

ECOLOGICAL DISTRIBUTION, MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF ZYGOPHYLLACEAE FROM DIVERSE ECOLOGICAL ZONES OF BALOCHISTAN, PAKISTAN

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Abstract. The study was aimed to characterize and document the wild plants of Zygophyllaceae in arid and semi-arid regions of Balochistan. The morphology, ecology and molecular characteristics and taxonomic implications of many taxa were subject to analysis. The field survey was carried out during 2017-18 in the diverse elevation zones of Balochistan selected due to the presence of the highest number of Zygophyllaceae in these regions. Morphological and molecular characterization was carried out by using, agglomerative hierarchical clustering and Principal Component Analysis. A total of 17 plants of Zygophyllaceae were documented from Sibi, Mashkaf, Noshki, Naal, Dalbandeen, Punjgur, Hingol National Park, Bella, Uthal and Quetta region. Commonly distributed taxa were *Peganum hermala* and *Tribulus terrestris*. Furthermore, seven species of *Fagonia*, five species of *Tribulus* and four species of *Zygophyllum* were also assessed. The present study documents and contributes detailed significant information of naturally occurring medicinal plants of Zygophyllaceae from diverse remote zones of Balochistan. It also provides a base line for identification of controversial taxa on morphological and molecular basis. Especially the data provided may further be utilized for novel drug development. Furthermore, the ecological distribution may be useful in management of conservation of endangered and endemic taxa.

Keywords: *Fagonia, Tribulus, Zygophyllum, Peganum, arid and semi-arid zones, PCA, AHC*

Introduction

Balochistan is the largest province comprising 44% of the total area of Pakistan. Geographical region comprised of arid to semi-arid structure ranging from cool temperate to coastal tropics. The diverse ecological conditions have an impact on the diversity of flora found. Local communities utilize wild plants as folk medicines in large amount. Selected areas are blessed by botanical diversity and endemism. Diverse taxa of the region include herbs, shrubs, and trees including evergreen Juniper forest. Aromatic, non-aromatic flowering and non-flowering plants contributes in more than sixteen hundred wild plant species. Total of 44 species are endemic to the Balochistan (Saeed et al., 2014).

The Zygophyllaceae consists of diverse habits of wild flora including succulents, herbs, undershrub, shrubs and small trees. The habitat of these plants is predominantly desert or saline areas of temperate and tropical regions around the globe (Beier et al., 2003; Bellstedt et al., 2008). *Tribulus*, *Fagonia*, *Zygophyllum* and *Peganum* considered being the main genera. Folk medicinal System is a precious resource, hence utilized as potent safe drugs (Hussain and Sher, 1998). Many members of the family are being used ethno medicinally for centuries in folk medicines. Taxa like *Peganum hermala*, *Fagonia indica*, *F. ovalifolia*, *F. bruguieri*, *Tribulus terrestris*, *T. longipetalus* etc traditionally used by local communities to cure fever, cough, inflammation of organs, gonorrhoea, obesity, urinary tract infection (UTI) etc.

(unpublished data). Many medicinal plants extract constituted various types of chemicals, each have a property to control a variety of biological and pharmacological activities such as antimicrobial, anti-parasitic, anti-diabetic, antioxidant, anti-inflammatory and anticholinesterase. These chemicals may have a combined effect in controlling various diseases (Rates, 2001; Houghton et al., 2007).

For proper identification and standardization of crude drugs, accurate anatomical and morphological description is necessary, and this description must take into account all the diagnostic features. The evaluation of crude drug involves a number of methods such as organoleptic, microscopic, chemical, physiological and biological. Organoleptic evaluation includes shape, size, odor, taste, texture, and color of crude drug along with external marking. On the basis of gross morphology, drugs may be grouped as leaves, bark, root, rhizome, and so forth. Microscopic features such as spines, trichomes, spores, and epidermal structures may be examined which are used as diagnostic features in the identification of plant drugs (Fransworth and Soejarto, 1988; Fazal et al., 2013). As the selected plants are medicinally important and utilized in folk medicine system, their authentication is essential on morphological and molecular basis that may be utilized for novel drug discovery as a future prospect.

During the past few decades, the theoretical framework of population genetics and empirical data gathered with the help of molecular genetic methods have been widely used in conservation biology. Given a haploid nature and a low frequency of genetic recombination, molecular markers of organelle DNA have long been used for phylogenetic reconstruction at various taxonomic levels, conservation genetics, and assessing the migratory routes of species (Nite-Kang and Yong, 2014; Yong and Nite-Kang, 2015).

Molecular marker system used to assess genetic variability within and among populations. Several marker systems were being used for genetic diversity analysis. Randomly Amplified Polymorphic DNA (RAPD) markers are appropriate for DNA fingerprinting of unknown sequences and they are rapid and easy to assess, (Kernodle et al., 1993). The Inter Simple Sequence Repeat (ISSR) did not need prior sequence and was less vulnerable (Adams et al., 2003). Universal Rice Primer (URP) markers were first time used by Kang et al. (2002). These are repeated chains that were extracted of genome bank of the rice of local Korea. URP markers with suitable PCR condition, produce high polymorphs. Additionally, it has been proved that these markers are useful tools for genetic analysis in between and within species (Rashmi et al., 2008; Abbas et al., 2015). Combined marker system used to assess genetic diversity within and among the population of different wild plants which were not sequenced earlier (Saeed et al., 2015, 2017).

In this view the present study was designed to provide comprehensive information about the medicinally important and highly controversial taxa of Zygophyllaceae used in folklore from different ecological zones of Balochistan, based on morphological and molecular characterization.

Material and Methods

Study Sites

The field survey was carried out during 2017-18 in various regions viz., Quetta valley, Sibi, Mashkaf, Noshki, Naal, Dalbandeen, Punjgur, Hingol National Park, Bella and Uthal. These areas of Balochistan were selected due to the presence of high number of taxa of Zygophyllaceae (*Figure 1*).

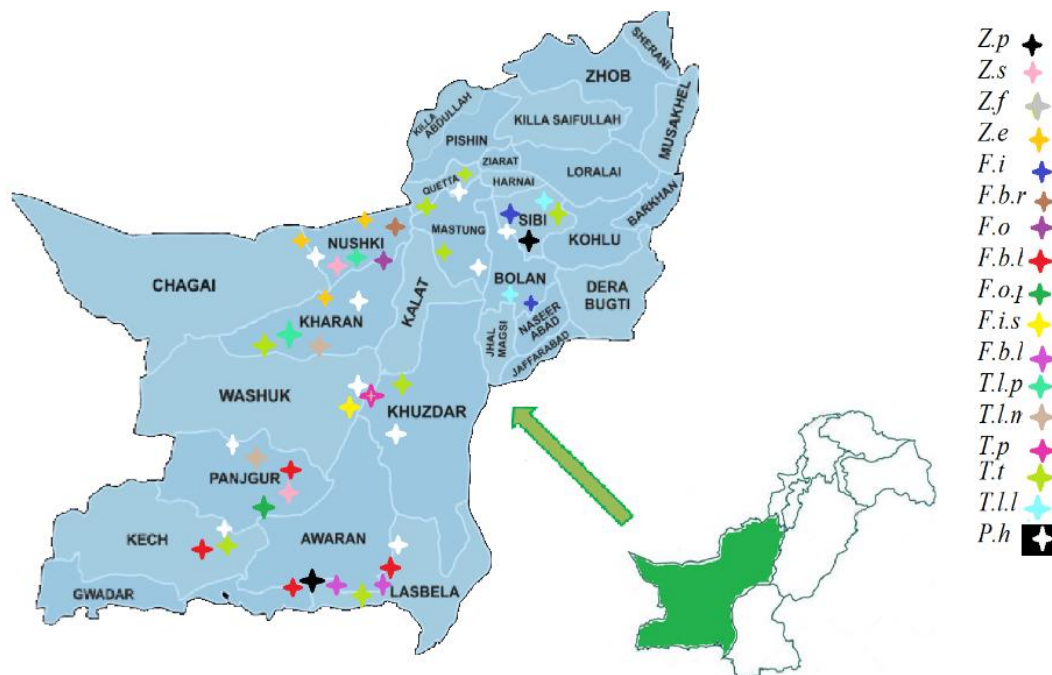


Figure 1. Map of Balochistan (Pakistan) illustrating the sampling site areas with geographical coordinates

Morphological Analysis

Total 58 characters were observed during the morphological analysis. Qualitative and Quantitative morphological characters were appraised and subjected to Principal Component Analysis (PCA) and Agglomerative Hierarchical Clustering (AHC).

Molecular Analysis

This study was carried out using three different marker systems namely, RAPD, ISSR and URP's (Table 1). Combined markers RAPD, ISSR and URP were used to amplify for cluster analysis.

DNA Extraction

Genomic DNA was isolated by using CTAB (Cetyl Trimethyl Ammonium Bromide) Doyle and Doyle (1987). Quantified on a spectrophotometer at 260 nm and Checked DNA Quality on 0.8% gel by electrophoreses.

PCR Amplification

PCR reaction for amplification was carried out. Total 20 µl volume reaction was prepared. Enzymomics 2X TOPsimple DyeMIX-nTaq, Master Mix was used. PCR mixture contains 5 µl Master Mix (2X), 0.4 µl Primer (5 pmol/ µl), 4 µl DNA template (0.1 ng/µl). PCR amplification was performed using thermal cycler (Applied Biosystems 96 well USA). For RAPD primers, PCR was performed with initial denaturation at 94°C for 4 min, Denaturation 94°C for 4 min and then 36 cycles with annealing temperature 33°C for 1 min, extension step at 72°C for 2 minutes. An additional extension step for 7 minutes at 72°C in the final cycle was also added. For

URP primers, cycles increased to 40. ISSR primers performed 40 cycles. The amplified products were checked by electrophoresis in 1.8% agarose gels. Gel stained with ethidium bromide (0.5 µg / ml) in 1X TAE buffer. The product was visualized by Gel documentation under UV light and the size of markers were estimated by comparing to the standard ladder (100 bp BIORON 0.2 mg/ml) in the gel.

Table 1. List of primers includes name of primers, and sequence of the primers used in the study

S. No.	Primer Name	Sequence (5'-3')
RAPD		
1	OPA-01	CAGGCCCTTC
2	OPA-02	TGCCGAGCTG
3	OPA-03	AGTCAGCCAC
4	OPA-04	AATCGGGCTG
5	OPA-07	GAAACGGGTG
6	OPA-09	GGGTAACGCC
7	OPC-19	GTTGCCAGCC
8	OPC-26	CACGTTATCGCA
9	OPD-13	GGGGTGACGA
10	OPD-20	ACCCGGTCAC
11	OPD-69	CGCTCAAAT
12	OPF-21	AGCAACAATC
13	OPF-22	AAGATCAAAGAC
14	OPG-04	AGCGTGTCTG
15	OPG-09	CTGACGTCAC
16	OPG-13	CTCTCCGCCA
17	OPH-02	TCGGACGTGA
18	OPH-04	GGAAGTCGCC
19	OPP-03	CTGATACGCC
20	OPT-02	GGAGAGACTC
21	OPT-05	GGGTTTGCCA
22	OPU-03	CTATGCCGAC
23	OPW-04	CAGAAGCGGA
24	OPX-17	GACACGGACC
25	OPAJ-20	ACACGTGGTC
26	S-03	AGCGGGGTCA
27	S-05	GGTCAACCCT
28	S-11	GAGGCGCTGC
29	S-23	CGGCCACCGT
30	UBC-181	ATGACGACGG
31	UBC-194	AGGACGTGCC
32	UBC-733	GGGAAGGGAG
URPS		
01	URP-25F	GATGTGTTCTTGGAGCCTGT
02	URP-1F	ATCCAAGGTCCGAGACAACC
03	URP-2R	CCCAGCAACTGATCGCACAC
04	URP-17R	AATGTGGGCAAGCTGGTGGT
ISSR		
01	INC-6	CGCGATAGATAGATAGATA
02	INC-7	GACGATAGATAGATAGATA
03	INC-8	AGACAGACAGACAGACGC
04	INC-14	CTCCTCCTCCTCCTT
05	INC-16	TCTCTCTCTCTCTCA

Gel Analysis by Software

Then amplified products were checked by electrophoresis in 1.5% agarose gels in 1X TAE buffer. Further gel was stained in ethidium bromide. The product was visualized by Gel documentation system under UV light and the size of markers was estimated by 100-1500 bp DNA ladder (RTU) containing orange G & xylene cyanol FF tracking dyes in the gel. Gel analysis was carried out by using UVI-Soft UVI-Band Map. Similarity coefficients Nie & Li (Dice) were performed by UPGMA. Dendrogram generated based on homology.

Results

In this project seventeen wild species of Zygophyllaceae were reported from diverse and remote ecological zones of Balochistan including endemic taxa (*Figure 2*). Voucher specimen of all collected species was submitted in www.openherbarium.edu. Geographical coordinates of these plants at their existing habitats were recorded and the Phenology (flowering period) of the taxa was also recorded (*Table 2*).

Table 2. Geographical coordinates, soil type and phenology of selected Taxa of Zygophyllaceae

S. No	Plant Name	Plant code	Voucher No.	Locality	Elevation (meter above sea level)	Soil type	Phenology (Flowering Period)
1	<i>Z. propinquum</i>	Z.p	QUETTA000208	Hingol National Park, Mushkaf, Sibi	100-350	Sandy, common carmonishius saline sandy loam soil	March -April
2	<i>Z. simplex</i>	Z.s	QUETTA000218	Panjgur, Noshki	950-990	Sandy Rocks	March -April
3	<i>Z. fabago</i>	Z.f	QUETTA000090	Quetta	1650-1700	Sandy loam	July-Aug
4	<i>Z. eurypterum</i>	Z.e	QUETTA000157	Noshki, Dalbandeen, Kharan	990-1000	Sandy rock	March-April
5	<i>F. indica</i>	F.i	QUETTA000207	Mushkaf, Sibi	100-200	Sandy	Feb-Aug
6	<i>F. bruguieri</i> var <i>rechingeri</i>	F.b.r	QUETTA000222	Noshki (Stone)	990-1000	Sandy rock	March-May
7	<i>F. oliveri</i>	F.o	QUETTA000221	Noshki (Janglat area)	1000	Sand	March-May
8	<i>F. bruguieri</i> var. <i>bruguieri</i>	F.b.b	QUETTA000220	Panjgur, Uthal, Hingol National Park, Turbat	970	Sand	February-April
9	<i>F. ovalifolia</i> var. <i>pakistanica</i>	F.o.p	QUETTA000219	Panjgur	980	Sandy stones	March-April
10	<i>F. indica</i> var. <i>schweinfurthii</i>	F.i.s	QUETTA000223	Naal	1200	Sandy Rock	April-September
11	<i>F. bruguieri</i> var <i>laxa</i>	F.b.l	QUETTA000224	Uthal, Hingol National Park	0-150	Sandy Rock	February-April
12	<i>T. longipetalus</i> subsp. <i>pterophorus</i>	T.l.p	QUETTA000215	Noshki	993	Sandy rock	March-May
13	<i>T. longipetalus</i> subsp. <i>macropterus</i>	T.l.m	QUETTA000216	Panjgur, Kharan	970	Sand	February-May.
14	<i>T. pentandrus</i>	T.p	QUETTA000217	Naal	1200	Sandy Rock	February-August.
15	<i>T. terrestris</i>	T.t	QUETTA000085	Bella, Punjgur, Noshki, Sibi, Hingol National Park, Quetta	100-1650	Sand	April-October
16	<i>T. longipetalus</i> subsp. <i>longipetalus</i>	T.l.l	QUETTA000209	Mushkaf, Sibi	130-150	Sand	March-October
17	<i>P. hermala</i>	P.h	QUETTA000002	Uthal, Khuzdar, Bella, Punjgur, Noshki, Kharan, Sibi, Hingol National Park, Naal, Quetta	100-1700	Sand	April- August.

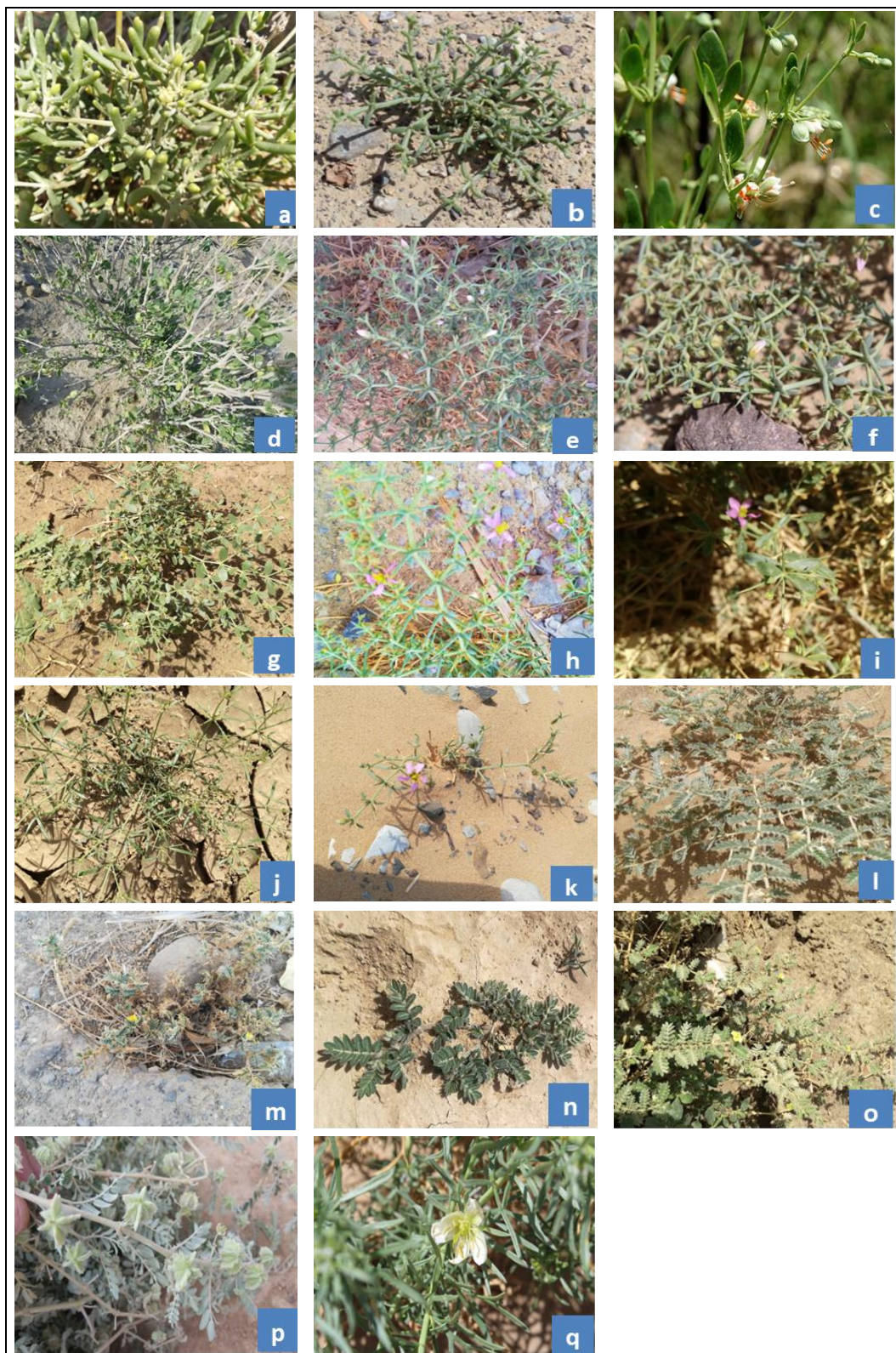


Figure 2. Plants at their habitat **a)** *Z. propinquum*, **b)** *Z. simplex*, **c)** *Z. fabago*, **d)** *Z. eurypterum*, **e)** *F. indica*, **f)** *F. bruguieri* var. *rechingeri*, **g)** *F. oliveri*, **h)** *F. bruguieri* var. *bruguieri*, **i)** *F. ovalifolia* var. *pakistanica*, **j)** *F. indica* var. *schweinfurthii*, **k)** *F. bruguieri* var. *laxa*, **l)** *T. longipetlus* sub.sp. *pterophorus*, **m)** *T. longipetalous* sub.sp. *macropterus*, **n)** *T. pentandrus*, **o)** *T. terrestris*, **p)** *T. longipetalus* sub.sp *longipetlus*, **q)** *P. hermala*

Morphological Analyses

Morphological evaluation of 17 taxa of Zygophyllaceae found in study area was carried out by total of fifty-nine morphological characters. Out of which thirty-one were qualitatively measured and twenty-eight were quantitative characters.

Qualitative Analyses

Life cycle

Studied taxa exhibited different life cycles. All the taxa of *Tribulus* were annual except the *T. pentandrus* was biannual. Perennial taxa were *P. hermala*, *Z. fabago*, *Z. propinquum*, *Z. eurypterum* and *F. bruguieri* var. *laxa*. *F. indica* var. *schweinfurthii* and *F. indica* were annual herbs all other taxa of *Fagonia* were Bi-annual.

Plant nature

Plant nature varied in all studied species of Zygophyllaceae. *T. longipetalus* subsp. *pterophorus* and *T. longipetalus* subsp. *macropterus* were prostrate-erect. *T. longipetalus* subsp. *longipetalus*, *T. terrestris*, *T. pentandrus*, *F. bruguieri* var. *rechingeri*, *F. bruguieri* var. *bruguieri* were prostrate in nature. *Z. simplex* was the only taxa having sub-erect plant nature. *F. ovalifolia* subsp. *pakistanica* was unique in nature i.e. Procumbent. *F. indica* var. *schweinfurthii* was ascending in nature. *F. indica* was decumbent and *F. olivieri* was sub-prostrate. All remaining were erect in nature.

Plant surface

Plant surface of *T. longipetalus* subsp. *macropterus* and *T. pentandrus* were densely hairy while *T. longipetalus* subsp. *longipetalus*, *T. terrestris* and *Z. propinquum* were sparsely hairy. *F. bruguieri* var. *laxa* was sparsely glandular whereas *F. indica* var. *schweinfurthii*, *F. olivieri* and *F. bruguieri* var. *bruguieri* were glandular. Other taxa had glabrous plant surface.

Hair shapes

Hair shapes of *T. longipetalus* subsp. *pterophorus*, *T. longipetalus* subsp. *macropterus*, *T. longipetalus* subsp. *longipetalus*, *T. terrestris*, *T. pentandrus*, *Z. propinquum* and *F. ovalifolia* subsp. *pakistanica* were glandular.

Trichome stalk

In most of the taxa trichome stalk were absent while present only in all genera of *Tribulus*.

Stem nature

Nature of the stem varies from woody to herbaceous in selected taxa of Zygophyllaceae. *Z. eurypterum* was characterized in woody stem nature. All genera of *Tribulus*, *P. hermala* and *Z. simplex* were having herbaceous stem. The stem in all genera of *Fagonia* were woody at the base.

Stem outline shape

Significant variations were found in stem outline shape of different taxa of Zygophyllaceae. *T. terrestris*, *T. pentandrus*, *P. hermala* and *Z. propinquum* were terete outline shape of stem. *Z. fabago*, *Z. simplex* and *Z. eurypterum* were striate. *F. indica* var. *schweinfurthii* and *F. indica* were terete and striate, while all other were quadrangular.

Stipule

Stipule was present in selected taxa of Zygophyllaceae. Nature of stipule varies within the selected taxa. Stipule of *T. pentandrus* was bristles while other four genera of *Tribulus* and *Z. eurypterum* were foliaceous. Stipule nature of *P. hermala* was also bristles. *Z. fabago*, *Z. propinquum* and *Z. simplex* stipule were scarious and all the *Fagonia* species have spinescent stipule nature.

Leaves/leaflets arrangement

Leaves/leaflets arrangements were alternate in *T. longipetalus* subsp. *pterophorus* and *F. indica* var. *schweinfurthii*, whereas all other taxa were having opposite Leaves/leaflets arrangements.

Leaf-blade\leaflets

Leaf-blade\leaflets of all the studied taxa of *Tribulus* were in paired while other taxa leaves were not paired.

Leaf structure

Variation was found in leaf structure of studied taxa. Leave of *T. longipetalus* subsp. *pterophorus* was paripinnat abruptly. Leave structure of *T. longipetalus* subsp. *macropterus*, *T. longipetalus* subsp. *longipetalus*, *T. terrestris*, *T. pentandrus*, *Z. propinquum* and *Z. fabago* were bifoliate. *P. hermala* and *Z. eurypterum* leaves were found simple. *Z. simplex* leaves succulent. *F. bruguieri* var. *laxa* leaves was trifoliate below and unifoliate above. All other taxa of *Fagonia* were had unifoliate leaf structure.

Leaf petiole

Leaf petiole showed variation among all selected taxa. *Z. fabago*, *Z. propinquum*, *Z. eurypterum*, *F. ovalifolia* subsp. *pakistanica*, *F. bruguieri* var. *bruguieri*, *F. bruguieri* var. *laxa*, *F. indica* var. *schweinfurthii*, *F. indica* and *F. olivieri* were petiolate. *T. terrestris* was sub-sessile while other taxa were sessile.

Leaf\leaflet shape

Leaf\leaflet shape also showed variation in all studied taxa. *T. terrestris* had ovate shape. *T. longipetalus* subsp. *pterophorus*, *T. longipetalus* subsp. *macropterus*, *T. longipetalus* subsp. *longipetalus*, *T. pentandrus* and *Z. simplex* shape were oblong. *Z. propinquum* was semi-cylindrical. *P. hermala*, *F. bruguieri* var. *rechingeri*, *F. olivieri* and *F. bruguieri* var. *bruguieri* were found in lanceolate shape. *F. indica* was ovoid, *F. indica* var. *schweinfurthii* was trifoliate, *F. bruguieri* var. *laxa* was linear lanceolate, and *F. ovalifolia* subsp. *pakistanica* was oblanceolate shape leaflets.

Leaf apex

Leaf apex of *P. hermala*, *F. indica* var. *schweinfurthii* and all taxa of *Tribulus* were acute. *Z. fabago* was oval apex. *Z. propinquum* and *Z. simplex* had obtuse apex. *Z. eurypterum* was found with spatulate, *F. ovalifolia* sub sp *pakistanica* was minutely mucronate. *F. bruguieri* var. *rechingeri*, *F. bruguieri* var. *bruguieri*, *F. bruguieri* var. *laxa*, *F. indica* and *F. olivieri* had mucronate apex.

Sepal shape

Shape of sepal of *F. bruguieri* var. *rechingeri*, *F. bruguieri* var. *laxa* and all species of *Tribulus* were lanceolate. *P. hermala* had linear sepal shape. *Z. simplex* was elliptic-oblong, *F. olivieri* was oblong. Whereas ovate sepal shape were found in *F. indica*, *F. indica* var. *schweinfurthii*, *F. bruguieri* var. *bruguieri*, *F. ovalifolia* subsp. *pakistanica*, *Z. propinquum*, *Z. fabago* and *Z. eurypterum*.

Sepal apex

Sepal apex was obtuse in *Z. fabago* and *Z. propinquum*, retuse in *Z. eurypterum* and acute in all remaining taxa.

Sepal surface

Sepal surface varies from glabrous to hairy. Sepal surface of *T. longipetalus* subsp. *longipetalus* was sparsely hairy. *F. ovalifolia* subsp. *pakistanica*, *F. indica* and *F. olivieri* had glandular sepal surface. *T. longipetalus* subsp. *pterophorus*, *T. longipetalus* subsp. *macropterus*, *T. terrestris*, *T. pentandrus* and *F. indica* var. *schweinfurthii* were having hairy sepal surface. While all other remaining taxa sepal were having glabrous surface.

Sepal and petal persistence at fruit maturity

Sepal in *T. longipetalus* subsp. *pterophorus*, *T. longipetalus* subsp. *macropterus*, *T. longipetalus* subsp. *longipetalus*, *T. terrestris*, *T. pentandrus*, *P. hermala*, *Z. propinquum*, *Z. simplex*, *F. bruguieri* var. *rechingeri*, *F. bruguieri* var. *laxa* and *F. indica* were persistent at the time of maturity. While in other taxa it was not persistent. Petals in all the studied taxa were not persistence at the time of fruit maturity.

Petal shape

Petal shape varies in these studied taxa. Obovate shape of petal was found in *T. longipetalus* subsp. *pterophorus*, *T. longipetalus* subsp. *macropterus*, *T. longipetalus* subsp. *longipetalus*, *T. terrestris*, *Z. fabago*, *Z. eurypterum*, *F. ovalifolia* subsp. *pakistanica* and *F. indica* var. *schweinfurthii*. *T. pentandrus* and *P. hermala* were oblong. *Z. propinquum*, *Z. simplex*, *F. bruguieri* var. *rechingeri*, *F. bruguieri* var. *bruguieri*, *F. bruguieri* var. *laxa*, *F. indica* and *F. olivieri* were spatulate.

Petal colour

Petal colour of all studied taxa showed variation. Petals of *P. hermala*, *Z. propinquum* and *Z. simplex* were yellowish white. Petal of *Z. fabago* was yellowish above orange at the base and *Z. eurypterum* was pale white. *F. ovalifolia* subsp. *pakistanica* and *F. bruguieri* var. *bruguieri* were light pink in colour. *F. bruguieri* var. *rechingeri* was

purple, *F. bruguieri* var. *laxa* was pale pink *F. indica* var. *schweinfurthii* pinkish purple and *F. olivieri* was pinkish white. Petals of all species of *Tribulus* were yellow in colour.

Petal apex

Retuse petal apex was found in *Z. fabago*. In *Z. propinquum* it was reported spathulate/margin serrate. *Z. simplex* was having serrate margin apex. The apex of petal *T. longipetalus* subsp. *pterophorus*, *T. longipetalus* subsp. *macropterus*, *T. longipetalus* subsp. *longipetalus* and *T. terrestris* were clawed. While in remaining taxa obtuse petal apex were found.

Ovary surface

Ovary surface was hairy in all selected species of *Tribulus* and as well as all selected species of *Fagonia*. While the surface glabrous in *P. hermala* and all selected species of *Zygophyllum*.

Fruit type

Fruit type of *T. longipetalus* subsp. *pterophorus*, *T. longipetalus* subsp. *macropterus*, *T. longipetalus* subsp. *longipetalus* and *T. terrestris* were schizocarpic. Fruit of *Z. eurypterum* was capsule oblong-spherical. Loculicidal capsule type of fruits was found in all the other remaining taxa.

Fruit surface

Fruit surface in *P. hermala*, *Z. fabago*, *Z. propinquum* and *Z. eurypterum* were glabrous. *Z. simplex* fruit surface was rugulose. While all the other studied fruits type surface was Hairy to sparsely hairy.

Fruit shape

Fruit shapes vary in all selected studied taxa of Zygophyllaceae. Shape of fruits in all species of *Fagonia* was pyramidate and not winged. *Z. eurypterum* was oblong-spherical and winged. *Z. simplex* fruit shape was oblong and not winged. *Z. propinquum* fruit was oblong pyramidate and not winged. *Z. fabago* was oblong-cylindric and not winged. Fruit shape of *P. hermala* was found trigonous and not winged. *T. terrestris* was also not winged and sub spherical. All other four species were winged and discoid.

Seed shape

Seed shape in all species of *Tribulus* and *P. hermala* were triangular. *Z. eurypterum* seed shape was reniform. *Z. fabago*, *Z. propinquum* and *Z. simplex* was oblong/compressed. Seed shape of *F. indica* var. *schweinfurthii*, *F. indica* and *F. olivieri* was broadly oblong. While oblong shape type of seeds were reported in three species of *Fagonia* i.e *F. ovalifolia* subsp. *pakistanica*, *F. bruguieri* var. *bruguieri* and *F. bruguieri* var. *rechingeri*. However seeds of *F. bruguieri* var. *laxa* were obovoid.

Quantitative Analysis of Morphological Characters

Morphological characterization was also performed on the basis twenty-eight quantitative characters in seventeen species of Zygophyllaceae. Quantitative characters were further subjected to analyses through Principle component analyses (PCA).

Principle Component Analyses (PCA)

Eigenvalues

Out of 16 principal components (PCs), three viz. PC-1, PC-II, PC-III, PC-IV, PC-V and PC-VI had Eigen values >1 and contributed for 89.41% of total cumulative variability among different species (Table 3). The contribution of PC-I towards variability was highest (35.96%), PC-II contributed (15.42%), PC-III showed (13.72%) variability, PC-IV gives (13.28%), PC-V and PC-VI (6.05% and 4.92%) variability, respectively.

Table 3. Eigenvalues of Principle component analyses

	F1	F2	F3	F4	F5	F6
Eigenvalue	10.069	4.319	3.843	3.719	1.707	1.379
Variability (%)	35.960	15.424	13.725	13.281	6.095	4.924
Cumulative %	35.960	51.384	65.109	78.390	84.485	89.409

The PC-I showed 17 positive factor loadings (Figure 3) for Plant length, Petiole, Flower pedicle, Sepal length, Sepal width, Petal length, Petal width, Stamens number, Filament length, Style number, Style length, Stem internode, leaf length, leaf width, Fruit length, Fruit width, Fruit wings size, Fruit pedicel, Seed length, Seed width. PC-II indicated 15 positive factor loading for stipule width, Leaf-blade\leaflets no, flower pedicle, sepal length, Petal length, Petal width, Sepal No, Stamens number, Style number, Fruit width, Fruit wings size, Mericarp edge no, Fruit pedicel, Seed length. Quantitative characters which contributed 17 positive factor loadings towards PC-III were Plant length, Stipule width, Leaf-blade\leaflets no, Petiole, Sepal width, Petal No, Filament length, Style number, Stem internode, leaf width, Fruit length, Fruit width, Fruit wings size, Mericarp edge no, Number of fruit locules, Seed Length, Seed width. Plant length, Stipule width, Leaf-blade\leaflets no. leaflet petiole size, Petiole size, Sepal length, Petal length, Petal No, Sepal No, Stamens number, Filament length, Style number, Style length, Stem internode, leaf length, leaf width, Mericarp edge no, Number of fruit locules, Fruit pedicel were contributed 20 positive factor loadings towards PC-IV. 19 positive factor loadings towards PC-V were Plant length, Stipule length, Leaf-blade\leaflets no, leaflet petiole size, Petiole size, Flower pedicle, Sepal length, Sepal width, Petal width, Petal No, Sepal No, Filament length, Style length, Stem internode, leaf width, Fruit length, Fruit width, Fruit wings size, Mericarp edge no., and PC-VI included 17 positive factor loadings Plant length, Stipule length, Leaf-blade\leaflets no, leaflet petiole size, Petiole size, Flower pedicle, Sepal length, Petal length, Petal width, Stamens number, Style number, Fruit length, Fruit width, Number of fruit locules, Fruit pedicel, Seed size Length, Seed width.

Cluster Analysis

Clustering of studied taxa based on quantitative characters only is presented in (Figure 4). Cluster analysis grouped all taxa into 3 clusters which are further divided as Cluster-I comprised T.l.p, Cluster-II comprised of 15 taxa which are further divided into sub clusters a & b. Cluster a comprised of P.h and Z.s. Cluster b further divided into c & d. Cluster c comprised of Z.e. Cluster d have two more groups e and f. Cluster e comprised of T.t. Z.p. Z.f. T.l.m and T.l.l. while cluster f comprised of all *Fagonia* species. Cluster-III comprised of T.p.

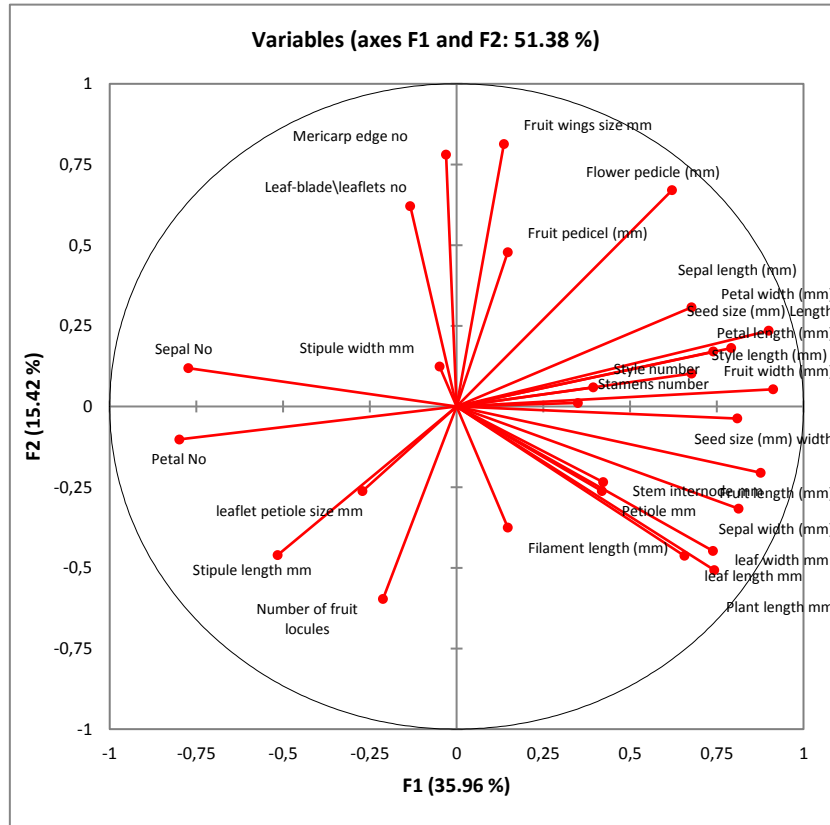


Figure 3. Correlations chart of 17 taxa of Zygophyllaceae plotted according to the first two principal components, obtained from morphological and quantitative traits

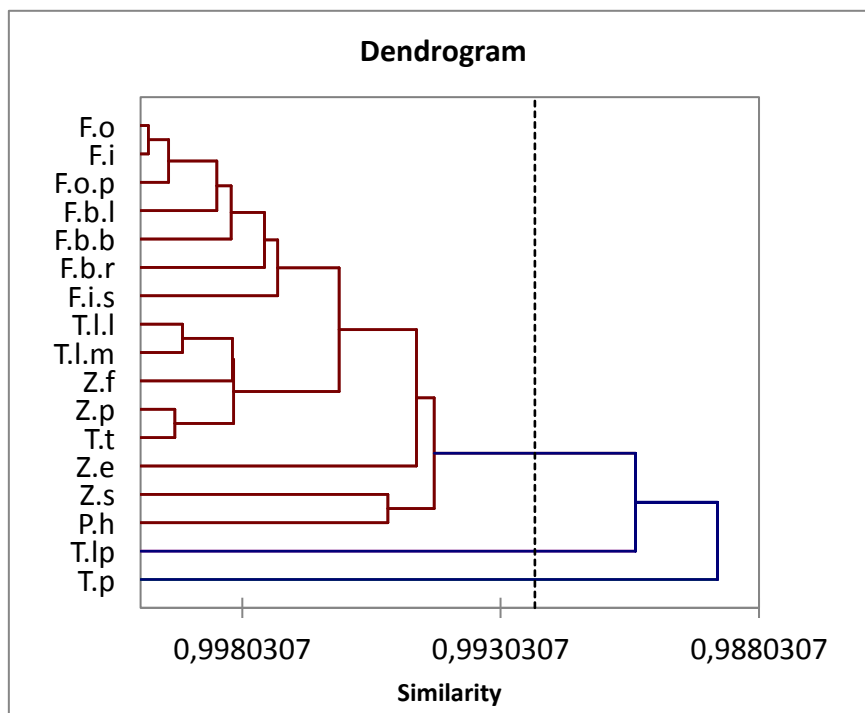


Figure 4. Tree diagram based on 28 quantitative characters in all selected taxa of Zygophyllaceae

Molecular Characterization

Using different molecular markers may target different regions of genome and could help in removing errors for the detection of polymorphism. Out of forty-one tested primers, thirty two RAPD and five ISSR revealed polymorphism, and exhibited reproducible bands among all selected taxa of Zygophyllaceae. The URP's did not give amplification in selected taxa. These taxa were not characterized earlier by different markers combination so this study would give the amended pattern of genetic variation.

Dendrogram based on molecular data grouped all genera into three main clusters A, B and C (Figure 5). Cluster A grouped into four sub-clusters a,b,c,d. Cluster a comprised of three species i.e *T. longipetalus* subsp. *longipetalus*, *T. longipetalus* subsp. *pterophorus* and *T. longipetalus* subsp. *macropterus*. Cluster b comprised of four species i.e *P. hermala*, *Z. simplex*, *Z. propinquum* and *Z. fabago*. Cluster c comprised of *F. olivieri*, *F. bruguieri* var. *rechingeri*, *F. bruguieri* var. *bruguieri*. Cluster comprised of *T. terrestris* and *T. pentandrus*. Cluster B having sub-clusters f and e contained *Z. eurypterum*, *F. indica*, and *F. indica* var. *schweinfurthii* while on cluster C *F. bruguieri* var. *laxa* collected from Uthal (Zero point) and Hingol National Park both plants were identified with same species showing interspecific diversity that may be due to change in elevation.

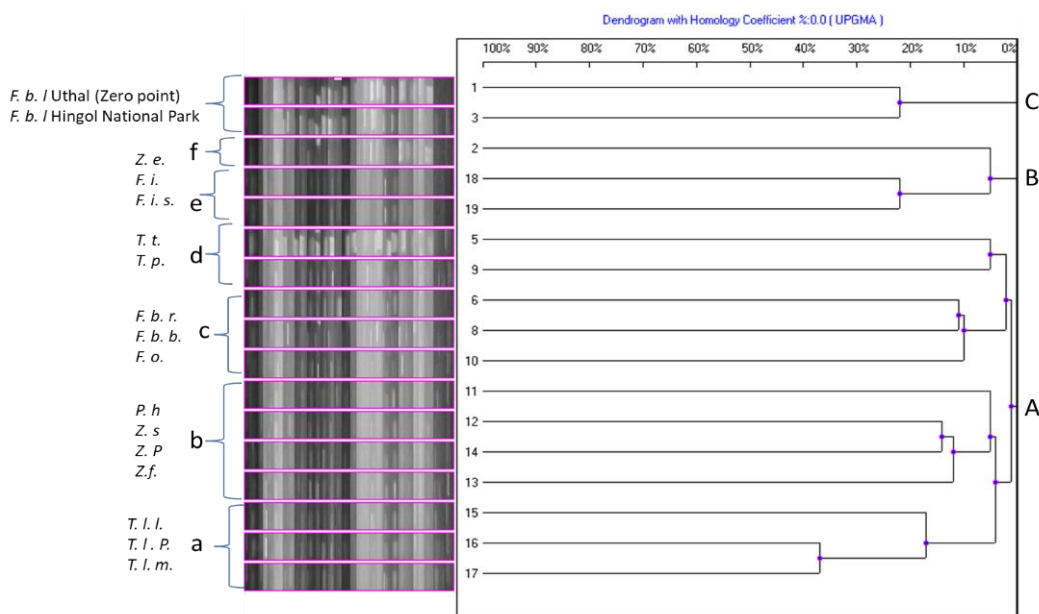


Figure 5. Cluster analysis UPGMA based on Homology Coefficient among taxa of Zygophyllaceae generated by combined tested markers

Discussion

Ecological Distribution

The species of Zygophyllaceae are widely distributed in scattered populations usually at diverse ecological zones from low to high elevations in Balochistan Pakistan. Earlier reported by Ghafoor (1974) that the plants are widespread in tropical, subtropical and warm temperate, often in drier areas and represented by 8 genera and 22 species. The present study revealed that *P. hermala* is widely distributed in all studied

sites. From high-elevation to low-elevation zones, High elevation includes Quetta, Naal, Noshki, Low elevation includes Uthal, Hingol National Park, Mushkaf, Sibi. Environmental conditions play a key role in determining the distribution and functional distinctiveness of the species occupying a particular region (Ricklefs and Latham, 1992; Noman et al., 2012). Previously, few ethnobotanical and phytochemical studies of Zygophyllaceae were reported from Balochistan by Tareen et al. (2010), Perveen and Qaiser (2008), Zaidi and Crow (2005) and Manzoor et al. (2017). *F. ovalifolia* subsp. *pakistanica* is the Endemic species to Pakistan collected from Panjgur (Ghafoor, 1974). *F. bruguieri* was earlier reported from Johan and Kalat area of Balochistan by Goodman and Ghafoor (1992) whereas in present study three sub.species of *F. bruguieri* i.e. *F. bruguieri* var. *rechingeri* from Noshki sandy Rocks, *F. bruguieri* var. *bruguieri* were collected from Panjgur, *F. bruguieri* var. *laxa* were found from Hingol National Park and Uthal. *F. indica* var. *schweinfurthii* were collected from Naal. *F. indica* were collected from Mushkaf earlier reported in plant biodiversity of Dureji in southern Balochistan Province by Perveen and Qaiser (2008). *F. olivieri* were collected from Noshki, Near Janglat area, *T. terrestris* were collected from Quetta, Mushkaf, Lasbella and Noshki earlier Goodman and Ghafoor (1992) reported from Kalat and Khuzdar. *T. longipetalus* subsp. *pterophorus* were collected from Noshki. *T. longipetalus* subsp. *macropterus* from Panjgur, *T. longipetalus* subsp. *longipetalus* from Sibi and Mushkaf, *T. pentandrus* were collected from Naal area. *Z. fabago* were found from Quetta earlier also reported by Zaidi and Crow (2005) from Quetta. *Z. eurypterum* from Dalbandeen and Noshki Goodman and Ghafoor (1992) earlier reported from Mastung, Naal and Kharan. *Z. simplex* collected from Punjgur, Naal and Noshki. *Z. propinquum* were found in Hingol National Park and Mushkaf earlier work has been done on *Z. propinquum* from Dureji in Southern Balochistan by Perveen and Qaiser (2008). Manzoor et al. (2017) also collected *Z. propinquum* from Hub District Balochistan. It was observed that the studied sites most dominated plants were succulents, often xerophytic and spiny as the area were mostly deserted. The areas have dry and warm climatic conditions, with low rate of precipitations.

Morphological Characterization

Morphological characters play an important role identification and documentation of the taxa. Earlier different studies were conducted explaining morphological identification of different taxa. Pollen morphology of the family has also been examined by Perveen and Qaiser (2006). Sheahan and Cutler (1993) investigated the vegetative anatomy of 37 species in 19 genera of Zygophyllaceae. In present study based on 57 morphological characters were recorded to study the interspecific relationships between taxa present in Balochistan, Pakistan. Plants of Zygophyllaceae reported earlier for its great medicinal importance so the morphological characterization leads towards the validation of taxa used in folk medicines. The results of cluster and principal component analysis revealed that all collected species of *Fagonia* form a well-distinguished group earlier also reported by Kadry (2012). *Fagonia* species indicated the *F.o* and *F.i* closely related and clustered with *F.o.p.* *F.b.l.*, *F.b.r.*, *F.i.s.* *Tribulus* genus was also reported from the region. Previously El-Hadidi (1975, 1977) classified the species of *Tribulus*. Mohamed (2006) studied seed morphology, proteins and Iso-enzymes in *Tribulus*. Kadry (2012) also characterized different species of *Tribulus*. In present study *T.p* clustered separately. Its closed group was *T.l.p.* while *T.l.l* and *T.l.m* were clustered together. Its closed group was *T.t.* Van Zyl (2000) presented morphological analysis of

the genus *Zygophyllum* in South Africa. Kadry (2012) also characterized different species of *Zygophyllum*. *Z.p.* grouped with *Z.f.*, *Z.e.* grouped separately, may be because of its diverse habit, plant height and woody stem. *Z.s.* grouped with *P.h.* In some previous reports, *Peganum* was placed in a distinct family (Takhtajan, 1969; El Hadidi, 1975). However, Takhtajan (1980) and El Hadidi and Fayed (1995) placed it in Zygophyllaceae. Kadry (2012) grouped *Peganum* with species of Zygophyllaceae as in present study. Shamso et al. (2013) concluded *Zygophyllum* and *Fagonia* linked in one group based on many characters.

Molecular Characterization

The molecular analysis confirmed the characterization of different genus of Zygophyllaceae. Earlier characterizations with in different genera of Zygophyllaceae have been reported from many regions of the world. Al-Arjany et al. (2014) worked on molecular diversity among three genera of Zygophyllaceae they displayed the genetic relationships between *Zygophyllum*, *Tribulus* and *Fagonia* species. Their work is in agreement with the present work here we also included *Peganum* with these three genera. All species of *Fagonia* has different pattern as they give amplification on very different pattern. To the knowledge of the authors no earlier work has been reported on the characterization of *Fagonia* species. Bakatoushi and Ahmed (2017) represented the genetic diversity in different wild populations of *Peganum* from desert of Egypt. Molecular analysis of present data of *Fagonia* exhibited no amplification in two species by the selected markers that may be due to varying base pair patterns. Similar findings were earlier reported by Al-arjany et al. (2014). Thus these two species need to be studied with additional diverse molecular markers. As the *F. ovalifolia* var. *pakistanica* is the endemic taxa of the region no earlier report has been found. No molecular characterization data on *Peganum* has been reported earlier from the region. Patil et al. (2014) characterized three species of *Tribulus*. Hammad and Qari (2010) worked on twelve populations of *Zygophyllum* and explained the genetic diversity. Wu et al. (2015) characterized different species of Zygophyllaceae. Few reports also explained the molecular characterization of Zygophyllaceae by Godoy-Bürki et al. (2018) and Kadry (2012). Finding of present study is in agreement with earlier reported work. Molecular characterization plays an important role in characterization of taxa at inter specific and infraspecific level. As these important plants used in folk medicines their authentication is very important. Furthermore, molecular characterization for evaluating genetic diversity has widely been used in conservation biology.

Conclusion and Recommendations

The selected zones of Balochistan have diverse distribution of different genera of Zygophyllaceae. These plants are well adapted in suitable habitats and soil types of these regions. Enormous morphological and molecular markers provide a better understanding in authentication of some controversial taxa that could further be exploited for a novel drug discovery. Furthermore, the unsustainable utilization of these taxa may cause a serious decline. It is thus recommended that conservation strategies must be implemented including cultivation techniques of these significant medicinal plants to fulfill the needs of local and International herbal market.

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