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

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Abstract

The phylogeny of the species of *Potentilla* in Kurdistan Region-Iraq was investigated by using six in-group species and one out-group related genus *Sibbaldia parviflora*, based on *trn*L-F intergenic region of chloroplast DNA and internal transcribed spacer of nuclear ribosomal DNA. In addition, the pollen morphology of the six species of *Potentilla* was examined by using Light Microscope (LM) and Scanning Electron Microscope (SEM). Individual and combined analysis of *trn*L-F and ITS sequence data indicated monophyly of the genus *Potentilla*, the results of bayesian and maximum parsimony displayed three clades of *Potentilla* with high supports (bs=100%, pp=1.00). The pollen grains appeared tricolporate and prolate-spheroidal, oblate-spheroidal, or oblate; their sizes were small to medium; the outlines varied from circular to ellipsoid; the exine sculpturing type was striate and consists of shallow, fingerprint-like ridges and predominately parallel arranged.

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

1. Introduction

Rosaceae family involves 3200 species throughout the world which distributed on 115 genera (Core, 1955); 2800 species on 95 genera (Sipson, 2006) and in Iraq, involves 53 species distributed on 19 genera (Al-Rawi, 1964). In Iraq, the genus *Potentilla* L. 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 of Iraq (except *P. supina*). The species of the genus *potentilla* which grow naturally and mainly in Kurdistan are: *P. speciosa* Willd., *P. hirta* L., *P. kurdica* Boiss. et Hoh. ex Boiss., *P. pannosa* Boiss. et Hausskn ex Boiss., *P. reptans* L. and *P. lignosa* Willd.

The first practical use of molecular data in Rosaceae was done by Takhtajan (1997). Integrating some of the possible results of the first molecular phylogenetic study of proper relationships across Rosaceae, traditionally recognized twelve subfamilies (Morgan *et al.*, 1994). However, several reliable sources of molecular data adequately support the monophyly of Rosaceae (Morgan *et al.*, 1994, Evans *et al.*, 2000, Potter *et al.*, 2002, Faghir *et al.*, 2014).

Potter *et al.* (2002), shows the phylogenetic relationships within 37 genera of Rosaceae, which are traditionally divided into 4 subfamilies and 3 related genera based on two specific regions of chloroplast DNA, the *matK* and the *trn*L-F genes. In addition, Potter *et al.* (2007), accurately classified 88 distinct genera of Rosaceae constructed on phylogenetic relationships based on nucleotide sequence data of six nuclear (18S, *gbssi1*, *gbssi2*, ITS, *pgip*, and *ppo*) and four chloroplast (*matK, ndhF, rbcL, and trnL-F*) regions.

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Eriksson *et al.* (2003), made a good phylogenetic definition of Rosaceae based on the sequences of internal transcribed spacer ITS of nuclear ribosomal DNA and *trn*L-F region of chloroplast DNA. In the same way, Eriksson *et al.* (2015), representing that *Sibbaldia* is a polyphyletic assemblage and it falls into five separate clades of Potentilleae, three with Fragariinae and two with *Potentilla*.
Dobes and Paule (2010), reconstructed the phylogenetic relationships among 98 species of the genus *Potentilla* and 15 related genera based on three chloroplast DNA markers *trnS-ycf9*, *trn*L-F, and *trnC-ycf6*. Moreover, several natural occurring hybrids in *Potentilla* have been reported by Topel *et al.* (2011), based on two nuclear ribosomal DNA the internal transcribed spacer ITS and external transcribed spacer ETS and two regions of chloroplast DNA *trn*S-G and *trn*L-F.

The completed last accurately reconstructed complex relationships among the tribe Potentilleae have been typically seen in the study of Feng *et al.* (2017), they identified three major clades of the Potentilleae: the subtribe Fragariinae, the genera *Argentina*, and *Potentilla*.
Pollen grains are important character for taxonomic purposes. In addition to the taxonomic value of the pore structure the variations in the pollen grain sculptures are important features for species classification (Reitsma, 1966). Comparative studies of pollen morphology have provided useful characters for demarcating genera and species and determining relationships in several ancestries of Rosaceae (Reitsma, 1966, Eide, 1981, Hebda *et al.*, 1991, Hebda and Chinnappa, 1994, Oybak Dönmez, 2008, Wronska-Pilarek, 2011).

Based on the pollen grains, the genus *Potentilla* is tricolporate (Reitsma, 1966, Eide, 1979a, Eide, 1981). Sánchez *et al.* (1998), used LM and SEM for 26 taxa of *Potentilla* and clarified the taxonomic and phylogenetic relationships among the species on the base of the pollen characters and they mentioned that *Potentilla* pollens appear stenopalynous under light microscope. Similarly, Kołodziejek and Gabara (2008), identified tricolporate sculpture type of 5 species of *Potentilla. Faghir et al. (2012),* found that two main exin sculpture types of 28 species of the *Potentilla* and 6 related genera.

The aim of the present study is to investigate the relativeness between the species of *Potentilla* based on the phylogenetic relationships and comparing with the nearest genus. For this purpose, two regions were selected, the first region is chloroplast DNA *trn*L-F and the second is nuclear ribosomal DNA ITS. Furthermore, to investigate the morphology of the pollen grains of 6 species of the genus *Potentilla* in order to use them in the separation of the species under study.

2. Material and Methods

2.1. Taxon Sampling

The plant taxa used in the present study were collected from the different districts of Kurdistan region-Iraq that preserved in the Herbarium of College of Education/ Salahaddin University (Table 1). Seven distinct taxa consist of six ingroup taxa and one out group *Sibbaldia parviflora* were used in the analysis.

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 some

modification (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 ddH_2O), 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 Taberlet *et al.* (1991), and ITS-A and ITS-B of White *et al.* (1990) for *trn*L-F intergenic spacer and ITS region respectively (Table 2). The primers were ordered from (IDT) company-Skokie, Illinois-USA. The total volume of amplification reactions was 25 µl and the Master Mix made up of 10.8 µl of ddH_2O, 2.5 µl ThermoPol reaction buffer, 2.5 µl $MgCl_2$, 5 µl dNTPs, 2 µl template, 1 µl from each primers, 0.2 µl DNA polymerase (Taq polymerase). The PCR-Thermal cycler started with 2 min for initial denaturation at 94 C° followed by 30 cycles: 30 sec. for *trn*L-F and ITS for denaturation at 94 C°; 60 sec. for annealing at 54 C° for *trnL-F* and 52 C° for ITS; 60 sec. for *trnL-F* and 90 sec. for ITS for extension at 72 C°; and 180 sec. for final extension at 72 C°. The resultant PCR products were checked on 1.5% agarose gel run in TAE buffer. The gel was stained with EtBr and photographed under UV transilluminator.

Table 1: Specimen numbers of *Potentilla* 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
P. hirta	7633	ESUH	Halgurd M.	2.7.2017
P. kurdica	7640	ESUH	Hasarost M.	26.6.2016
P. lignosa	7451	ESUH	Qandil M.	25.8.2016
P. pannosa	7617	ESUH	Sakran M.	7.6.2017
P. reptans	7624	ESUH	Tawela village	15.5.2016
P. speciosa	7635	ESUH	Gara M.	12.7.2017

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

Primer	Direction	Sequence 5'---- 3'	Resources
ITS-A	Forward	GGAAGGAGAAGTCGTAACAAGG	(White *et al.*, 1990)
ITS-B	Reverse	CTTTTCCTCCGCTTATTGATATG	(White *et al.*, 1990)
trn-C	Forward	CGAAATCGGTAGACGCTACG	(Taberlet *et al.*, 1991)
trn-F	Reverse	ATTTGAACTGGTGACACGAG	(Taberlet *et al.*, 1991)

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.

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 out group species.

2.5. Phylogenetic Analyses

a. Maximum Parsimony Analysis

The reconstruction of the phylogenetic relationships was based on Maximum Parsimony (MP) methods. The analysis was carried out for separate and combined 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, 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 both combined and ITS regions, while F81+G 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 Education College/Salahaddin 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 morphological features 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

Taxon	Polar axis (µm) Mean	Equatorial axis (µm) mean	P/E	Pollen Shape (PS)	Polar view	Equatorial view	Aperture Shapes & numbers
P. hirta	(15-17-18) 16.6 ± 0.88	(12-15-18) 15.00 ± 1.73	1.10	Prolate spheroidal	circular	oblate	3 - Colporate
P. kurdica	(20-21-22) 21.00± 0.57	(18-20-22) 20 .00± 1.15	1.05	Prolate spheroidal	circular	oblate	3- Colporate
P. lignosa	(10-12-13) 11.66 ± 0.88	(12-13-15) 13.33 ± 0.88	0.87	Suboblate	triangular	oblate	3 - Colporate
P. pamnosa	(21-25-30) 25.33 ± 2.60	(22-25-30) 25.66 ± 2.33	0.98	Oblate spheroidal	circular	oblate	3 - Colporate
P. reptans	(10-12-15) 12.33 ± 1.45	(11-12-13) 12.00 ± 0.57	1.02	Prolate spheroidal	circular	Oblate	3 - Colporate
P. speciosa	(11-12-13) 12.00 ± 0.57	(10-11-12) 11.14 ± 0.57	1.07	spheroidal	circular	oblate	3 - Colporate

The numbers inside bracts represent the minimum and maximum limit and that outside bracts represent the average.

Table 4: A summary of alignment and tree statistics of trnL-F, ITS and combined analyses.

Parameters/Regions	*trn*L-F	ITS	Combined
Aligned length	374	467	841
Number of parsimony informative characters	100	38	261
Number of variable parsimony uninformative characters	51	67	118
Number of constant characters	51(%26)	362(%77)	426 (%54)
Tree length (steps)	425	145	578
CI (Consistency Index)	0.908	0.855	0.882
RI (Retention Index)	0.873	0.604	0.811
RC (Rescaled Index)	0.793	0.516	0.716
HI (Homoplasy index)	0.092	0.145	0.118
Model	F81+G	GTR+G	GTR+G

3.2. Phylogenetic relationships within Potenetilla species

Three major clades were recovered within *Potentilla* in both nuclear ribosomal (Reitsma) and plastid (*trn*L-F) tree, although the positions of these clades are varied (fig. 1, 2 and 3). The analyses were carried out for separate and combined regions, consisted of six ingroups and one outgroup taxa. The tree topology of the maximum parsimony showed same results with bayesian analysis.

The clades of trnL-F region are as follow: Clade A consists of only *P. lignosa* with bootstrap support (bs=100%, pp=0.93); the clade B consists of *P. kurdica* and *P. hirta* and are highly supported (bs=100%, pp=1.00); while the clade C consists of *P. pannosa*, *P. reptans* and *P. speciosa* and are highly supported (bs=100%, pp=0.98).
The clades of ITS region are as follow: Clade A consists of only *P. lignosa* with bootstrap support (bs=72%, pp=0.75); Clade B consists of *P. kurdica*, *P. Pannosa* and *P. hirta* (bs=92%, pp=1.00); and Clade C consists of *P. reptans* and *P. speciosa* (bs=96%, pp=1.00).
The clades of combined regions are as follow: Clade A consists of only *P. lignosa* with bootstrap support (bs=100%, pp=1.00); the clade B consists of *P. kurdica* and *P. hirta* and are highly supported (bs=100%, pp=1.00); and clade C consists of *P. pannosa*, *P. reptans* and *P. speciosa* and are highly supported (bs=100%, pp=1.00).

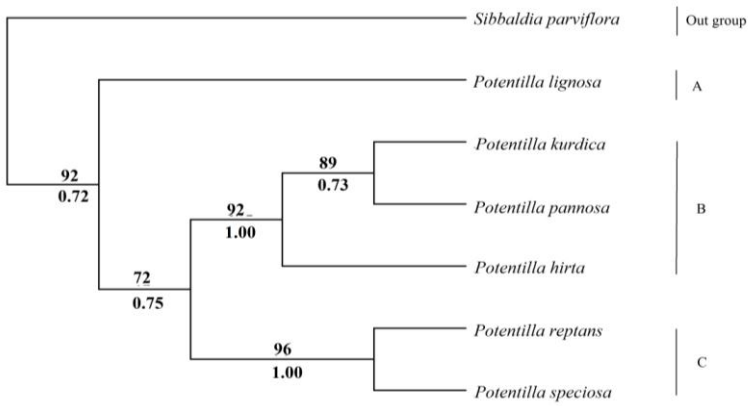

Figure 1: Strict consensus tree of most parsimonious tree resulting from phylogenetic analysis of the cpDNA *trn*L-F sequences with heuristic search using maximum parsimony analysis. (Tree length of 425 steps, CI = 0.908, RI = 0.873, RC = 0.793 and HI =0.092). Numbers on the branches indicate bootstrap support and numbers below branches are Bayesian posterior probability values and clades are identified by letters

3.3. Palynology

The *Potentilla* pollen grains are tricolporate, radially symmetrical monads. The shape classes of pollen grains are based on P/E ratio are studied, various shapes found in polar view from prolate-spheroidal (1.10, 1.05, and 1.02 µm) as in *P. hirta*, *P. kurdica* and *P. reptans* (Plates 1A, C and 2C) respectively, oblate-spheroidal (0.98) as in *P. pannosa* and spheroidal (1.07) as in *P. speciosa* (Plate 2A and 2E), and suboblate (0.87) as in *P. lignosa* (Plate 1E). While, the outline of pollen grains is ellipsoid in equatorial view (Plate 2 C and D) and circular (Plate 1 B, D, and E). The aperture includes three ectocolpi and three endopores. The size of the pollen grains ranged from small to medium. The exine sculpturing type includes striate pattern and consists of shallow, fingerprint-like ridges and predominately parallel arranged. (Plates 3-4).

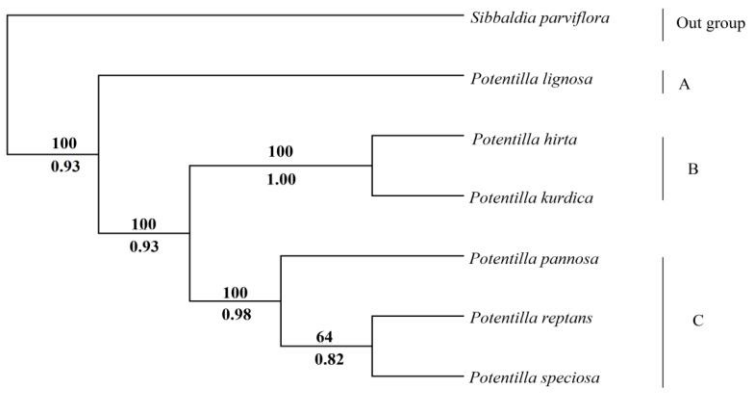

Figure 2: Strict consensus tree of most parsimonious tree resulting from phylogenetic analysis of the nrDNA ITS sequences with heuristic search using maximum parsimony analysis. (Tree length of 145 steps, CI = 0.855, RI = 0.604, RC = 0.516 and HI =0.145). Numbers on the branches indicate bootstrap support and numbers below branches are Bayesian posterior probability values and clades are identified by letters.

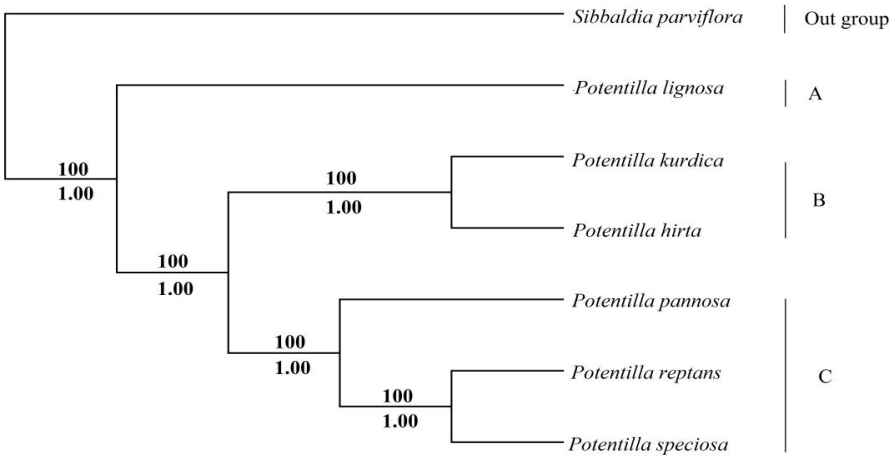

Figure 3: Strict consensus tree of most parsimonious tree resulting from phylogenetic analysis of the combined sequences with heuristic search using maximum parsimony analysis. (Tree length of 578 steps, CI = 0.882, RI = 0.811, RC = 0.716 and HI =0.118). Numbers on the branches indicate bootstrap support and numbers below branches are Bayesian posterior probability values and clades are identified by letters.

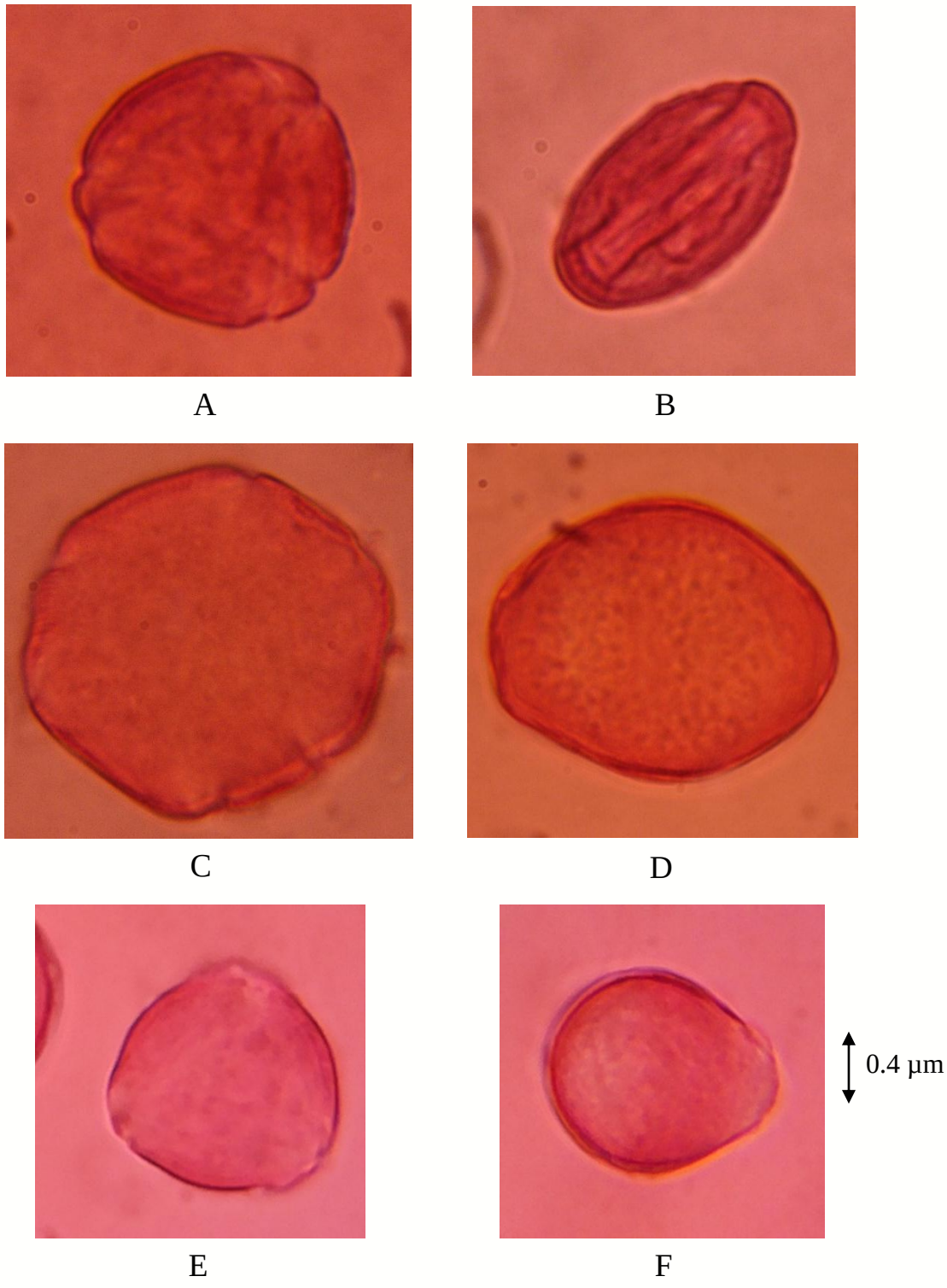

Plate (1): Pollen grains of *Potentilla* species (LM): A&B. *P. hirta*; C&D. *P. kurdica*; E&F. *P. lignosa*; A, C, and E = polar view; B, D, and F = equatorial view

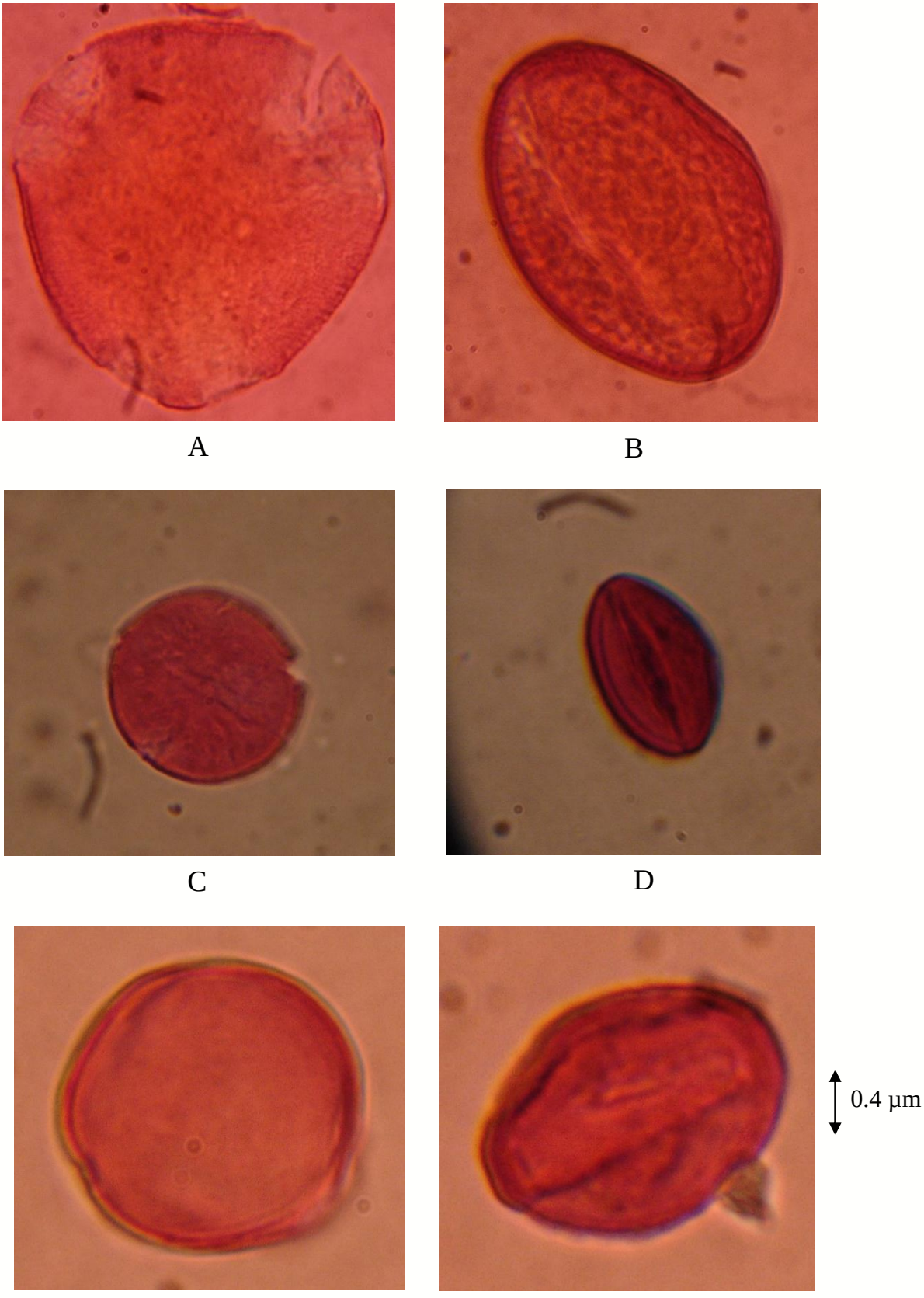

Plate (2): Pollen grains of *Potentilla* species (LM): A&B. *P. pannosa*; C&D. *P. reptans*; E&F. *P. speciosa*; A, C, and E = polar view; B, D, and F = equatorial view

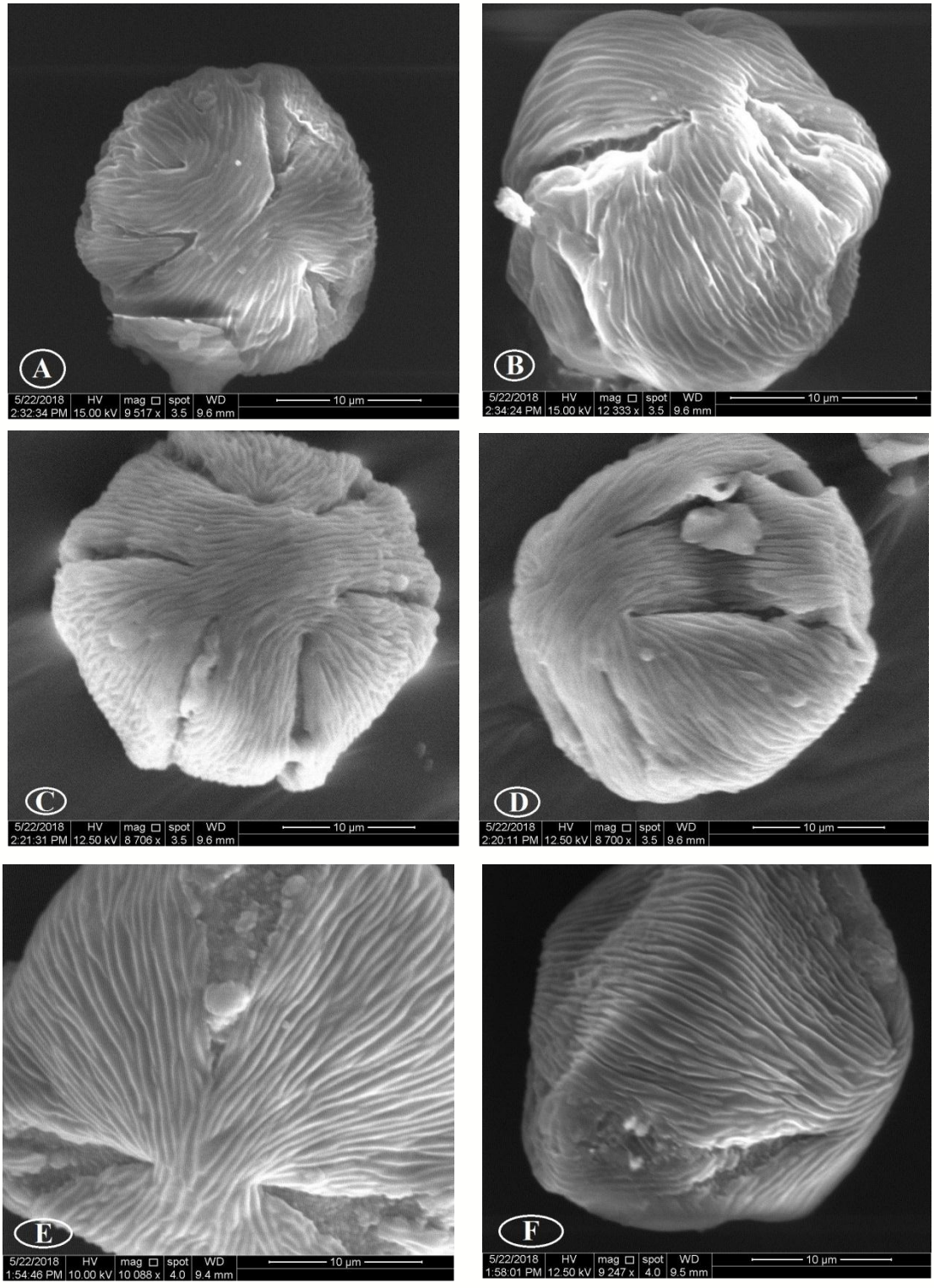

Plate (3): Pollen grains of *Potentilla* (SEM): A&B. *P. hirta*; C&D. *P. kurdica*; and E&F. *P. pannosa*; A, C, and E= polar view and B, D, and F= equatorial view

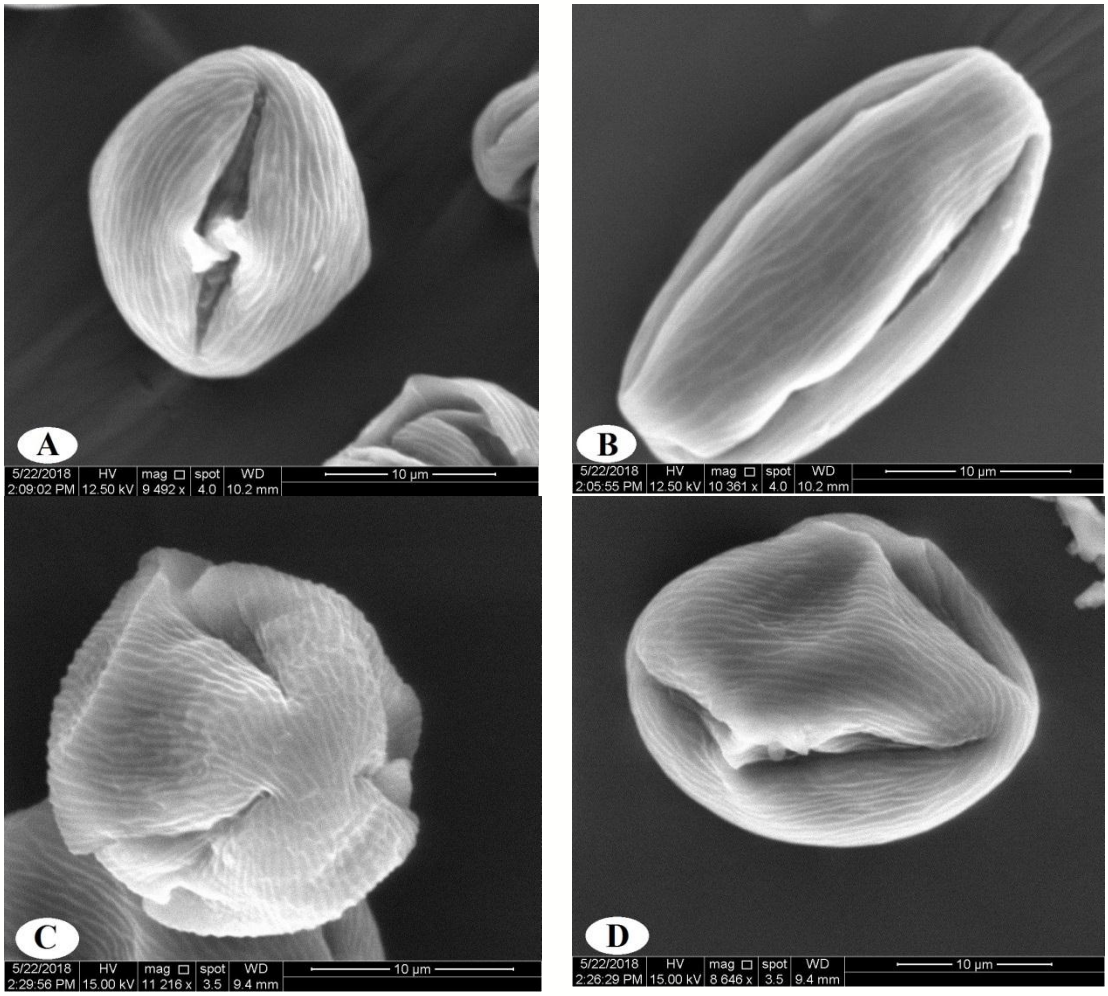

Plate (4): Pollen grains of *Potentilla* (SEM): A&B. *P. reptans* and C&D. *P. speciosa*; A and C= polar view & B and D= equatorial view

4. Disccusion

4.1. Phylogenetic Analysis

The tree depending on *trn*L-F gene sequences data in (Figure 2) showing three clades with high supports (bs=100%, pp=1.00): The clade A consists of only *P. lignosa* with supports (bs=100%, pp=0.93), due to the special occurrence and habit, it's the only shrub species within the other *Potentilla* species; while the clade B consists of sister species *P. kurdica* and *P. hirta* with high supports (bs=100%, pp=1.00), because they are compound dichasium inflorescence and similar in there pollen grain shapes; and clade C consists of *P. pannosa* and sister species *P. reptans* and *P. speciosa* with supports (bs=100%, pp=0.98), because they have 30 stamens, triseriate, and the flowers are bisexual, actinomorphic, pediculate and hypogenous.

Matthews (1972), identified 53 species of *Potentilla* and divided them into four groups, he separated *P. lignosa* and *P. fruticosa* from the other species in a single special group because both them are shrubs, and this is a support for the current study.

On the other hand, the results of Eriksson *et al.* (2015), showed that *P. lignosa* is strongly supported as a sister group with the rest of the sampled species. Also, this finding was congruent with the results of Topel *et al.* (2011).

The tree based on ITS region sequences data in (Figure 2) showing three clades with high supports (bs=96%, pp=1.00), and the results are slightly differ from *trn*L-F in which *P. pannosa* become a sister species with *P. kurdica*, because they are similar in the arrangement of vascular bundle in midrib, both are collateral and they have villous hairs.

Faghir et al. (2011), identified two types of vascular bundles within 27 species of *Potentilla* and four related genera. According to the author the *P. pannosa* and *P. kurdica* have a collateral vascular bundle. Furthermore, Faghir *et al.* (2014), based on ITS region found that *P. kurdica* and *P. pannosa* located in the same clade.

The tree based on combined regions in (Figure 3) represents the same result as *trn*L-F tree with high supports (bs=100%, pp=1.00).

The phylogenetic analysis based on chloroplast noncoding *trn*L-F and nuclear ribosomal ITS regions are showed to be monophyletic by Dobes and Paule (2010), Faghir *et al*. (2014), and Feng *et al.* (2017).

4.2. Pollen grains study

It is obvious that the pollen grains of *Potentilla* are generally tricolporate and prolate-spheroidal, oblate-spheroidal, or oblate. The size of the grains according to Erdtman (1952), are small to medium. The largest pollen grains were seen in *P. pannosa*. While, the smallest one is found in *P. speciosa*. The outlines of species are varied from circular to ellipsoid.

Tricolporate pollen grains are founded in previous works of authors (Reitsma, 1966, Eide, 1979b, Eide, 1981, Sánchez *et al*., 1998, Hesse *et al*., 2009, Faghir *et al*., 2012). With respect to quantitative data, the pollen morphological data of the present study were dissimilar to that of previous authors (Faghir *et al*., 2012), especially in P, E, and P/E mean values in *P. kurdica*, *P. reptans*, and *P. pannosa* where Faghir *et al*. (2012), reported larger pollen sizes. However, P/E means value of *P. hirta* and *P. reptans* are similar to previous authors (Sánchez *et al*., 1998).

The apertures are consisting of three ectocolpai and three endoporus. The operculum is another feature which covers the pores, in some species the operculum is located at the middle of aperture and partially covers it as in *P. pannosa* (Plate 3E). The exine sculpturing type includes striate pattern and consists of shallow, fingerprint-like ridges and predominately parallel arranged.

Exine sculpture types are important characters for identification between the members of family Rosaceae (Ueda and Tomita, 1989). Striate exine sculpture in *Potentilla* is found in previous investigations of authors like (Reitsma, 1966, Hebda and Chinnappa, 1990, Faghir *et al*., 2012). Perveen and Qaiser (2014), identified striate exine ornamentation in 17 species of *Potentilla* and also they found the variation between exine ridge intervals and on the basis of this variation the species are categorized into three categories: coaurse, medium, and fine striate.

Any pores on the surface of exine sculpture have not seen due to the low resolution of the images of SEM. While, the microperforate-striate exine type is the predominant feature in most species of *Potentilla* (Hebda and Chinnappa, 1990, Chung et al., 2010, Hebda and Chinnappa, 1994). (Faghir et al. (2012)), found the

striate perforate with round pore in *P. reptans* and striate perforate with linear pore in *P. pannosa*.

5. Conclusions

In the present study three major clades within the species of *Potentilla* were identified. In the *trn*L-F tree the species *P. hirta* and *P. kurdica* placed in the same clade, while in the ITS tree the species *P. hirta* replaced by the species *P. pannosa*. The analysis of both *trn*L-F and combined regions are appeared similar in the arrangement of the taxa.

The study of the pollen grains by using Light and Scanning Electron Microscope showed that the palynological data of *Potentilla* species are not representing more variation among the species. One reason may be that *Potentilla* pollen grains appears stenopalynous under light microscope, and another reason due to the limited numbers of *Potentilla* species in Kurdistan region – Iraq. However, the present study revealing different pollen shapes among species.

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