**Materials and methods**

***Sampling***

Three sampling sites of *Blepharis*, where the genus was recorded and observed to be abundant, were chosen for this study. These included: lower slopes of Kufranjah valley, Ajlun (32º14´872"N; 35º37´007"E, altitude - 169 m below sea level), Dead Sea Valley (31º41´364"N; 35º34´736"E, altitude - 397 m below sea level), and Al-Yotm valley (29º35´098"N; 35º09´462"E, altitude 623 m above sea level) (Fig. 1). *Blepharis* in these sites was readily recognized by their general growth habit and inflorescence on stony hilltops, arid and semiarid slopes, and in wadi beds on rocky grounds, runnels and fissures (Fig. 2). From a total of 19 randomly selected *Blepharis* plants in each site, shoots bearing mature inflorescences and healthy fully expanded leaves were collected during April, 2013 and used for morphological characterization, and young leaves dried in silica gel were harvested from the same plants and used for PCR-ISSR DNA studies. The latter samples were stored at -20 °C until use. Voucher specimens from all sampling sites were preserved in the Herbarium at the Department of Biological Sciences, Yarmouk University.

***Morphological studies***

Shoots collected from the three *Blepharis* populations were described and evaluated for quantitative morphological characters by counting and manually using a ruler. Measured variables included bract length and width, number of veins per bract, number of lateral spines per bract margin, length of the longest lateral spine, ratios of the longest spine length to bract width, distance between successive spikeletes, leaf length and width, number of teeth per leaf margin, length of filament and anther for each of the dimorphic anterior and posterior stamens, and appendage length of the anterior stamens (Figs.). Bracts and stamens were quantified under a dissecting scope (Wild Heerbrugg, Switzerland) and photographed using an eyepiece digital camera (OptikaSrl, Ponteranica, Italy).

***Multivariate analysis***

To test for morphological differences among sites, we used the Linear Discriminant Analysis (LDA) as implemented in the MASS package (ver. 7.3-45) in R (ver. 3.3.2). All variables were transformed to a mean of zero and unit standard deviation before analysis. Detailed methods with raw data and original R code are available through the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.**XXXX**).

**Statistical analysis**

**Univariate** **analysis**

***Molecular methods***

**DNA extraction**

Total genomic DNA was extracted from silica-dried leaf samples using a GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher-SCIENTIFIC, MA, USA). Leaves were ground to a fine powder in liquid nitrogen using a prechilled mortar and pestle and DNA was extracted following the manufacturer’s instructions. The quantity and quality of DNA were evaluated using the Nanodrop Spectrophotometer (Thermo Scientiﬁc) and on 1 % agarose gel electrophoresis for integrity.

**ISSR fingerprinting**

ISSR analysis was performed according to Bornet and Branchard (2001) using 8 primers (Table X). Amplification was carried out in 25 μl reaction mixture contained 30 ng of genomic DNA, 0.3 μM of the primer, 1X Taq DNA polymerase reaction buffer, 1.5 unit of Taq DNA polymerase and 0.2 mM of each dNTP. Amplifications were performed in Bioer-XP thermal cycler (BIOER) programmed for an initial denaturation step of 5 min at 94°C, followed by 45 cycles composed of 30 s at 94°C, 45 s annealing at 52°C, and 90 s at 72°C, and a final extension of 72°C for 5 min. Amplified products of ISSR were separated on 1.5% agarose gels in 1X TBE buffer. Fragment size was estimated using a Quick-Load® 100 bp DNA Ladder (New England BioLabsinc.) and detected by staining with ethidium bromide (10 mg/ml) according to Sambrook et al., (1989). Then, the PCR products were visualized by UV-transilluminator and photographed using gel-documentation system (BioDocAnalyze (Biometra).

**Data analysis**

For each primer, the gel was analyzed by scoring the presence or absence of ISSR bands. The presence of an amplified fragment was scored as 1, while the absence was scored as zero. All unclear bands were ignored. Primer effecincy was calculated by the number of markers obtain from each primer by the total number of markers generated by all primers. Discrimination power was calculated by dividing polymorphic markers produced from each primer by the total polymorphic markers produced (Khierallah et al.,2011). The data obtained by scoring ISSR profiles were subjected to the calculation of similarity matrix using Jaccard’s coefficients. The similarity values were used for cluster analyses. Sequential agglomerative hierarchical nonoverlapping (SAHN) clustering was applied using unweighted pair group method with arithmetic averages (UPGMA) method. Dendrograms were plotted using NTSYSpc 2.02 software (Rohlf, 1998).

**Results and Discussion**

***General morphology and distinguishing features***

*Blepharis* plants observed in their natural habitats and collected for this study showed morphological characters typical of these previously described by Feinbrun-Doathan (1978) and Vollesen (2000) (Fig.2). Generally, they were spiny, annual or perennial herbs, with erect, semi-erect to decumbent branches and bearing few basal leaves at maturity (Fig.). They were plants were distinctive in having spike-type inflorescence and (Fig.). However, spikes of plants from Kufranjah and Dead Sea valleys were lax with longer internodes than their counterparts from Al-Yotm valley, which were markedly much branched from base with compact spikelets (Fig.). Leaves were flat, lanceolate, leathery, subsessile to shortly petiolated, with dentate margins and shiny green color adaxially, and whitish-green color abaxially. Closer assessment of the leaf arrangement revealed a pseudo-whorl type phyllotaxy, where two pairs of leaves at a node are situated just above each other (Vollesen, 2000). For true whorled phyllotaxy, three or more leaves at a node must be initiated simultaneously (Lee et al., 2009).

On the plant, regardless of the site, green spikes with different stages of flower and seed development in addition to dead spikes from prior years containing mature capsules were observed (Fig.; Gutterman, 2002). In addition, green fertile spikes re-sprouting distal to older spikes were seen on plants found in Kufranjah and Dead Sea valleys (Fig.). This feature has not been observed in plants collected from Al- Yotm valley.

Flowers were violet, purple, mauve, rarely white, subtended by leathery, recurved, canaliculate, veined, and spiny bracts (Figs.). In *Blepharis* plants gathered from Kufranjah and Dead Sea valleys, bracts were narrower, longer, and with mostly less number of stout lateral spines compared to their counterparts in plants from Al-Yotm valley (Fig., quantified below). Also, the longest lateral spine in the former was seen longer than the bract width compared to the latter, in which spine length did not exceed in length (occasionally shorter) the bract width (see quantitative data below). Our observations are consistent with the diagnostic characters previously outlined by Feinbrun-Doathan (1978).

The morphology of stamens in *Blepharis* is a principal distinctive features at the species level (Vollesen, 2000). Two pairs of stamens were found, the anterior pair were laterally flattened, hairy toward the base, and bearing a notable, flattened-like appendage distally. By contrast, the posterior pair were slightly narrower, curved with knee-like at the base and unappendaged (Fig.).

The fruits were explosively-dehiscent ellipsoid capsules (Fig.). Each is 2- loculated, with a pair of seeds borne on retinacula (arrowhead). Seeds were flat, ovate, and coated with hygroscopic hairs that swell up, outspread, and become mucilaginous when wetted (Fig.). This adaptation has been regarded as an efficient strategy that facilitates dispersal and germination of *Blepharis* when there is adequate rainfall, thus contributing to its success in desert habitats (Vollesen, 2000; Gutterman, 2002).

***Multivariate analysis***

Our LDA successfully distinguished morphological features of plants collected from all three sites, with a significant difference among group means along axis 1 (*F* = 509.4, *P* < 0.001). Plants from the Al-Yotm valley were morphologically distinct primarily due to larger bract widths and shorter internodes, with no overlap in 95% ellipses (Fig.). Plants from the Kufranjah and Dead Sea valleys were significantly different along axis 2 (*F* = 14.0, *P* < 0.001). On average, Kufranjah plants possessed more veins per bract, with longer anterior appendage but shorter anterior filament and shorter spine than plants from the Dead Sea valley. However, 95% ellipses also overlap for these populations (Fig.).