*Pak. J. Bot.*, 50(2): 575-578, 2018.

# CHEMOTAXONOMY AND SEED MORPHOLOGICAL STUDIES OF THE GENUS *MELILOTUS* MILL. FROM PAKISTAN

**RUBINA ABID[1*], SYED ABID ALI[2], ELSA SAEED[1], IQRA MUNIR[2], SYEDA UROOSA HASHMI[2], AFSHEEN ATHER[1] AND M. QAISER[1]**

*[1] Department of Botany, University of Karachi, Karachi-75270, Pakistan*
*[2] HEJ Research Institute of Chemistry, International Centre for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi-75270, Pakistan*
*[*]Corresponding author's email: rubinaku@yahoo.com*

**Abstract**

The genus *Melilotus* Mill. of the family Fabaceae is investigated for its seed characters and chemotaxonomy. The seed morphological data, distinct proteins, peptide fingerprinting and elemental composition provide the additional tools for the specific delimitation within the genus *Melilotus* from Pakistan.

**Key words:** *Melilotus*, Pakistan, Seed morphology, Proteins, Elemental analysis.

## Introduction

The genus *Melilotus* belongs to the tribe Trifolieae of the sub family Papilionoidae (Leguminosae) (Berchtold & Presl, 1820; Polhill & Raven, 1981). Since last few decades, seed morphological characters were significantly used to strengthen the taxonomic delimitation at various levels (Nasir, 1985, 1986; Qaiser, 1987; Abid *et al*., 2015, 2016). Moreover, Capitaine (1912) studied the family Fabaceae for the seed characters and concluded that these characters were much useful for classification and identification of taxa at different levels. Similarly, Güneş & Cirpici (2011) examined seed characteristics including surface patterns in some taxa of the genus *Lathyrus* from Turkey. While, Özbek *et al.,* (2014) also studied the seed morphology of *Melilotus* species from Turkey. Seed characters were also used to find out the phenetic relationship between the genera of the family Fabaceae (Salimpour *et al.,* 2013; Khandani *et al.,* 2016). Moreover, elemental analyses of the seeds of various species were also carried out, such as, Juknevičius & Sabienė (2007) reported the mineral elements of the various species of the family Fabaceae. While, Bahadur *et al.* (2011) and Ghani *et al.* (2014) studied the elemental analysis of fodder and medicinally important plants including the some members of the family Fabaceae. Likewise, protein and peptide fingerprinting were also found useful for the identification of various species within the family Fabaceae. (Jackson *et al.,* 1967; Mathesius *et al.,* 2002). Inspite of the above mentioned reports, there is no detailed account present on seed morphology and chemotaxonomy of the genus *Melilotus*. Therefore, present study was under taken to provide the strength to the taxonomic delimitation of the genus *Melilotus* from Pakistan.

## Materials and Methods

**Seeds:** Mature seeds of *Melilotus alba, M. indica, M. messanensis* and *M. officinalis* were collected from field as well as from herbarium specimens. Mostly 10 to 15 seeds/plants and 10 plants/species were studied (depending on the availability of material) and examined under stereomicroscope (Nikon XN Model) and scanning electron microscope (JSM-6380A). For scanning electron microscopy dry seeds were directly mounted on metallic stub using double adhesive tape and coated with gold for a period of 6 minutes in sputtering chamber and observed under SEM. The terminology used is in accordance to Berggren (1981) and Stearn (1983) with slight modifications. Seed macro and micro morphological characters viz., shape, surface patterns, colour, size and position of hilum were observed. Elemental characterization was performed by energy dispersive X-ray spectrometry (EDXS) mode which can count emission peaks to 9335 counts per second. Data was analyzed by EDS-SEM associated analytical program EDS Analysis Station.

**Extraction and estimation of proteins and peptides:** Seeds (10-50) were crushed on a watch glass and transferred into 2 ml Eppendorf tubes. After grinding seeds were directly subjected for extraction in extraction buffer (i.e., 50% ACN and 1% TFA in Milli-Q water). Each extraction was performed for 1 hr at 350 rpm at 37ºC using a Thermomixer comfort (Eppendorf Hamburg, Germany). The extracts were centrifuged at 14,000 rpm for 15 min (Smart 15 Micro-centrifuge Hanil, Korea) and the supernatant was collected. The supernatants were completely air dried using Speedvac concentrator-5301 (Eppendorf Hamburg, Germany) and directly reconstituted in equilibration buffer (0.1% TFA-$H_2O$, Buffer-A). Total protein concentration of all extracts was measured by the modified dye-binding assay of Bradford (1976) using bovine serum albumin (Merck Munich, Germany) as a standard. Measurements were performed in triplicate in a microplate reader (Sunrise-Tecan Salzburg, Austria).

**SDS PAGE & RP FPLC analysis:** For the proteins and peptides fingerprinting, crude seed extracts (*ca.* 20 µg) was subjected to SDS-PAGE analysis under dissociating and denaturing conditions (pH 8.8 in the presence of SDS and β-mercaptoethanol). The proteins and peptides were separated in 14% resolving and 5% stacking gels at 140 V for 1.5 hr and at the end of electrophoresis the gel was stained with 0.2% Colloidal Commassie Blue G-250 as recently described by Abid *et al.* (2014). For complementing the SDS-PAGE results, same samples (*ca.*

100 µg) were also subjected for reversed phase fast protein liquid chromatography (AKTA-design Amersham Biosciences, Buckinghamshire, UK) using a reversed phase FPLC column (µRPC C2/C18, ST 4.6/100; Amersham Biosciences, UK). The column was equilibrated in 0.1% TFA-$H_2O$ (Buffer-A) and eluted with a short linear gradient (60% B in 20 min) of 100% ACN containing 0.1% TFA (Buffer-B). The flow rate was maintained at 1 min/ml and the elution was monitored at 280 nm. The data was analyzed by the automated software UNICORN 5.0 (Amersham Biosciences, UK).

**Observations**

**General seed characters of the genus *Melilotus*:** Seeds sub-orbicular, obliquely oblong or broad elliptic, 1.9-2 x 1-1.8mm, apex rounded, obtuse or truncate, base rounded or obtuse, brown, yellowish brown or greenish brown, surface ruminated colliculate, colliculate or verrucate, hilum lateral in position (Fig. 1; Table 1).

Represented by 4 species *M. alba* Desr., *M. indica* (L.) All., *M. messanensis* (L.) All. and *M. officinalis* (L.) Pall.

**Key to the species**

1 + Seeds yellowish brown, surface ruminated colliculate ……... 2
- Seeds greenish brown or brown, surface verrucate or colliculate ……..……..... 3
2 + Seeds sub-orbicular ……..…….. *M. alba*
- Seeds broad elliptic ……...……... *M. officinalis*
3 + Seeds sub-orbicular with verrucate surface pattern ……..…… *M. indica*
- Seeds obliquely oblong with colliculate surface pattern ……...……..….. *M. messanensis*

**Table 1. Seed morphological characters of the genus *Melilotus* Mill.**

| Name of taxa | Size(mm) | | Shape | Apex | Base | Colour | Surface | Hilum |
|---|---|---|---|---|---|---|---|---|
| | Length | Breadth | | | | | | |
| *Melilotus alba* | 2 | 1.8 | Sub-Orbicular | Truncate | Rounded | Yellowish brown | Ruminated colliculate | Lateral |
| *M. indica* | 1.9 | 1.8 | Sub-Orbicular | Truncate | Obtuse | Greenish brown | Verrucate | Lateral |
| *M. messanensis* | 1.9 | 1 | Obliquely Oblong | Rounded | Rounded | Brown | Colliculate | Lateral |
| *M. officinalis* | 2 | 1.8 | Broad elliptic | Obtuse | Rounded | Yellowish brown | Ruminated colliculate | Lateral |

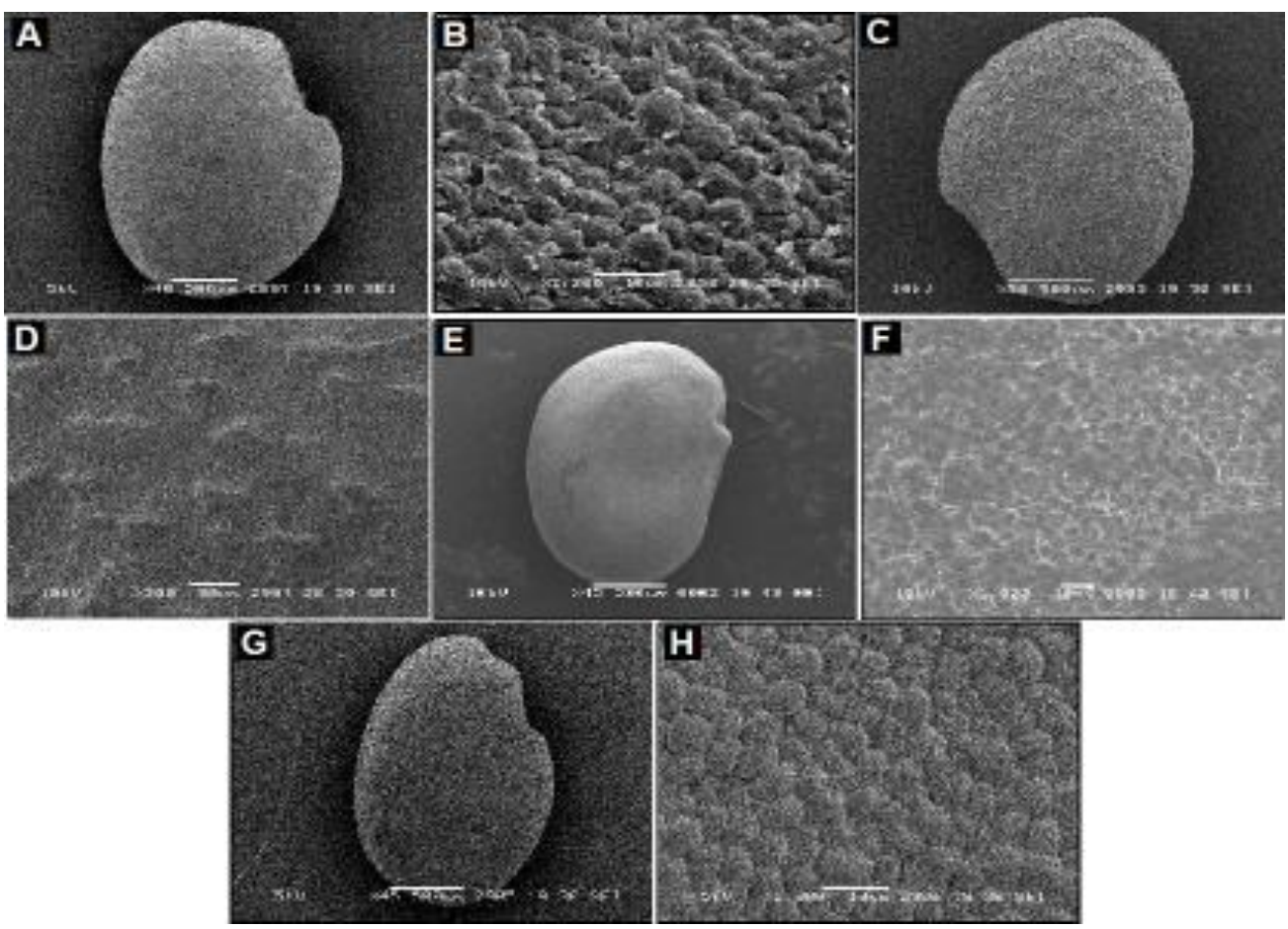


Fig. 1. Scanning electron micrographs. *Melilotus alba:* A, seed; B, surface. *M. indica:* C, seed; D, surface. *M. messanensis:* E, seed; F, surface. *M. officinalis:* G, seed; H, surface (Scale bars: A, C, E, G=500 µm; D=50 µm; B, F, H=10 µm).

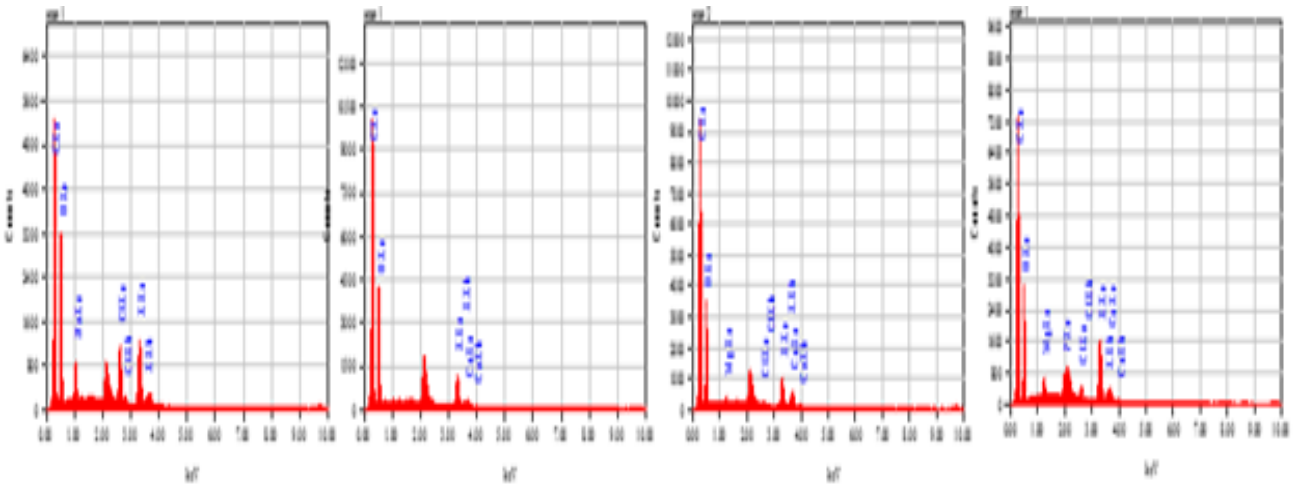

Fig. 2. EDX spectroscopic analysis of the seeds of *Melilotus* species.

## Discussion

The infra familial classification of the family *Fabaceae* has been the matter of interest to various scientists. To resolve the taxonomic status of the taxa within family Fabaceae several studies were carried out on different aspects such as seed morphology (Salimpour *et al.,* 2013; Khandani *et al.,* 2016) and chemotaxonomy like elemental analysis, protein and peptide finger printing (Jackson *et al.,* 1967; Mathesius *et al.,* 2002; Juknevičius & Sabienė, 2007; Ghani *et al.,* 2014). On the basis of seed morphological data, four species of the genus *Melilotus* were splitted into two groups. First group, having yellowish brown seeds with ruminated colliculate surface pattern comprises *M. alba* and *M. officinalis* (Fig. 1; Table 1). While, the remaining 2 species viz., *M. indica* and *M. messanensis* are grouped by having brown or greenish brown seeds with verrucate or colliculate surface pattern. The grouping of species based on seed morphology may be well correlated with gross morphology of the genus *Melilotus.* Such as, *M. alba* and *M. officinalis* were reported with 3.5-7 mm long corolla and entire stipules (Ali, 1977).Whereas, *M. indica* and *M. messanensis* are characterized by having dentate stipules or entire stipules with c.2 mm long corolla (Ali, 1977). Furthermore *M. indica* and *M. messanensis* may also remain distinct having different seed shape and surface pattern, such as, *M. indica* is characterized with sub-orbicular seeds with verrucate pattern while, *M. messanensis* have obliquely oblong seeds with colliculate surface pattern (Fig. 1; Table 1). Likewise, *M. alba* could be separated from *M. officinalis* by having sub-orbicular seeds and white flowers (Ali, 1977) while *M. officinalis* have broad elliptic seeds and yellow flowers (Ali, 1977). The data of elemental analysis was also useful to some extent for specific delimitation as *M. messanensis* has P and Cu (Fig. 2; Table 2) while these elements were totally absent from rest of the species. Similarly, Na and Si were present in *M. indica* and *M. officinalis* but missing from *M. alba* and *M. messanensis.* However, the concentrations of these elements show slight variation but could not be significant enough for specific delimitation.

**Table 2. Elemental composition of the seeds of *Melilotus* species determined using energy-dispersive X-ray spectroscopy (EDXS) using the associated analytical program EDS analysis station.**

| Elements | *M. indica* | *M. alba* (% mass)** | *M. officinalis* | *M. messanensis* |
|---|---|---|---|---|
| C | 47.12 | 49.71 | 50.23 | 48.76 |
| O | 42.07 | 45.10 | 43.20 | 36.65 |
| Na | 2.84 | - | 0.63 | - |
| Mg | 0.45 | 0.41 | 0.56 | 1.41 |
| Al | 0.54 | 0.86 | 0.78 | 0.4 |
| Si | 0.64 | - | 1.86 | - |
| Ca | 1.57 | 0.92 | 1.86 | 2.67 |
| K | 4.22 | 3.69 | 2.67 | 8.25 |
| Cl | 2.44 | 0.42 | 0.38 | 1.14 |
| P | - | - | - | 0.71 |
| Cu | - | - | - | 1.49 |

**Results presented are means of three independent measurements

**Seed proteins and peptides finger printing:** In order to gain biochemical insight, mature seeds of *M. indica, M. alba* and *M. officinalis* were subjected to proteins and peptides finger printing analysis using 14% SDS-PAGE under dissociating-denaturing conditions (Fig. 3a). Subtle variations in proteins and peptides band pattern in molecular weight ranges from 6.5 to 66.2 kDa were observed among all the studied species (Fig. 3a). However, *M. alba* having more bands as compared to *M. indica* and *M. officinalis* respectively. Unfortunately, due to the small size and low protein contents in *M. messanensis* we could not get some results for comparison but we anticipate high intra-species variations. In order to complement the SDS-PAGE data, the same samples were also subjected to RP FPLC analysis. Very well-resolved proteins and peptides separation profiles were observed (Fig. 3b) which confirmed our aforementioned results. The three species *M. indica, M. alba* and *M. officinals* could be ideally distinguished from each other at the end of elution profiles by high molecular mass protein fractions (retention time 15-20 min). Likewise, the subtle differences among the three species have also been observed in the peak pattern of hydrophilic peptides in the unbound region (i.e. retention time 0–7 min).

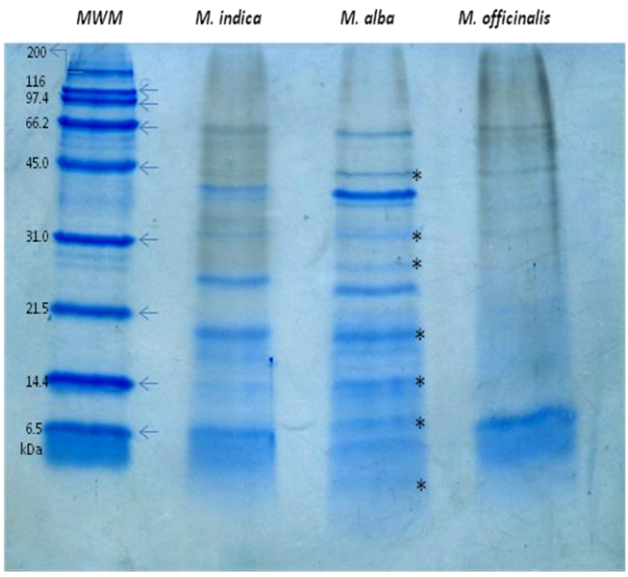


Fig. 3a. Proteins and peptides figure printing of the seeds of *Melilotus* species using 14% polyacrylamide gel electrophoresis under dissociating and denaturing (pH 8.8 in presence of SDS and ß-mercaptoethanol) conditions.

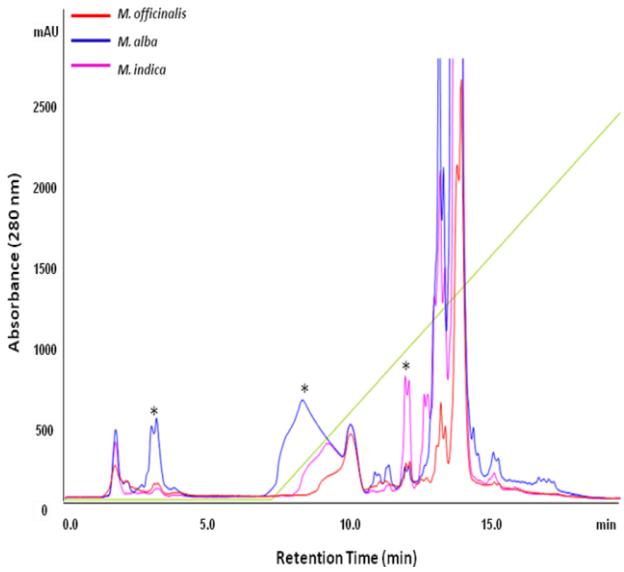


Fig. 3b. RP FPLC analysis of the same samples on µRPC C2/C18 column. Asterisks indicates the subtle variations in band pattern and their elution profiles, respectively.

**References**


Abid, R., A. Ather and M. Qaiser. 2016. The Seed Atlas of Pakistan-. Papaveraceae. *P. J. Bot.*, 48(3): 1035-1044.

Abid, R., D. Kanwal and M. Qaiser. 2015. The Seed Atlas of Pakistan-X. Cucurbitaceae. *Pak. J. Bot.*, 47(2): 429-436.

Abid, R., S.A. Ali, I. Munir and M. Qaiser. 2014. Hybridization in *Sida ovata* complex (Malvaceae) III. Evidences from seed micro-morphology and seed protein analysis. *Plant Biosys*., 148(5-6): 1027-1031.

Ali, S.I. 1977. Papilionaceae In: (Eds.): E. Nasir & S.I. Ali. Flora of Pakistan. No. 100. Department of Botany, University of Karachi and NARC, Islamabad.

Bahadur, A., Z. Chaudhry, G. Jan, M. Danish, A. Rehman, R. Ahma, A. Khan, S. Khalid, Irfan Ullah, Z. Shah, F. Ali T. Mushtaq and F.G. Jan. 2011. Nutritional and elemental analyses of some selected fodder species used in traditional medicine. *Afr. J. Pharm. & Pharm.*, 5(8): 1157-1161.

Berchtold, F.G.V. and J.S. Presl. 1820. Tribe Trifolieae. O. Prirozenosti Rostlin. Praha: K. W. *Endersa*, pp: 230.

Berggren, G. 1981. *Atlas of seeds, and small Fruit of Northwest European plant species, Salicaceae-Cruciferae. Part 3.* Swedish Museum of Natural History, Stockholm.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72: 248-254.

Capitaine, L. 1912. *Les graines des Légumineuses*. Paris, Larose-Lechevalier.

Ghani, A., Z. Ali, T. Islam, S. Sanaullah and S. Saeed. 2014. Nutrient evaluation and elemental analysis of four selected medicinal plants of soon valley Khushab, Punjab, Pakistan. *Pak. J. Pharm. Sci.*, 27(3): 597-600.

Güneş, F. and A. Cirpici. 2011. Seed characteristics and testa textures some taxa of genus *Lathyrus* L. (Fabaceae) from Turkey. *Int. J. Agric. Biol.*, 13 (6): 1814-9596.

Jackson, P., J.M. Milton and D. Boulter. 1967. Fingerprint patterns of the Globulin fraction obtained from seeds of various species of the Fabaceae. *New Phytol.*, 66: 47-56.

Juknevičius, S. and N. Sabienė. 2007. The content of mineral elements in some grasses and legumes. *Ekologija*, 53(1): 44-52.

Khandani, S., M. Assadi, T. Nejadsatari and I. Mehregan. 2016. Phenetic analysis of the genera Medicagoid *Trigonella, Medicago and Melilotus* (Fabaceae) on seed coat in Iran. *Biodiversitas*, 17(1): 162-171.

Mathesius, U., N. Imin, H. Chen, M.A. Djordjevic, J.J. Weinman, S.H. Natera, A.C. Morris, T. Kerim, S. Paul, C. Menzel, G.F. Weiller and B.G. Rolfe. 2002. Evaluation of proteome reference maps for cross-species identification of proteins by peptide mass fingerprinting. *Proteomics*, 2(9):1288-303.

Nasir, Y.J. 1985. Seed Studied in the *Androsace* L. (Primulaceae) species Found

Nasir, Y.J. 1986. Seed Studied in the *Primula* species (Primulaceae) Found in Pakistan with special reference to taxonomy. *Willdenowia*. 15: 475-483.

Özbek, F., M.U. Özbek and M. Ekici. 2014. Morphological, anatomical, pollen and seed morphological properties of Melilotus bicolor Boiss. & Balansa (Fabaceae) endemic to Turkey. *A. J. C. S*., 8(4):543-549.

Polhill, R.M. and P.H. Raven 1981. Advances in Legumes Systematics, R. Bot. Gard. Kew.

Qaiser, M. 1987. Studies in the seed morphology of the family Tamaricaceae from Pakistan. *Bot. Jour. Lin. Soc*., 94: 469-484.

Salimpour, F., M. S. Ardebili and F. Sharifnia. 2013. Phylogenetic relationships between *Trigonella* species (Bucerates section) using ITS markers and morphological traits. *J. Bio. & Env. Sci.*, 3(12): 116-124.

Stearn, T.W. 1983. *Botanical Latin, 3rd edition* David & Charles Britain.


(Received for publication 15 January 2017)