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Molecular study in some species of family Papaveracea and Fumariaceae in Iraq and Iran by used *matK* gene and ITS4/5

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Abstract
The position between the three families Papaveraceae, Fumariaceae and Hypecoaceae is one of the most problematic taxonomic. To understand the phylogeny relationship of these genera thirty six samples of Papaveraceae *s.l.* were extracted for DNA analysis. Three primers based on ITS of nrDNA ITS3,5 ITS4,5 and *matk* chloroplast gene were used in PCR amplification. Phylogenetic relationships among genera and species of Papaveraceae *s.l.* based on Neighbor-joining (NJ), UPGMA and ML-DTR-GL 1000 models were drowning. The clades of phylogenetic relationship clearly support relationships of Fumariaceae to the Fumarioideae in the family Papaveraceae *s.l.*
Keywords: Papaveraceae, Fumariaceae, classification, ITS, *matk*

Introduction

The family Papaveraceae took a big attention from many scientists because it is one of the most interesting families having medicinal properties and taxonomic problems. The relationships between the three families Papaveraceae, Fumariaceae and Hypocoaceae are still a matter of discussion (Taia, 2009). Papaveraceae has long been known by human civilization since ancient times because of their benefit in medicine as narcotics and hallucinogenic agents (Hassan Dar *et al*., 2010).The family is well known the commercial and medicinal importance, *Papaver* is the most important genus of the family for its producing the opium alkaloids including morphine, codeine and heroine which are of many important medicinal applications. The family Papaveraceae is a medium sized family comprising of more than 40 genera and 770 species. It distributed mainly in the northern temperate region of the world (Kadereit, 1993a).

In Iraq the family is distributed throughout the country, 5 genus and 26 species Cullen (1980) provided a taxonomic account for the family in Iraq in its strict sence based only gross morphology,
and treated Fumariaceae as a separated family.

Recent studies on different aspects of taxonomical evidences such as anatomy, chemistry and molecular characteristics (Kadereit *et.al*., 1995; Hoot *et.al.*, 1997; Heywood, 2007) showed different opinions on Papaveraceae classification, they clearly supporting the *sensu lato* concept of the Papaveraceae based on DNA analysis. Due to lacking of any detailed taxonomical studies on the Papaveraceae *s.l.* in Iraq, the present study aims to: Comparing species by using PCR and DNA analysis to resolving the controversial between the Papaveraceae and Fumariaceae by used some Iraqi and Iranian species.

Materials and methods:

The molecular study carried out in the department of Agronomy and Plant Breeding, and the Central Laboratory of College of Agriculture and Natural Resources, University of Tehran, Karaj-Iran, materials were used which were collected during the sampling trip (carried out during the spring months (February to May) in 2013 and 2014 for collection and identification of plants from different cities in Iraq) with dry samples from National Herbarium of Iraq (BAG), Herbarium of Basra University at Science College (BSRA), Herbarium of Bagdad University (BUH) and National Herbarium of Iran (TARI).

DNA Extraction

DNA was extracted from dried leaves. The total DNA was extracted in CTAB (cetyltrimethyl ammonium bromide) isolation buffer according to the modified protocol of Doyle and Doyle (1987) and Costa-Sánchez *et al* for herbarium samples. (2006).

Two primer pairs namely ITS4, ITS5, and *matk* were used as. These primers were used for PCR amplification and direct double-stranded DNA sequencing. The sequence of these primers is as follows:

Primers	Origen			Sequences (5' ⟶ 3')
matk	Micro gene Company (South Korea)	390F	F	5'-CGATCTATTCATTCAATATTTC-3'
		1326R	R	5'-TCTAGCACACGAAAGTCGAAGT-3'
ITS		ITS5	F	5'- GGAAGTAAAAGTCGTAACAA -3'
		ITS4	R	5'- TCC TCC GCT TAT TGA TAT GC -3'

Reaction conditions for the *matk* region were : denaturation at 94 °C for 3 min followed by 30 cycles of 1 min at 94 °C for, 1 min at 51 °C, 1 min at 72 °C and a final extension at 72 °C for 7 min.

PCR amplification for ITS region was achieved using touchdown PCR strategy involved: denaturation at 94 °C for 3 min followed by 30 cycles of 1 min at 94 °C for, 1 min 58-51 °C (over the first eight cycles with the remaining cycles at 52 °C) 1 min at 72 °C and a final extension at 72 °C for 7 min. PCR products were purified for sequencing by using GeneAll Exin kit following the protocol recommended by the manufacturers.

Results

1- DNA Extraction

DNA extracted from 36 samples of dry leaves by used CTAB, the modified method (Doyle and Doyle, 1987) and (Cota-Sánchez *et al*., 2006). In this study used two methods for extraction DNA, the first included samples collected during trips field where its period of collection does not exceed more than one year, the second method included herbarium samples collected from different herbarium. The quantity and quality of the DNA, in general, is better in recent samples than from the herbarium samples, but this depends on the preservation and storage of samples, whether recent or old samples. The results have shown that DNA fragments size were between 750 - 900 bp in all species.

Analysis of *matk*

As shown in Fig. 1 phylogenetic analysis was carried out by using 16 species belong to four genera as outgroup which includes *Meconopsis csmbrics*, *Papaver triniifolium*, *Papaver nudicsule*, *Papaver radicstum*, *Papaver alpinum*, *Papaver hultenii*, *Papaver lapponicum*, *Papaver mcconnellii*, *Papaver dahlianum*, *Papaver comwallisense*, *Fumaria cspreolata*, *Fumaria muralis*, *Fumaria purpurea*, *Fumaria bastardii*, *Fumaria officinalis* and *Eomecon chionantha*.

The results of phylogenetic relationships appeared the topology of *matk* trees designed based on Neighbor-joining (NJ) for a bootstrap 50% (bootstrap percentage; BP) the tree involved 30 species of Papaveraceae and Fumariaceae, 18 species were grouped into 6 clades, whereas the remaining 12 species separated without gathered in a particular clade and these species included *P.rhoeas*, *F.bracteosa*, *P.macrostmum*, *P.macrostmum* T, *C.oppositifolia*, *P.macrostmum* S, *P.bornmueleri*, *P.somniferum*, *P.dubium*, *P.orientale*, *F.cilicica*, *E.californica*.

Clade 2 gathered the four species of section *Miltantha*, while *P.cylindricum* nested with *P.tenuifolium* and *F.densiflora*. Both species of section *Argemonidium* (*Argemonorhoeades*) *P.argemone* and *P.hybrida* nested with *Roemeria hybridum* in clade 3. *H. geslinii* and *H. pendulum* gathered in clade 4 in 98 BP, while *H.imberbe* nested with genus *Papaver* in clade 1. As shown in Fig. 1.

The conducted analysis by ML- DTR- GI 1000 model, composed 7 clades which gathered 20 species, whereas the remaining 10 species separated without gathered in a particular clade and this species included *P.macrostmum* S, *P.macrostmum* T, *P.macrostmum*, *F.bracteosa*, *P.bornmueleri*, *P.rhoeas*, *P.oriental*, *P.somniferum*, *C.oppositifolia*. *F.cilicica*. they were also gathered with different species from genus *Papaver* section *Miltantha* as appeared in clade 1 with 57 BP.

Furthermore as above in NJ model, genus *Fumaria* gathered with genus *Glaucium* in clade 6 and with *Papaver* in clade 7. As for *H. imberbe* was collected with genus *Papaver* in clade 2, while clade 3 clustered genus *Roemeria* with *Papaver* section *Argemonidium* as shown in Fig 1.

Conducted analysis by UPGMA model Fig.2, composed 5 clades which were gathered 29 species, only *E.californica* was separated without gathered in a particular clade. Clade 1 is the main clade which consists of 10 species that nested from different genera and included *Papaver*, *Hypecoum*, *Fumaria* and *Corydalis*. Also, clade 5 gathers species from different genera which involve *Papaver*, *Hypecoum*, *Fumaria* and *Glaucium*. Results

noticed *F. parviflora* is a sister group with *G. corniculatum* in 100 BP and *F. densiflora* is a sister group with *P.tenuifolium* in 83 BP.
The similar to both models NJ and ML, genus *Roemeria* clustered with *Papaver* section *Argemonidium* in clade 4, and *F.cilicica* gathered with genus *Papaver* section *Miltantha* in clade 2 with high support 100 BP. But this model different from the previous two other models in the overlapped of species *P.somniferum* with different section in genus *Papaver* in clade 3, as shown in Fig.3.

Analysis of ITS4 and ITS5

As shown in Fig. 4 the results of phylogenetic relationships the topology of ITS 4 and ITS 5 trees designed based on Neighbor-joining (NJ) for a bootstrap 50% (bootstrap percentage; BP) majority-rule agreement tree for 28 species of Papaveraceae and Fumariaceae, 22 species were grouped into 5 clades and 6 species unaffiliated with a particular clade which included *P. rhoeas, P. persicum, P. dubium, P.somniferum, P. orientale, P.bracteatum*. ITS4 and ITS5 sequence numbers were obtained from Gene Bank.

Species that group in one clade do not always display morphological groupings, for example clade 5 include species in different genera from *Papaver*, *Fumaria*, *Roemeria* and *Hypecoum*. The out-group included nine species from four genera, *Meconopsis integrifolia*, *Hypecoum procumbens, F. officinalis, P.pilosum, P.alpinum, P.nudicaula, P.pavonium, P. commutatum* and *P.pseudoorientale.*

Similarly, the conducted analysis by Maximum-Likelihood (ML-K2P) model Fig. 5, included five clades. Also 22 species were grouped into 5 clades and 6 species nonaffiliated with a particular clade which included *P. dubium, P.somniferum, P. persicum, P. rhoeas, E.californica* and *C.oppsitifolia*.
With some minor differences, in the ML tree the relationships among species were similar as see with NJ analysis. The variation was in bootstrap percentage between them. The only differences observed were the grouping of *E.californica* and *C.oppsitifolia* in Clade 1 in NJ tree.

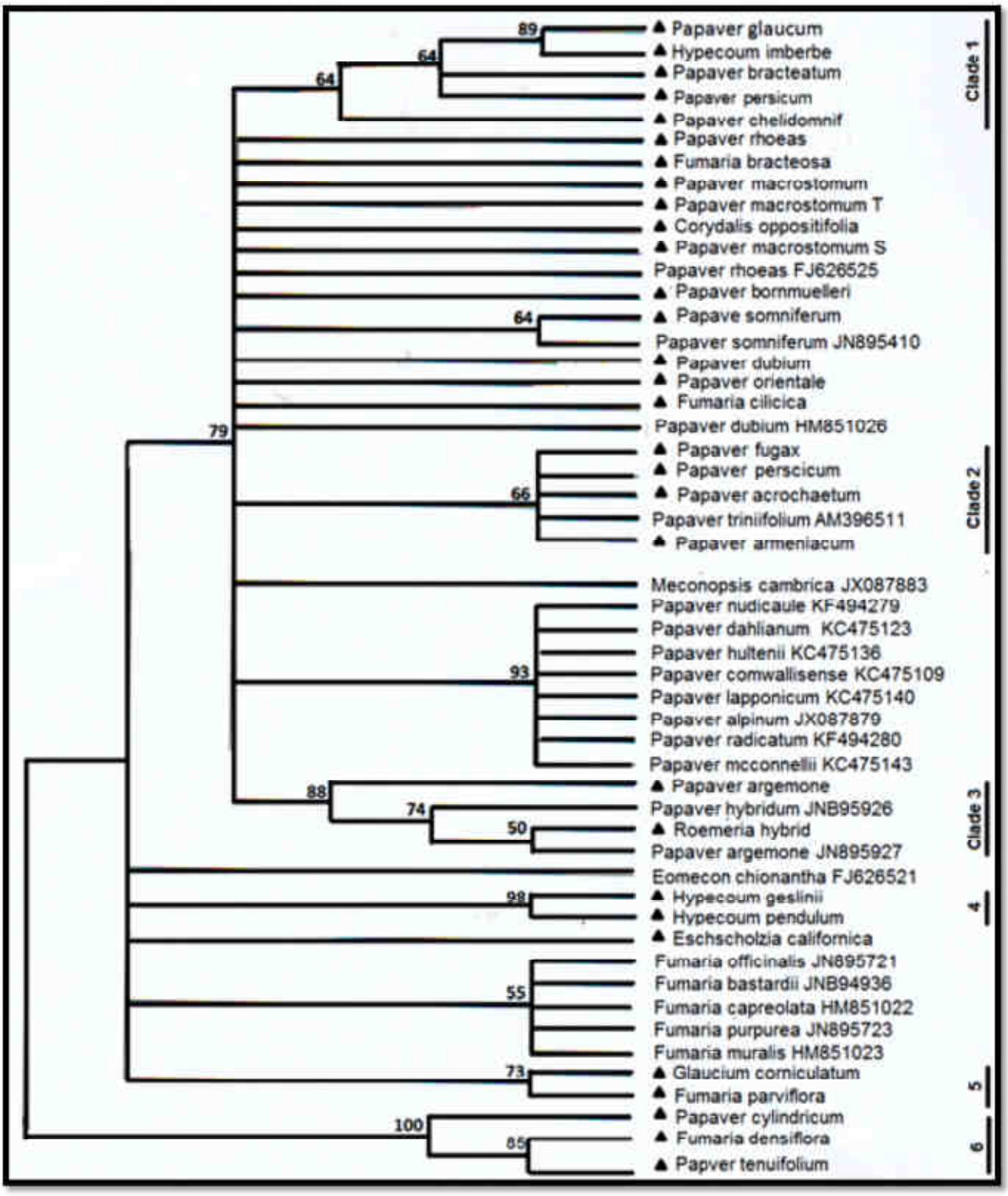

Fig. 1: Phylogenetic relationships among Papaveraceae and Fumariaceae using *matk* based on Neighbor-joining NJ model

▲ **Species under study / NCBI samples ex. *Papaver macrostomum* DQ250275**

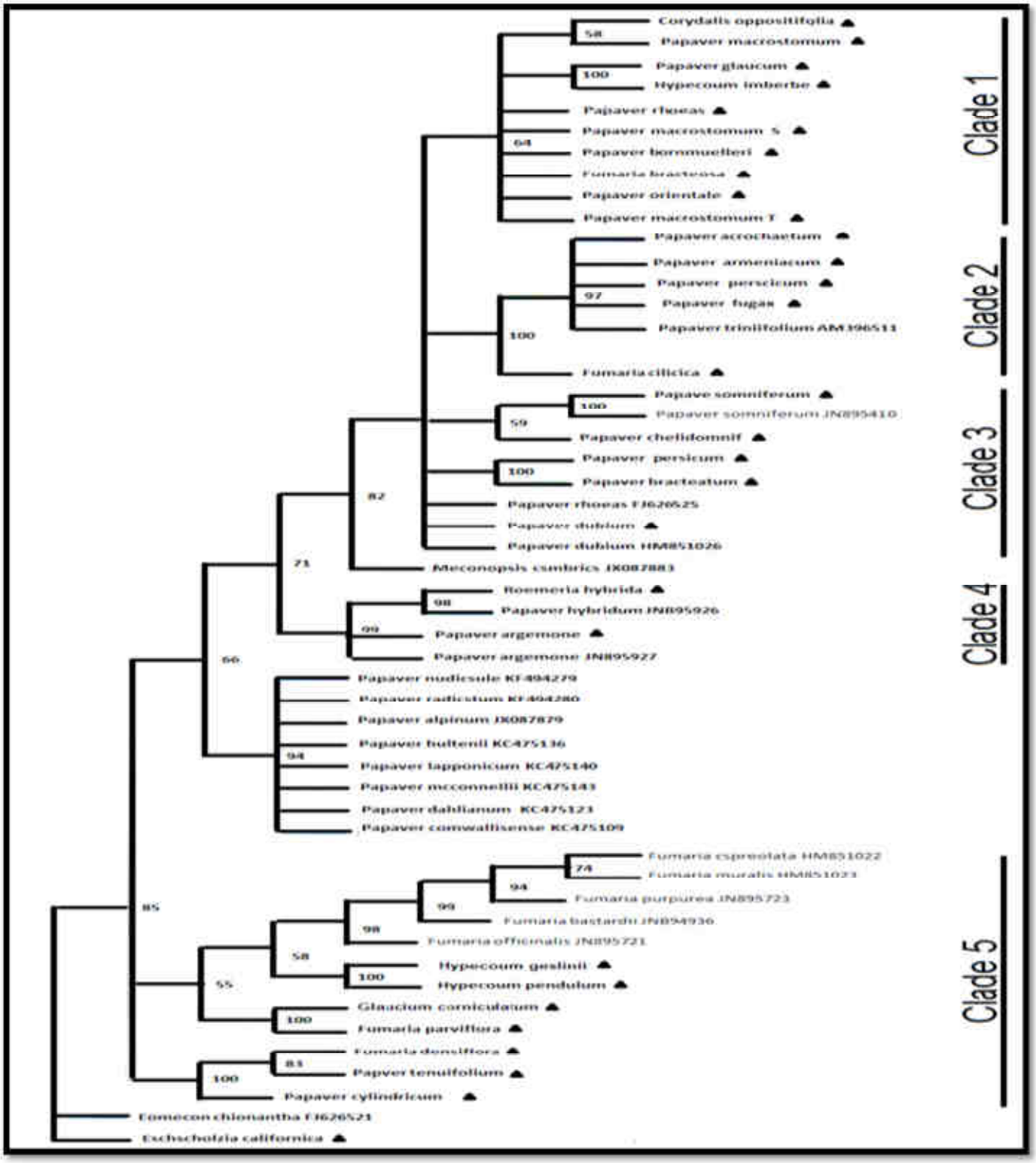

Fig. 2: Phylogenetic relationships among Papaveraceae and Fumariaceae using *matk* based on UPGMA model

▲ **Species under study / NCBI samples ex. *Papaver macrostomum* DQ250275**

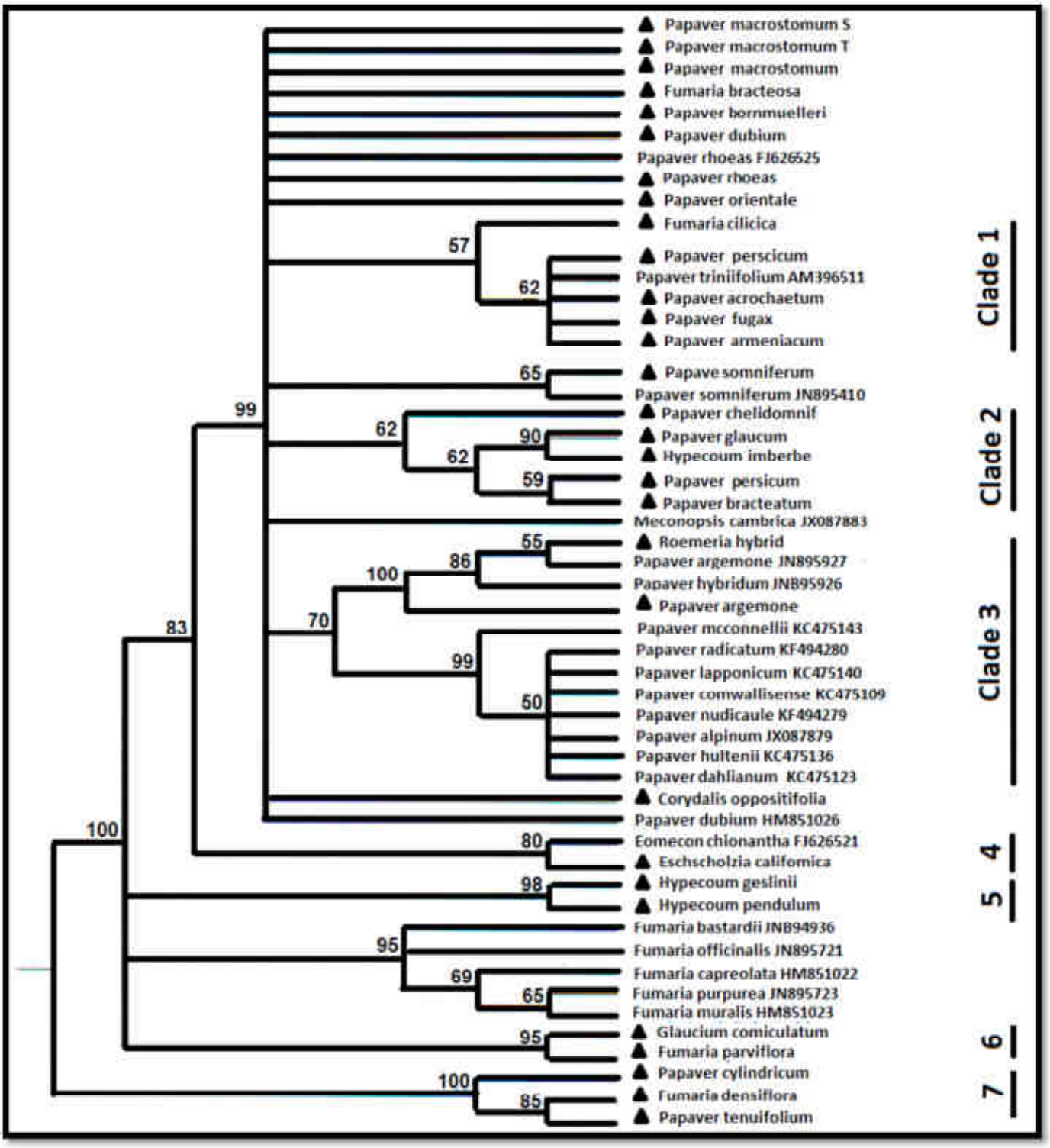

Fig. 3: Phylogenetic relationships among Papaveraceae and Fumariaceae using *matk* based on ML- DTR-GI
1000 model
▲ **Species under study / NCBI samples ex. *Papaver macrostomum* DQ250275**

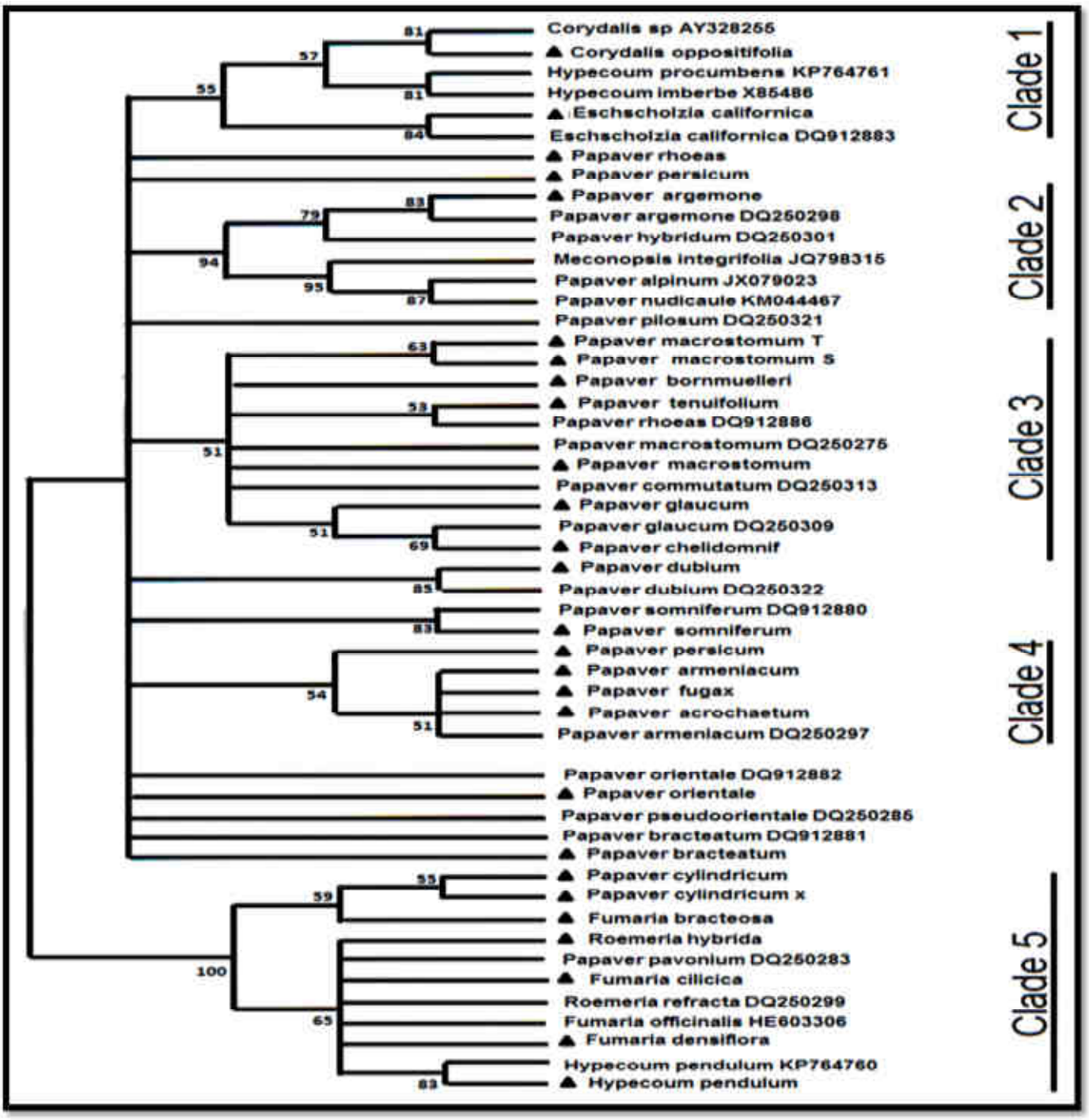

Fig. 4: Phylogenetic relationships among Papaveraceae and Fumariaceae using ITS4 & ITS5 based on
Neighbor-joining NJ model
▲ Species under study / NCBI samples ex. *Papaver macrostomum* DQ250275

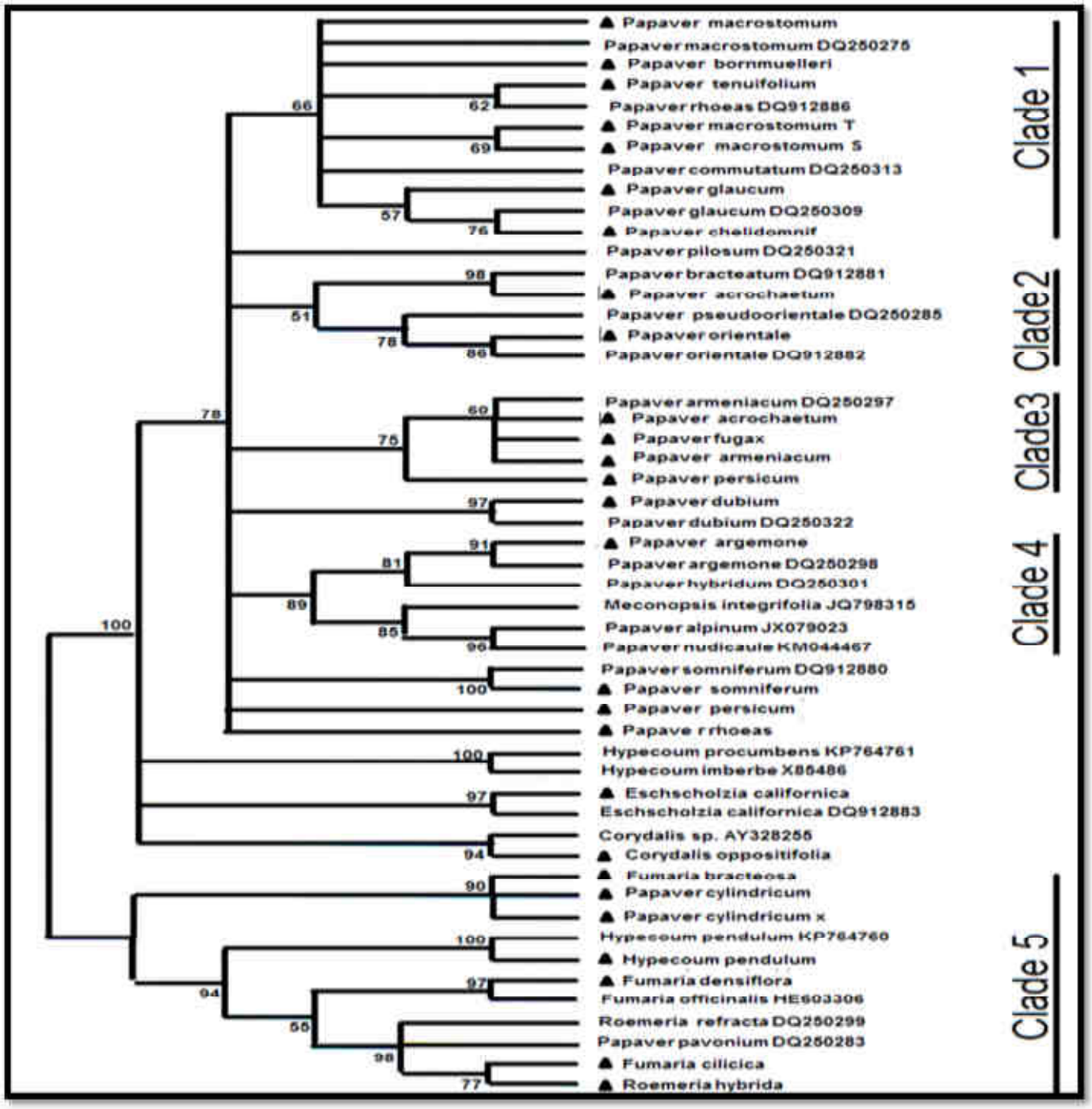

Fig. 5: Phylogenetic relationships among Papaveraceae and Fumariaceae using ITS4 & ITS5 based on
Maximum-Likelihood (ML-K2P) model
▲Species under study / NCBI samples ex. *Papaver macrostomum* DQ250275

Discussion

Extraction of DNA from a fresh material is perfect for getting good quality of DNA, but it can be extracted from each fresh, lyophilized, and dried sample. The choice of a protocol for DNA extraction depends on the quality and quantity of DNA that we needed, the samples nature, and the natural substances presence that may intervene with the extraction and latter analysis (Semagn *et al*., 2006). For this reason, this study has followed two methods of extraction one of them for herbarium samples and the other for the samples that collected during the time of this study and the modified protocol because some samples are very old or not preserved in good conditions. Therefore, we must test several protocols to find out the best one that serves for the species under search.

The DNA intensity of absorbance was measured by Spectrophotometer at 260/280 nm wavelength, which shows the existence of protein contaminants but it does not determine if the DNA is degraded or not, in this stage agarose gel it is used to check whether the DNA is degraded or not.

Then the size of bands were measured by using the molecular ladder, but DNA concentration is estimated by visually distinguished band density of the extracted DNA with a ladder of known concentration depending on the person who works (Semagn *et al*., 2006). *matK* showed the highest level of universality performed better in gymnosperms than in angiosperms, while ITS performed relatively well in angiosperms and detection of sequence quality indicates that high-quality sequences were obtained 60.2% for *matK*, and 58.6% for ITS (Li *et al*., 2011).

In this study, *matK* and ITS4/ITS5 have been effectively utilized in distinguishing morphologically similar species and in solve the dubious species; furthermore in resolving the controversial between the Papaveraceae and Fumariaceae by drawing trees.

The phylogenetic tree is a result of phylogeny reconstruction, which can be either rooted or unrooted. In the unrooted tree, groupings are detected; it only displays the relationships between the taxons. While the rooted tree involving directionality in time and shows the relationships with regard to an out-group. The choice of out-group can be on the basis of the most informative and the actual sister group (Michu, 2007).

Utilization of (cpDNA) has been widely applied to understand plant phylogenies at various taxonomic levels (Gielly and Taberlet, 1994). *matk* gene which encodes a chloroplast in the *trnK* intron has become more common for plant molecular systematic because this gene has a comparatively great substitution rate which shows that it may be suitable for systematic studies at lower taxonomic levels (Li *et al*., 1999). *matk* is a region that exists universally in land plants and only a few exceptions of a secondary loss or reorganizations (Wicke and Quandt, 2009).

From this study has clarified that *matK* highest sequence quality for recovered samples and more phylogenetically informational sites than ITS. In *matK* we used NJ, ML and UPGMA analysis. In the three models, the species *F. parviflora* was isolated with *G. corniculatum* with high bootstrap support in all models and reached to (100%) in UPGMA model, while species *F.densiflora* was a sister group with *P.tenuifolium* with bootstrap support in all models that reached to (85%) and that these two species are sister group to the *P.cylindricum*; and this support for a monophyletic origin of *Fumaria* with *Papaver* and *Glaucium*.

R. hybrida was a sister group with *P. argemone* in the trees NJ and ML models with weak bootstrap support in (50%) and (55%) respectively, while in UPGMA model very strong bootstrap support in (98%) between *R. hybrida* and *P .hybridum*; this agrees with (Kadereit & Sytsma, 1992) and (Hoot *et al*., 1997) who place *Roemeria* near *Papaver* sec. *Argemonidium* particularly *P.hybridum*.

Section *Argemonidium* in genus *Papaver* shares some character tics with *Roemeria*, most species of *Papaver* have 3 colpate pollen grains, sect. *Argemonidium* and *Roemeria* have pollen grains with polyporate, and the similarity is in capsule morphology. Also, most species in the family have sepals with a flap - like lobe on their upper left-hand margin while in sect. *Argemonidium* and *Roemeria* the sepals with this lobe are on the right-hand margin (Ernst, 1962).

One of the problems noted with ITS where universal primers hampered PCR and sequencing did not succeed. Our results confirmed that ITS had minimum amplification and sequencing success compared with the *matK*; match with what mentioned by Li *et al*., (2011) that ITS region had lower amplification and sequencing success when compared with the three plastid DNA regions (*rbcL*, *matK*, *trnH–psbA*), especially in gymnosperms.

In ITS4 and ITS5 the present molecular phylogeny clearly indicates the monophyly of genus *Fumaria* with *Papaver* as shown in Fig. 4. The (NJ) analysis supported that *F.bracteosa* is nested within *P.cylindricum* in clade 5, also *F.cilicica*, and *F.densiflora* gathered with genera *Papaver*, *Roemeria* and *Hypecoum*; whereas genus *Corydalis* is gathered with genera *Hypecoum* and *Eschscholiza* in clade 1.

However, in the other trees using (ML) Fig 5, essentially similar results were obtained which *F.bracteosa*, *F.cilicica*, and *F.densiflora*n are nested with genera *Papaver*, *Roemeria* and *Hypecoum*. But *Corydalis oppositifolia* nonaffiliated with a specific clade but it was a sister species with *Corydalis sp.*

In figs (3 and 4) clades 3 and 4 consecutively *Fumaria* is sister to *Hypecoum* with BP 95% and 88% respectively, while in UPGMA analysis *Fumaria* and *Hypecoum* are separated into two clades and the relationship between them is strongly supported by 99 BP. Also genus *Corydalis* with genus *Hypecoum* were separated as sister group in clade 1 in NJ model, Hoot *et al*., (1997) mentioned in their study that the genera *Corydalis* and *Hypecoum* from the Fumarioidae are closely related on the basis of *rbcL*, *atpB* and *trnK* sequences and they supported the separation in family Fumariaceae. In the current study analyzed three specimens from *P.macrostomum*. These similar species were nested in the same clade with the two specimens (*P.macrostomum* S) and (*P.macrostomum* T) the relation between them are weakly supported as a sister group. *P.macrostomum* is characterized by branched, 15-40 cm and petals scarlet with black blotches at the base. From field observation (*P.macrostomum* T) was distinguished by differing in petals color between the white, pink, peach with black blotches at the base and its distribution restricted in MSU in Qala Chulan village. While (*P.macrostomum* S) is distinguished by differing in the length ranging from 7-12cm and the stems solitary and its distribution in MSU and MRO. Therefore it may be treated as separated species or at least a subspecies within *P.macrostomum*.

Several shared morphological features such as leaf hispid setose, plant covered with setose hair, bud glabrous and capsule oblong-ellipsoid which support their affinity. This is based on our results in ITS4 and ITS5 which distinguish these 3 specimens and separated them as various species.

In the dubious species *P.cylindericum* (herbarium specimens) and *P.cylindericum* x (collected by the researcher) they nested in one clade in both trees of ITS4/ITS5. Because the overlapping characters between the species in section *Miltantha*, the researcher decided to conduct molecular analysis to verify the classification.

Molecular analysis has shown that the classification of this sample that collected from the Haj Umran in MRO was closer to be *P. cylindericum.* Regarding to the species *P.somniferum* it grouped with reference species of *P.somniferum* from NCBI in all trees with various bootstrap which reached to 100 BP in both *matk* gene in the model UPGMA and ITS4/ITS5 in the model ML and this clarified that both species are the same.

From this molecular study we noted there is a clear overlap between the species of Fumariaceae and Papaveraceae and this shows the monophyly between these two families, this matches with Kadereit *et al.*, (1994) and Hoot *et al*., (1997) who considered *Fumaria* as subfamily Fumarioidae in Papaveraceae.

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