

## PHYLOGENETIC RELATIONSHIPS WITHIN THE COSMOPOLITAN FAMILY RHAMNACEAE USING *atpB* GENE PROMOTER

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### Abstract

Eighteen species of *Rhamnaceae* were collected from different geographical regions of Pakistan to resolve its controversial phylogenetic position using morphological and molecular analysis. The phylogenetic tree based on 71 different micro and macro-morphological characters using Paleontological and statistical software (PAST) with Dice's coefficient showed an overall genetic diversity of 32%. Further, in each species the *atpB* gene promoter was amplified, purified, sequenced and the dendrogram was constructed using Molecular evolutionary genetic analysis (MEGA7) tool which divided the sequences into two main clades showing a narrow genetic diversity of 0.05% with well supported bootstrap's values (95-100%). Pairwise's distance ranged from 0.12 to 0.73 with a mean value of 0.396. The phylogenetic study confirmed the work done by earlier phylogeneticist with additional reports of some new species, *Berchemia pakistanica*, *Berchemia edgeworthii*, *Berchemia floribunda*, *Helinus lanceolatus* and *Rhamnella gilgitica* which are indigenous to Pakistan. The analysis of *Cis*-regulatory elements and its mapping via Plant *cis*-acting regulatory DNA element (PLACE) and Domain graph (DOG) revealed numerous elements including 50 common and 28 unique, showing variation in copy numbers and locations. It was observed that *Berchemia pakistanica* and *Berchemia edgeworthii* have the unique features possessing diverse *cis*-regulatory elements with diverse functions.

**Key words:** *Rhamnaceae*, Phylogenetic analysis, *atpB* promoter, Genetic diversity, Morphological characters, *Cis*-acting elements.

### Introduction

The plant family *Rhamnaceae* Juss. comprises of 11 tribes, 60 genera and 900 species, cosmopolitan in distribution mostly found in warm temperate regions, with an epicenter of its diversity in Southern hemisphere (Burge *et al.*, 2011, Jehangir *et al.*, 2018). The largest number of species (250 out of 950), represented by ~ 21 different genera are found in Australia. Pakistan is hosting six genera and 21 species, mostly found in Azad Jammu Kashmir, Balakot, Gilgit, Galyat, Abbottabad, Peshawar, Swat, Upper Dir, Waziristan, Kurram Agency, Rawalpindi and Salt range having most of the species still unexplored (Qaiser & Nazimuddin, 1981). The family is famous for its significant importance in pharmaceutical industry acting as a source of various biological compounds like carbohydrates, vitamins, starch, secondary metabolites with strong therapeutic potentials against cancer, hepatitis (HBV, HCV), diabetes, asthma, stomach ulcer and leishmaniasis (Parmar *et al.*, 2012; Iqbal *et al.*, 2017; Iqbal *et al.*, 2018; Abbasi *et al.*, 2018). The first phylogenetic relationship of *Rhamnaceae* using *trnL-F* and *rbcL* plastid DNA sequences demonstrated that *Rhamnaceae* is a monophyletic family closely related to *Dirachmaceae* and *Barbeyaceae* (Richardson *et al.*, 2000a). Recent taxonomic revisions considered that *Rhamnaceae* is a monophyletic family and divided it into three major groups: rhamnoid (300 species), ziziphoid (600 species) and ampelozizyphoid (10 species) (Hauenschild *et al.*, 2016). Previously, different studies have significantly emphasized the systematics and taxonomy of *Rhamnaceae* by reporting some new insights about its classification (Niamat *et al.*, 2012). In past, chloroplast genome was used to study the phylogenetic relationship of *Ziziphus* (Mill.), *Helinus*, E. Mey. ex Endl. *Sageretia*, Brongn. *Rhamnella*, Miq. *Rhamnus*, L. and *Berchemia*, Neck. ex DC. (Richardson *et*

*al.*, 2000b), *Ceanothus* (Burge *et al.*, 2011), *Colletieae* (Aagesen *et al.*, 2005), *Pomadereae* (Kellermann & Udovicic, 2007), *Cryptandra* (Kellermann & Rye, 2008), and *Paliurus* (Islam and Simmons, 2006). The above studies have provided some new insights into its classification, but due to the addition of new species and ecotypes from different geographical regions of the world need further studies.

The discovery of new and advance markers provided an opportunity to study plant phylogeny at a whole new level which includes morphological, biochemical and molecular analysis (Zahra *et al.*, 2016; Channa *et al.*, 2018; Shah *et al.*, 2018; Sohail *et al.*, 2018; Akbar *et al.*, 2019). However molecular markers are more reliable due to their stability, adaptability and less susceptibility to environmental changes, while others are potentially prone to environmental and developmental plasticity. Chloroplast DNA provides an excellent opportunity for studying phylogeny at intra- and inter-species level due to its high genetic potential and conserved nature as compared to nuclear genome (Rasheed *et al.*, 2012). There are several studies wherein the cpDNA markers have been employed to resolve the phylogenetic and taxonomic issues (Shinwari, 1998; Shinwari, 2000; Shinwari, 2002; Mahmood *et al.*, 2010; Zeb *et al.*, 2011; Shinwari *et al.*, 2014; Zahra *et al.*, 2016) The *atpB* gene positioned on the chloroplast DNA codes the beta ( $\beta$ ) subunit of ATP synthase enzyme and was used to elucidate phylogenetic relationship among different taxa due to its relatively slow rate of nucleotide substitution as compared to other genes in chloroplast genome (Magee *et al.*, 2010). According to our knowledge no earlier study has been reported by any research group using *atpB* gene promoter on any single molecular phylogenetic aspect for the members of *Rhamnaceae*. So keeping in view the importance of *Rhamnaceae* and its controversial phylogenetic position,

the present study was designed to find the phylogenetic relationship among its six genera using morphological as well as molecular (*atpβ* gene promoter) data and to investigate functionally important *cis*-regulatory elements present in these promoters.

## Materials and Methods

**Morphological data analysis:** Young fresh and herbarium specimens of eighteen species of *Rhamnaceae* (from six different genera) were collected from different geographical regions of Pakistan and the voucher specimens were deposited in Plant Biochemistry and Molecular Biology Laboratory, Quaid-I-Azam University Islamabad (Table 1). Morphological study of 5 different *Ziziphus* and 3 *Berchemia* species was conducted by studying its different micro- and macro-morphological characters along with information taken from Flora of Pakistan and previous research papers. Detailed information about these characters along with their key is given in (Table 2). The data was arranged in Microsoft Excel Sheet by giving proper code to each character and similarity-based dendrogram was constructed with PAST (Ver. 3.11) (Hammer *et al.*, 2001) using Dice's coefficient, which divided the species into various clades and clusters.

**Molecular data analysis:** For molecular analysis, DNA was extracted from 6 different species of *Ziziphus* and 2 *Berchemia* species using Cetyltrimethylammonium bromide (CTAB) method (Richard, 1997) and PCR was carried out using *atpβ* gene promoter primers in a total volume of 25 μL containing 16.2 μL of nano pure water, 2.5 μL of 10X PCR buffer, 1.5 μL of 2 mM dNTPs, 1.5 μL of 25 mM MgCl<sub>2</sub>, 1 μL of each primer, 0.3 μL of Taq polymerase (5 U) and 1 μL of template DNA using PCR MultiGene Thermal Cycler (Labnet). Annealing temperature was optimized for each primer ranging from 56 to 61°C. The PCR cycling conditions were as follow: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 1 min at 60°C, 1 min at 72 C and final extension of 20 min at 72 °C. The products were purified (GeneJET PCR Purification kit, ThermoScientific), sequenced (Macrogen, South Korea) and the sequences were deposited to GenBank, National Center for Biotechnology Information (NCBI)

(Accession numbers mentioned in figure 2) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic analysis was conducted using MEGA7 (Tamura *et al.*, 2013a) via neighbor-joining tree-making method with a bootstrap's (BS) value of 100. Similarly, Tajima's neutrality test of selection was performed to find nucleotide diversity ( $\pi$ ) with the same software.

**Regulatory elements analysis:** *Cis*-regulatory elements in *atpβ* gene promoter of each species were found on both strands (+ and -) using PLACE (<http://www.dna.affrc.go.jp/PLACE>) online server and their corresponding positions were mapped with the help of DOG2 (Domain Graph Ver. 2.0) software (van Dijk and Bonvin, 2009), searched for the most common and unique elements along with their putative roles in gene regulation.

## Results

**Morphological analysis:** Phylogenetic analysis based on 71 different micro- and macro-morphological characters given in table 2 divided the species into two main clades (Fig. 1). The species revealed an overall homology of 68% with morphological diversity of 32%. Clade 1 contained a single species of *Helinus lanceolatus* revealing a high divergence pattern while clade 2 contained the rest of 17 species showing an overall homology of 72%. Clade 2 was again divided into cluster 1 comprised of 15 species and cluster 2 which consisted of 2 species (*Sageretia thea* and *Sageretia thea* var. *brandrethiana*). In cluster 1 *Rhamnella gilgitica* has made an out-group with the rest of species. In genus *Rhamnus*, *Rhamnus purpurea* has made an out group with the remaining species, indicating possible ancestral role of the genus *Rhamnus*. Generally, all the species falls into their respective genera. Similarly, the genus *Berchemia* (3 species) and *Ziziphus* (5 species) revealed the same pattern by showing 77% and 84% similarity at morphological level. Overall, greater similarity was observed among *Rhamnus* and *Berchemia*'s species which form close relationship with *Ziziphus* species. However, the distant relationship was observed for genus *Helinus* and *Sageretia* (Fig. 1).

**Table 1. List of selected species along with their voucher numbers, geographic coordinates and area of collection.**

S. No.	Species name	Voucher number	Location	Geographic coordinates
1.	<i>Ziziphus spina-christi</i> (L.) Desf	HPBMBL-16-064	Islamabad	33° 42' 0" North, 73° 10' 0" East
2.	<i>Ziziphus jujuba</i> Mill.	HPBMBL-16-065	Mardan	34° 11' 54" North, 72° 2' 45" East
3.	<i>Ziziphus nummularia</i> (Burm.f.) Wight & Arn.	HPBMBL-16-066	Islamabad	33° 42' 0" North, 73° 10' 0" East
4.	<i>Ziziphus rugosa</i> Lam.	HPBMBL-16-067	Hyderabad	25° 26' 0" North, 68° 32' 0" East
5.	<i>Ziziphus mauritiana</i> Lam.	HPBMBL-16-068	Peshawar	34° 0' 28" North, 71° 34' 24" East
6.	<i>Ziziphus mauritiana</i> var. <i>spontanea</i> (Edgew.) R.R. Stewart ex Qaiser & Nazim	HPBMBL-16-069	Peshawar	34° 0' 28" North, 71° 34' 24" East
7.	<i>Berchemia edgworthii</i> Lawson	HPBMBL-16-070	Malakand	34° 37' 0" North, 71° 58' 17" East
8.	<i>Berchemia pakistanica</i> Browicz	HPBMBL-16-071	Waziristan	32° 59' 12" North, 70° 16' 24" East
9.	<i>Rhamnus triquetra</i> (Wall.) Brandis	HPBMBL-16-072	Upper Dir	35° 12' 21" North, 71° 52' 32" East
10.	<i>Rhamnus purpurea</i> Edgew	HPBMBL-16-073	Kashmir	33° 8' 35" North, 73° 44' 51" East.
11.	<i>Rhamnus virgata</i> Roxb.	HPBMBL-16-074	Kashmir	33° 8' 35" North, 73° 44' 51" East.
12.	<i>Rhamnus pentapomica</i> R. Parker	HPBMBL-16-075	Balakot	34° 33' 0" North, 73° 21' 0" East
13.	<i>Rhamnus prostrate</i> Jacq.	HPBMBL-16-076	Kashmir	33° 8' 35" North, 73° 44' 51" East.
14.	<i>Rhamnus persica</i> P. Lawson	HPBMBL-16-077	Quetta	30° 12' 0" North, 67° 0' 0" East
15.	<i>Sageretia thea</i> (Osbeck) M.C. Johnst.	HPBMBL-16-078	Chitral	35° 50' 32" North, 71° 46' 55" East
16.	<i>Sageretia thea</i> var. <i>brandrethiana</i> (Aitch.) Qaiser & Nazim	HPBMBL-16-079	Islamabad	33.7444° North, 73.0417° East
17.	<i>Helinus lanceolatus</i> Brandis	HPBMBL-16-080	Lehtar	33° 36' 0" North, 73° 4' 0" East
18.	<i>Rhamnella gilgitica</i> Mansf. & Melch.	HPBMBL-16-081	Gilgit	35° 55' 0" North, 74° 18' 0" East

**Table 2. List of different micro- and macro-morphological characters used in morphological analysis along with characters' key. S/N: serial number, Char. Studied: character studied. Different numbers represent the arbitrary scale used for data scoring in dendrogram construction.**

S. No.	Char. studied	Key
1.	Plant habit	Tree (0), Shrub (1), Climber (2), Herb (3)
2.	Plant nature	Evergreen (0), Deciduous (1)
3.	Stem Succulence	Succulent (0), Non succulent (1)
4.	Stem nature	Herbaceous (0), Woody (1), Herbaceous + woody (2)
5.	Stem indumentums	Present (0), Absent (1)
6.	Xerophytic nature	Absent (0), Present (1)
7.	Spines	Present (0), Absent (1)
8.	Type of leaf	Simple (0), Compound (1)
9.	Leaf orientation	Palmate (0), Pinnately nerved (1),
10.	Leaf with lateral nerves	3-4 pairs (0), 3-5 pairs (1), 6-8 pairs (2), 7-8 pairs (3), 8-11 pairs (4), 6-10 pairs (5), 4-5 Pairs (6), 2-5; Pairs (7)
11.	Leaf arrangement	Alternate (0) ,Opposite to Sub-opposite (1), Opposite (2)
12.	Leaf disc	Present (0), Absent (1)
13.	Leaf disc nature	Fleshy (0), Nectariferous (1) Disc remnants (2)
14.	Leaf disc appearance	Glabrous (0), Velutinous (1)
15.	Leaf Petiole	Petiolate (0), Sessile (1)
16.	Petiole length	1-1.5 cm (0), 1-2 mm (1), 2-3 mm (2), 2-4 mm (3), 1-3 mm (4), 3-5 mm (5), 5-10 mm (6), 8-15 mm (7), 5-6 mm (8), 4-8 mm (9), 3-12 mm (10), 0.8-1.5 cm (11) 8-20 mm (12), 1-2 cm (13), 0.5-2 cm (14), 3-4 mm (15), 3-5 mm (16),
17.	Leaf shape	Cuneate (0), Narrow oblong (1), Ovate (3), Elliptic lanceolate (4), Elliptic (5), Oblong-ovate (6), Oblanceote (7), Obvate oblong (8) Sub-orbicular (9)
18.	Leaf margin shape	Ovate (0), Ovate-orbicular (1), Ovate-elliptic (2), Oblong-ovate (3), Ovate-lanceolate (4), Elliptic (5), Obovate (6), Elliptic-lanceolate (7), Sub-ovate (8)
19.	Leaf stipules	Stipulate (0), Exstipulate (1)
20.	Stipule size	1.5-2 mm (0), 1-2 mm (1), 1-3 cm (2), 2 cm (3), 3 cm (4), 5-7 mm (5), 1 cm (6), 3-6 mm (7), 5 mm (8), 2-3 mm (9)
21.	Stipules shape	Traingular (0), Awl shaped (1), Triangular (2), Lanceolate (3), Long and straight (4)
22.	Stipules nature	Caduceus (0), persistent (1),
23.	Flower sex	Unisexual (0), Bisexual (1)
24.	Flower symmetry	4-merous (0), 5-merous (1), 6-merous (2), 5-6 (3)
25.	Pedicel length	1-1.5 mm (0), 2-3 mm (1), 2-4 (2), 3-5 (3), 1-2 mm (4), 4-5 (5), 6-7 (6), 3-6 (7), 5-8 mm (8), 3-6 mm (9)
26.	Flower diameter	2-3 mm (0), 3-4 mm (1), 4-6 mm (2), 2-4 mm (3), 4-5 mm (4), 2 mm (5), 5 mm (6), 3 mm (7), 12-15 cm (8), 2.5 3 mm (9), 2.5 mm (10)
27.	Flower color	Greenish yellow (0), Yellowish (1), Rarely brightly colored (2)
28.	Flower axis	Sessile (0), Sub-sessile (1), Pedunculate (2) Pedicellate (3)
29.	Floral Bracts	Bracteates (0), Ebracteate (1) , Bracteoles (2)
30.	Sepals	Present (0), Absent (1),
31.	Number of sepals	5 (0), 5-6 (1)
32.	Aestivation of sepals	Valvate (0), Imbricate (1)
33.	Calyx keel	Present (0), Absent (1)
34.	Petals	Present (0), Absent (1)
35.	Number of petals	5 (0), 6 (1), 0 (2), 4 (3)
36.	arrangement of floral parts	Obhaplostemonous (0), Not obhaplostemonous (1)

Table 2. (Cont'd.)

S. No.	Char. studied	Key
37.	Ovary position	
38.	Number of locules per ovary	1 (0), 2 (1), 3 (2), 1-2 (3), 2-4 (4), 2-3 (5)
39.	Number of ovule per locule	1 (0), 2 (1), 3 (2), 2-4 (3), 2-3 (4)
40.	Ovary shape	Globose (0), Glabrous (1), Completely immersed in disk (2)
41.	Fruit shape	Globose (0), Globose-oblong (1), Obovoid (2), Obovate to oblong (3), Ovoid (4), Ovoid to globose (5), Ovate to globose (6)
42.	Fruit type	Drupaceous (0), Capsule (1), fleshy (2), Samaroid (3)
	Mesocarp	Fleshy (0), Without-fleshy (1)
43.	Endocarp	Cartilaginous (0), Woody (stony) or (Corky) (1), Fragile-crustaceous (2), Thickly leathery (3)
44.	Endosperm	Copious (0), Scanty (1), Fleshy (2), Absent (3)
45.	Fruit splitting	dehiscent (0), Indehiscent (1)
46.	Seed	Present (0), Absent (1)
47.	Seed shape	Plano-convex (0), Globose (1), Ovoid (2), Obovoid (3), Heart shape (4) Oblong (5)
48.	Seed color	Red brown (0), Reddish brown (1), Orange black (2), Dark brown (3), Yellow brown (4), Light brown (5) purple red (6), Purple black (7)
49.	Seed albumin	Thin albumin (0), Oily albumin (1), Exalbuminous (2), Stoney albumen (3)
50.	Inflorescence	Cymes (0), Panicle (1), Racemes (2), Spikes (3), Tomentose (4), Fasciculate (5)
51.	Inflorescence Position	Axillary (0), Terminal (1)
52.	Perianth	Present (0), Absent (1)
53.	Involucre	Present (0), Absent (1)
54.	Petiole size	8-20 mm (0), 5-13 mm (1), 3-12 mm (2), 5-10 mm (3), 2-3 mm (4), 1-1.5 cm (5), 2-4 mm (6), 3-5 mm (7), 1-6 mm (8), 1-2 cm (9), 0.5-2 mm (10), 5-6 mm (11), 4-8 mm (12)
55.	Leaf blade	Present (0), Absent (1)
56.	Leaf blade shape	Ovate (0) Elliptic (1) Rarely subrounded (2), Ovate or ovate-elliptic (3), Obovate-lanceolate (4), Obovate-oblong (5), Elliptic to oblong (6), Lanceolate (7)
57.	Leaf blade length	2.5-6 cm (0), 1-2 cm (1) 2-6 cm (2), 8-11(3), 5-12 cm(4),3-7 cm (5), 10-30 mm (6),7-15 cm (7), 2-10 cm (8), 1.8-6 cm (9),5-20 mm (10), 1-4 cm (11), 1.5-2.5 cm (12), 2.5-6 cm (13), 5-15 cm (14)
58.	Leaf blade width	1.5-4.5 cm (0), 0.5-2 cm (1), 1-4 cm (2), 4.5-9.5 cm (3), 2.5-4 cm (4), 2-5 mm (5), 2-20 mm (6), 2-8 cm (7), 1-6 cm (8), 1-4 cm(9), 4-8 cm (10), 5-2 cm (11), 8-12 (12)
59.	Tendrils	Present (0), Absent (1)
60.	calyx keel	Present (0), Absent (1)
61.	Arrangements of floral parts	Obhaplostemonous (0), Not obhaplostemonous (1)
62.	Ovary position	Superior (0), (1), Inferior (2)
63.	Pollen	Presence (0), Absence (1)
64.	Pollen shape	Sub-prolate (0), Oblate-spheroidal (1), Prolate (2)
65.	Pollen class	Tricolporate (0), Non-Tricolporate (1)
66.	Aperture	Long elliptic (0), Acute ends (1),
67.	Tectum	Striate (0), Finally striate (1), Reticulate (2), Finally reticulate (3), Striate reticulate (4), Strio-Rugulate (5), Regulate-striate(6), Regulate7
68.	P/E ratio	Semi- erect to sub-transverse (0), Semi erect (1), Sub-transverse to semi- erect (2), Erect to semi- erect (3)
69.	Ornamentation	Tectum medium to finely reticulate (0), Tectum psilate (1), Tectum striate – regulate (2) Tectum rugulate to rugulate-striate (3), Tectum striate (4)
70.	Exine	Sexine thicker than nexine (0), Sexine thinner than nexine (1), Sexine thicker than nexine or as thick as nexine (2)
71.	Exine thickness (µm)	1.25 µm (0), 3.94 µm (1), 2.6 -2.68 µm (2), 8.36 µm (3), 1.25 µm (4), 2.25 µm (5), 4.75 µm (6), 1.79 µm (7), 2.5 µm (8), 2.63 µm (9)

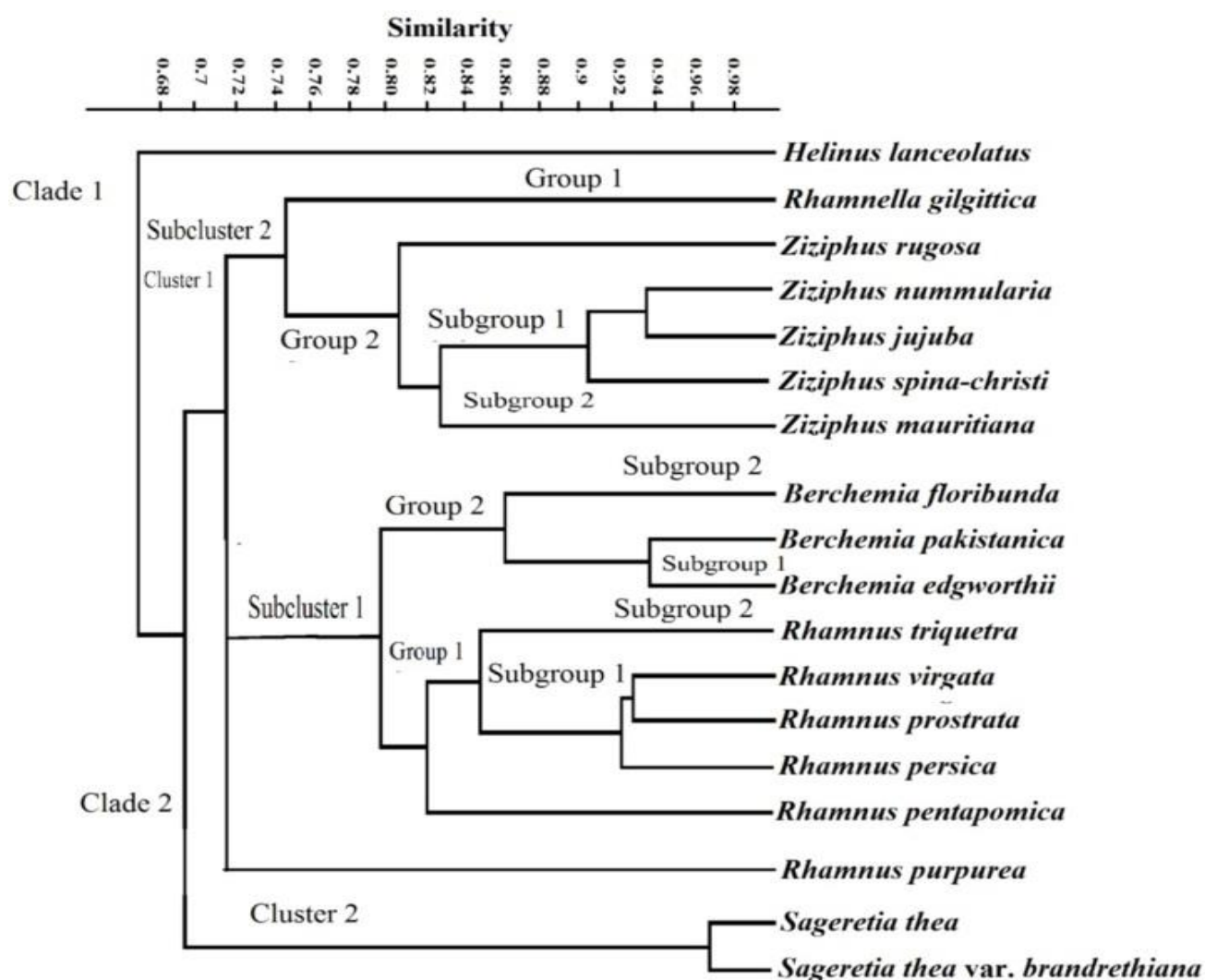


Fig. 1. Phylogenetic analysis of selected species of *Rhamnaceae* using 71 morphological characters. Tree was constructed using Paleontological and Statistical Software (PAST) (ver. 3.11) with Dice's coefficient. Range of similarity is given as percentage. Each node is labeled which divide the species into various clusters, subclusters and groups etc.

**Molecular data analysis:** Molecular analysis based on *atpβ* gene promoter divided the species into clade 1 having 11 species and clade 2 containing 7 species with well supported BS values of 95-100% and 99-100% respectively, showing an overall genetic diversity of 0.05%. Clade 1 and 2 were again divided into species-based clusters showing similarity among members of the same genus. Overall, greater similarity was observed among member of *Ziziphus*, *Sageretia* and *Berchemia* genera, while *Rhamnus* form a separate group. However, *Rhamnella* form a sister group with cluster 1 and two species and *Helinus* form an out-group with *Berchemia* species. Similarly, two species of *Ziziphus* (*Ziziphus jujuba* Mill. *Ziziphus nummularia* Burm.f.) form close relationship with *Rhamnus* species instead of *Ziziphus* (Fig. 2).

**Regulatory elements analysis:** *Cis*-regulatory elements analysis revealed large number of elements with diverse functions among which 50 were common and 28 were unique. Functionally, most of the reported elements in all species belong to flavonoid biosynthesis, light responsiveness and seed storage. The first five elements with highest copy numbers belong to flavonols biosynthesis (422), bacterial response regulator (356), seed storage

(284), photosynthesis controlling elements (249) and Dof protein (230). Other elements with highest copy number were involved in guard-cell specific expression (136), pollen development and maturation (98), circadian rhythm (70) and elements related to various environmental stresses (30). However, elements with low copy numbers and important functions include auxin, gibberellin, abscisic acid (ABA), cold and wound-inducible elements. Overall, highest number of elements was found in *Helinus lanceolatus* (283) and *Rhamnella gilgitica* (272) while lowest were found in *Ziziphus jujuba* and *Berchemia edgeworthii* (219). The name of each sequence, factor name (Fig. 3) copy number, different functions and associated reference are given in Table 3. Beside, 28 unique elements were also found which play significant role in various plant processes. Largest number of unique elements were found in *Berchemia pakistanica* (14), *Berchemia edgeworthii* (13), *Rhamnus triquetra* (12) *Helinus lanceolatus* (12), *Ziziphus mauritiana* (11), *Rhamnus pentapomica* (11), *Ziziphus mauritiana* (10) *Rhamnus virgata* (10) *Ziziphus rugosa* (9), *Sageretia thea* var. *brandrethiana* (9) *Ziziphus spina-christi* (8) *Ziziphus jujuba* (8) *Rhamnus persica* (8) *Ziziphus nummularia* (6) and *Rhamnus purpurea* (5). Detailed information about these elements is given in Table 4.

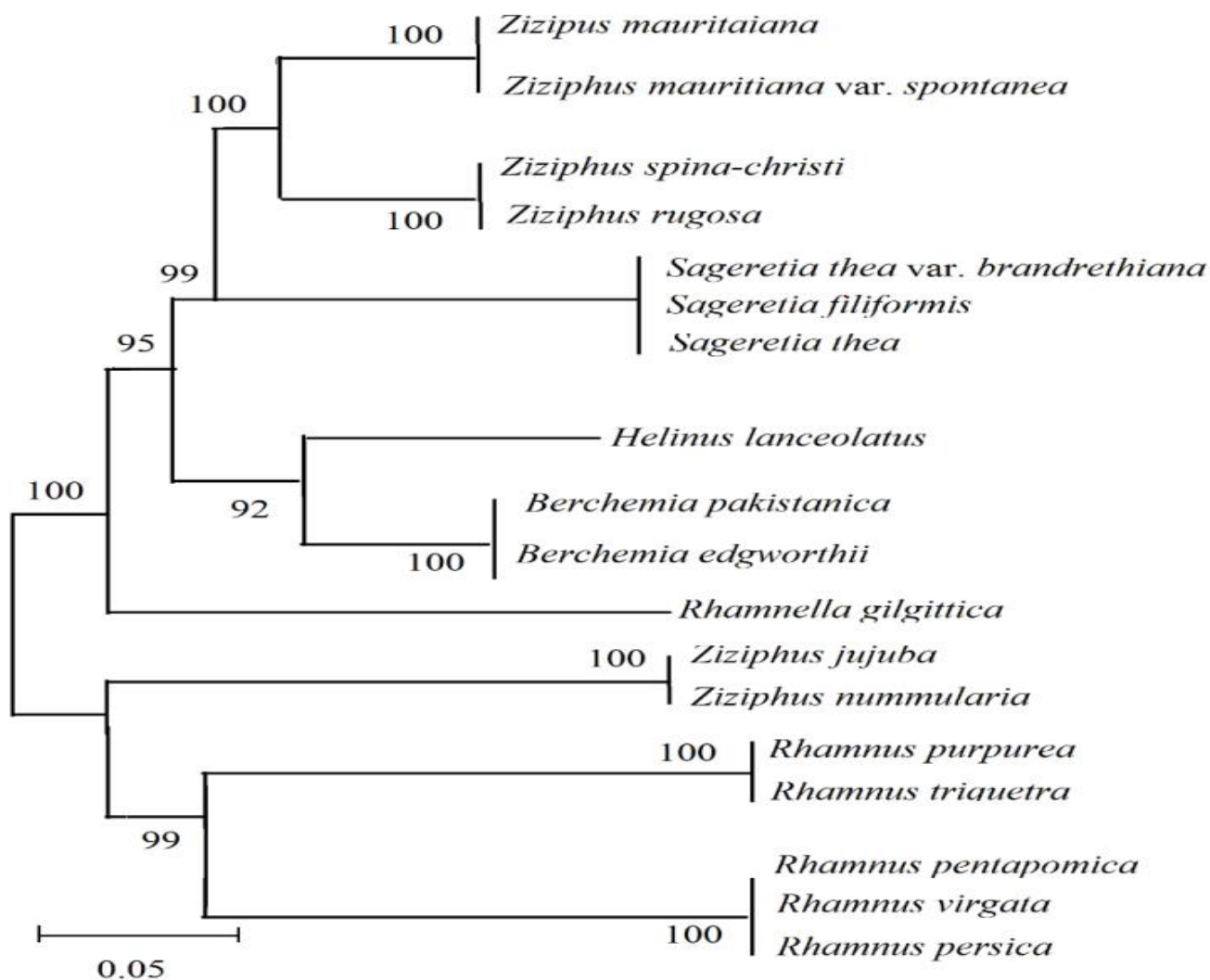


Fig. 2. Phylogenetic analysis of selected species of family *Rhamnaceae* using *atpβ* gene promoter. The tree was constructed using Molecular and evolutionary genetic analysis (MEGA7) software with neighbor's joining method. Each node is labeled with bootstrap's value (in percentage) using 1000 replicate. Accession numbers are *Rhamnus purpurea* (KP121447), *Berchemia pakistanica* (KP121456), *Rhamnus pentapomica* (KP121448), *Ziziphus mauritiana* var. *spontanea* (KP121446), *Ziziphus jujuba* (KP121449), *Rhamnella gilgittica* (KP121457), *Ziziphus mauritiana* (KP121450), *Sageretia thea* var. *brandrethiana* (KP121458), *Rhamnus virgata* (KP121451), *Sageretia filiformis* (KP009593), *Ziziphus spina-christi* (KP121452), *Sageretia thea* (KP009594), *Berchemia edgeworthii* (KP121453), *Rhamnus triquetra* (KP009595), *Helinus lanceolatus* (KP121454), *Rhamnus persica* (KP009596), *Ziziphus nummularia* (KP121455), *Ziziphus rugosa* (KP009597) and showing a narrow genetic background of 0.5% with well supported bootstrap values.

## Discussion

**Morphological data analysis:** Phylogenetic analysis based on morphological characters can be used as a useful strategy to find and analyze the relationship of selected taxa (Kellermann & Barker, 2012; Onstein *et al.*, 2015). Previously, Islam & Guralnick (2015) studied 30 morphological characters of *Rhamnaceae* species by examining the evolution of vegetative and reproductive characters. Presently, greater similarity was observed among studied genera using 71 characters which are due to similarity in their morphology largely governed by the same geographical and environmental conditions. The present study is similar to the phylogenetic reconstructions of *Rhamnaceae* by Richardson, (2000) and Onstein *et al.*, (2015), which was based on molecular data including additional morphological, anatomical and geographical

information by employing 20 and 15 morphological characters respectively. Similarly, Thulin *et al.*, (1998) used 22 morphological characters (also including anatomy) which divided the species into two main clades along with molecular study employing *rbcL* and *trnL-F* genes primers. However, in the present study *Helinus* showed a very distant relationship with other genera which may be due to variation in its morphology and genetic makeup through the course of evolution (Richardson *et al.*, 2000, Richardson *et al.*, 2004). *Sageretia* is the most primitive species serve as an ancestor for *Rhamnus*, *Berchemia*, *Ziziphus* and *Rhamnella* (Onstein *et al.*, 2015; Hauenschild *et al.*, 2016). The greater similarity of *Ziziphus*, *Berchemia* and *Rhamnus* may be contributed to the fact that most of these species have drupaceous fruits, and belong to the same geographical region, resulting similarities at their morphological level but the distant relationship of





Table 3. Comparative analysis of common *Cis*-regulatory elements identified in the *atpβ* gene promoter sequences of selected species of *Rhamnaceae*.

Signal sequence	Factor name	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	Functions
TATTCT	10PEHVPSBD	1	2	3	2	1	3	3	1	2	3	2	1	1	2	1	3	2	2	Encodes chlorophyll-binding protein of photosystem II (Thum <i>et al.</i> , 2001)
NGATT	ARR1AT	22	17	17	17	21	22	22	21	17	17	22	22	16	22	21	16	22	22	A response regulator (Sakai <i>et al.</i> , 2000)
TGTCA	BIHD1OS	1	2	4	1	2	4	4	2	1	4	2	1	1	2	4	3	3	4	Activation of homeodomain transcriptional factors (Luo <i>et al.</i> , 2005)
CAAT	CAATBOX1	20	10	15	21	18	10	10	18	21	15	11	20	20	10	15	21	11	18	Found in legA seed storage protein (Shirsat <i>et al.</i> , 1989)
YACT	CACTFTPPCA1	10	16	16	15	16	10	10	16	15	16	16	10	11	15	10	16	15	16	Engaged in C4 photosynthesis (Gowik <i>et al.</i> , 2004)
RYCGAC	CBFHV	2	2	1	1	1	2	2	1	1	1	2	2	2	1	2	1	2	1	Regulate of cold responsive genes (Xue, 2002)
CCAAT	CCAATBOX1	5	2	3	5	3	2	2	3	5	3	3	2	5	2	3	5	4	3	Regulate flowering of Arabidopsis (Wenkel <i>et al.</i> , 2006)
CAANNATC	CIACADIANLELHC	6	2	3	6	4	3	3	4	6	3	2	3	2	6	6	2	6	3	Circadian expression of tomato LHC Gene (Piechulla <i>et al.</i> , 1998)
AAAG	DOFCOREZM	12	18	11	8	17	11	11	17	8	11	18	11	18	8	17	18	16	16	Binding of D of proteins in maize (Yanagisawa, 2000)
CANN TG	EBOXBNNAPA	18	10	10	14	8	16	16	8	14	8	16	15	8	11	8	15	8	14	Flavonoid production (Hartmann <i>et al.</i> , 2005)
GATA	GATABOX	6	5	9	11	2	5	5	2	11	9	5	6	5	6	9	11	9	8	Light-dependent control of transcription (Reyes <i>et al.</i> , 2004)
GRVAVV	GT1CONCENSUS	14	3	9	8	11	7	7	11	8	9	3	14	14	3	9	8	8	11	Consensus GT-1 binding site in many light-regulated genes (Zhou, 1999)
GTAC	CURECORECR	2	2	1	2	3	2	2	3	2	1	2	2	1	2	2	1	1	2	Core region of Cyc6 genes stand against cytochrome c (6) deficiencies (Quinn <i>et al.</i> , 2000)
CCGAAA	LTREIHVBLT49	1	1	2	1	2	2	2	2	1	1	2	2	2	2	3	2	1	2	Crucial against environmental stress (Dunn <i>et al.</i> , 1998)
AACCCA	SEF3MOTIFGM	2	1	2	2	1	2	2	1	2	1	2	2	1	2	1	2	2	1	Role in embryo development (Allen <i>et al.</i> , 1989)
AACCAA	REALPHALGLHCB21	2	2	2	3	2	2	2	2	3	1	2	2	3	2	2	2	1	3	Lennagibba gene regulation (Degenhardt and Tobin, 1996)
AATCCAA	RBCSCONCENSUS	1	1	2	2	2	1	1	2	2	1	2	1	1	2	3	1	3	2	1, 5-bisphosphate carboxylase expression (Donald & Cashmore, 1990)
AATAAT	POLASIG1	5	3	5	4	7	4	4	7	4	5	3	3	2	4	2	6	6	2	Involved in polyadenylation (Joshi <i>et al.</i> , 1987)
WAACCA	MYBIAT	1	2	1	1	1	1	1	1	1	2	1	2	1	2	1	3	2	5	Involved in ABA and drought-induced expression of rd22 gene (Abe <i>et al.</i> , 2003)
TTWTWTWTT	MARTBOX	3	1	1	1	3	2	2	3	1	1	3	2	3	4	3	2	3	1	Play discrete genome functions (Gasser <i>et al.</i> , 1989)
YTCANTYY	INRNTPSADB	2	0	1	1	1	2	2	1	1	1	1	0	3	2	4	2	4	5	Initiator sequence in many light-regulated genes (Nakamura <i>et al.</i> , 2002)
GANTTNC	ECCRCAHI	10	5	6	6	7	10	10	7	6	4	5	10	5	4	7	2	10	7	Encoding periplasmic carbonic anhydrase (Yoshioka <i>et al.</i> , 2004)
TTATTT	TATABOX	8	4	6	5	7	6	1	1	2	1	3	5	6	5	8	1	5	4	Found in glutamine synthetase gene (Tjaden <i>et al.</i> , 1995)
AAACAAA	ANAERO1CONSENSUS	1	1	2	1	2	1	1	2	1	2	3	3	2	1	2	3	3	1	Involved in fermentative pathways (Mohanty <i>et al.</i> , 2005)
TTGAC	WBOXATNPR1	2	3	3	1	2	3	3	2	1	3	3	1	1	2	2	2	3	4	Defense specific regulatory element (Chen & Chen, 2002)
CTGACY	WBOXNTCHN48	1	1	2	1	1	1	1	1	1	1	2	4	2	1	2	3	2	1	Defense transcription mechanism (Yamamoto <i>et al.</i> , 2004)



Table 3. (Cont'd.).

Signal sequence	Factor name	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	Functions
TGAC	WRKY710S	4	6	5	4	4	6	6	4	4	5	6	4	4	6	5	4	5	6	Gibberellins repression pathway (Eulgem <i>et al.</i> , 1999)
TGACT	WBOXHVISO1	2	2	3	3	3	2	2	3	3	2	2	4	3	2	2	3	6	4	Encodes isoamylase I promoter (Sun <i>et al.</i> , 2003)
TGACY	WBOX	2	2	3	2	3	2	2	3	2	3	2	3	4	4	3	2	4	2	Wound response (Nishiuchi <i>et al.</i> , 2004)
TATTAAT	TATABOX3	1	2	2	1	1	3	3	1	1	2	2	1	1	2	3	2	2	3	Regulation of transcription initiation (Kim & Ivor, 2004)
CNAACAC	CANBNNAPA	1	1	0	1	2	1	1	2	1	0	2	3	2	5	2	1	1	2	Embryonic gene expression (Ellerstrom <i>et al.</i> , 1996)
CAANNNATC	CIACADIANLELHC	3	3	7	4	8	3	3	8	4	7	3	3	8	6	3	3	2	2	Tomato circadian expression (Piechulla <i>et al.</i> , 1998)
ACACNNG	SOOO292	3	3	1	2	3	3	3	3	2	1	3	3	3	2	1	2	3	3	Embryo and ABA-signalling (Lopez-Molina & Chua, 2000)
TGTCA	BIHDIOS	2	3	2	2	3	2	2	3	2	2	3	2	3	2	2	3	3	2	Binding site for rice BELL homeodomain transcription factor (Luo <i>et al.</i> , 2005)
TGAC	WRKY710S	1	3	6	3	7	1	1	7	3	6	3	1	1	3	7	1	1	4	Gibberellins repression pathway (Eulgem <i>et al.</i> , 1999)
TAAAG	TAAAGSTKST1	10	7	5	9	6	5	6	9	5	7	10	7	6	10	9	8	8	9	Guard-cell specific gene expression (Plesch <i>et al.</i> , 2001)
ATATT	ROOTMOTIFTAPOX1	6	4	11	8	3	2	2	3	8	9	11	4	6	6	4	11	11	8	Roots and vascular tissues elongation (Elmayan & Tepfer, 1995)
CAACA	RAV1AAT	1	4	6	2	5	1	1	5	2	6	4	6	1	4	5	6	6	3	DNA binding transcription factor of Arabidopsis (Kagaya <i>et al.</i> , 1999)
CANN TG	EBOXBNNAPA	28	20	20	24	28	20	22	23	20	24	28	28	20	28	22	20	24	27	Biosynthesis of flavonols (Hartmann <i>et al.</i> , 2005)
GTGA	GTGANTG10	2	6	11	3	7	2	2	7	3	11	6	2	6	11	3	7	7	2	Pollen development (Rogers <i>et al.</i> , 2001)
CTCTT	NODCON2GM	1	6	4	5	4	1	1	4	5	4	6	1	1	6	4	5	6	1	Required for putative signal peptide and DNABP (Sandal <i>et al.</i> , 1987)
AACGG	MYBCOREATCYCB1	1	1	1	2	1	1	1	2	3	1	2	1	2	1	2	2	2	2	Reporter gene activation (Planchais <i>et al.</i> , 2002)
CCWACC	MYBPZM	1	1	1	2	1	1	2	1	1	2	1	1	2	1	1	2	2	1	Encode for flowers red pigmentations (Grotewold <i>et al.</i> , 1994)
AAAGAT	NODCON1GM	8	6	8	4	8	8	8	8	4	8	6	7	7	5	7	6	7	8	Encodes for putative signal peptide (Sandal <i>et al.</i> , 1987)
ACTTTA	NTBFF1ARROLB	2	2	2	1	2	2	3	2	2	1	2	2	2	3	1	1	2	3	Auxin and tissue specific expression (Baumann <i>et al.</i> , 1999)
CTCTT	NODCON2GM	1	6	5	4	1	5	5	1	4	5	6	1	1	2	5	4	5	5	Putative signal peptide and DNABP (Sandal <i>et al.</i> , 1987)
AATAAA	POLASIG1	10	7	7	10	11	7	8	7	11	10	7	7	10	11	6	7	11	9	Poly A signal is found in legA gene of pea (Joshi, 1987)
CACCTG	RAV1BAT	1	1	1	1	1	1	1	1	1	2	2	1	1	2	1	2	3	1	Binding sequence of Arabidopsis transcription factor, RAV1 (Kagaya <i>et al.</i> , 1999)
GATA	GATABOX	6	5	9	11	2	4	5	4	2	11	9	5	6	6	5	4	5	7	Light-dependent control of transcription (Reyes <i>et al.</i> , 2004)
GATAA	IBOXCORE	4	2	3	4	2	1	2	1	2	4	3	2	4	2	3	4	4	1	Light-regulated transcription (Terzaghi & Cashmore, 1995)

S1: *Ziziphus spina-christi*, S2: *Ziziphus jujuba*, S3: *Ziziphus nummularia*, S4: *Ziziphus rugosa*, S5: *Ziziphus mauritiana*, S6: *Ziziphus mauritiana* var. *spontanea*, S7: *Berchemia edgeworthii*, S8: *Berchemia pakistanica*, S9: *Rhamnus irriqueira*, S10: *Rhamnus purpurea*, S11: *Rhamnus virgata*, S12: *Rhamnus pentapomica*, S13: *Rhamnus prostrata*, S14: *Rhamnus persica*, S15: *Sageretia thea*, S16: *Sageretia thea* var. *brandrethiana*, S17: *Helinus lanceolatus*, S18: *Rhamnella gligittica*

Table 4. Comparative analysis of unique *Cis*-regulatory elements identified in the *atpf* gene promoter sequences of selected species of *Rhamnaceae*.

Signal sequence	Factor name	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	Functions
CTGACY	WBOXNNTCHN48	-	-	1	1	-	-	-	1	2	-	-	-	-	-	-	-	1	1	Elicitor-responsive transcription of defense genes in tobacco (Yamamoto <i>et al.</i> , 2004)
ATGGTATT	S1FSORPL21	-	-	-	-	1	2	-	1	1	-	2	-	-	-	1	-	-	1	Down regulating RPL21 promoter activity (Lagrange <i>et al.</i> , 1993)
GTTAGGTT	MYB26PS	1	-	-	-	-	-	1	-	1	-	-	-	-	-	1	-	-	-	Expression of phenylpropanoid biosynthetic genes (Uimari & Strommer, 1997)
GATAAG	IBOX	-	-	-	-	-	1	1	-	-	-	-	-	-	1	1	-	-	1	Act as a transcriptional activator (Rose <i>et al.</i> , 1999)
WTTSSCSS	E2FCONSENSUS	-	1	1	-	-	-	-	1	-	-	1	1	-	2	-	1	-	-	Regulate S phase of cell cycle (Vandepoelle <i>et al.</i> , 2005)
CCCTTT	PYRIMIDINEBOXRAMY1A	1	-	-	1	2	-	-	-	-	-	-	2	1	-	1	-	-	-	Specific to sugar repression and Gibberellins response gene (Mena <i>et al.</i> , 2002)
TGGGCV	SITEIIATCYTC	-	1	-	-	-	1	-	-	1	-	-	1	-	-	-	-	-	1	Required for oxidative phosphorylation (Welchen & Gonzalez, 2006)
GGTTAA	GT1CORE	1	1	-	-	-	1	2	1	-	-	-	-	-	-	1	-	2	-	Cell-type specific transcriptional modulation (Villain <i>et al.</i> , 1996)
AAACAAA	ANAERO1CONSENSUS	-	-	-	-	-	-	-	1	-	-	-	-	1	-	-	-	1	1	Involved in the fermentative pathway (Mohanty <i>et al.</i> , 2005)
TGTCTC	ARFAT	1	-	-	-	-	-	1	-	-	1	2	1	-	-	1	-	1	-	Involved in auxin regulated expression (Goda <i>et al.</i> , 2004)
ACCGAC	DRE2COREZMRAB17	-	-	1	-	-	-	2	1	-	-	-	-	-	-	-	-	1	-	Stress response related cis-regulatory element (Dubouzet <i>et al.</i> , 2003)
YGTGCWC	SEBFCONSSTPR10A	-	-	-	1	1	2	1	-	-	-	-	-	-	-	-	-	-	1	Defense-specific regulatory element (Boyle & Brissson, 2001)
GTGGGCCCG	GBP2ZMGAPC4	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1	Heterologous anaerobic gene expression (Geffers <i>et al.</i> , 2000)
ATGGTATT	S1FSORPL21	1	-	-	-	-	2	1	-	-	1	-	-	-	-	-	-	-	1	Plastid ribosomal protein regulatory elements (Lagrange <i>et al.</i> , 1993a)
CGAACTT	AMMORRESIVDCRNIAI	-	1	-	1	-	-	-	-	-	1	2	1	-	-	-	-	-	-	Specific to suppress Nia1 gene expression (Lopes & Radoux, 1999)
ACTCAT	PREATPRODH	-	-	-	1	-	-	2	-	-	-	1	-	-	-	-	-	-	-	Required for pre, pro or hypo-osmolarity (Satoh <i>et al.</i> , 2002)
GNATATNC	PIBS	-	1	-	-	1	2	-	-	-	-	-	1	-	-	-	-	2	-	Phosphate (Pi) deprivation (Rubio <i>et al.</i> , 2001)
GTGGWWHG	SV40COREENHAN	-	-	-	1	2	-	-	-	-	1	1	-	-	-	1	-	-	1	SV40 core enhancer affecting transcription (Weiher <i>et al.</i> , 1983)
GCCGCC	GCCCORE	-	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	Role in pathogen-responsive and ethylene-responsive genes (Chakravarthy <i>et al.</i> , 2003)
AGCGGG	BS1EGCCR	-	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1	Vascular expression of the cinnamoyl CoA reductase gene and for protein-DNA complex development (Lacombe <i>et al.</i> , 2000)
CCGTCC	HEXAMERATH4	-	-	1	1	-	-	-	1	-	1	1	-	-	-	1	1	-	-	Motif of <i>Arabidopsis thaliana</i> histone H4 promoter (Chaubet <i>et al.</i> , 1996)
GGATA	MYBSTI	-	-	-	-	-	1	1	1	-	-	1	1	-	-	-	-	2	-	DNA binding and transcription activator activity (Baranowski <i>et al.</i> , 1994)
TACTATT	SP8BFIBSP8BIB	1	-	-	1	1	-	1	1	-	-	-	-	-	1	1	-	-	-	Specific to polygalacturonic acid supply in tuberous roots (Ishiguro & Nakamura, 1994)
VCGCGB	CGCGBOXAT	-	-	-	-	2	1	-	1	1	-	-	1	-	-	-	-	-	1	Response to ABA, ethylene and light signalling (Yang & Poovaiah, 2002)
KCACGW	RHERPATEXPA7	-	-	-	1	-	-	-	-	1	-	-	1	1	-	-	-	-	1	Expression of genes involved in hair distribution patterns (Kim <i>et al.</i> , 2006)
RCCGAC	DRECRTOREAT	1	1	-	-	1	1	-	-	1	-	-	-	-	-	-	-	-	1	Core motif of DRE/CRT cis-acting element, identified in many genes in <i>Arabidopsis</i> (Suzuki <i>et al.</i> , 2005)
CCGAC	LTRECOREATCOR15	-	-	1	1	-	-	-	-	1	-	-	1	-	1	1	1	-	-	Cold induction of BN115 gene from winter <i>Brassica napus</i> LTRE (Jiang <i>et al.</i> , 1996)
CAACTC	CAREOSREPI	1	1	-	-	-	-	-	-	1	-	-	-	-	1	1	1	-	1	Gibberellin-upregulated proteinase expression in rice seeds (Sutoh and Yamauchi, 2003)

S1: *Ziziphus spina-christi*, S2: *Ziziphus jujuba*, S3: *Ziziphus nummularia*, S4: *Ziziphus rugosa*, S5: *Ziziphus mauritiana*, S6: *Ziziphus mauritiana* var. *spondanea*, S7: *Berberchia edgeworthii*, S8: *Berberchia pakistanica*, S9: *Rhamnus triquetra*, S10: *Rhamnus purpurea*, S11: *Rhamnus virgata*, S12: *Rhamnus pentapomica*, S13: *Rhamnus prostrata*, S14: *Rhamnus persica*, S15: *Sageretia thea* var. *brandreithiana*, S17: *Helinus lanceolatus*, S18: *Rhamnella gilgitica*.

Several other molecular markers have been used to evaluate the phylogenetic relationship of the members of *Rhamnaceae*. Islam & Guralnick (2015) evaluated the degree of similarity and diversity among different genera of *Rhamnaceae* with two loci from the nuclear genome internal transcribed spacers (*nrITS*) and 26S rDNA and two loci from the chloroplast genome (*trnL-trnF* intergenic spacer (*trnL-F*), and *trnQ-5'rps16* intergenic spacer (*trnQ-rps16*). They found relatively higher genetic homologies between members of *Ziziphus* (*Ziziphus rugosa* and *Ziziphus jujuba*) with a BS score of 100%. Furthermore, Onstein *et al.*, (2015) worked on the molecular analysis of *Rhamnaceae* (280 species and seven subspecies) with reference to morphological studies. They examined the evolution of the vegetative and reproductive characters using six chloroplast markers *matK*, *rbcL*, *trnL-F* and intergenic spacer, *psbA* and *psbA-trnH* intergenic spacer, *ndhF* spacer, *rpl16* gene and intron and one nuclear marker *ITS* gene marker have shown close relationships between taxa with more than 80% BS support. However, no significant studies based on molecular markers have been carried out for the members of *Rhamnaceae* using cpDNA in Pakistan and many of these species are being studied and reported for the first time from Pakistan.

**Regulatory elements analysis:** The presence of large number of regulatory elements in studied species represents its diverse function. Highest number of these elements are involved in the regulation of flavonols biosynthesis genes which are in accordance to the observed role of this family in diverse medicinal proposes (Hartmann *et al.*, 2005). It may also be due the fact that *Rhamnaceae* play an important role against various diseases. The presence of these elements in all species may be contributed to its common evolutionary history and similar properties. Other important elements were related to light responsiveness and seed storage showing the important role of these promoters in related processes. Similarly, other elements like CACTFTPPCA1 play an important role in the photosynthesis of C4 plants (Gowik *et al.*, 2004), CIACADIANLELHC in circadian expression (Piechulla *et al.*, 1998), ROOTMOTIFTAPOX in the elongation of vascular tissues (Elmayan and Tepfer, 1995), GTGANTG10 in development of Pollen (Rogers *et al.*, 2001), MYBPZM code for flowers red colorations (Grotewold *et al.*, 1994). Further, *in silico* analysis revealed the presence of several other functional elements like, NTBBF1ARROLB which regulate tissue specific expression (Baumann *et al.*, 1999), WRKY710S in repression pathway of gibberellin (Eulgem *et al.*, 1999), SOOO292 in ABA-signalling pathway (Lopez-Molina & Chua, 2000), IBOXCORE in light-regulated transcription (Terzaghi and Cashmore, 1995). Furthermore, four unique elements, including ANAERO1CONSENSUS is involved in the fermentation pathway (Mohanty *et al.*, 2005), SITEIIATCYTC is required for oxidative phosphorylation (Welchen & Gonzalez, 2006), SEBFCONSSTPR10A a defense-specific regulatory element (Boyle & Brisson, 2001), and CGCGBOXAT regulate ethylene, ABA and light signalling (Yang & Poovaiah, 2002) were identified. The highest number of these elements were found in

*Helinus lanceolatus* and *Rhamnella gilgitica* promoter showed its importance regarding crop biotechnology. The presence of these unique elements showed its more diverse functions through the course of evolution during which nature accumulated more novel elements regarding their demanding function. A more detailed analysis is presented in table 3 and 4 which provides information about the possible regulatory role of these elements in different parts of the plant in response to various stresses.

## Conclusion

The present study is the first to investigate phylogenetic relationships of the entire *Rhamnaceae* members from Pakistan using both morphological and molecular data. Many genera, particularly *Rhamnella*, *Helinus* and *Berchemia* are endemic to Pakistan and have been poorly sampled for evolutionary studies. Hence, species from this region will provide critical information on phylogenetic relationships and will allow for a comprehensive re-evaluation of the taxonomy of different taxa. Future studies can focus further on the examination of the morphology, anatomy, phytochemistry, genetics and evolution of this diverse family. Our *atpβ* gene promoter analysis also elucidated the occurrence of important novel *cis*-acting elements from the different species of *Rhamnaceae* which is helpful in various stresses and can have application in saving genetic resources and breeding. However, to determine the precise role of these *cis*-regulatory elements, *In vivo* studies are needed.

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