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Phylogenetic and Palynological Study of the Genus *Epilobium* L. (Onagraceae) in Kurdistan Region-Iraq

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Abstract

The phylogeny of the species of *Epilobium* in Kurdistan Region-Iraq was investigated by using six in-group species: *E. anatolicum* Hausskn. subsp. *anatolicum*, *E. hirsutum* L., *E. obscurum* Schreber, *E. parviflorum* Schreber, *E. rechingeri* Raven., *E. tetragonum* L. and one out-group related genus: *Ludwingia grandiflora* (Michx.) Greuter & Burdet, based on *trn*L-F intergenic region of chloroplast DNA and internal transcribed spacer (ITS) of nuclear ribosomal DNA. In addition, the pollen morphology of seven species of *Epilobium* (the previous mentioned species with *E. ponticum* Hausskn.), was examined by using Light Microscope (LM) and Scanning Electron Microscope (SEM). Individual analysis of *trn*L and ITS sequence data indicated monophyly of the genus *Epilobium*, the results of bayesian and maximum parsimony displayed two clades of *Epilobium* with high supports (bs=100%, pp=1.00). The pollen grains usually tetrads, radially symmetrical, isopolar, numerous, yellow, five species appeared oblate whereas *E. rechingeri* and *E. tetragonum* were suboblate; the polar view was triangular and the equatorial view was ellipsoid; their sizes were medium (medium-large in *E. hirsutum* and *E. parviflorum*). The apertures were triporate. The exine sculpturing were not obvious.

Keywords: Phylogenetic, Palynological, *trn*L-F, ITS, *Epilobium*, Kurdistan, Iraq

1. Introduction

Onagraceae are a well-defined family of flowering plants, consisting of seven tribes, 16 genera, and approximately 652 species of worldwide distribution (Raven, 1988). Onagraceae are highly distinctive, forming a monophyletic group defined by the following synapomorphies: the presence of abundant raphides in the vegetative cells (Dickison, 1975, Metcalfe and Chalk, 1950); "paracrystalline beaded" pollen ektexine (Raven, 1976); viscin threads or ektexinous strands on the proximal pollen wall (Hoch et al., 1993, Patel et al., 1984)

In Iraq, the genus *Epilobium* has not been dealt with extensive studies as the other Iraqi plant genera which they also in need to phylogenetic and palynological studies, where the Flora of Iraq is still in need to this type of study. However, all of its species grow in Kurdistan region of Iraq. The species of the genus *Epilobium* which grow naturally and mainly in Kurdistan are: *E. anatolicum* subsp. *anatolicum*, *E. hirsutum*, *E. obscurum*, *E. parviflorum*, *E. ponticum*, *E. rechingeri* and *E. tetragonum.*

The relationship between Epilobieae and the rest of Onagraceae was considered enigmatic (Baum et al., 1994), but recent cladistic analyses of both molecular and morphological data support a close relationship between Epilobieae and the other estipulate tribe, Onagreae (Hoch et al., 1993, Sytsma et al., 1991, Chase et al., 1993). Indeed, apart from the basal position of Ludwigia (supported by all cladistic

analyses), an Onagreae plus Epilobieae clade is the best supported phylogenetic relationship in the family (Levin et al., 2004, Conti et al., 1993)

Over the past few decades, the family has developed as a model system for studying plant evolution. Comparative studies of cytology, embryology, palynology, anatomy, morphology, reproductive biology, and chemistry have all been completed for various groups within the family (Raven, 1988). Unfortunately, a limitation of these previous studies has been the absence of a robust phylogenetic framework within which to examine the evolution of these traits.

The genus *Epilobium* is remarkable for its morphological, ecological and cytological diversity. This variation is manifested primarily at the sectional level with eight sections being recognized. The sections are highly distinctive showing variation in vegetative and floral morphology, anatomy, palynology, cytology, phytochemistry, biogeography, ecology, and breeding systems (Raven, 1976). However, in the absence of a phylogeny for *Epilobium* the pattern of evolution of these characters cannot be resolved (Baum et al., 1994).

The genus *Epilobium* is distinguished from the rest of the family by the combination of dot like, heteropycnotic chromosomes found also in Ludwigia (Skvarla et al., 1975)

Comparative studies of pollen morphology have provided useful characters for delimiting genera and species and resolving relationships (Yurtseva et al., 2014)

Recent studies in the Onagraceae family using Scanning Electron Microscopy (SEM) revealed a range of exine sculpturing patterns (Eide, 1981, Chung et al., 2010, Hebda and Chinnappa, 1990). that were informative for resolving phylogenetic relationships (Wen and Nowicke, 1999) and for delimiting genera and/or species.

The genus *Epilobium* with about 185 species throughout the world (Raven, 1976), it is a very difficult group taxonomically because of its fairly uniform external appearance and the high possibility of hybridization among almost all species (Akbari and Azizian, 2006). In addition to phenetic characteristics, pollen features have been employed as important characters within the genus *Epilobium* (Akbari and Azizian, 2006). In recent years the relationships among *Epilobium* species have been explored in various studies, most of them focused on pollen features. Pollen grains of Onagraceae received much attention in early works (Erdtman, 1952). but these studies considered only brief stages. Recent palynological studies on *Epilobium* focus mainly on the development of microspores in mature pollen grains (Keri and Zetter, 1992), and exine structure (Rowley and Claugher, 1996).

Pollen taxonomy of *Epilobium* have been done by (Makbul et al., 2008). However, little attention has been paid to pollen characteristics of Onagraceae in Iraq. There are several challenges in taxonomy of Onagraceae such that SEM techniques have been used to study the evidence related to Iraqian Onagraceae (Yurtseva et al., 2014).

The present study aimed to examine the phylogenetic and palynological properties of Iraqi *Epilobium* and to evaluate their importance as taxonomic characters.

2. Materials and Methods

2.1. Taxon sampling

The plant taxa used in the present study were collected from the different districts of Kurdistan region-Iraq, as well as the preserved specimens in the Herbaria of College of Education and College of Science/Salahaddin University. Seven distinct

taxa consist of six ingroup taxa and one out group *Ludwigia grandiflora* were used in the analysis. The outgroup sequence was obtained from gene bank (Accession number KX168324 and KX168324.1 for *trn*L-F gene and ITS region respectively).

2.2. DNA extraction

Total DNA was extracted from the collected specimens. The extraction method was based on the CTAB protocol of Doyle and Doyle (1990) with somemodification (1X CTAB: 10 mL of 1.0 M Tris-HCl, PH 8; 4 mL of 0.5 M EDTA, PH 8; 28 mL of 5 M NaCl; 2% CTAB; 2 g PVP; and 158 ddH2O), the washing process of the DNA pellet has been conducted twice with 0.5 mL of 80% ethanol, then DNA was dissolved in 25 µl TE-buffer.

2.3. PCR and DNA sequencing

The two noncoding regions of nrDNA and cpDNA were amplified by using the primers trn-C and trn-F of Chen *et al*. (2010), and ITS-A and ITS-B of Taberlet *et al.* (1991) for trnL-F intergenic spacer and ITS region respectively (Table 2). The primers were ordered from Macrogen Company, Seoul, Korea. The total volume of amplification reactions was 25 µL and Master Mix made up of 12.5 µL, 3 µL genomic DNA extract, 2 µL of each primer, 5.5 µL free nuclease water. The PCR-Thermal cycler for trnL-F gene started with 5 min for initial denaturation at 94 C° followed by 35 cycles: denaturation at 94 C° for 30 sec.; annealing at 54 C° for 60 sec.; extension at 72 C° for 60 sec. and the final extension at 72 C° for 5 min. While, the PCR program for ITS gene started with 5 min for initial denaturation at 94 C° followed by 35 cycles: denaturation at 94 C° for 30 sec.; annealing at 56 C° for 20 sec.; extension at 72 C° for 20 sec. and the final extension at 72 C° for 5 min. The resultant PCR products were checked on 1.5% agarose gel run in TAE buffer. The gel was stained with Safe red dye and photographed under UV transilluminator. PCR products were purified by using Kits (Promega company-Madison-USA). The purified PCR products were sent to the National Science and Technology Development Agency (NSTDA) in Thailand for sequencing

Table 1: Specimen numbers of *Epilobium* species which their DNA and pollen grains have been studied, and their preserved locations in the Herbarium of College of Education/ Salahaddin University with collection date

Species	Specimen number & Herbarium symbol		Specimen location	Date of collection
E. anatolicum subsp. *anatolicum*	7594	ESUH	Hasarost M.	12.7.2017
E. hirsutum	7977	ESUH	Kory valley	7.11.2021
E. obscurum	7984	ESUH	Qandil M.	25.8.2016
E. parviflorum	7991	ESUH	Gali Ali Beg	9.10.2021
E. ponticum	7998	ESUH	Halgurd M.	1.9.2020
E. rechingeri	8005	ESUH	Balayan valley	4.8.2020
E. tetragonum	8012	ESUH	Hasarost M.	14.9.2021

Table 2: list of primers and their sequences that have been used in the study

Primer	Product size	Direction	Sequence 5'---- 3'	Resources
trn-C	600 bp	Forward	ATT TGA ACT GGT GAC ACG AG	(Chen *et al.*, 2010)
trn-F		Reverse	CGA AAT CGG TAG ACG CTA CG	(Chen *et al.*, 2010)
ITS-A	400 bp	Forward	ATG CGA TAC TTG GTG TGA AT	(Taberlet *et al.*, 1991)
ITS-B		Reverse	TCC TCC GCT TAT TGA TAT GC	(Taberlet *et al.*, 1991)

2.4. Sequence alignment

All the DNA sequences were edited and aligned with ClastalW option available in BioEdit, Version 7.0.4.1 (Hall, 2001) and manual adjustment, there are 7 accessions for each *trn*L-F and ITS regions, including the outgroup species.

2.5. Phylogenetic analysis

a. Parsimony analysis

The reconstruction of the phylogenetic relationships was based on Maximum Parsimony (MP) methods. The analysis was carried out for separate regions. MP analysis was performed by using PAUP* version 4.0a164 (Swofford, 2000). Using heuristic search with 100 replicates of random taxon additions, Tree-Bisection-Reconnection (TBR) branch swapping, MulTrees on, and steepest decent off was performed. The maximum numbers of saved trees were 100 for each replicate. The bootstrap values were calculated from 100 replicates, the consistency index (CI), retention index (RI), rescaled consistency (RC), and homoplasy index (HI) were measured (Felsenstein, 1985).

b. Bayesian analysis

Bayesian analysis was carried out by using MrBayes version. 3.2 (Ronquist and Huelsenbeck, 2003). The parameters and evolutionary models were selected by assistant of MrModeltest2 version 2.3 (Nylander *et al.*, 2004), based on Akaike Information Criterion (AIC), which selected GTR+G model for ITS region, while GTR+G+I was selected for *trn*L-F. Two independent analyses were run 1000000 generations with four chains (one cold and three heated) for each generation and the temperature parameter set to 0.1. Trees were sampled every 100th generations. After that (25% of initial tree sampled) were removed by burn-in period samples, a tree with maximum 50% (majority rule consensus tree) was plotted. The value of posterior probability (PP) was calculated and the final tree was plotted by using FigTree software version 1.4.3 (Rambaut, 2016).

2.6. Palynological Study

a. Light Microscope (LM)

For light microscope, a mature anther has been taken from a fresh specimen and has put in a clean hour glass, a drop of safranine-glycerine stain has added to it (Al-Mayah, 1983). The anther has been opened by two dissected needles, then the pollens have pull with the stain by using a special dropper for each species, and have put on a clean slide, then covered by the cover slip slightly, at this stage the slide was ready for examine.

In the present study, the data have been taken from (10-15) specimens for each species. The slides have examined under Olympus-compound microscope, and photographed by using Sony-digital camera in Faculty of Science /Soran University.

b. Scanning Electron Microscope (SEM)

For scanning electron microscope, the anthers were washed with sterilized distil water in eppendorff tubes and then dehydrated with alcohol series 50%, 70%, 80%, 85%, 90%, 95% and three times 100%, followed by three times of 100% aceton for 30 min each time, then left to dry in the room temperature to eliminate the large amount of impurities that might hinder vision of the structural characteristic of the wall. Finally, the grains were mounted on metal stup with double-side cellophane tape and then coated with a film of gold palladium by the aid of sputtering chamber, after that the coated samples were viewed under scanning electron microscope (INSPECT S50) in (College of Science / Kufa University). The pollen terminology used in accordance of Erdtman (1952) and Ueda and Tomita (1989).

3. Results

3.1. Data matrix, tree statistics and pollen morphological features

The main pollen grains dimensions of the studied species and the characteristics of each data matrix and tree statistics of *trn*L-F and ITS regions are summarized in (Tables 3 and 4).

Table 3: Pollen grain featurs

Table (2): Pollen grains dimensions of *Epilobium* species in micrometer*

Species	**Pollen Grains**		
	Polar axis length	Equatorial axis length	Al(P/E)
E. anatolicum subsp. *anatolicum*	(25.00-32.50)29.16	(35.00-45.00)40.00	0.729
E. hirsutum	(25.00-35.00)30.00	(40.00-70.00)55.00	0.545
E. obscurum	(30.00-37.50)30.75	(40.00-45.00)42.50	0.723
E. parviflorum	(25.00-40.00)32.50	(40.00-55.00)47.50	0.684
E. ponticum	(30.00-37.50)32.50	(40.00-50.00)45.62	0.712
E. rechingeri	(30.00-40.00)34.37	(40.00-50.00)45.62	0.753
E. tetragonum	(30.00-42.50)36.87	(35.00-50.00)44.37	0.830

* The dimensions have been taken from (10-15) specimens, the numbers inside bracts represent the minimum and maximum limit and that outside bracts represent the average. Al (P/E) = average length (Polar view /Equatorial view)

Table 4: A summary of alignment and tree statistics of trnL-F and ITS

Parameters/Regions	*trn*L-F	ITS
Aligned length	619	352
Number of parsimony informative characters	226	1
Number of variable parsimony uninformative characters	323	74
Number of constant characters	70	277
Tree length (steps)	898	75
CI (Consistency Index)	0.912	1.00
RI (Retention Index)	0.727	1.00
RC (Rescaled Index)	0.663	1.00
HI (Homoplasy index)	0.088	0
Model	GTR+G+I	GTR+G

Phylogenetic relationships within Epilobium species

Only one clade was recovered within *Epilobium* in *trn*L-F and two major clades were found in nuclear ribosomal (RNA) tree (Fig. 1 and 2). The analyses were carried out for separate regions, consisted of six ingroups and one outgroup taxa. The tree topology of the maximum parsimony showed same results with bayesian analysis.

The clade of *trn*L-F region is monophyletic and bootstrap support was (bs=81%, pp=0.71) for *E. anatolicum* subsp. *anatolicum* and *E. obscurum*, the other species nearly about (bs=100%, with different ranged between PP=0.82 -0.99). The clades of ITS region are as follow: Clade A consists of *E. hirsutum; E. parviflorum and E. tetragonum* with bootstrap support between (bs=85% - 90%, pp=0.95 – 0.100); Clade B consists of *E. obscurum*; *E. anatolicum* subsp. *anatolicum* and *E. rechingeri* (bs=89% - 90%, pp=0.87 – 0.92).

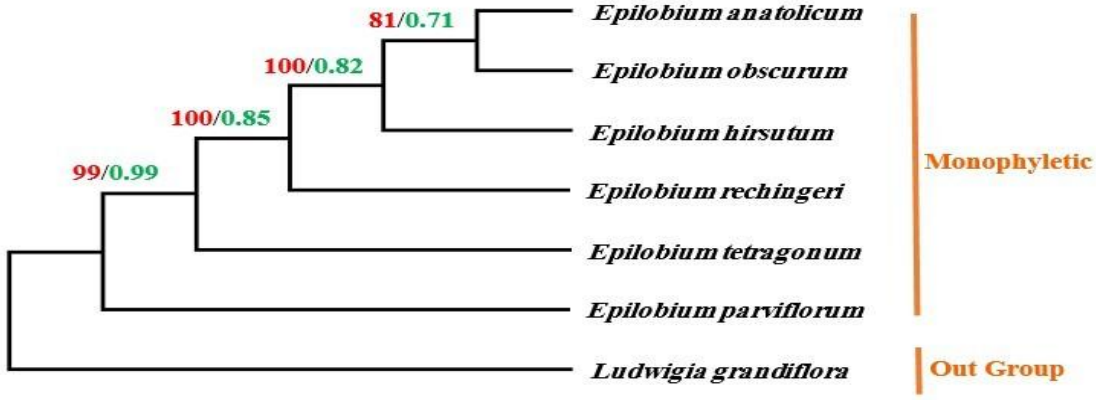


Figure 1: Strict consensus tree of most parsimonious tree resulting from phylogenetic analysis of the cpDNA *trn*L-F sequences with heuristic search using maximum parsimony analysis. (Tree length of 898 steps, CI = 0.912, RI = 0.727, RC = 0.663 and HI =0.088). Numbers in red color indicate bootstrap support and numbers in green color are Bayesian posterior probability values.

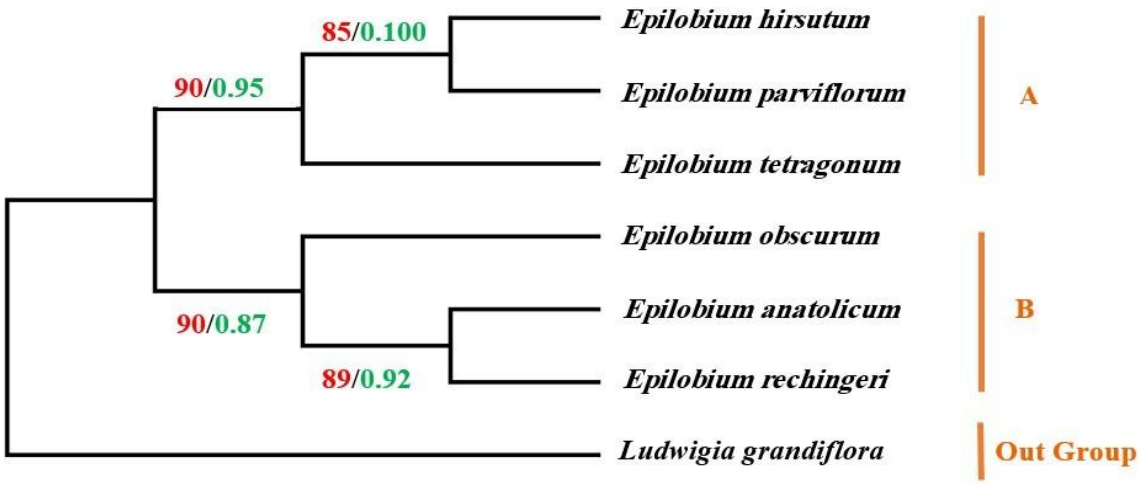


Figure 2: Strict consensus tree of most parsimonious tree resulting from phylogenetic analysis of the nrDNA ITS sequences with heuristic search using maximum parsimony analysis. (Tree length of 75 steps, CI = 1.0, RI = 1.0, RC = 1.0 and HI = 0). Numbers in red color indicate bootstrap support and numbers in green color are Bayesian posterior probability values and clades are identified by letters.

3.3. Palynology

The *Epilobium* pollen grains are usually tetrads, radially symmetrical, isopolar, numerous, yellow. The shape classes of pollen grains are based on P/E ratio are studied where five species appeared oblate whereas *E. rechingeri* and *E. tetragonum* were suboblate, the polar view was triangular and the equatorial view was ellipsoid (Plates1-6). The apertures were triporate. The sizes were medium (medium-large in *E. hirsutum* and *E. parviflorum*). The exine sculpturing were not obvious (baculate in *E. hirsutum* and striate in *E. ponticum*); 2-3 thin threads project from pollens surface seen in polar view called viscin threads.

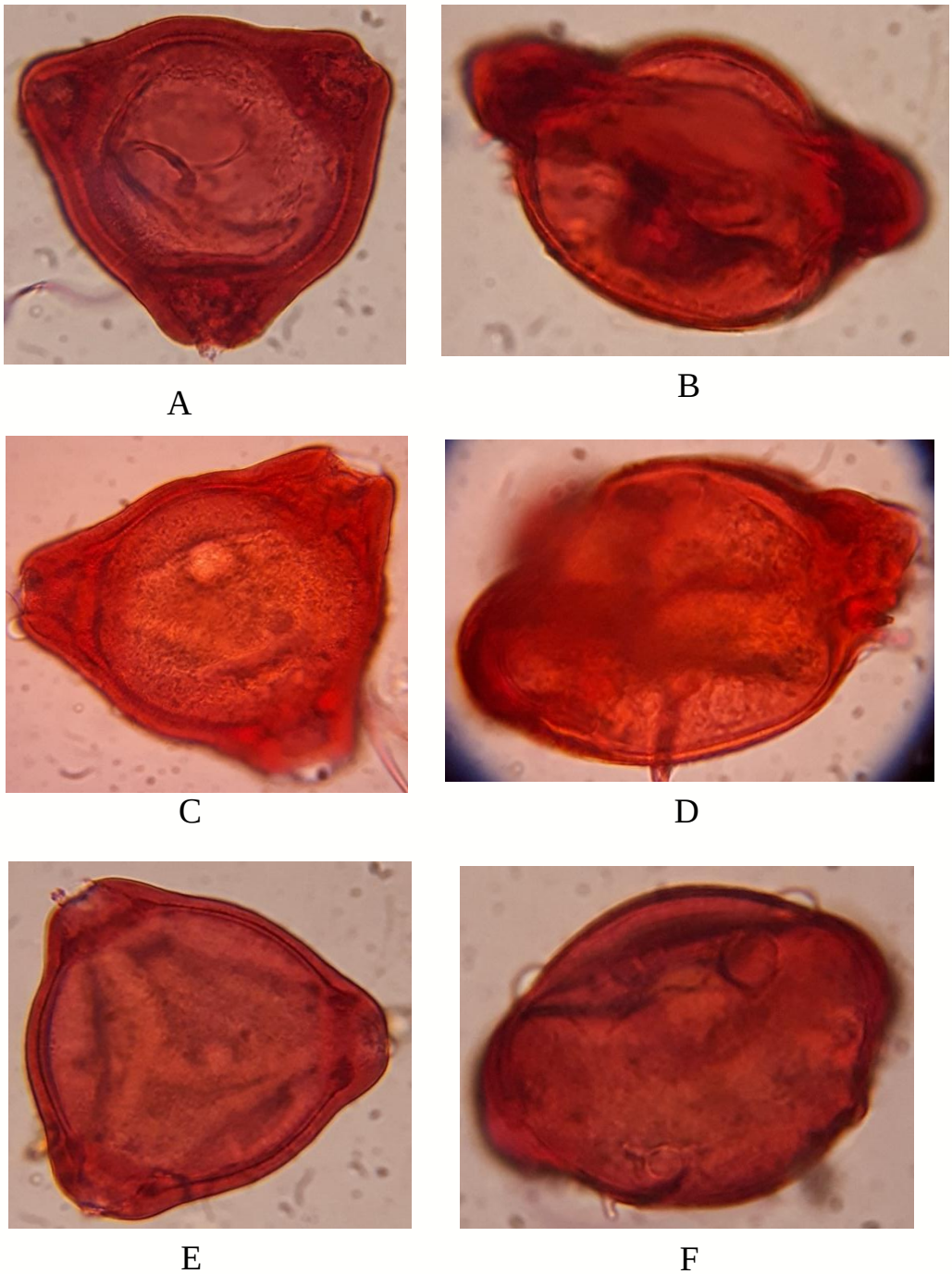


Plate (1): Pollen grains of *Epilobium* species (LM) x100: A&B. *E. anatolicum* subsp. *anatolicum*; C&D. *E. hirsutum*; E&F. *E. obscurum*; A, C, and E = polar view; B, D, and F = equatorial view

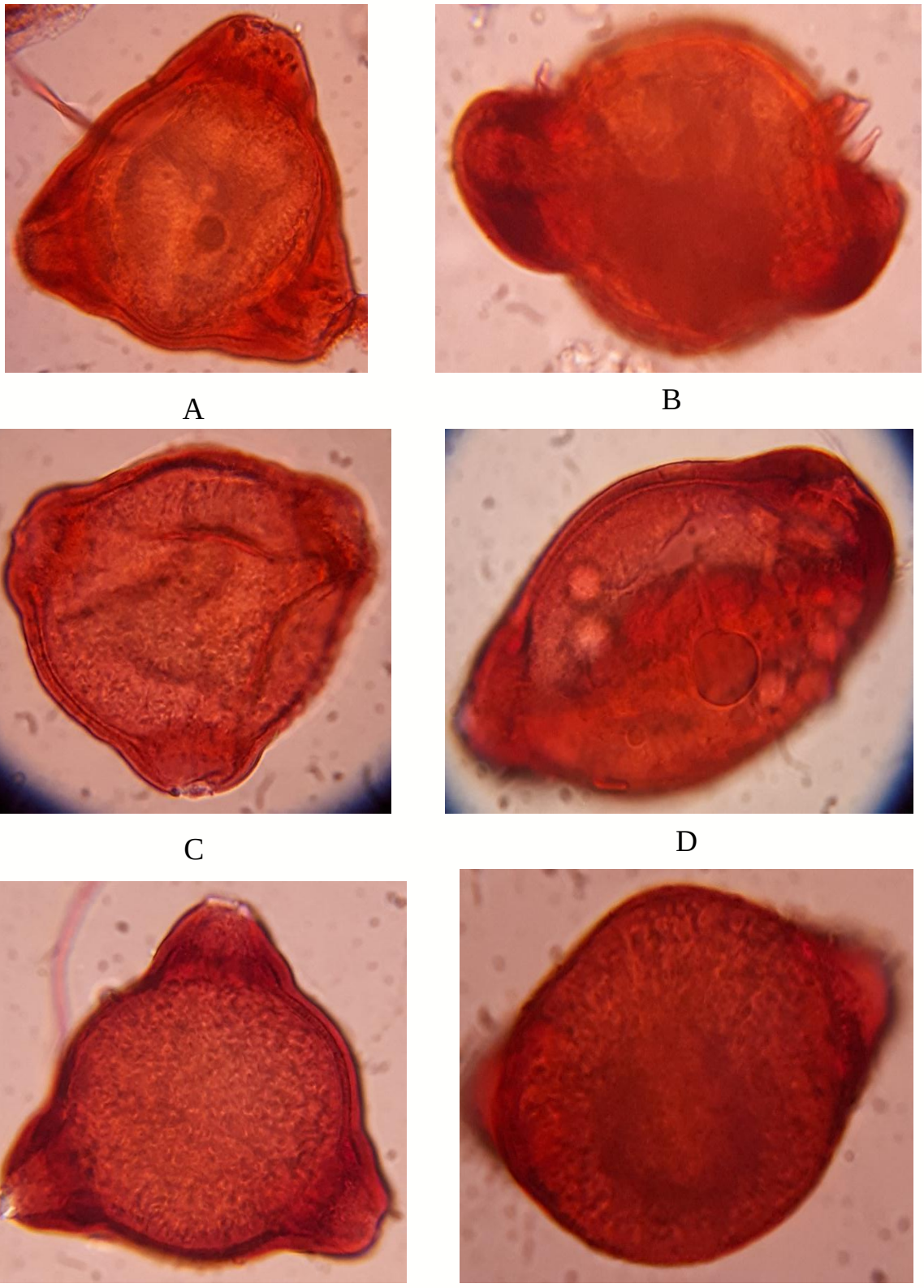


Plate (2): Pollen grains of *Epilobium* species (LM) x100: A&B. *E. parviflorum*; C&D. *E. ponticum*; E&F. *E. rechigeri*; A, C, and E = polar view; B, D, and F = equatorial view

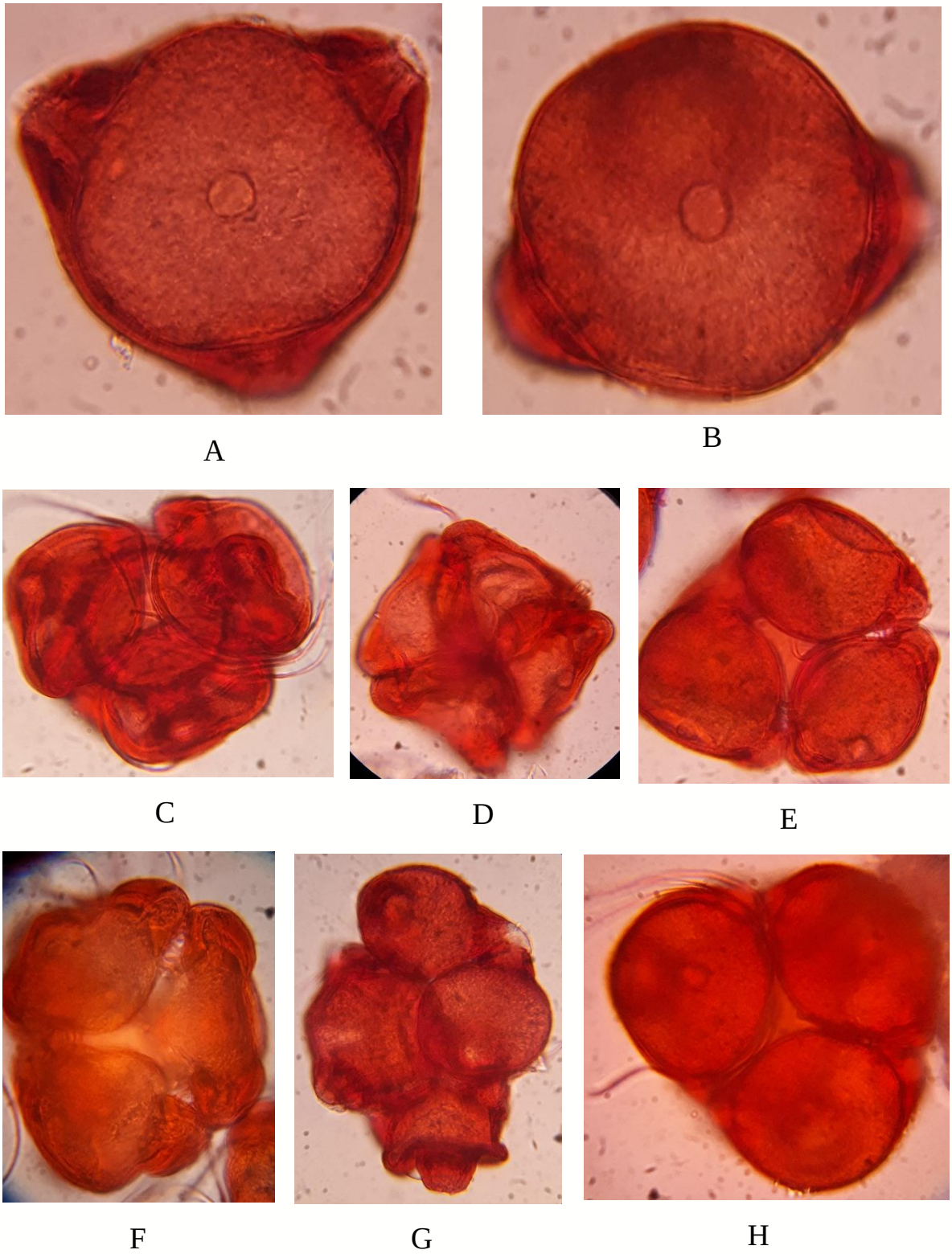
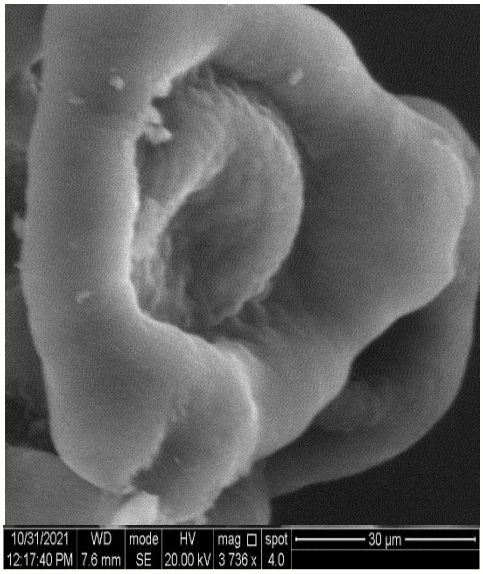
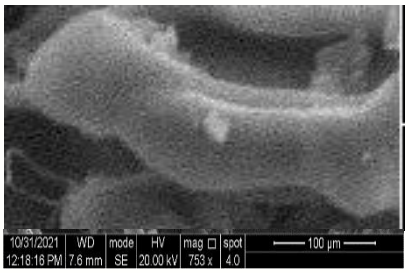
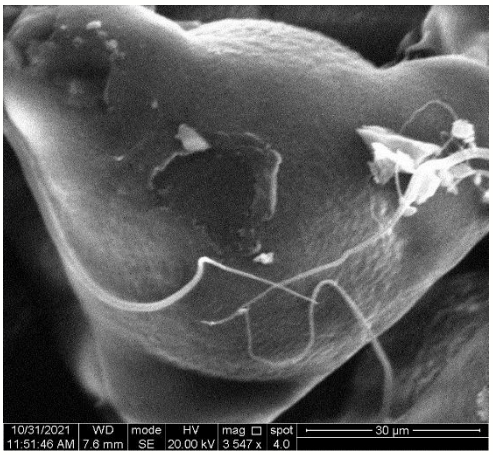


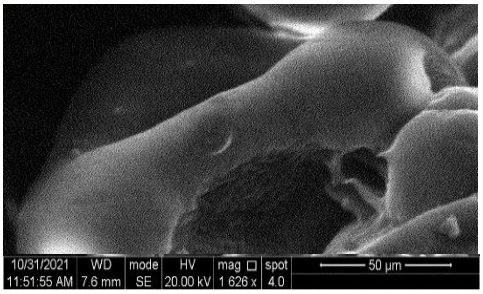
Plate (3): Pollen grains of *Epilobium* species (LM) x100: A&B. *E. tetragonum*; C. *E. anatolicum* subsp. *anatolicum*; D. *E. hirsutum*; E. *E. obscurum*; F. *E. parviflorum*; G. *E. rechigeri*; H. *E. tetragonum*; A, C, and E = polar view; B, D, and F = equatorial view; C-H =Tetrads&Viscin threads



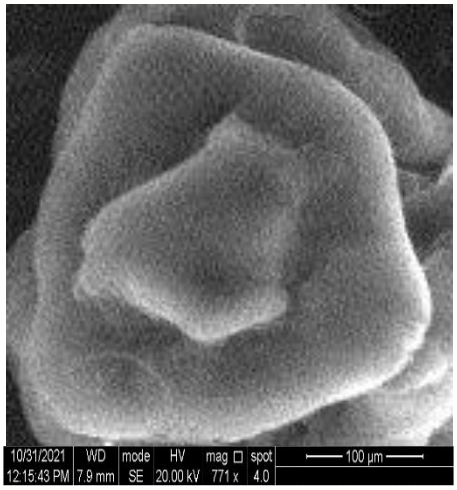


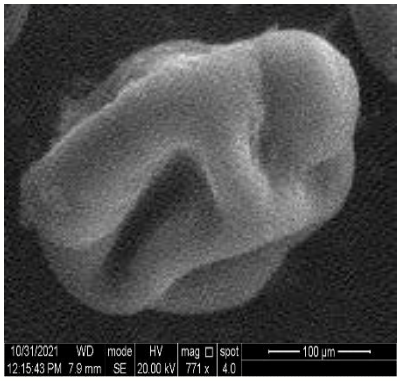
E. anatolicum subsp. *anatolicum*





E. hirsutum

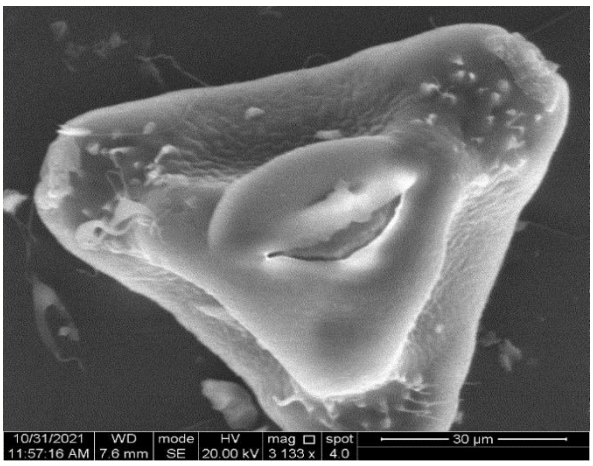


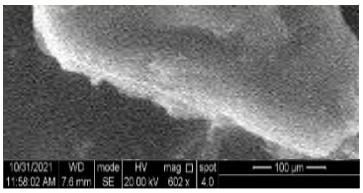


E. obscurum

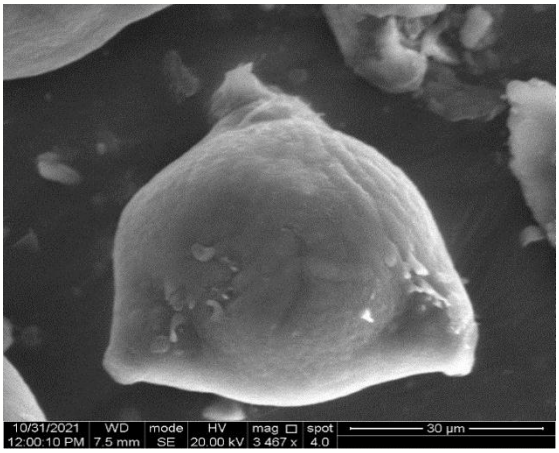
A B

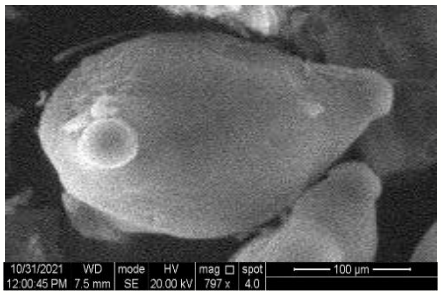
Plate (4): Pollen grains of the studied species A: Polar view; B: Equatorial view



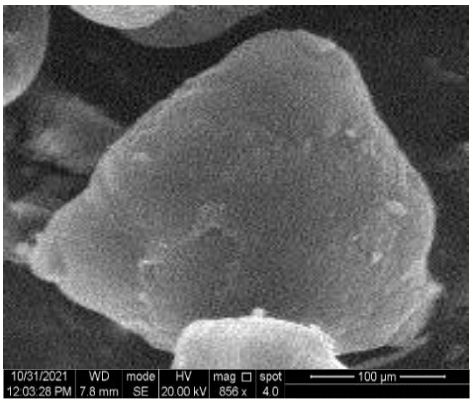


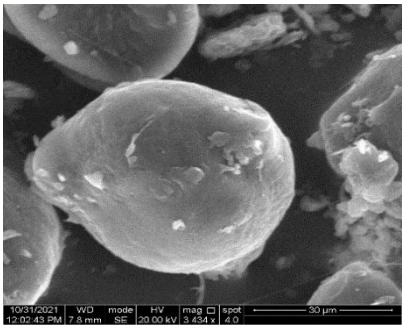
E.parviflorum





E. ponticum

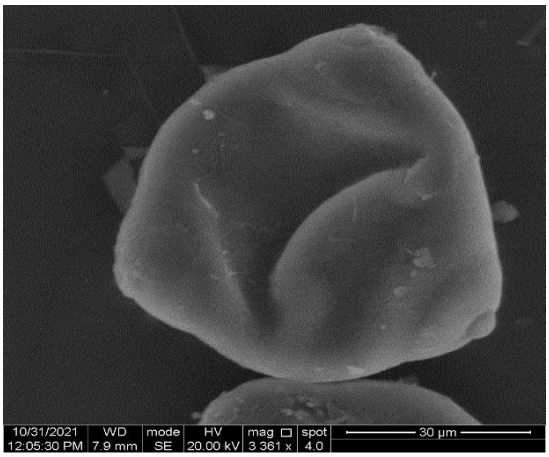




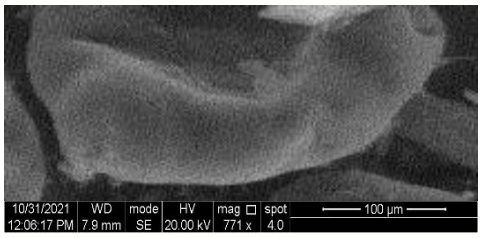
E. rechigeri

A B

Plate (5): Pollen grains of the studied species A: Polar view; B: Equatorial view

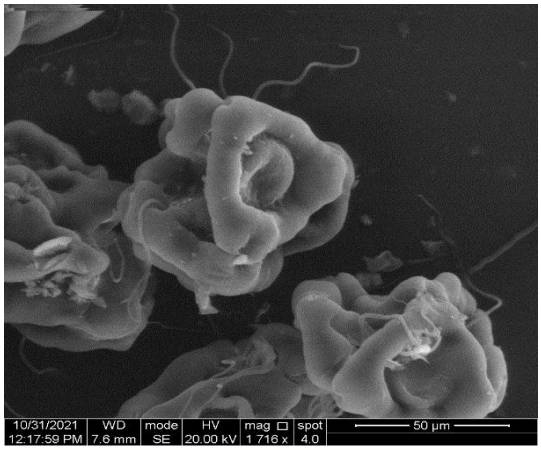


A

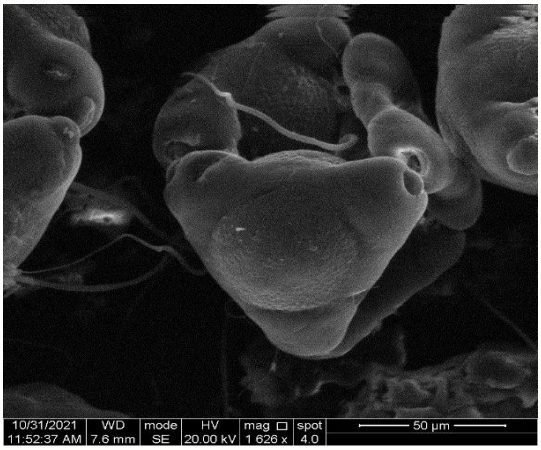


E. tetragonum

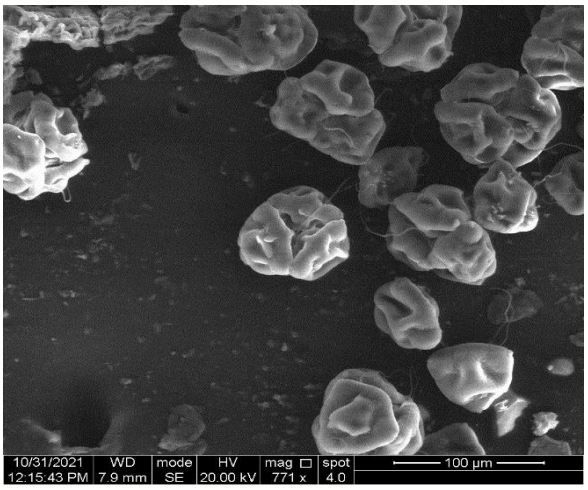
B



E. anatolicum subsp. *anatolicum* C



E. hirsutum



E. obscurum

Plate (6): Pollen grains of the studied species A: Polar view; B: Equatorial view; C: Tetrads

4. Discussion

4.1. Phylogenetic analysis

The trnL-F sequences revealed a close phylogenetic relationship among the species (Figure 1). The tree depending on ITS gene sequences data in (Figure 2) showing two clades, the clade A with supports (bs=90%, pp=0.95) consists of *E. tetragonum* and *E. hirsutum* with sister species *E. parviflorum* with supports (bs=85%, pp=0.1), due to having villous hairs and and 4-lobed stigma, while the clade B with supports (bs=90%, pp=0.87) consists of *E. obscurum* and sister species *E. anatolicum* subsp. *anatolicum* and *E. rechingeri* with high supports (bs=89%, pp=0.92), due to having glandular hairs and leaves lanceolate or narrowly ovate with distinct petiole. Both the ITS and trnL-F trees showed to be monophyletic. Raven (1976) delimited sections in Epilobium based on morphological (including palynological and anatomical) and cytological characters.

The results of krajshek, (2008), showed that *E. hirsutum* is strongly supported as a sister group with *E. parviflorum*. Also, this finding was congruent with the results of (Fulton et al., 2000).

4.2. Pollen grains study

It's clear that Pollen grains of *Epilobium* are usually tetrads, radially symmetrical, isopolar, numerous, yellow. The shape of five species appeared oblate whereas *E. rechingeri* and *E. tetragonum* were suboblate, the polar view was triangular and the equatorial view was ellipsoid (Plates1-6). The apertures were triporate. The sizes were medium (medium-large in *E. hirsutum* and *E. parviflorum*). The exine sculpturing were not obvious (baculate in *E. hirsutum* and striate in *E. ponticum*). Pollen grains of *E. obscurum* are single (sometimes tetrads), triporate, oblate in equatorial view, triangular in polar view; 2-3 thin threads project from pollens surface seen in polar view called viscin threads: acetolysis resistant threads arising from the exine (Hesse, et al., 2009), (Sardar and Al-Edhari, 2017) and (Sardar, 2018).

Rahimi et al., (2018) reviewed that Polar axis ranged between 30 and 80 µm. Equatorial axis showed variation from 40 to 90 µm in all *Epilobium, Circaea, Oenothera* species.

Brown, (1967) stated that in the Epilobieae tribe, which also includes the genus *Epilobium*, the pollen grains are seen as either monads or tetrahedral tetrads. The pollens of tetraploid taxa are larger than the pollen of other taxa. Makbul et al., (2008) studied the anatomical and pollen morphological characters of the genus *Epilobium* from Northwest Anatolia.

Punt et al., (2003) and Makbul et al., (2008) determined that the pollen shape of the *Epilobium* taxa were suboblate or oblate and the pollen shape contributed significantly to the differentiation of the *Epilobium* taxa. Erdtman, 1960; Punt et al., 2003 revealed that there is a fine connection between the tetrad and monad pollen spreads by breaking this connection with the acetolysis method. Rahimi et al., (2018) determined that radial symmetrical, tetrahedral tetrad, trizonoporate, rugulate, granulate, verrucate, and striate ornamentations were observed in pollens and that these family members did not differ significantly in terms of characteristics such as pollen size, shape, and pore structure. Exine sculpturing, showed three distinct types of surface structures: Baculate (the common type), baculate-rugulate (*E. anatolicum*), and rugulate *(E. hirsutum*) (Basher et al., 2021)

5. Conclusions

In the present study two major clades within the species of *Epilobium* were identified in the ITS tree, while in the *trn*L-F a close phylogenetic relationship among the species has been present. The study of the pollen grains by using Light and Scanning Electron Microscope showed that the palynological data of *Epilobium* species are not representing more variation among the species. One reason may be that *Epilobium* species are morphologically similar, and another reason due to the limited numbers of *Epilobium* species in Kurdistan region – Iraq.

6. References

AKBARI, R. S. & AZIZIAN, D. 2006. Seed morphology and seed coat sculpturing of *Epilobium* L. species (Onagraceae Juss.) from Iran. Turkish Journal of Botany, 30, 435-440.

BASHER, B., NAZLı, A. & KÜRŞAT, M. 2021. Using of light and scanning electron microscopy for pollen morphology of some *Epilobium* (Onagraceae) taxa and Its taxonomic importance. *Bitlis Eren University Journal of Science and Technology*, 11**,** 36-45.

BROWN, C. A. 1967. Pollen morphology of the Onagraceae. *Review of palaeobotany and palynology*, 3**,** 163-180.

BAUM, D. A., SYTSMA, K. J. & HOCH, P. C. 1994. A phylogenetic analysis of *Epilobium* (Onagraceae) based on nuclear ribosomal DNA sequences. Systematic Botany, 363-388.

CHASE, M. W., SOLTIS, D. E., OLMSTEAD, R. G., MORGAN, D., LES, D. H., MISHLER, B. D., DUVALL, M. R., PRICE, R. A., HILLS, H. G. & QIU, Y.-L. 1993. Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene rbcL. Annals of the Missouri Botanical Garden, 528-580.

CHEN, S., YAO, H., HAN, J., LIU, C., SONG, J., SHI, L., ZHU, Y. MA, X., GAO, T. & PANG, X. 2010. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species.

CONTI, E., FISCHBACH, A. & SYTSMA, K. J. 1993. Tribal relationships in Onagraceae: implications from rbcL sequence data. Annals of the Missouri Botanical Garden, 672-685.

DICKISON, W. C. 1975. The bases of angiosperm phylogeny: vegetative anatomy. Annals of the Missouri Botanical Garden, 590-620.

DOYLE, J. J. & Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. Focus, 12, 13-15.

EIDE, F. Y. 1981. Key for northwest European Rosaceae pollen. Grana, 20, 101-118.

ERDTMAN, G. 1952. On pollen and spore terminology. Journal of Palaeosciences, 1, 169-176.

FELSENSTEIN, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39, 783-791.

FULTON, T., GRANDILLO, S., BECK-BUNN, T., FRIDMAN, E., FRAMPTON, A., LOPEZ, J., PETIARD, V., UHLIG, J., ZAMIR, D. & TANKSLEY, S. 2000. Advanced backcross QTL analysis of a Lycopersicon esculentum× Lycopersicon parviflorum cross. *Theoretical and Applied Genetics*, 100**,** 1025-1042.

HALL, R. E. (2001). The Stock Market and Capital Accumulation. *American Economic Review*, 91**,** 1185-1202.

HEBDA, R. J. & CHINNAPPA, C. 1990. Studies on pollen morphology of Rosaceae in Canada.

HESSE, M., Halbritter, H., Zetter, R., Weber, M., Buchner, R., Frosch-Radivo, A. and Ulrich, S. (2009). Pollen Terminology, An illustrated handbook. Springer - Verlag/Wien, Austria: 219

HOCH, P. C., CRISCI, J. V., TOBE, H. & BERRY, P. E. 1993. A cladistic analysis of the plant family Onagraceae. Systematic Botany, 31-47.

KERI, C. & ZETTER, R. 1992. Notes on the exine ultrastructure of Onagraceae and Rhododendroideae (Ericaceae). Grana, 31, 119-123.

LEVIN, R. A., WAGNER, W. L., HOCH, P. C., HAHN, W. J., RODRIGUEZ, A., BAUM, D. A., KATINAS, L., ZIMMER, E. A. & SYTSMA, K. J. 2004. Paraphyly in tribe Onagreae: insights into phylogenetic relationships of Onagraceae based on nuclear and chloroplast sequence data. Systematic Botany, 29, 147-164.

MAKBUL, S., TÜRKMEN, Z., COSKUNÇELEBI, K. & BEYAZOGLU, O. 2008. Anatomical and pollen characters in the genus Epilobium L. (Onagraceae) from Northeast Anatolia. Acta Biologica Cracoviensia Series Botanica, 50, 51-62.

METCALFE, C. R. & CHALK, L. 1950. Anatomy of the Dicotyledons: leaves, stem, and wood, in relation to taxonomy, with notes on economic uses. Anatomy of the Dicotyledons: leaves, stem, and wood, in relation to taxonomy, with notes on economic uses.

NYLANDER, J. A., Ronquist, F., Huelsenbeck, J. P. & Nieves-Aldrey, J. L. (2004). Bayesian phylogenetic analysis of combined data. Syst Biol, 53, 47-67. Review of Palaeobotany and Palynology, 64, 103-108.

PATEL, V. C., SKVARLA, J. J. & RAVEN, P. H. 1984. Pollen characters in relation to the delimitation of Myrtales. Annals of the Missouri Botanical Garden, 858-969.

RAVEN, P. H. 1976. Generic and sectional delimitation in Onagraceae, tribe Epilobieae. Annals of the Missouri Botanical Garden, 326-340.

RAMBAUT, A. (2016). ‹http://tree.bio.ed.ac.uk/software/figtree)/ Accessed 6 January 2016.

RAVEN, P. H. 1988. Onagraceae as a model of plant evolution. Plant evolutionary biology. Springer.

RONQUIST, F. & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19, 1572-4.

ROWLEY, J. R. & CLAUGHER, D. 1996. Structure of the exine of Epilobium angustifolium (Onagraceae). Grana, 35, 79-86.

SARDAR, A. Sh. & AL-Edhari, A. H. (2017). Taxonomic Study for the New Record Epilobium obscurum Schreber (Onagraceae) in Iraq. Asian Journal of Research, 6(6): 127-129.

SARDAR, A. Sh. (2018). Taxonomic Study of the New Record *Epilobium anatolicum* Hausskn. subsp. anatolicum (Onagraceae) in Iraq. Al-Kufa University Journal for Biology, 10(3): 15-23

Swofford, D. L. (2000). PAUP*. Phylogenetic Analysis Using Parsimony, Version 4, Sunderland, Massachusetts, Sinauer Associates: 6-10.

SYTSMA, K. J., SMITH, J. F. & BERRY, P. E. 1991. The use of chloroplast DNA to assess biogeography and evolution of morphology, breeding systems, and flavonoids in Fuchsia sect, Skinnera (Onagraceae). Systematic Botany, 257-269.

TABERLET, P., GIELLY, L., PAUTOU, G. & BOUVET, J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol Biol*, 17**,** 1105-9.

WEN, J. & NOWICKE, J. W. 1999. Pollen ultrastructure of Panax (the ginseng genus, Araliaceae), an eastern Asian and eastern NorthAmerican disjunct genus. American Journal of Botany, 86, 1624-1636.

YURTSEVA, O., SEVEROVA, E. & BOVINA, I. Y. 2014. Pollen morphology and taxonomy of *Atraphaxis* (Polygoneae, Polygonaceae). Plant Systematics and Evolution, 300, 749-766.

UEDA, Y. & Tomita, H. (1989). Morphometric analysis of pollen exine patterns in roses. J. Japan. Soc. Hort. Sci., 58, 211-220.