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Research Article

# Detection and phylogenetic analysis of new Iranian isolates of *Cucumber mosaic virus* on *Achillea* species

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**Abstract:** Cucumber mosaic virus (CMV) is one of the most important viruses that globally cause disease outbreaks in horticultural, vegetable, and agronomic crops. To investigate the incidence of viruses infecting *Achillea* species (yarrows), 350 samples with mosaic, yellowing, and deformation symptoms were collected from different Markazi province locations during the spring seasons of 2017, 2018. CMV infection was detected by using DAS-ELISA. Five samples were selected for phylogenetic studies based on sampling areas and yarrow's species. A 657 bp fragment of coat protein was amplified using specific primers of CMVCPr and CMVCPf in RT-PCR, cloned and sequenced. Obtained sequences of this study were compared with the retrieved sequences from GenBank. The identities of the CP at the nucleotide and amino acid levels were 95% and 100%, respectively, and are close to other Iranian isolates of CMV. Constructed phylogenetic tree showed that five Iranian isolates from yarrows were placed in the subgroup IB. This finding is the first genetic analysis of CMV from yarrow in Iran.

**Keywords:** Coat protein, Phylogenetic tree, Subgroup IB, Yarrow

## Introduction

*Achillea* species is one of the most important genera of the family Asteraceae (Compositae) (Kiumarsi *et al*., 2009). Research shows that the various therapeutic and pharmacological effects of *Achillea* species are due to the phytochemicals' diversity and complexity (Trumbeckaite *et al*., 2011).

*Cucumber mosaic virus* (CMV) is the type virus of the genus *Cucumovirus* in the family *Bromoviridae*. CMV can cause significant economic losses in many vegetable and horticultural crops (Escriu *et al*., 2003). Genetic variation among CMV isolates is very high, and isolates are classified into two subgroups I and II according to serological relationships, nucleic acid hybridization assays, peptide mapping of the coat protein, and sequence similarity of the genomic RNAs. Further analysis of the CP gene and the 5' non-translated region (NTR) of RNA 3 has led to further division of subgroup I into IA and IB. The subgroup I shared 91 to 99%, and subgroup II isolates shared 76 to 84% sequence identity. In total, the isolates belonging to subgroup I have a much higher distribution than subgroup II, so that in some cases, 80% of all detected isolates belonged to subgroup I, and also the frequency of subgroup IA was found to be higher than that of IB (Palukaitis *et al*., 2003; Gallitelli, 2000). In Iran, CMV is an

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economically significant and widely spread plant virus and has been reported from many different host plants such as banana, bean, turnip, peanut, pumpkin, tomato, mint, hollyhock, gladiolus, lily, olive, eggplant, soybean, pea and Bougainvillea (Ghotbi and Bananej, 2005; Nematollahi *et al*., 2012; Forghani *et al*., 2014; Farzadfar *et al*., 2014; Hosseinzadeh *et al*., 2012; Shahmohammadi *et al*., 2015). There are limited molecular studies of Iranian CMV isolates. Rasoulpour and Izadpanah (2008) investigated two isolates of this virus in Isfahan and Fars provinces, as well as some isolates from north and northwest of Iran (Bashir *et al*., 2006 and 2008; Nematollahi *et al*., 2012), Khorasan Razavi province (Golnaz *et al*., 2009) and CMV, isolates from a tomato from different regions of the country are molecularly classified (Arafati *et al*., 2013). CMV isolates obtained from different world areas are different in biological, serological, and physicochemical properties (Phan *et al*., 2014). Recently, research has been conducted to identify the host range of CMV among medicinal plants in France. This research showed that Rosemary isolates were different from all isolates based on biological and molecular characteristics. Unlike other isolates, these isolates have a limited host range that does not belong to any IA, IB, and II phylogenetic subgroups (Tepfer *et al*., 2016). Determining the subgroups of CMV isolates is vital in the epidemiological study of the virus. Detection of CMV isolates and studying their genetic diversity is a practical step in controlling viral diseases primarily through genetic engineering (Yu *et al*., 2005).

In the present study, the occurrence of CMV on *Achillea* species is reported for the first time from Iran. Molecular analysis of these CMV isolates' coat protein genes has been done to study the isolates' genetic diversity.

## Materials and Methods

### Plant materials and virus

During the spring seasons of 2017-2018, surveys were conducted in the main *Achillea* species growing areas in Markazi province to evaluate the CMV infection status (Table 1). The total number of 1050 leaf samples with virus-like symptoms, including chlorotic local lesions, fern leaf/ shoestring, mottle, mosaic, vein necrosis, and yellows, were collected. (Table 1)

**Table 1** Infection percentage of *Achillea* species to CMV in Markazi province in 2017-2018.

| Year | Entries | Host species | | | |
|---|---|---|---|---|---|
| | | AN | AT | AB | AW |
| 2017 | No. of collected samples | 94.0 | 315.0 | 42.0 | 66.0 |
| | No. of infected samples | 8.0 | 190.0 | 7.0 | 10.0 |
| | Infection (%) | 0.8 | 60.3 | 16.6 | 15.2 |
| 2018 | No. of collected samples | 65.0 | 250.0 | 53.0 | 165.0 |
| | No. of infected samples | 40.0 | 77.0 | 21.0 | 61.0 |
| | Infection (%) | 61.5 | 30.8 | 39.6 | 36.9 |
| | Total infection (%) | 30.2 | 47.3 | 29.5 | 30.7 |

AN*: Achillea nobolis* L subsp.neilrechii (Kerner) Formanek, AT: *A. tenuifolia* Lam, AB: *A. bibersteinii* Afan, AW: *A. wilhelmsii* C. Koch.

### Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was carried out according to the method of Sasaya and Yamamoto (1995). For this assay, the sap from the plant sample was extracted in extraction buffer (1:10 ratio) [% 2 PVP2400 in PBST buffer]. Plant samples were tested using rabbit anti-CMV polyclonal antibody (1:1000) (Shiraz University). Goat anti-rabbit IgG alkaline phosphatase conjugate (1:3000) (Promega, USA) was used as the secondary antibody. The wells were developed in 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP) (Sigma-Aldrich), and the absorbance was measured using an ELISA reader Anthos 2,020 at 405 nm. The mean of three samples was considered. If the samples' optical density (OD) were higher than R (R = X + 3 SD, X being the mean of the negative control, SD is the standard deviation of the negative control), the sample was considered as positive.

### Viral cDNA synthesis and PCR

According to the manufacturer's instructions, total RNAs were extracted from healthy and CMV-

infected plants using the column RNA isolation kit (DENAzist, Tehran, Iran). The reverse transcription was performed using the hyperScript RT premix kit described by the manufacturer (GeneAll, South Korea). The PCR reaction was performed in a total volume of 20 µl containing 1 µl of 10 µM each of forward and reverse primers, CMVCPr: 5'-GCTTCTCCGCGAG-3', CMVCPf: 5′-GCCGTAAGCTGGATGGAC-3′. The PCR reaction was carried out in a SensQuest thermocycler (Germany) as follows: initial denaturing at 94° C for 2 min, 35 cycles of 94 ° C for 40 s, annealing temperature at 50° C for 30 s, extension at 72° C for 90 s, then followed by one cycle at 72° C for five min as a final extension. Amplified fragments were resolved by electrophoresis on 1% (w ⁄ v) agarose gel and stained with ethidium bromide at a final concentration of 0.5 µg/ml. The PCR products were purified by QIAquick PCR purification kit (Qiagen).

**Cloning, sequencing, and phylogenetic analyses**

About 20 ng of each PCR product was ligated into 50 ng of the T/A cloning plasmid, pTZ57R/T (Fermentas, Lithuania), in a 10 µl reaction volume. Cloning was performed using the InsT/Aclone PCR product cloning kit described by the manufacturer (Fermentase). After selecting desired colonies on IPTG/ X-Gal plate, they were subjected to colony PCR using M13 primers. Then, plasmids from two clones of each sample (isolate) were extracted by plasmid DNA isolation kit and sequenced using an ABI 3730XL sequencer (Macrogene, South Korea).

Our isolates' sequences were compared with the type strains of species from the *Cucumovirus* using the BLAST homology search program. With the GenBank sequences used for phylogenetic analyses, newly obtained DNA sequences were aligned by Clustal X2 (http://www.clustal.org/) using the default parameters. Phylogenetic trees were constructed using MEGA7 software to show the phylogenetic relationships and determine the selective pressure on CMV CP.

## Results

**Sampling and enzyme-linked immunosorbent assay**

Results showed that the CMV was present about 3.1% in *Achillea nobilis* subsp. *neilrechii*, 47.4% in *Achillea tenuifolia* Lam, 29.4% in *Achillea bibersteinii* Afan and 30.7% in *Achillea wilhelmsii* C. Koch in different regions of Markazi province. The results also showed that the highest rate of virus distribution (47.4%) during 2017 and 2018 occurred in the *Achillea nobilis* subsp. *neilrechii* (Table 1).

According to the results, the abundance (incidence**)** of CMV infection in 2017 was higher than in 2018 (although the infection rate in *Achillea tenuifolia* was higher in 2018). Most infection was observed in Shazand (42%) and Kamijan (39%) areas. On the other hand, the lowest virus distribution (Zero%) was recorded in the Delijan and Saveh (Table 2).

**Table 2** Percentage of CMV distribution in different cities of Markazi province in 2017-2018.

| Name of city | Infection rate | |
|---|---|---|
| | 2017 | 2018 |
| Ashtian | 0.33 | 0.30 |
| Arak | 0.29 | 0.24 |
| Tafresh | 0.35 | 0.33 |
| Khomein | 0.09 | 0.07 |
| Khondab | 0.25 | 0.23 |
| Delijan | 0.03 | 0 |
| Saveh | 0.04 | 0 |
| Shazand | 0.42 | 0.39 |
| Komijan | 0.39 | 0.41 |
| Mamuniyeh | 0.08 | 0.04 |
| Mahallat | 0.37 | 0.32 |
| Farmahin | 0.18 | 0.14 |

**Molecular detection and analysis of viral sequences**

During the analysis of the RT-PCR products, the expected size amplicons of ~650 bp were obtained by CMV-specific primers from naturally infected samples. The amplified segments of five

CMV isolates were sequenced, and the obtained data were analyzed to eliminate any ambiguity. The sequence data of consensus 650 bp sequence from each isolate was deposited in GenBank under Acc. Nos. MK488059 to MK488063 and designated as CMV-Ach1 to CMV-Ach5 isolates.

BLASTn analysis of CMV-Ach1 to CMV-Ach5 isolates (MK488059 to MK488063) revealed the highest nucleotide sequence identities with the CMV isolate JK (EF153737). The phylogenetic relationship derived from Neighbor-joining analysis of the coat protein sequences of the five strains from the present study with 55 sequences of CMV subgroups is shown in Fig. 1. The phylogenetic tree showed that strains CMV-Ach1 to CMV-Ach5 (isolated from Arak, Shazand, Khendab, Kamijan, and Fermin specimens, respectively) clustered with members of the IB subgroup with high posterior probability value (Fig. 1).

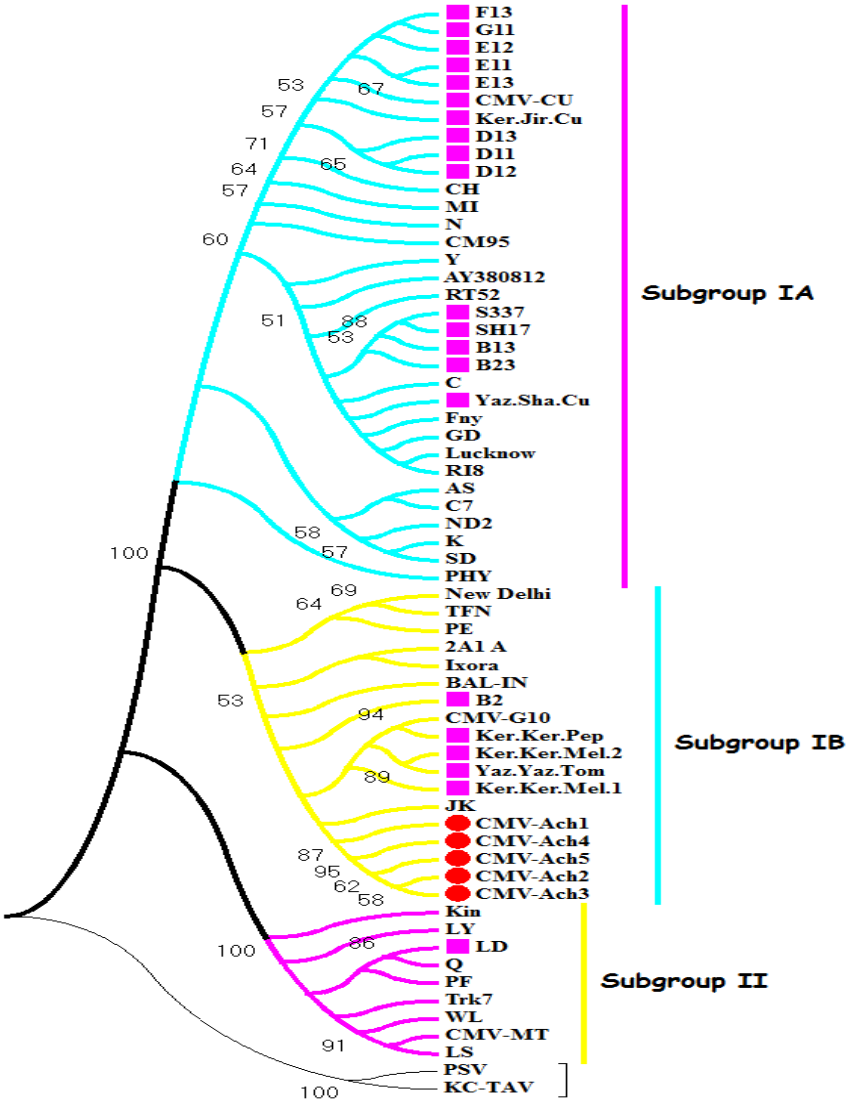


**Figure 1** Phylogenetic analysis of CMV, including new isolates from Iran based on genes encoding the coat protein. Bootstrap values are shown above the branches. Red circles show new Iranian isolates. Strains of *Peanut stunt virus* (PSV) and *Tomato aspermy virus* (TAV) were designated the outgroup. Characteristics of the other isolates in the phylogenetic tree are listed in Table 3.

Estimation of Tajima's D factor and dN / dS ratio (dN: 0.019, dS: 0.17) using MEGA7 software showed that dN / dS ratio in coat protein was less than 1 (0.112), and the Tajima's D test was also negative (-2.2), which proves adverse selection in this area.

**Table 3** Accession numbers, abbreviations, and geographical origins of CMV strains/isolates used for the phylogenetic analyses.

| Accession No. | Isolate | Origin | Subgroup |
|---|---|---|---|
| D10538 | FNY | USA | IA |
| AJ810258 | RT52 | USA | IA |
| HU916354 | GB | Austria | IA |
| AM183119 | RI8 | Spain | IA |
| AY871068 | SH17 | Iran | IA |
| AB188236 | CM95 | Japan | IA |
| AB188230 | Mi | Japan | IA |
| JX112018 | Ker.Jir.Cu | Iran | IA |
| AY380812.1 | - | Brazil | IA |
| JX112022 | Yaz.Sha.Cu | Iran | IA |
| AB261174.1 | CH | Japan | IA |
| D00462 | C | USA | IA |
| D12499 | Y | Japan | IA |
| D28486 | N | Japan | IA |
| DQ295914.1 | Lucknow | India | IA |
| EF620777.1 | CMV-Cu | Iran | IA |
| AY871069.1 | S337 | Iran | IA |
| AY871070.1 | B13 | Iran | IA |
| AY871071.1 | B23 | Iran | IA |
| DQ002876.1 | DI1 | Iran | IA |
| DQ002877.1 | DI2 | Iran | IA |
| DQ002879 | DI3 | Iran | IA |
| DQ002885.1 | G11 | Iran | IA |
| AY871068.1 | SH17 | Iran | IA |
| DQ002880.1 | E11 | Iran | IA |
| DQ002881.1 | E12 | Iran | IA |
| DQ002882.1 | E13 | Iran | IA |
| DQ002883.1 | F13 | Iran | IA |
| Y16926 | TFN | Italy | IB |
| GU111229 | New Delhi | India | IB |
| DQ412732 | Phy | China | IB |
| EU414786 | ND2 | China | IB |
| AB008777 | SD | China | IB |
| JX112023 | Yaz.Yaz.Tom | Iran | IB |
| JX112019 | Ker.Ker.Mel.1 | Iran | IB |
| JX112021 | Ker.Ker.Pep | Iran | IB |
| JX112020 | Ker.Ker.Mel.2 | Iran | IB |
| AF268597.1 | PE | China | IB |
| AF013291.1 | AS | South Korea | IB |
| D42079.1 | C7-2 | Japan | IB |
| AF127977.1 | K | Japan | IB |
| EF153737.1 | J&K | India | IB |
| AB069971.1 | B2 | India | IB |
| AJ271416.1 | 2A1-A | USA | IB |
| U20219.1 | Ixora | USA | IB |
| Y16926.1 | Tfn | Italy | IB |
| MK488059 | CMV-Ach1 | Iran | IB |
| MK488060 | CMV-Ach2 | Iran | IB |
| MK488061 | CMV-Ach3 | Iran | IB |
| MK488062 | CMV-Ach4 | Iran | IB |
| MK488063 | CMV-Ach5 | Iran | IB |
| AY541691 | CMY-G10 | Egypt | II |
| JF279609 | Bal-In | India | II |
| AB189917.1 | CMV-MT | Japan | II |
| AB368501.1 | PF | Japan | II |
| LS AF127976 | LS | USA | II |
| AF198103 | LY | USA | II |
| D00463 | WL | USA | II |
| L15336 | Trk7 | Hungary | II |
| M21464 | Q | Australia | II |
| Z12818 | Kin | UK | II |
| EF050074.1 | LD | Iran | II |
| AJ237849.2 | TAV | - | - |
| NC-002040 | PSV | - | - |

## Discussion

In this study, CMV was observed for the first time on *Achillea* species in Iran, and the average infection rate of different *Achillea* species with CMV was determined 39.4% in Markazi

province (Table 1). This finding is the first report of molecular detection of CMV on *Achillea* species in the world. To our knowledge, in previous studies, the identification of medicinal plant viruses has been based on serological tests. BLASTn analysis of these isolates (CMV-Ach1 to CMV-Ach5) revealed 84–86% nucleotide sequence identities with various CMV isolates. Adams *et al*. (2005) determined the extent of species and genera in the *Bromoviridae* family by phylogenetic studies. Accordingly, isolates with more than 76% identity in the nucleotide sequence and more than 82% identity in the amino acid sequence of the coding protein-encoding gene (CP) are classified as CMV species. Studies have shown that the CMV coat protein gene isolated from different hosts such as other cucumoviruses is highly conserved (Roossinck, 2001). Therefore, the phylogenetic analysis of CMV isolates in this study was performed based on the coat protein's coding region. The phylogenetic evaluation confirmed that the five strains reported in this study from *Achillea* species were classified in subgroup IB. Nematollahi *et al*. (2012) have also shown that the Iranian CMV strains isolated from different host plants belong to subgroup IB. In the phylogenetic tree based on the CP gene's nucleotide sequences (Fig. 1), the newly sequenced isolates of the present research were placed in the IB cluster sharing 92−96% nt (and 94− 100% aa) identities with other Iranian isolates of subgroup IB. These isolates have 96% nucleotide identity to Iranian pepper isolates, 94% nucleotide identity to Iranian melon isolates, and 92% nucleotide identity to Iranian tomato isolates. The CP nucleotide and amino acid identities of isolates characterized in this study and that of other IB isolates were both 91−99%. It also appeared that the Iranian isolates, particularly IB isolates, had the highest similarities to the Asian isolates of CMV based on the CP sequences. Most IB isolates are distributed in Asia, whereas IA and II are found worldwide (Palukaitis and Zaitlin, 1997; Roossinck, 2002). Recombination is more likely to occur in RNA viruses than other viruses. Recombination involves the exchange of genetic material between two related viruses during the coinfection of a host cell. The development of viruses with new antigenic determinants by either type of recombination may allow viruses to infect and cause disease in previously immune hosts. This phenomenon is unavoidable in the evolutionary process of viruses (Worobey and Holmes, 1999). The distribution of aphids as an insect vector for CMV has become widespread in most regions of Iran. Strong winds in the growing season also cause widespread dispersal of vector aphids from infected farms to healthy farms, which increases the spread of the virus, and therefore the role of vectors in the transmission of CMV virus is detrimental (Adams *et al*., 2005). The wide distribution of the CMV has provided for its evolution and ability to infect more hosts of different plant families. As shown in the results, the prevalence of CMV infection in 2017 was higher than in 2018. The highest prevalence of CMV was observed in Shazand, and the lowest prevalence was recorded in Delijan and Saveh. Environmental factors such as minimum temperature, relative humidity, and leaf moisture play an essential role in CMV occurrence. Studies have shown that the prevalence of CMV at temperatures between 25 and 30 °C under greenhouse conditions causes severe necrosis. Planting date also dramatically influences the frequency and acquisition of this virus (Bashir *et al*., 2008). Shazand's weather is more moderate than Saveh's weather, while Saveh is a tropical city. Therefore, because yarrow is cultivated in mild months of the year, its prevalence has been lower in warm regions. According to the meteorological data, the planting and growth period of *Achillea* species in Shazand falls within the favorable temperature range of CMV prevalence. The low prevalence of the virus in Khomein and Delijan may be due to climate, the population of *Myzus persicae* (Sulzer) and *Aphis gossypii* Glover, the prevalence of weeds, and neighboring fields of cucurbitaceous plants and vegetables.

Detection and distribution of plant viruses in different hosts can select the appropriate strategy for their control. One of the most

important ways to control plant viruses is to use resistant cultivars. The genetic diversity of Iranian virus isolates needs to be studied before the relative resistance of cultivars or transgenic plants is considered. Determining genetic diversity in a viral group and understanding the mechanisms and factors affecting this diversity and variability is vital for determining and applying resistance genes.

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**Declaration of conflicting interests**

The authors state that there is no conflict of interest.

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# ردیابی و آنالیز فیلوژنتیکی جدایه جدید ویروس موزائیک خیار از گیاه بومادران

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**چکیـــده:** ویـروس موزائیـک خیـار Cucumber mosaic virus (CMV) از مهم‌تـرین ویروس‌هـای آلوده‌کننده محصولات مختلف زراعی، باغی و سبزیجات در سراسـر جهـان محسوب می‌شود. بـه‌منظـور بررسی وضعیت آلودگی ویروسی گیاه دارویی بومـادران تعـداد ۳۵۰ نمونـه در بهـار سـال‌هـای ۱۳۹۶ و ۱۳۹۷ از شهرستان‌های مختلف استان مرکزی جمع‌آوری گردید. آلودگی به CMV با استفاده از آزمـون ساندویچ دوطرفه الیزا (DAS-ELISA) مورد بررسی قرار گرفت. براساس مناطق نمونه‌برداری و گونه گیاه بومادران، پنج نمونه جهت مطالعات فیلوژنتیکی انتخاب شد. با استفاده از آزمون RT-PCR و آغازگرهای اختصاصی CMVCPr و CMVCPf قطعه‌ای به اندازه ۶۵۷ نوکلئوتید، مربوط به چارچوب ژنـی پـروتئین پوششی ویروس تکثیر، همسانه‌سازی و تعیین ترادف گردید. مقایسه توالی‌ها نشان داد کـه جدایـه‌هـای ایرانی CMV جدا شده از بومادران در سطح نوکلئوتیدی و آمینواسیدی (۱۰۰-۹۵ درصد) با یـک‌دیگـر برابری قابل‌توجهی دارند و به دیگر جدایه‌هـای ایرانـی CMV نزدیـک هسـتند. در درخـت فیلـوژنتیکی ترسیم شده پنج جدایه ایرانی از بومادران در زیرگروه IB واقع گردیدند. ایـن اولـین گـزارش از بررسـی فیلوژنتیکی چندجدایه CMV جدا شده از گیاه بومادران در ایران است.

**واژگان کلیدی:** پروتئین پوششی، درخت فیلوژنی، زیرگروه IB، بومادران