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IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF SELECTED MEDICINAL PLANT SPECIES FROM PAKISTAN: DNA BARCODING APPROACH

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Abstract

Proper identification of medicinally important plant species is useful for their efficient utilization as food and medicine. Seventy eight species belonging to different plant families were characterized through chloroplast DNA gene sequences of *rbc*L, *mat*K and intergenic spacer region *psb*A-*trn*H. These species were used to search possible relationships through phylogenetic analysis. The phylogenetic tree based on *mat*K (~800 bp), *rbc*L (~1400 bp) and *psb*A-*trnH* (~500 bp) sequences showed 6, 8 and 4 diverged groups, respectively. Our analysis suggests that *Ajuga bracteosa, Salvia aegyptiaca, Bupleurum falcatum* and *Acorus calamus* are highly diverged from rest of seventy four different species. Based on the results of medicinal plants from Pakistan, we validate that *mat*K and *rbc*L are more useful as barcode for studying large population of plant species. The present study is useful for authentic identification of new plant species and clearly shows high degree of intra-specific and inter-specific evolutionary relation among all tested species from different medicinally important plant families. Our findings suggest that proper identification of some of the plant species is still challenging and these unique species need further studies. The present study can serve as a model to identify and differentiate new medicinal plant species from Pakistan and neighboring countries through similar methods.

Key words: DNA Barcode, Chloroplast DNA, Phylogenetics, Medicinal plants.

Introduction

Identification of important medicinal plant species is a difficult task and it needs expert taxonomists. At present 391000 vascular plant species are known to science of which about 94% are flowering plants (Anonymous., 2016). However, large number of plant species are yet to be identified. About 2000 species are being described every year. Therefore, limited number of species has been identified through conventional identification and classification methods (Chase & Fay, 2009; May, 1992). These medicinal plant species are mostly collected by the local untrained collectors based on the indigenous knowledge. During the collection process, sometimes the closely resembling undesirable species are also collected and sold with the same name. Therefore, it is necessary to collect proper species with the help of expert taxonomists from different regions of the country/state. In addition, proper identification and classification would be useful to preserve the threatened or endangered plant species that seem decreasing day by day worldwide especially in developing country like Pakistan (Hussain *et al.*, 2009).

DNA barcode is one of the advanced molecular marker-based methods that identify target plant species in short duration. The purpose of DNA barcoding is nucleotide sequence based identification of multiple plant species with accuracy and is one of the widely accepted technology (Group *et al.*, 2009). It is an efficient, quick, low-cost and standard method for evaluation and identification of different plant species (Khan *et al.*, 2015). In addition, this method can efficiently identify unknown species and/or species having complex morphometric behaviors (Hebert *et al.*, 2004). This technique is also used to study both inter-specific and intra-specific variations. Initially DNA barcoding was used to identify some metazoans species through cytochrome oxidase 1 (*CO1*) gene sequences. Later barcode regions like *rbc*L, *mat*K, *ITS* and *trnH-psb*A were used to identify flowering species (Kress *et al.*, 2005; Hollingsworth *et al.*, 2011). The DNA barcode is an improved and efficient method to differentiate among plant species (Zahra *et al.*, 2016; Zahra *et al.*, 2014; Khan *et al.*, 2015; Jamil *et al.*, 2014; Shinwari *et al.*, 2014). Although, the selection of an ideal sequence for DNA barcoding is a key to discover new plant species however, a single ideal barcode sequence is not discovered yet. Therefore two or multiple plastid sequences are used for discrimination/identification of plant species (Frezal & Leblois, 2008). Several different plant barcode sequences were recommended by different researchers. These include *rpoC*1 + *mat*K+ *trnH-psb*A or *rpoC*1 + *rpo*B + *mat*K, *rbc*L+ *trnH-psbA*, *mat*K + *atp*F/H + *trnH-psb*A and *mat*K + *atp*F/H + *psb*K/I (Kress & Erickson, 2007). According to CBOL (Consortium for the Barcode Life) the cp DNA regions *rbc*L and *mat*K play vital role for rapid identification of medicinally important plant species.

The *rbc*L (1400 bp) and *mat*K (1500 bp) plastid DNA sequences are considered important to study the similarity and differences found among different plant species and widely used to solve evolutionary and taxonomic issues (Janzen, 2009; Neuhaus & Link, 1987). These medicinal plants play key role in controling and treatment of many diseases (Khan *et al.*, 2017; Habiba *et al.*, 2016, Qasim *et al.*, 2016; Hayat *et al.*, 2016; Ullah *et al.*, 2017; Jan *et al.*, 2015). However, limited data is avalible to diffentiate medicinally important plant species from Pakistan. Therefore, comprehensive study was designed to test

*rbc*L, *mat*K and *trnH-psb*A regions for authentication and phylogenetic investigation of various medicinal plant species from different regions of Pakistan.

Material and Methods

Plant material collection and storage: Selected native plant species were collected from different regions of Pakistan. However, two species of *Mentha* viz. *M. aquatica* and *M. suaveolens*, grown were collected from Qarshi Medicinal Plants garden, Hattar, Pakistan. These plants were collected during their flowering seasons. Herbarium sheets were prepared and the voucher samples were deposited to the Molecular Systematics and Applied Ethnobotany Lab (MoSAEL), Quaid -i-Azam University Islamabad, Pakistan. The plant material was kept in sealed plastic bags added with the silica gel to absorb the moisture content.

DNA extraction, amplification, and sequencing: Total genomic DNA was extracted from specimens by grinding the tissue in liquid nitrogen and DNA was isolated using 2% CTAB (Cetyltrimethyl ammonium bromide) method (Richards, 1997) with few modifications. The obtained pellet was washed with 70% ethanol and allowed to dry at room temperature. The DNA pellet was dissolved in TE buffer (10 mMTris–HCl, pH 8.0, 1 mM EDTA) to a final concentration of 20–30 ng/µL. For the amplification of the desired loci, PCR reaction was carried out in 0.5 ml PCR tubes. The desired loci were amplified using KAPA3G Plant PCR Kit (Kapa Biosystems, Woburn, Massachusetts, USA) as described by Zahra *et al*., (2016). The cycling conditions used for *rbc*L were: 95°C 10 min; 50 cycles: 95°C 20 s, 58°C 15 s, 72°C 90 s; and final elongation at 72 °C 90 s. The *rbc*L forward primer 1F (Fay *et al.*, 1998) and 1460R reverse (Cuenoud *et al.*, 2002) were used in this experiment. Same PCR parameters were used for *mat*K 390F/1360R primers (Cuenoud *et al.*, 2002) with 50°C annealing temperature for 40 cycles (Schori *et al.*, 2013; Shinwari *et al.*, 2014). A touchdown program was carried out for *psbA-trnH* using the PsbAF/PsbHR primers (Sang *et al*., 1997; Tate & Simpson, 2003), where the annealing temperature was 58°C for initial 11 cycles followed by touchdown to 48°C for 29 cycles (Schori *et al.*, 2013; Shinwari *et al*., 2014; Zahra *et al.*, 2016). PCR products were examined through gel electrophoresis using 1% agarose gel. PCR products were cleaned with the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, Wisconsin, USA). The purified PCR products were sequenced using ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA). The sequencing product was purified using Big DyeX Terminator Purification Kit (Applied Biosystems). For sequencing of *mat*K gene and *psbA-trnH* spacer, same primers were used as were for amplification while in case of the sequencing of *rbc*L gene, internal primers (636F, 724R) (Fay *et al.*, 1997), and external primers (1F, 1460R) were used (Zahra *et al.*, 2016).

Data analysis: The generated sequences were submitted to GenBank under the accession numbers provided in Table 1. Sequence data from previous studies of same authors was also incorporated in the analysis. For analysis, target nucleotide sequences were aligned by using Mega 7.0 (Sudhir *et al.*, 2015). The final alignments were analyzed separately by Neighbor Joining (NJ) method using Mega 7.0 and final consensus phylogenetic trees were developed for *rbc*L, *mat*K and *psbA-trn*H. Bootstrap support was accessed by 1000 replicates.

Results and Discussion

In the current study a total of 78 medicinally important species collected from different regions of Pakistan were assessed for identification and classification using DNA barcoding data (Fig. 1). Although, the general protocol for the isolation and amplification of DNA remained the same but in some cases the PCR conditions were optimized. Rigorous optimizations for the isolation of DNA and the PCR annealing temperature for family Rosaceae were performed. Among the total selected species 54 *mat*K, 72 *rbc*L and 25 *psbA-trn*H nucleotide sequences were found in good order to be further used for the phylogenetic analysis. All these sequences were submitted to GenBank database with the accession numbers illustrated in Table 1.

The phylogenetic analysis based on *maturase* K (*mat*K) gene classified the target plant species into six diverged groups (Fig. 2). The first group had 6 sub-groups. The first sub-group contained seven different species. Among these species *Mentha longifolia is* closely related to *Mentha pulegium* and *Mentha x piperita* as compared to *Mentha spicata*. The second sub-group included three different species. *Lallementia royleana* and *Salvia aegyptiaca* are closely related as compared to *Nepeta cataria*. The third sub-group had four diverged species including *Melissa officinalis*, *Salvia moorcroftiana*, *Rosmarinus officinalis* and *Salvia plebeia*, respectively. The sub-group IV included four species from genus *Ocimum* and all accessions are in close relation with one another. The sub-group V had three diverse species i.e. *Stachys byzantina*, *Leucas cephalotes* and *Rydingia limbata* (= *Otostegia limbata*). The sub-group VI had one unique species as *Ajuga bracteosa* and this sub-group was highly diverged from other five sub-groups. The group II included species from four different genera i.e. *Withania, Solanum, Datura* and *Physalis*. The *Withania* and *Physalis* species are highly dissimilar from one another within the group. However, the speciess from same genus are in close relation as compared to species from different genera.The group III had six distinct species such as *Bupleurum falcatum*, *Scandix pectin-veneris, Heracleum candicans, Apium graveolens, Foeniculum vulgare* and *Trachespermum ammi*, respectively. The group IV included six different species from genus *Tamarix*. The group V had five species from four different genera like *Ricinus*, *Chrozophora*, *Euphorbia* and *Triadica*. In last group VI predominantly included three species from genus *Potentilla* and one species each from genus *Triadica*, *Eriobobtrya* and *Dasiphora* (Fig. 2).

Table 1. List of different medicinally important plant species (n = 78) of Pakistan studied through DNA barcode methods.

Sr. No.	Species	*Accession No.	Sr. No.	Species	*Accession No.
1.	*Acacia modesta*	KC336419	40.	*Nepeta cataria*	KP172059, KP172038
2.	*Acacia nilotica*	KC417041, KC417042	41.	*Ocimum africanum*	KP172062, KP218940
3.	*Acorus calamus*	D28865	42.	*Ocimum basilicum*	KP172060, KP172061, KP172036, KP172037, KP21893, KP218939
4.	*Ajuga bracteosa*	KP172049, KP172044, KP218929	43.	*Ocimum tenuiflorum*	KP172063, KP172072
5.	*Albizia lebbeck*	KC417043	44.	*Physalis divaricata*	JX996066, JX996060
6.	*Albizia procera*	KC417044	45.	*Polygonatum humile*	AB009947
7.	*Apium graveolens*	KP900771	46.	*Potentilla desertorum*	KT808473
8.	*Bupleurum falcatum*	KP900772	47.	*Potentilla indica*	KT808472
9.	*Chrozophora tinctoria*	KM273031, KM273036	48.	*Potentilla nepalensis*	KT808474
10.	*Clintonia borealis*	D17372	49.	*Prosartes lanuginosa*	D17374
11.	*Convallaria majalis*	D28334	50.	*Prosartes maculata*	D17375
12.	*Coriandrum sativum*	KP900778	51.	*Pyrus pashia*	KT808470
13.	*Dasiphora arbuscula*	KT808475	52.	*Ricinus communis*	KM273034, KM273038, KM273039
14.	*Datura inoxia*	JX996065, JX996059	53.	*Rosmarinus officinalis*	KP172074, KP218942
15.	*Datura stramonium*	JX996064, JX996058	54.	*Rydingia limbata*	KP172073, KP218941
16.	*Disporopsis pernyi*	D17373	55.	*Salvia aegyptiaca*	KP172075, KP218943
17.	*Disporum nantouense*	D17377	56.	*Salvia aethiopis*	KP172081
18.	*Disporum sessile*	D17376	57.	*Salvia moorcroftiana*	KP172082
19.	*Eriobotrya japonica*	KT808471	58.	*Salvia plebeia*	KP172076
20.	*Erythronium japonicum*	D28156	59.	*Scandix pecten-veneris*	KP900775
21.	*Euphorbia prostrata*	KM273037	60.	*Scilla scilloides*	D28161
22.	*Euphorbia pulcherrima*	KM273032	61.	*Scoliopus bigelovii*	D28162
23.	*Foeniculum vulgare*	KP900773	62.	*Senegalia modesta*	KP218946
24.	*Gloriosa superba*	D28867	63.	*Solanum melongena*	JX675575
25.	*Heracleum candicans*	KP900774	64.	*Solanum nigrum*	JX996062, JX996056
26.	*Isodon rugosus*	KP172080	65.	*Solanum virginianum*	JX996063, JX996057
27.	*Lallemantia royleana*	KP172070	66.	*Stachys byzantina*	KP172077, KP218945
28.	*Leucas cephalotes*	KP172050, KP218930	67.	*Streptopus lanceolatus*	D17381
29.	*Maianthemum dilatatum*	D17378	68.	*Tamarix aphylla*	KC840667, KC505173, KC840661
30.	*Maianthemum hondoense*	D17380	69.	*Tamarix indica*	KC840662, KC840663, KC505169, KC505168, KC840656, KC840657
31.	*Medeola virginiana*	D28158	70.	*Tamarix pakistanica*	KC840664, KC505170, KC840658
32.	*Melissa officinalis*	KP172051, KP172046	71.	*Tamarix passerinoides*	KC840665, KC840666, KC505172, KC505171, KC840659, KC840660
33.	*Mentha aquatica*	KP172053, KP172048, KP218932	72.	*Trachyspermum ammi*	KP900776
34.	*Mentha arvensis*	KP172052, KP218931	73.	*Triadica sebifera*	KP900777, KM273040, KM273035
35.	*Mentha longifolia*	KP172054, KP172043, KP218933	74.	*Tricyrtis affinis*	D17382
36.	*Mentha piperita*	KP172055, KP172042, KP218934	75.	*Uvularia sessilifolia*	AB009948
37.	*Mentha pulegium*	KP172056, KP218935	76.	*Vachellia nilotica*	KP218947
38.	*Mentha spicata*	KP172058, KP172039, KP218937	77.	*Withania coagulans*	KC291707, KC291706, KC291705
39.	*Mentha suaveolens*	KP172057, KP172040, KP218936	78.	*Withania somnifera*	JX996055

*The nucleotide sequences obtained in the current study has been submitted to GenBank

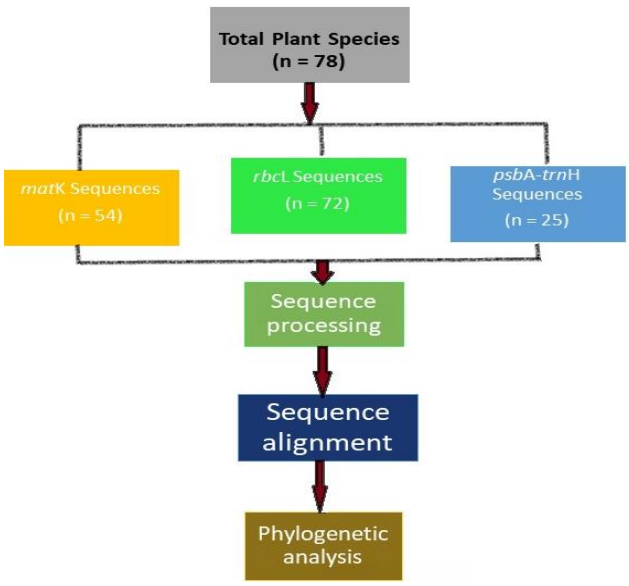

Fig. 1. Illustration of the general methodology used in the current work. DNA was extracted from the plant specimens and was processed for the nucleotide sequencing and post-sequence analysis.

The phylogenetic tree based on *rbc*L gene classified all species into eight major groups (Fig. 3). The group I consisted nine further small sub-groups. The sub-group I had all five species from *Mentha*. Among these species *Mentha longifolia* is grouped with *Mentha suaveolens* and *Mentha spicata* as compared to *Mentha aquatica* and *Mentha x piperita*. The sub-group II included two species from genus *Salvia* and are closely related to each other and sub-groups III, IV, V each had one species which are *Rosmarinus*, *Salvia* and *Melissa*, respectively. Sub-group VI contained two species e.g. *Nepeta* and *Salvia*. Genus *Ocimum* is included predominantly in sub-group VII. While, sub-group VIII included two genera, *Rydingia* and *Stachys* followed by a high diverse sub-group IX which included genus *Ajuga* only. *Withania*, *Physalis*, *Datura* and *Solanum* are the part of group II. *Solanum virginianum* has a close relation with *Solanum nigrum* as compared to other species e.g. *Datura inoxia*, *Physalis divaricata* and *Withania somnifera*. The group III a highly divergent group contains only genus *Bupleurum*.

The group IV contains further four sub groups in which sub group I has three genera including *Daucus* and *Scandix* (Fig. 3). *Heracleum* is the only genus present in the sub group II. The sub group III and IV contain *Foeniculum*, *Anethum* and *Trachyspermum* and *Bunium*, respectively. In group V the sub-group I contained genus *Tamarix* predominantly followed by sub group II which contained *Ricinus*, *Chrozophora* and *Triadica*. *Pyrus*, *Eriobotrya* and *Dasiphora* are present in sub group III, while sub group IV has *Acacia* and *Albizia* (Fig. 3).

The group VI had one unique species (*Acorus calamus*) and it is highly diverse from rest of groups based on the fact that it is not from Pakistan. The group VII contains five genera; *Maianthemum*, *Polygonatum*, *Disporopsis*, *Convallaria* and *Scill*a. The last group VIII included three sub-groups in which sub- group I had *Disporum*, *Uvularia* and *Gloriosa* species. There are three genera in sub-group II (*Clintonia*, *Medeola* and *Erthronium)* and four genera in sub-group III (*Tricyrtis*, *Prosartes*, *Streptopus* and *Scoliopus*) as shown in Fig. 3.

The genetic tree based on *psbA-tm*H gene sequences grouped all available 25 species into four main groups (Fig. 4). The group I, II and II had 13, 4 and 8 species respectively. This group is further divided into two sub-groups. The sub-group I had seven different *Mentha* species and two other different species from *Rosmarinus* and *Salvia*. These finding showed that a strong resemblance exist among these three genera (*Mentha*, *Rosmarinus* and *Salvia*). The sub-group II contained three different species from genus *Ocimum* and one accession from genus *Salvia*. The group II is highly diverged from rest two and had four different species from genera *Ajuga*, *Stachys*, *Leucas* and *Rydingia*. The group III is the smallest group having two genotypes from genera *Senegalia* and *Vachellia*. The last group IV is least variable that contained all 8 species from genus *Tamarix* (Fig. 4).

The proper identification and evaluation of biologically important plant families play a key role to understand evolutionary history of many important plant species. These methods enable researchers to find similarity and differences among different plant families. Among these methods DNA barcode is an inexpensive, standard and rapid method that can be used by researchers to identify and classify different organisms (Ikram *et al.*, 2015; Khan *et al.*, 2015; Shinwari & Shinwari, 2010). Phylogenetic study of important plant species is useful to identify new species (Onstein *et al.*, 2015). Several genes are used for DNA barcode studies like *rbc*L, *mat*K, *trnH-psbA*, *ndh*F, *trn*L-*trn*F, and *ITS* separately or in combinations (Moylan *et al.*, 2004).

The present study revealed the phylogenetic relationship of seventy eight selected species from important plant families collected from different regions of Pakistan. We described molecular systematics, to study genomic diversity among different medicinally important plant species. The study was conducted on the basis of three standard plant DNA barcode regions i.e., *mat*K, *rbc*L and *psbA-trnH-*. The findings of our study confirm that species from the same family are clustered together when nucleotide sequences are assessed by phylogenetic analysis. However, it was observed that identification and differentiation of some of the plant species is still challenging. We observed some diverged species that show unique sequence pattern (Figs. 2-4). These unique species need further investigations. Previously, *Euphorbiaceae* was classified into different groups using *rbc*L, *atp*B, *mat*K, 18S rDNA, *ITS*, *ndh*F and *trn*L-F (Yang & Berry, 2011). Yang & Berry (2011) studied the origin of *Euphorbia* through *ITS* and *ndhF* markers with genetic diversity of (0.05%). Peirson *et al.*, (2014) recorded the taxonomic relationship of some new world leafy species of genus *Euphorbia* via nuclear genome nr*ITS* and plastid *ndh*F regions with overall genetic diversity of 0.03%. Cardinal-McTeague & Gillespie (2016) identified species of Euphorbiaceae family by using *ITS* and plastid *psb*A-*trn*H DNA sequences as molecular markers. According to Group *et al.*, (2009) the chloroplast markers *rbc*L and *mat*K serve as universal plant barcodes. They also urged that other new additional markers single or in different combination must be used to screen further genetic relationships. In addition, McDade *et al.*, (2000) who envisaged the family Acanthaceae through *trnL-trnF* chloroplast region; found that *Ruellia* has maximum similarity with *Hygrophila* supported by 79.5, 96 and 74.5 BS value respectively.

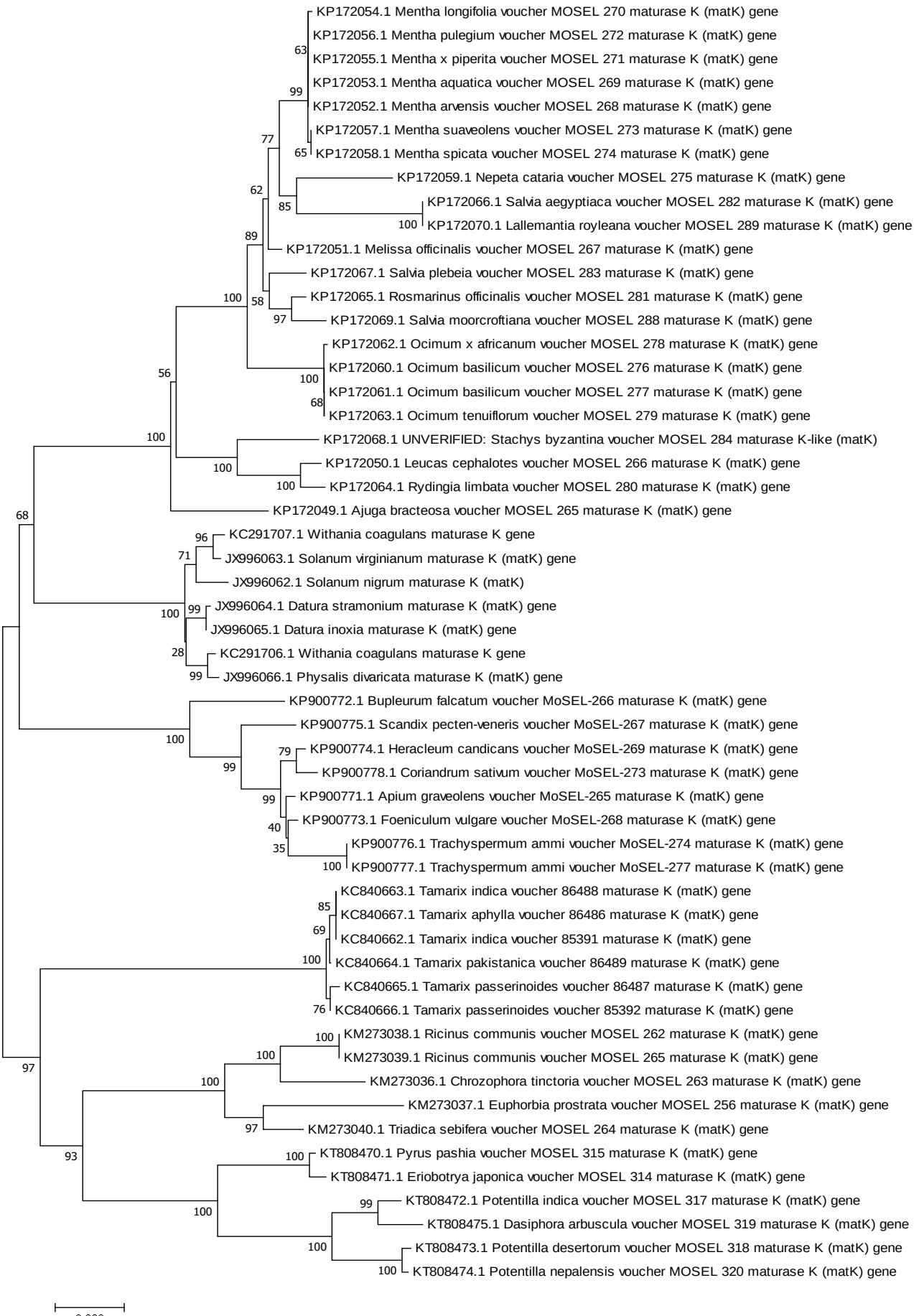

Fig. 2. Phylogenetic analysis of selected species from different medicinally important plant families using *mat*k gene marker (n = 54).

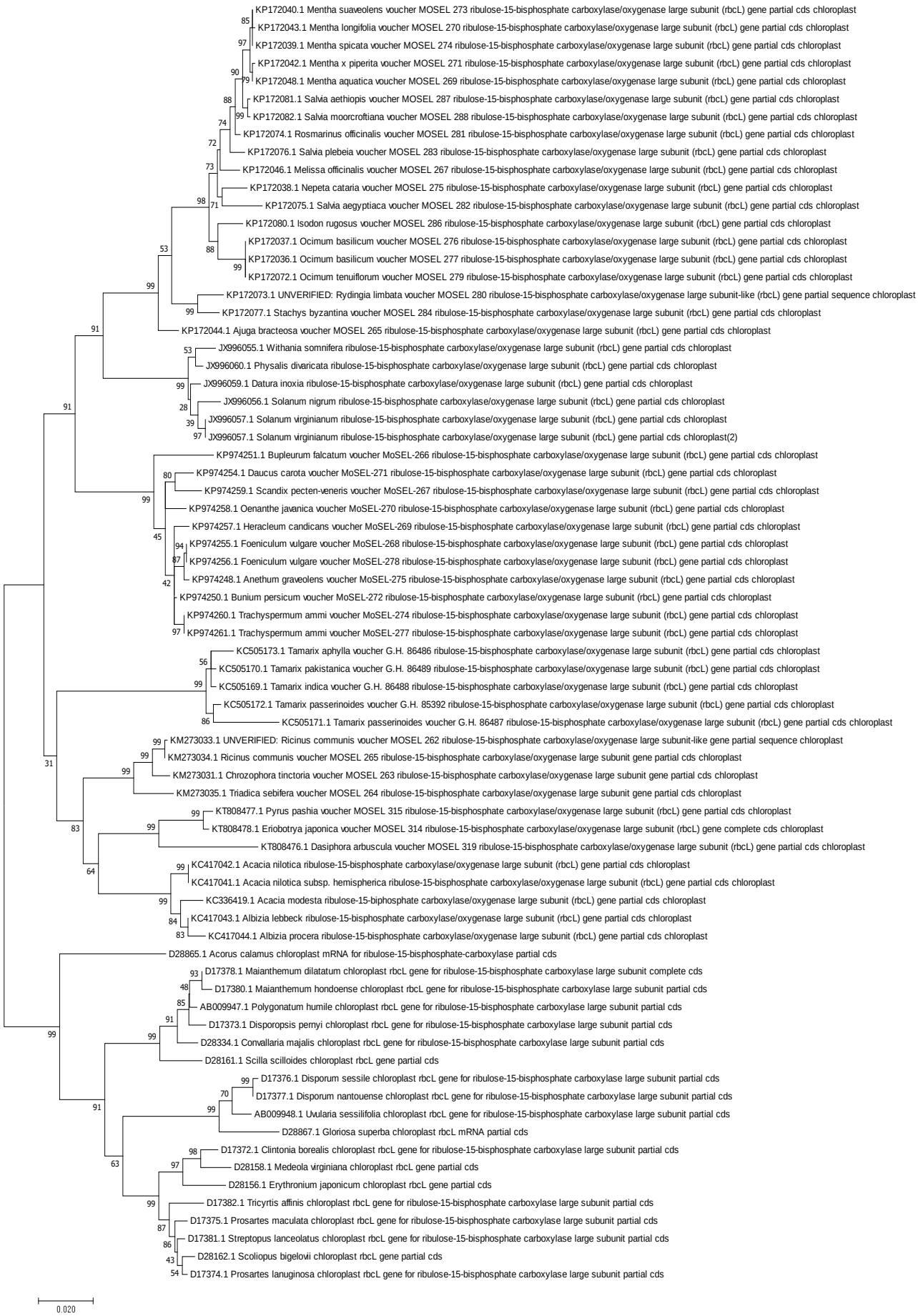

Fig. 3. Phylogenetic analysis of selected species from different medicinally important plant families using *rbc*L gene marker (n = 74).

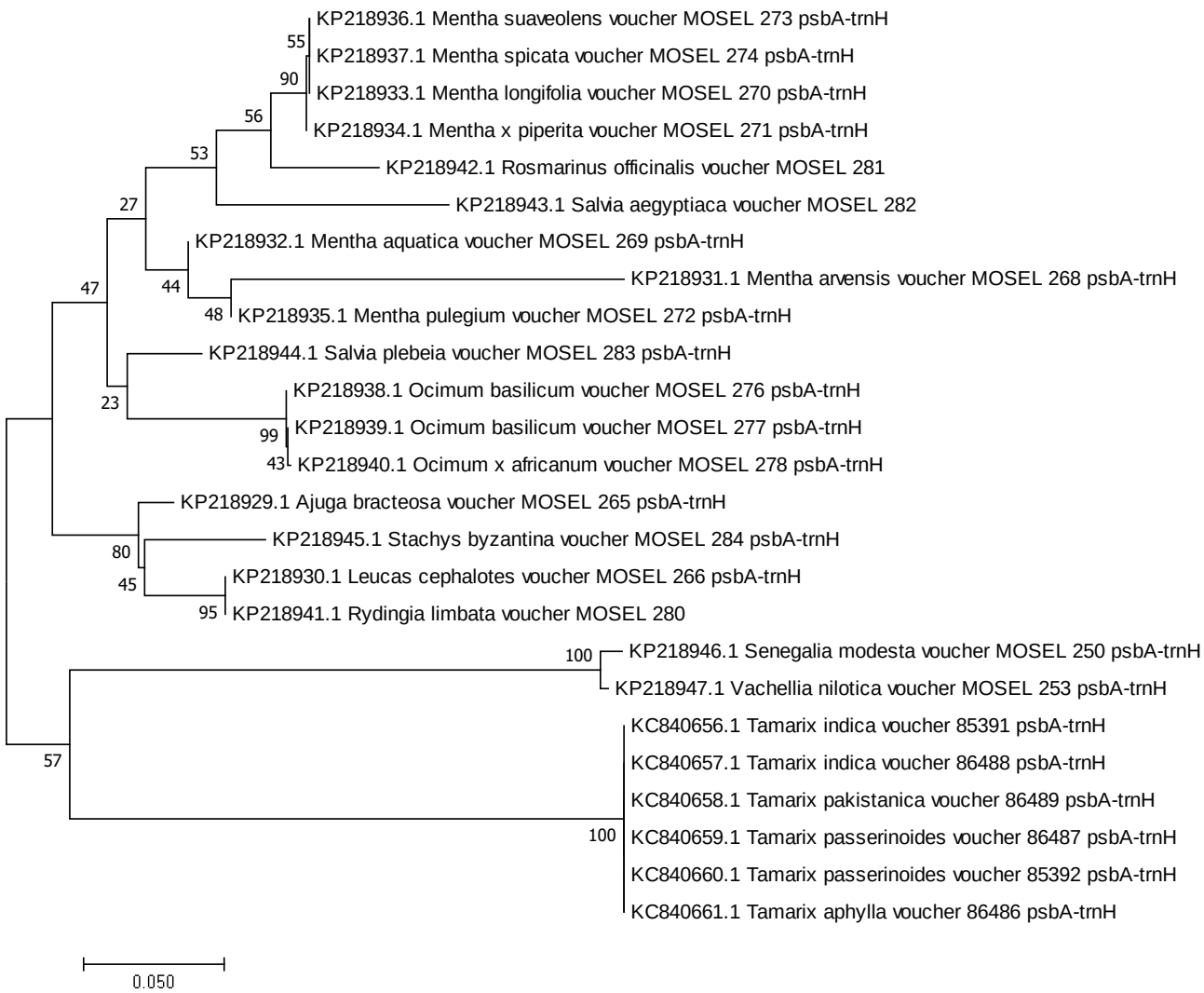

Fig. 4. Phylogenetic analysis of selected species from different medicinally important plant families using *psbA-trnH* marker (n = 25).

Conclusions

In present study the possible evolutionary relationship among 78 different plant species from Pakistan was analyzed through advanced DNA barcode methods. This study is the first of its kind to assess molecular markers based identification and classification of a large set of medicinal plants from different regions of Pakistan. The new identified sequences from multiple plant families provide critical data for further evolutionary study and these should be tested with other related Asian plant families to broaden its genetic background. Further DNA barcode studies are needed to characterize some new medicinal plant species from other diverse families.

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References

Anonymous. 2016. State of the World's Plants, Royal Botanic Gardens, Kew, United Kingdom. https://news.mongabay. com/ 2016/05/many-plants-world-scientists-may-now-answer/

Cardinal-McTeague, W.M. and L.J Gillespie.2016.Molecular phylogeny and pollen evolution of *Euphorbiaceae* tribe Plukenetieae. *Syst. Bot.*, 41(2): 329-347.

CBOL. Plant Working Group. 2009. A DNA barcode for land plants. *Proc. Nat. Acad. Sci.*, 106: 12794-12797.

Chase, M.W. and M.F. Fay. 2009. Barcoding of plants and fungi. *Sci.*, 325(5941): 682-683.

Cuenoud, P., S.V.L. Chatrou, M. Powell, R. Grayer and M.W. Chase. 2002. Molecular phylogenetics of Caryophyllales based on nuclear 18S rDNA and plastid rbcL, atpB, and matK DNA sequences. *Amer. J. Bot.*, 89: 132-144.

Fay, M., C. Bayer, W. Alverson, A. DeBruijn and M. Chase. 1998. Plastid rbcL sequence data indicate a close affinity between Digodendron and Bixa. *Taxon.*, 47: 43-50.

Frezal, L. and R. Leblois. 2008. Four years of DNA barcoding: Current advances and prospects. *Infec. Genet. Evol.*, 8: 727-736.

Group, C.P.W., P.M. Hollingsworth, L.L. Forrest, J.L. Spouge, M. Hajibabaei, S. Ratnasingham and D.L. Erickson. 2009.

A DNA barcode for land plants. *Proc. Nat. Acad. Sci.,* 106(31): 12794-12797.

Habiba, U., M. Ahmad, S. Shinwari, S. Sultana, Z.K. Shinwari and M. Zafar. 2016. Antibacterial and Antifungal Potential of Himalayan Medicinal Plants for Wound Infections. *Pak. J. Bot*., 48(1): 371-375.

Hayat, M.K., T. Kumar, N. Ahmad, W. Sajjad and Z. Rahman. 2016. *In vitro* antimicrobial activity of *Aloe vera* L. extracts against pathogenic bacteria and fungi. *Mycopath.,* 14(1): 21-27.

Hebert, P.D., M.Y. Stoeckle, T.S. Zemlak and C.M. Francis. 2004. Identification of birds through DNA barcodes. *PLoS Biol.,* 2(10): 312.

Hollingsworth, P.M., S.W. Graham and D.P. Little. 2011. Choosing and using a plant DNA barcode. *PLoS ONE., 6*(5): e19254.

Hussain, J., A.L. Khan, N. Rehman, K.F. Zainullah, S.T. Hussain and Z.K. Shinwari. 2009. Proximate and nutrient investigations of selected medicinal plants species of Pakistan. *Pak. J. Nut.,* 8(5): 620-624.

Ikram, A., N.B. Zahra, Z.K. Shinwari and M. Qaiser. 2015. Ethnomedicinal review of folklore medicinal plants belonging to family Apiaceae of Pakistan. *Pak. J. Bot*., 47(3): 1007-1014.

Jamil, I., S. Qamarunnisa, A. Azhar, Z.K. Shinwari, S.I. Ali and M. Qaiser. 2014. Subfamilial relationships within Solanaceae as inferred from *Atpβ-rbc*L intergenic spacer. *Pak. J. Bot.,* 46(2): 585-590.

Jan, S.A., Z.K. Shinwari, A. Zeb, A.T. Khalil and S.H. Shah. 2015. Ethnobotany and Research Trends in *Trachyspermum ammi* L. (Ajowan); A Popular Folklore Remedy. *American-Eurasian J. Agric. & Environ. Sci.,* 15(1): 68-73.

Janzen, D.H. 2009. A DNA barcode for land plants. *Proc. Nat. Acad. Sci.,* 106(31): 12794-12797.

Khan, I., S.A. Jan, Z.K. Shinwari, M. Ali, Y. Khan and T. Kumar. 2017. Ethnobotany and medicinal Uuses of folklore medicinal plants belonging to family Acanthaceae: An updated review. *MOJ Biol. Med.*, 1(2): 00009.

Khan, M.Q., A.T. Khalil, K. Rehman and Z.K. Shinwari. 2015. Searching for DNA Barcodes in Plants. *Global J. Biotechnol. Biochem.,* 10(1): 1-10.

Kress, W.J. and D.L. Erickson. 2007. A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding trnH-psbA spacer region. *PLoS ONE.,* 2(6): e508.

Kress, W.J., K.J. Wurdack, E.A. Zimmer, L.A. Weigt and D.H. Janzen. 2005. Use of DNA barcodes to identify flowering plants. *Proc. Nat. Acad. Sci. United States of Amer.*, 102(23): 8369-8374.

May, R.M. 1992. How many species inhabit the earth. *Sci. Amer*., 267(4): 42-48.

McDade, L.A., T.F. Daniel, S.E. Masta and K.M. Riley. 2000. Phylogenetic relationships within the tribe Justicieae (Acanthaceae): evidence from molecular sequences, morphology, and cytology. *Ann. Missouri Botanical Garden.,* 87(4): 435-458.

Moylan, E.C., J.R. Bennett, M.A. Carine, R.G. Olmstead and R.W. Scotland. 2004. Phylogenetic relationships among Strobilanthessl (Acanthaceae): evidence from ITS nrDNA, trnL-F cpDNA, and morphology. *Amer. J. Bot.,* 91(5): 724-735.

Neuhaus, H. and G. Link. 1987. The chloroplast tRNALys (UUU) gene from mustard (Sinapis alba) contains a class II intron potentially coding for a maturase-related polypeptide. *Curr. Genet.,* 11(4): 251-257.

Onstein, R.E., R.J. Carter, Y. Xing, J.E Richardson and H.P Linder. 2015. Do Mediterranean–type ecosystems have a common history?—Insights from the Buckthorn family (*Rhamnaceae*). *Evol.,* 69(3): 756-771.

Peirson, J.A., R. Riina, M.H. Mayfield, C.J. Ferguson, L.E. Urbatsch and P.E. Berry. 2014. Phylogenetics and taxonomy of the New World leafy spurges, Euphorbia section Tithymalus (*Euphorbiaceae*). *Bot. J. Linn. Soc.,* 175 (2): 191-228.

Qasim, M., M. Khalid, A. Sayyed, I. Din, K. Hayat and S.A. Jan. 2016. Phytochemical potentials and medicinal uses of twenty-four selected medicinal plants from Swabi, Pakistan. *J. Rural Develop. Agri*., 1(1): 49-58.

Richards, E., M. Reichardt and S. Rogers. 1997. Preparation of plant DNA using CTAB. *Short Protocols. Mol. Biol.,* 3(2): 10-12.

Schori, M., M. Appel, A. Kitko and A.M. Showalter. 2013. Engineered DNA polymerase improves PCR results for plastid DNA. *Appl. Plant Sci*., 1(2): 1-7.

Shinwari, Z. K. and S. Shinwari. 2010. Molecular data and phylogeny of family Smilacaceae. *Pak. J. Bot.*, 42 (SI): 111-116.

Shinwari, Z.K., K. Jamil and N B Zahra. 2014. Molecular systematics of selected genera of family Fabaceae. *Pak. J. Bot.,* 46(2): 591-59.

Sudhir, K., S. Glen and T. Koichiro. 2015. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0. *Mol. Biol. Evol*., 33: 1870-1874.

Ullah, I., A. Wakeel, Z.K. Shinwari, S.A. Jan, A.T. Khalil and M. Ali. 2017. Antibacterial and antifungal activity of *Isatis tinctoria* l. using micro-plate method. *Pak. J. Bot.,* 49(5): 1949-1957.

Yang, Y. and P.E. Berry. 2011. Phylogenetics of the Chamaesyce clade (*Euphorbia, Euphorbiaceae*): Reticulate evolution and long-distance dispersal in a prominent C4 lineage. *Amer. J. Bot.,* 98: 1486-1503.

Zahra, N.B., M. Ahmad, Z.K. Shinwari, M. Zafar and S. Sultana. 2014. Systematic significance of anatomical characterization in some Euphorbiaceous species. *Pak. J. Bot.,* 46(5): 1653-1661.

Zahra, N.B., Z.K. Shinwari and M. Qaiser. 2016. DNA barcoding: a tool for standardization of herbal medicinal products (HMPS) of Lamiaceae from Pakistan. *Pak. J. Bot.,* 48: 2167-2174.

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