrap of 98%.

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**Appendix Table 1.** Diagnosis codes used to categorize influenza-like illness encounters (4)

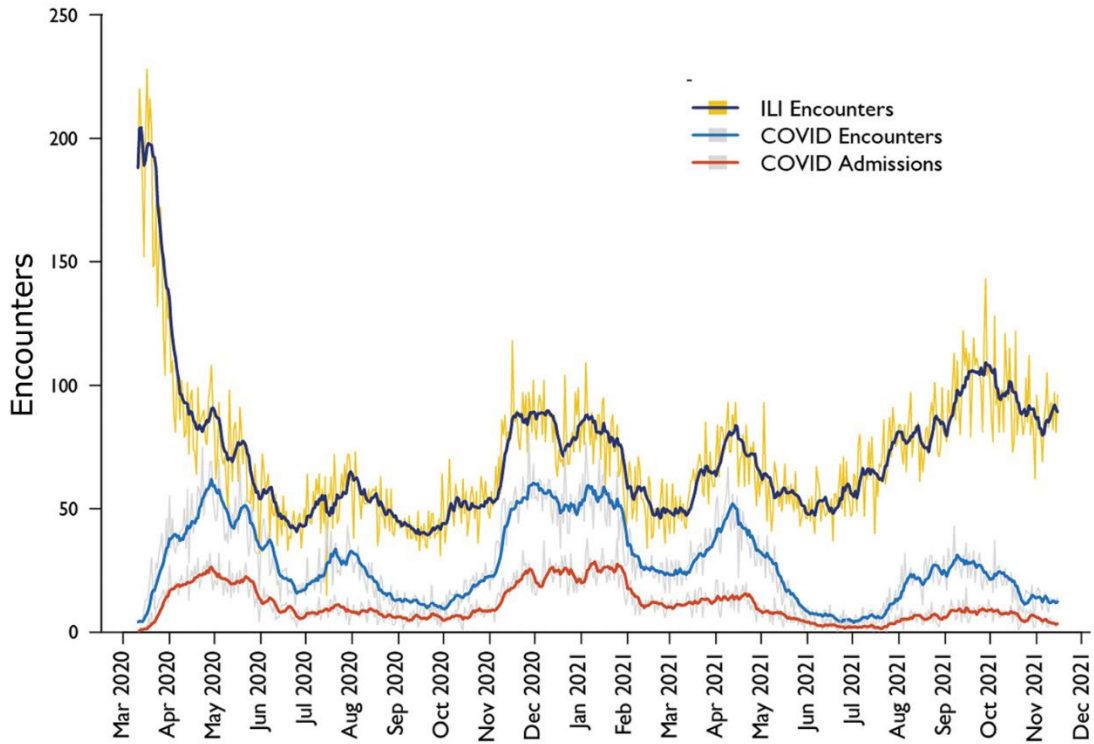
Code	Diagnosis
B97.89	Other viral agents as the cause of diseases classified elsewhere
H66.9	Otitis media, unspecified
J06.9	Acute upper respiratory infection, unspecified
J00	Acute nasopharyngitis; common cold
J01.9	Acute sinusitis, unspecified
J09.X	Influenza due to identified novel influenza A viruses
J10.0	Influenza due to identified novel influenza A viruses
J10.1	Influenza due to other identified influenza virus with other respiratory manifestations
J10.2	Influenza due to other identified influenza virus with gastrointestinal manifestations
J10.8	Influenza due to other identified influenza virus with other manifestations
J11	Influenza due to unidentified influenza virus
J12.89	Other viral pneumonia
J12.9	Viral pneumonia, unspecified
J18	Pneumonia, unspecified organism
J20.9	Acute bronchitis, unspecified
J40	Bronchitis, not specified as acute or chronic
R05	Cough
R50.9	Fever, unspecified
J22	Unspecified acute lower respiratory infection
B34[0–9]	Viral infection of unspecified site [adenovirus, enterovirus, coronavirus, parvovirus, papovavirus, other viral infections]
U07.1	COVID-19
R68.89	Diagnosis reported 'Flu-like symptoms' or 'Influenza-like symptom'

**Appendix Table 2.** Clinical and demographic characters of enterovirus infected patients in September and October 2021

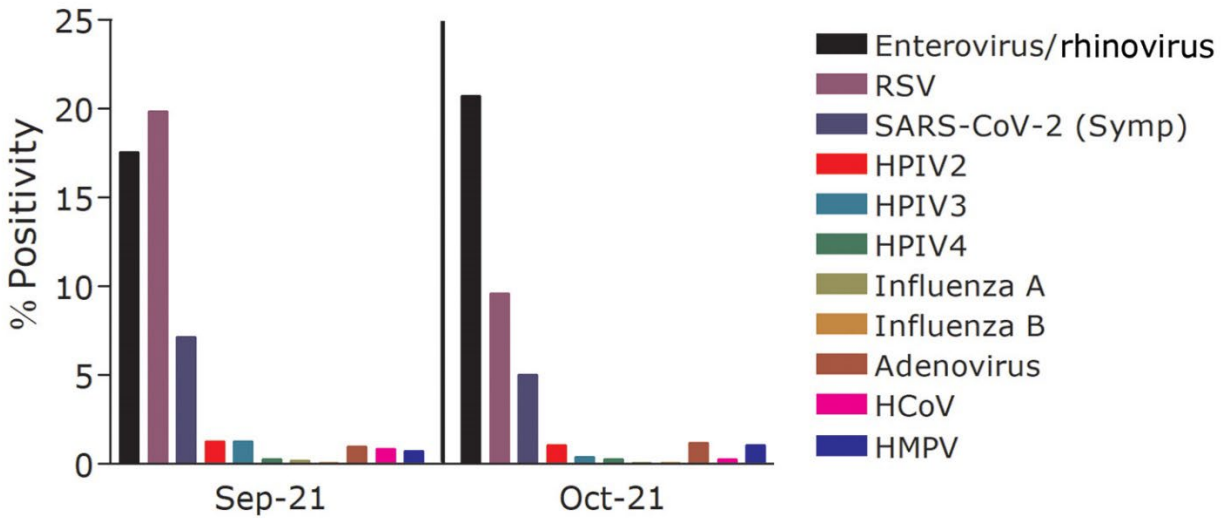
Genome ID	Enterovirus	Age range, y	Gender	Respiratory/viral Complaint	Admitted	ICU	Supplemental Oxygen
JH-EV-0001/2021	EV-D68	<1	Female	Breathing problem	Yes	Yes	Yes
JH-EV-0002/2021	EV-D68	45–48	Male				
JH-EV-0003/2021	EV-D68	<1	Female		Yes	Yes	Yes
JH-EV-0004/2021	EV-D68	8–10	Male	Viral illness	Yes	Yes	Yes
JH-EV-0005/2021	EV-D68	23–25	Female				

Genome ID	Enterovirus	Age range, y	Gender	Respiratory/viral Complaint	Admitted	ICU	Supplemental Oxygen
JH-EV-0006/2021	EV-D68	1–3	Male	Rhinorrhea			
JH-EV-0007/2021	EV-D68	<1	Male	Cough, Bronchiolitis, Acute respiratory distress	Yes		Yes
JH-EV-0008/2021	EV-D68	4–6	Female	Fever, loss of appetite, cough, runny nose			
JH-EV-0009/2021	EV-D68	<1	Female	Fever, irritability, congestion, rhinorrhea, sneezing, cough			
JH-EV-0010/2021	EV-D68	1–3	Male	Cough, rash			
JH-EV-0011/2021	EV-D68	1–3	Female	Respiratory distress, viral pneumonia, enterovirus bronchiolitis	Yes	Yes	Yes
JH-EV-0012/2021	EV-D68	1–3	Male	Cough and nasal congestion			
JH-EV-0013/2021	EV-D68	1–3	Male	Fever, productive cough, runny nose			
JH-EV-0014/2021	EV-D68	<1	Female	Nasal congestion, cough			
JH-EV-0015/2021	EV-D68	1–3	Male	Congestion and cough			
JH-EV-0016/2021	EV-D68	1–3	Male	Cough, rhinorrhea, fever, respiratory distress, pneumonia	Yes	Yes	Yes
JH-EV-0017/2021	EV-D68	1–3	Female	Nasal congestion and fever			
JH-EV-0018/2021	CV-A6	32–35	Male	Rash			

\*ICU, intensive care unit.

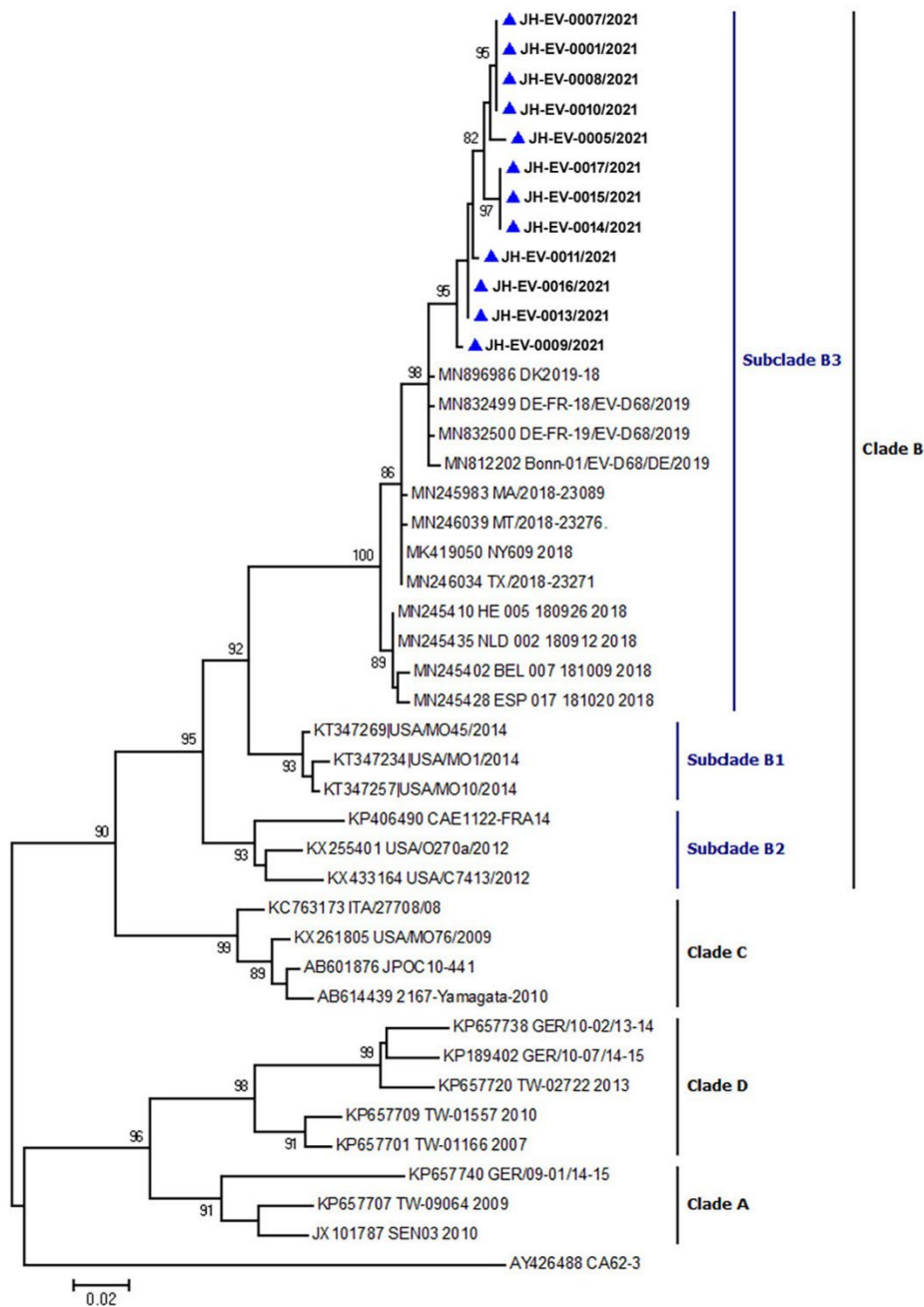


**Appendix Figure 1.** Encounters of influenza-like illness (ILI), COVID-19 encounters, and admissions since the beginning of the COVID-19 pandemic at Johns Hopkins Hospital system.



**Appendix Figure 2.** Positivity of respiratory viral targets at Johns Hopkins Medical Microbiology laboratory in September and October 2021. HPIV (human parainfluenza virus), RSV (respiratory syncytial virus), HMPV (human metapneumovirus), SARS-CoV-2 (symp) indicates symptomatic testing positivity.





**Appendix Figure 3.** Phylogenetic relationships of EV-D68 strains identified from the Johns Hopkins Medical Microbiology laboratory between September and October 2021 (marked with blue triangles). The phylogenetic tree constructed on complete 5' half of the genome of EV-D68 strains was generated using the Maximum Likelihood method based on the using the Tamura 3 parameter method in MEGA7. The phylogenetic tree is rooted by the oldest EV-D68 sequence in GenBank, the Fermon strain. We performed 1,000 bootstrap replicates to determine the consensus tree; support for nodes present in >70% of the trees are annotated.