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Genetic diversity and population structure of *Seriphidium* Sub-genus of *Artemisia* from different terrains of Balochistan, Pakistan

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Abstract. *Rehman A, Saeed S, Ahmed A. 2021. Genetic diversity and population structure of* Seriphidium *Sub-genus of* Artemisia *from different terrains of Balochistan, Pakistan. Biodiversitas 22: 2658-2664. Seriphidium* is one of the largest sub-genus of *Artemisia* growing in different geographical populations and terrains of Balochistan Pakistan. This genus is economically important and used as folk medicine. The genus is highly variable, besides morphological characterization, molecular authentication is also needed for novel drug discovery. Three significant species of the genus viz., *S. quettense, S. oliverianum,* and *S. maritimum* were selected for morphological and molecular characterization and phytochemical investigation. Genetic variability assessment was carried out by polymorphic bands analysis by using molecular markers in 18 accessions collected from two sites, namely, Ziarat and Hazarganji-Chiltan National Park, Balochistan, Pakistan. A combined molecular marker system by using 17 Randomly Amplified Polymorphic DNA (RAPD) and 9 Inter-Simple Sequence Repeats (ISSR) amplified 231 loci, of which 111 were polymorphic (48% polymorphism). Phenolic contents and Flavonoids were estimated for phytochemical assessment. Results revealed that the highest phenolic compounds were in *S. quettense* when compared with other investigated taxa. In conclusion, molecular marker profiling together with phytochemical variation of all three species exhibited genetic diversity and chemical variation. The findings of the current investigation can further be used as a baseline study for the implementation of conservation strategies. In addition, phytochemical assessment can be utilized for modern drug discovery.

Keywords: Genetic variability, Hazarganji, phytochemicals, *Seriphidium quettense, S. oliverianum, S. maritimum,* Ziarat

INTRODUCTION

Seriphidium is the sub-genus of *Artemisia* belongs to the family Asteraceae. The habit of the genus is mostly herbs and shrubs. They are found in temperate climatic conditions frequently in dry or semi-dry habitats around the world. *Seiphidium* is the largest group within the genus Artemisia, and is considered as ecologically and economically important (Malik et al. 2017). Many species of this genus are known for their medicinal and other ethnobotanical uses. The known compounds from the genus are sesquiterpene lactones and terpenoids, making their taste bitter, producing strong aroma, and discouraging herbivores. Few species of this genus are also used as flavoring agents, food and some are used to repel insects (Shafiq et al. 2013; Ali et al. 2017). Phytochemical survey revealed that the genus *Seriphidium* produces essential oils and releases strong aroma, indicating volatile organic compounds (VOCs) (Shao et al. 2013; 2018). In recent world, these compounds are used as insecticides (Rizvi et al. 2020), bioherbicide (Shao et al. 2018), antioxidant and anticancer (Shafiq et al. 2013; Mokhtar et al. 2017; Liang et al. 2020), and many taxa are anthelmintic (Xie et al. 2019; Hussain et al. 2020). These compounds are natural and have no side effects. Pollination in this genus is through the wind-dispersed.

Seriphidium quettense (Podlech) is locally known as Zher or Terkha sperah. Synonym of *Artemisia quettensis* is a perennial, woody, aromatic shrublet endemic to Balochistan Province of Pakistan. The plant is well known for its medicinal and ornamental uses (Saeed et al. 2020).

Seriphidium oliverianum (J. Gay ex Besser) Bremer & Humphries ex Y.R. Ling, Bull. is a synonym of *Artemisia oliveriana*. A densely arachnoid-tomentose to glabrescent or ± glabrous, suffrutescent shrublet. The plant perennial and is abundantly found in Balochistan, Pakistan. Earlier various antioxidants were isolated from this plant and different biological activities investigated. Many important compounds like alkaloids, saponins, tannins, terpenoids, etc. are isolated from this species. The plant is also known for its use as anti-urease and anti-bacterial activities (Ali et al. 2017; Shafiq et al. 2017).

Seriphidium maritimum (L.) Poljakov is a synonym of *Artemisia maritimum* also found in Blochistan, Pakistan. It is also perennial and known as Sea wormwood. This species is not much used as a medicine. But some other ethnobotanical uses are well known about this species (Kumar et al. 2011).

In recent years molecular marker techniques are used to assess the genetic diversity of plants. The technique is also used for correct plant identification and the conservation of medicinally important taxa (Ahmed et al. 2020). The PCR-based methods are preferable because of their easiness and utilizing less quantities of sample DNA. The polymerase Chain Reaction (PCR) can widely be modified to perform a wide range of genetic manipulations.

Studies on a number of RAPD and ISSRs markers provide a simple, potent, rapid, reproducible and low-cost means to assess genetic variability. RAPD and ISSR markers are the best, even if no prior information of the selected taxon is available. Further, such amplification does not require genome sequence information and leads to multilocus and highly polymorphic patterns (Sara et al. 2013).

This article deals with the genetic diversity in three *Seriphidium* species from different terrains of Balochistan, Pakistan. The species selected for molecular and phytochemical investigation are medicinally important, and the results may contribute to the conservation of threatened and endemic populations of the examined species.

MATERIALS AND METHODS

Field surveys

Field surveys were carried out over a two-year period (2018-2019) in different terrains of Balochistan, Pakistan, to investigate various *Seriphidium* species. Two mountainous ranges were selected for plant collection Ziarat (2,200-3,400 m asl.) and Hazarganji-Chiltan National Park mountain range from 1600-3300 masl (Figure 1). Field methods were followed as described by (Saeed et al. 2020).

Sample collection

Plant specimens were collected (in the replicates of three), identified and described morphologically. The voucher specimens were deposited in Botany Department, University of Balochistan, Quetta herbarium for future reference (Table 1).

Morphology

A total of 21 morphological characters were observed for morphological analysis listed in Table 3. Different magnifiers were used for observations of various parts.

Phytochemical variation

For phytochemical investigation, three samples were collected from each site. Total phenolic and flavonoid contents were estimated by using Double Beam Spectrophotometer (Hitachi u-2800).

Flavonoids

The method of Ordonez et al. (2006) was used for analysis of flavonoid contents. The amount of flavonoid measured at 420 nm absorbance. Total flavonoids contents were assessed by the quercetin (mg g^{-1}) equation Y=0.0255xR2=0.9812.

Total phenolic contents

The method by Slinkard and Singleton (1977) used to analyze total phenolic content was calculated at 765 nm absorbance. Total phenolic contents were expressed as mg g^{-1} tannic acid equivalent by the equation Y=0.1216xR2=0.9365.

Figure 1. Map of Balochistan, Pakistan illustrating two study sites of plant sampling. 1. Hazarganji-Chiltan National Park, 2. Ziarat

Table 1. Name of plant species, voucher no. coordinates of study sites

Plant name	Vern. name	Voucher no.	Locality	Elevation (m asl.)	Coordinates	Flowering period
S. quettense	Zher / Tarkha	QUETTA000025	Hazarganji	2800	30.1312°N; 66.4348°E	May-November
S. oliverianum	Tirkha	QUETTA000358	Ziarat	2500	30.3810°N; 67.7270°E	August-September
S. maritimum	Tirkha sperah	QUETTA000121	Ziarat	2800	30.3810°N; 67.7270°E	August-September

Genetic diversity

The DNA extraction was carried out by using the method of Doyle (1991) with few modifications The PCR reaction was carried out by using method of Saeed et al. (2017). A total of Twenty six primers (RAPD 17 and ISSR 09) were used (Table 2).

PCR reaction

The PCR reaction for amplification was carried out in total 20 µl volume. The PCR master mix was prepared to contain 3 mM MgCl2, 1X PCR buffer, 0.2 mM dNTPs mix, 20 pmol primers, 0.01% gelatin, 1U/rxn Dream Taq DNA polymerase and 25 ng DNA of template DNA. PCR amplification was performed using Applied Biosystems 96 well (USA), thermal cycler. For RAPD primers, PCR was programmed as mentioned below in table with initial denaturation at 94 ºC for 3 min and then 36 cycles with denaturation at 94 ºC for 1 min, annealing temperature at 36 ºC for 1 min, extension step at 72 ºC for 2 minutes and final extension for 5 minutes at 72 ºC. For ISSR primers, PCR was programmed as; initial denaturation at 94 ºC for 3 min and then 40 cycles with denaturation at 94 ºC for 1 min, annealing temperature 56 ºC for 1 min, extension step at 72 ºC for 2 minutes followed by an additional final extension for 5 minutes at 72 ºC. The amplified products were checked by electrophoresis in 1.8% agarose gels containing ethidium bromide (0.5 µg ml^{-1}) in 1X TAE buffer. The product was visualized by Gel documentation system under UV light and the size of markers was estimated by comparing it to the standard ladder (100 bp BIORON 0.2 mg ml^{-1}) in the gel.

Table 2. Names and sequences of RAPD and ISSR used in analysis of genetic diversity

Name of marker	Sequence	Size of nucleotide
RAPD		
OPA-3	AGTCAGCCAC	10
OPA-7	GAAACGGGTG	10
OPA-9	GGGTAACGCC	10
OPA-15	TTCCGAACCC	10
OPA-17	GACCGCTTGT	10
OPA-18	AGGTGACCGT	10
OPB-1	GTTTCGCTCC	10
OPB-4	GGACTGGAGT	10
OPB-6	TGCTCTGCCC	10
OPB-8	GTCCACACGG	10
OPB-17	AGGGAACGAG	10
OPC-06	GAACGGACTC	10
OPC-07	GTCCCGACGA	10
OPC-11	AAAGCTGCGG	10
OPC-13	AAGCCTCGTC	10
OPC-15	GACGGATCAG	10
OPC-16	CACACTCCAG	10
ISSR		
ISSR-1	(CAC)7T	22
ISSR-2	(GA)9C	19
ISSR-5	(CAC)7GT	23
ISSR-15	(GA)8C	17
ISSR-18	(CT)8G	17
ISSR-21	(CA)8G	17
ISSR-23	(GT)8C	17
ISSR-29	(AC)8C	17
ISSR-30	(AC)8G	17

Statistical analysis of data

For analysis of genetic diversity molecular markers, data were scored as 1 (present) and 0 (absent). RAPD and ISSR data were clustered and dendrograms based on similarity matrices were calculated by using Sequential Agglomerative Hierarchical and Non-overlapping (SAHN) algorithm by "Unweighted Pair Group Method with Arithmetic Mean" (UPGMA) through Numerical by using NTSYS 2.10 (Rohlf 1998). Plant secondary metabolites (PSMs) were analyzed statistically by using XLSTAT version (2007). In the graphs, values presented are mean ± SE. M. Morphological comparison was carried out by different qualitative and quantitative characters (Table 3). Three individuals per species were observed in the biometric analysis.

RESULTS AND DISCUSSION

The present study is the first comprehensive report on the morphological, chemical, and molecular diversity of three species of *Seriphidium* the one of the largest sub-genus *Artemisia groups.* The data was collected from two high mountainous regions of Balochistan, Pakistan, i.e., Ziarat and Hazarganji-Chiltan National Park.

Morphological diversity

Life cycle and plant nature

Genus *Seriphidium* is a perennial shrub. *S. quettense* is perennial, woody and aromatic erect shrub. *S. oliverianum* is also woody and much-branched as compared to other two species. *S. maritimum* is perennial temperate shrub and aromatic similar to wormwood.

Plant height and diameter

Height of *S. quettense* is 38 to 40 cm and its diameter is 101 cm. *S. oliverianum* is 40 to 60 cm tall with large diameter 125 cm. The *S. maritimum* height range of 50 to 65 cm and diameter shorter than other two species 90 cm.

Plant surface and stem nature

Plant surface presented in Figure 2. *S. quettense* is densely whitish hairy and color is pale green. Surface of *S. oliverianum* is densely arachnoid-tomentose to glabrescent and color is brownish yellow. The *S. maritimum* stem surface is also densely hairy and greenish in color.

Leaf length and width

Leaf length and width vary in all three species. Average leaf length and width of *S. quettense* is 2.7 x 1.4 cm respectively. The length and width of *S. oliverianum* is 3.9 x 2 cm and *S. maritimum* is 2.2 x 1.2 cm, respectively.

Leaf structure

Leaf structure varies as *S. quettense* leaves punctate-glandular covered with hair, greyish-green with petiole. Leaves of *S. oliverianum* are also long-petiole and oblong in shape. Leaves of *S. maritimum* are white and pinnate-sect with numerous segments.

Flower

Capitula of *S. quettense* is numerous and sessile, shape is ovate-oblong and arranged obliquely erect. *S. oliverianum* has sessile capitulum, oblong to narrowly ovate shape, arranged towards apices. *S. maritimum* inflorescence is generally in the form of a paniculate-raceme arrangement, flat and naked or covered by hair attached with small stalks Shape of flower is ovoid and aromatic.

Flower size

Seriphidium quettense flowers are 3.5-4.5 x 2 mm long, Flowers of *S. oliverianum* are 3-4 x 1.5 mm long and the *S. maritimum* have short flowers of 2.5 x 1 mm length.

Flowering period

Seriphidium quettense flower period is between May to November, which is a comparatively long period compared with other two species. Flowering period of *S. oliverianum* is very short between August and September. The flowering period of *S. maritimum* is between October to November.

Genetic diversity

The present work is based on two different molecular markers RAPD and ISSR. These primers are used for the first time on three species of this significant genus *Seriphidium* from Balochistan. The sizes of amplified bands were 250 to 1000 bp from RAPD, and 200 to 1700 bp from ISSR. Combined molecular marker system of RAPD and ISSR were used to generate a dendrogram and cluster analysis. The present approach revealed better results and low chance of error as described earlier by (Saeed et al. 2020; Saeed et al. 2017). Out of total of 35 used makers, 26 were amplified, 17 RAPD and 09 ISSR exhibited polymorphism revealed by reproducible bands amongst various *Seriphidium* species. Table 4 explains the characteristics of banding patterns obtained by primers. The amplified primers showed 231 loci and 111 were polymorphic (48% polymorphism). The average bands scored per primers were 4.27.

Figure 2. Morphological variation of three species. A. *Seriphidium maritimum*, B. *S. quettense*, C. *S. oliverianum*

Table 3. Morphological characteristics analysis of three species of *Seriphidium*

Characters	*S. quettense*	*S. oliverianum*	*S. maritimum*
Qualitative			
Plant length (cm)	38	56	60
Plant width (cm)	101	125	96
Leaf length (cm)	2.7	3.9	2.4
Leaf Width (cm)	1.4	2	1.2
Flower length (mm)	4	3.5	2.5
Flower width (mm)	2	1.5	1
Quantitative			
Plant color	Greenish	Greenish-yellow	Whitish green
Aromatic odor	High	Low	Medium
Stem color	Pale green	Brownish-yellow	Greenish
Stem texture	Wooly	Wooly	Wooly
Margin of leaf	Entire	Entire	Entire
Leaf stipules	Present	Present	Present
Leaf shape	Lobed	Oblong	Oblong
Leaf apex	Acute	Acute	Acute
Leaf surface	Wooly	Medium wooly	Medium wooly
Leaf color	Greyish green	Greenish-yellow	Whitish
Type of leaf lobes	Pinnate-sect	Pinnate-sect	Pinnate-sect
Shape of lobes	Oblong	Oblong	Oblong
Shape of head	Ovate-oblong	Oblong to narrowly ovate	Flat
Arrangement	Arranged obliquely erect	Arranged towards apices	Raceme arrangement
Flowering period	May to November	August and September	October to November

Based on the amplified bands, data were subjected to cluster analyses shown in Figure 3. Two clusters A and B were obtained. Cluster A represented the population of *S. quettense* collected from Hazarganji-Chiltan National Park. *S. quettense* is the endemic species of the region. While Cluster B comprised of two species collected from Ziarat, i.e., *S. oliverianum* and *S. maritimum*.

Chemical diversity

Three species of *Seriphidium* were assessed for phytoconstituents. The total phenolic content (TPC) expressed as tannin equivalent and flavonoids content as quercetin equivalent within and among populations (Figure 4). The TPC ranged from 51 to 53 mg g^{-1} within the population of *SQ*, 40 to 42 mg g^{-1} within population of *SO* and 40 to 43 mg g^{-1} within population of *SM* (Table 4). Mean concentration of TPC across the populations was 45.37±1.89 mg g^{-1} whereas flavonoids showed diverse patterns within and among population. It ranged from 137 to 138 mg g^{-1} within population of *SQ*, while in population of *SO* it ranged from 131-135 and population *SM* from 122-127 mg g^{-1}. Mean concentration of flavonoids across the populations was 132±3.15 mg g^{-1}.

Discussion

In the recent era, the indigenous folk medicines and traditional uses of herbs acquired importance in primary health care system. Most of the population of world depends upon medicinal plants and the phytochemicals isolated from these plants are being used for drug discoveries. Since the wild plants used in the drug discoveries, phytochemical screening and proper identification is required. Incorrect identification of plants due to similar morphological characters may cause loss in the production of herbal drugs.

Table 4. The number and type of amplified bands generated by seventeen RAPD and nine ISSR primers examined *Seriphidium* populations.

Name of marker	Annealing temp. (°C)	Total amplified bands	Polymorphic bands	Polymorphic bands percentage
RAPD				
OPA-3	34	5	3	60
OPA-7	34	8	3	38
OPA-9	36	11	4	36
OPA-15	36	7	4	57
OPA-17	36	5	2	40
OPA-18	36	12	4	33
OPB-1	34	6	3	50
OPB-4	34	15	8	53
OPB-6	36	6	4	67
OPB-8	36	7	3	43
OPB-17	38	10	8	80
OPC-06	36	8	4	50
OPC-07	34	8	2	25
OPC-11	36	13	4	31
OPC-13	36	7	3	43
OPC-15	34	5	1	20
OPC-16	34	10	4	40
ISSR				
ISSR-1	54	5	4	80
ISSR-2	52	8	6	75
ISSR-5	54	11	6	55
ISSR-15	50	7	3	43
ISSR-18	56	15	3	20
ISSR-21	52	10	6	60
ISSR-23	50	8	4	50
ISSR-29	56	16	10	63
ISSR-30	50	8	5	63
Total		231	111	48

Table 5. Phytochemical composition representing mean±SD values

Plant code	Total phenolic (mg g^{-1})	Flavonoids (mg g^{-1})
SQ1	42.15±0.78	117.35±0.54
SQ2	41.32±1.25	112.65±0.71
SQ3	40.18±1.44	115.55±0.91
SO1	43.74±1.31	102.25±1.24
SO2	42.58±0.86	106.84±1.95
SO3	45.54±0.21	101.51±3.12
SM1	51.64±1.19	135.11±1.72
SM2	45.86±0.82	130.82±1.94
SM3	46.29±2.14	127.51±1.27
Mean average	45.37±1.89	132±3.15

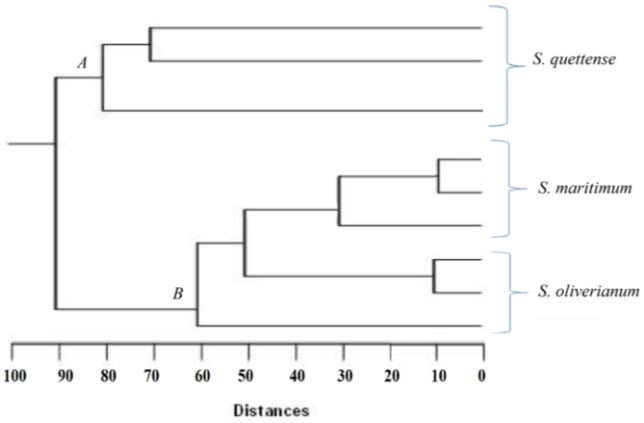

Figure 3. Cluster analyses based on molecular markers

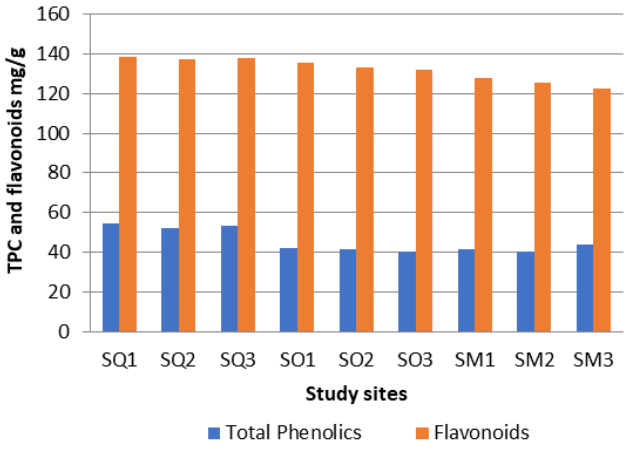

Figure 4. Patterns of phytochemical variations among different populations of *Seriphidium*

Molecular and morphological characterization resolves the misidentification of the taxa. Therefore, field surveys of *Seriphidium* plants have been undertaken in conserved region of Hazarganji-Chiltan National Park and Ziarat, Balochistan Pakistan. *S. quettense*, *S. oliverianum*, and *S. maritimum* were morphologically identified using taxonomic keys. The analysis of morphological criteria clearly reflects the differences among the populations of three Seriphidium species. Earlier *S. quettense* from various ecological zones of Balochistan was investigated (Saeed et al. 2020). Five species of *Seriphidium* from Balochistan Pakistan were characterized based on morphology (Peer et al. 2020). *S. maritimum* were characterized for its proper identification our finding is in agreement with its morphological characters (Kumar et al. 2011).

Genetic diversity plays an important role in the proper identification as well as planning of conservation of medicinally important taxa. The molecular marker system can be utilized to conservation medicinally important taxa (Saeed et al. 2016). Many researchers elucidated that change in the morphological characters may be due to harsh climate or weather. Plant adaptation property tends to alter their characteristics in response to any biotic factors. In the light of above-mentioned facts of morphological variation. The populations of *S. quettense* showed more variation within and among populations in comparison with other studied taxa that may be because the later is found in the same vicinity. This report contains genetic differences of three species of *Seriphidium* for the purpose of proper identification based on DNA fingerprinting by using RAPD and ISSR markers. A UPGMA tree exhibited the genetic distance amongst three populations of *Seriphidium* represented in Figure 2.

Results revealed a relatively high level of genetic distance of 0.80 of *S. quettens*e when compared with the populations of *S. oliverianum* and *S. maritimum* that diverged at 0.61 and are closely related. Previously, Saeed et al. (2020) presented the highest degree of genetic similarity (0.53) that occurred between *S. quettense* from two different ecological zones of Balochistan. Phylogenetic studies provided the logical basis for classification of species. In current project, morphological characterization carried out along with the phytochemical and molecular characterization of various populations.

The selected plants are found to be used for medicinal purposes, total phenols and phenolic components were present. The similar level of total flavonoids found in all three species in our study is consistent with the results obtained by a photometric method exhibiting no significant differences. Total phenolics compounds varied from 40.18-54.41mg g^{-1}. Although the average Phenolic contents and flavonoid content observed for our study species was similar to those reported (Saeed et al. 2020). Earlier different antioxidant and anticancer compounds were reported from genus *Seriphidium* (Deng et al. 2013; Mohamed et al. 2010). our results suggested that other phytochemical investigations included GC-MS or HPLC may carry out for the studied taxa for isolation of medicinally important compounds from these three species.

The purpose of phytochemical investigation is to provide information about the best and suitable species of *Seriphidium* for further analysis. Further sequence analysis is strongly recommended for the authenticity of the species.

In conclusion, the present work on three species of *Seriphidium* in Balochistan, Pakistan reported here is an initial attempt that needs to be expanded towards a broad survey to validate the distribution of other species of

Seriphidium. The ecological variations may have played a role in the genetic diversity of *Seriphidium* species in study area. Results of the study may be utilized for future sustainable conservation plans.

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