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TAXONOMIC REAFFIRMATION OF SOME MEMBERS OF FAMILY CANNABACEAE, MORACEAE, RHAMNACEAE, ROSACEAE AND URTICACEAE OF ORDER ROSALES USING DNA BARCODING MARKERS

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Abstract

DNA Barcode Analysis was carried out as new taxonomic approach to re-affirm the identity and the phylogenetic relationship of five families of order Rosales by using three molecular markers such as *rbcL*, *matK* and *trnH-psbA.* This study will serve as contribution to the previous taxonomic position of the order Rosales on the basis of morphological characters as well as DNA sequence data. According to the DNA Barcode data cladogram of order Rosales indicated that family Moraceae formed an individual clade in all Neighbour Joining trees formed on the basis of *rbcL*, *matK*, *trnH-psbA*, *rbcL+matK* and *rbcL+matK+trnH-psbA* while in previous studies it was sister to Urticaceae and Cannabaceae. Another change was also observed in the placement of Family Rosaceae that did not form a separate clade as it did in previous studies but it was sister to Cannabaceae, Rhamnaceae and Urticaceae and embedded among these families. Our study does not support the previous taxonomic position of order Rosales based on morphological characters and some DNA sequences which are not standard barcodes now. The PCR amplification success rate was found highest 96% for *rbcL* followed by *matK* 64% while lowest for *trnH-psbA* 58%. The gene sequencing tree was successfully constructed when markers were used both singly and in combinations. The phylogenetic tree analysis demonstrated that, separate clusters forming species with more than 50% bootstrap value were the discriminate species, indicating high reliability of evolutionary relationship among the taxa. In the present study it was found that all three DNA Barcode markers when employed in combination exhibited maximum discriminatory power amongst all the studied species in the families of order Rosales.

Key words: *rbcL, matK, trnH-psbA*, Molecular systematics, Order rosales.

Introduction

Plant taxonomy is mainly concerned with identification, description and classification and relationship of plants. However, in old days this was usually done on the basis of their morphological characters, however, with the advent of new techniques approaches like structural, cytological, chemical and molecular techniques are also used (Noshad *et al*., 2020). Dasgupta (2016) quoted the findings of Royal Botanic Gardens, Kew, United Kingdom, there are about 391,000 species of vascular plants currently known to science, of which about 369,000 species (94%) are flowering plants. In recent years, several publications under the DNA barcode title remarkably provide novel system for the identification of the species at rapid and authentic level and opens up new horizon in plant systematics (Hebert & Barrett, 2005; Noshad *et al*., 2021). This idea was purposed by Paul Hebert and his co-workers in 2003 that short sequence or piece of the DNA of any species can be utilized for the identification of the species. Barcodes of DNA contain 400-800 long base pairs (bps) which can be amplified through the Polymerase Chain Reaction (PCR) and variable sequences of the particular species can be studied form this information (Ali *et al*., 2014). While comparing the gene sequences obtained from other studies which help to find the close relationship or match to taxon. This molecular information emerged can be confirmed from the taxonomical keys or monographs to affirm the identity of the Molecular Operational Taxonomic Unit (MOTU) and possible close relationship to their close taxonomical allies in the form of phylogenetic tree (Floyd *et al*., 2002).

Phylogenetic tree developed from phylogenetic hypothesis, normally based upon the substitution of homologus nucleotides (bases), usually vary among the different taxa. In the phylogenetic trees different clades (grouping of two or more taxa) formed are based upon the length of the branches that ultimately connects taxa or clades with their common ancestral origin (Kress & Erickson, 2012). For detailed analysis of the plant species through DNA Barcode technique, scientists put emphasis in Consortium for Barcode of Life (CBOL) the chloroplast genes, *rbcL* and *matK* (Chase *et al*., 2007) which serve as the core barcodes for plant species, along with supplement barcodes *trnH-psbA* and ITS which serve as intergenic sequence and ribosomal gene respectively (Chase *et al*., 2005; Kress *et al*., 2005). The basic aim of CBOL was the standardization of a reference library related to DNA sequences and also to develop economical technologies regarding the correct and accurate identification of species thus benefiting the taxonomists and phylogenetists in processing the biodiversity surveys, to resolve issues on cryptic species and biological conservation of the specific site (Janzen *et al*., 2005).

The order includes some of the best known ornamentals and edible fruit plants in the temperate parts of the World. While studying the phylogenetic relationships of the order Rosales, Zhang *et al*., (2011) divided the order into three clades on the basis of the DNA analyses, the clade 1 had family Rosaceae only,

other one had four families, Rhamnaceae, Elaeagnaceae, Barbeyaceae and Dirachmaceae and the last clade had Ulmaceae, Cannabaceae, Moraceae and Urticaceae. The main problem of the families of this order was that the evolutionary trends and their proper placement along with close relatives were not discussed. According to APG-II (Angiosperm Phylogenetic Group-II) system of classification, several attempts were made in the past to resolve issues on its evolutionary relationships with its respective clades. The order was strongly considered Monophyletic in origin by Angiosperm Phylogeny Group (APG) on the basis of Phylogenetic Analysis of the DNA sequences. According to APG-III and APG-IV system of plant classification, the relationship of its families in this order is very uncertain although molecular studies based on two nuclear and ten chloroplast genes have tried to resolve this issue. In the present study 15 plant species belonging to 5 families of order Rosales were studied for their gene sequencing by employing DNA barcoding using three molecular markers such as *rbcL*, *matK* and *trnH-psbA* as a new taxonomic approach in collaboration with Guelph University, Ontario, Canada. In order to resolve this problem the present project was undertaken.

Materials and Methods

Plant sampling: Fifteen plant species belonging to the families Cannabaceae, Moraceae, Rhamnaceae, Rosaceae and Urticaceae of the order Rosales were collected from Lahore and its vicinity including Botanical Garden, GC University, Lahore (74°E and 31°N). The GPS coordinates of collected plants is given here (Table 2). The plants were identified with the help of Lahore District Flora (Kayshap & Joshi, 1936) and Flora of Pakistan (Qaiser, 1973; Qaiser & Nazimuddin, 1981; Ghafoor, 1981, 1985; Shah, 2009 and Rajput Tahir, 2017). The images of studied plants with their barcode markers are given in Fig. 7. The voucher specimens were deposited in Dr. Sultan Herbarium, G. C. University, Lahore.

For genomic DNA isolation, 1-5 fresh young leaves (each 0.5cm² in size) of each plant species were preserved in air tight plastic Ziploc bags having few crystals of silica gel under a Project named "GCUBG" on BOLD. In present study, we analyzed 53 sequences from which 51 of *rbcL*, 34 of *matK* and 33 of *trnH-psbA* belonging to 15 plant species of five families respectively. Among all collected plant species 9 species have both *rbcL* and *matK* gene markers but only four species out of 15 have *trnH-psbA* along with *rbcL* and *matK* (Table 1).

DNA extraction, amplification and sequencing: Following the standard protocol, 96 well box was loaded with leaf samples dried in silica gel using standard protocol. The tissue lysis and DNA extraction was performed following standard protocols (Ivanova *et al*., 2008).

PCR products for rbcL, matK and trnH-psbA were obtained. The primers rbcLa F (ATGTCACCACAAA CAGAGACTAAAGC) by (Levin *et al*., 2003) and rbcLa-R (GTAAAATCAAGTCCACCRCG) by (Kress and Erickson 2007) were used for sequencing of almost 552bp rbcL barcode sequence, 800bp trnH-psbA. A 773 bp long sequence of matK was obtained with primers, matK-3FKIM-f(CCCAGTCCATCTGGAAATCTTGGTTC) and matK-3FKIM-R (GTACAGTACTTTGTGTTT ACGAG). Purification and bidirectional sequencing of PCR products obtained was done by following Haibabaei *et al*., (2006).

Molecular analysis: The original sequences were edited, cleaned and assembled using codon code Aligner V3.0 (Codon code co., USA). The nucleotide sequences analysis was carried out by using parameters under the profile alignment option on MEGA 5. For Neighbour Joining Cluster Analysis of all three barcodes, i.e. *rbcL*, *matK* and *trnH-psbA* one consensus barcode of all species was obtained from "Consensus Barcode Generator" parameter of Taxon DNA because there was more than one sequence for all species in the data set (Meier *et al*., 2006). The Neighbour–Joining (NJ) analysis performed on MEGA5 visualized the patterns of divergence among taxa (Tamura *et al*., 2011). The assessment of node support was achieved by bootstrap test with 500 replicates on MEGA5 (Felsenstein, 1985). Barcode sequence distance was computed with Kimura 2-Parameter (K2P) evolutionary model (Kimura, 1980).

Table 1. PCR amplification of gene markers of Order Rosales.

Family	Plant species (15)	No.of samples (56)	*matK* (bp) (11)	*rbcL* (bp) (14)	*trnH-psbA* (bp) (8)
Cannabaceae	1. *Cannabis sativa* L.	4	807	550	NA
Rhamnaceae	1. *Sageretia thea* (Osbeck) M.C.	5	NA	550	400
	2. *Ziziphus mauritiana* Lam.	3	801	550	NA
	3. *Ziziphus nummularia* (Burm.f.) Wight & Arn.	4	865	550	375
Rosaceae	1. *Potentilla supina* L.	5	800	550	NA
	2. *Spirea cantoniensis* Lour.	5	850	550	370
Moraceae	1. *Artocarpus integrifolia* L.	5	0	550	NA
	2. *Artocarpus lacucha* Buch.Ham.	5	0	550	270
	3. *Ficus elastica* Roxb.ex Hornem	4	835	550	401
	4. *Ficus lyrata* Warb.	4	0	550	406
	5. *Ficus nerrifolia* Sm.	4	620	550	400
	6. *Ficus benghalensis* L.	2	801	550	NA
	7. *Morus macroura* Miq. Pl. Jungh.	3	800	551	NA
Urticaceae	1. *Boehmeria rugulosa* Wedd.	2	792	NA	497
	2. *Parietaria judaica* L.	1	800	550	NA

Results and Discussion

PCR amplification success: PCR Amplification successes along with sequence recoverability are the important steps in DNA Barcode. The successful sequencing from CBOL plant working group, Guelph University, Ontario, Canada, resulted in 53 sequences for three plant barcode markers, *rbcL*, *matK* and *trnH-psbA*. The PCR amplification success rate was highest for *rbcL*, i.e., 96% (51/53) followed by *matK* 64% (34/53) and less for *trnH-psbA* 62% (33/53) in species recoverability (Table 1). The results idicated aligned sequence length of *rbcL* 505-552bp, *matK* 724-846bp and of *trnH-psbA* 650-871bp. It was observed that *rbcL* sequence mostly showed no variation in its sequence length while *matK* and *trnH-psbA* showed significant variation which is very much in line with (Kress *et al*., 2005; Kress & Erickson 2007) as high sequence success with no variation in sequence length of *rbcL*. Moreover, the present work also supported the findings of (Zhang *et al*., 2012; Maia *et. al*., 2012) as they reported 100% PCR amplification and sequence success for *rbcL*.

By using only one DNA Fragment, maximum rate for species identification was obtained for *rbcL* i.e., 98% followed by *trnH-psbA* 96%, suggesting both *rbcL* and *trnH-psbA* more reliable for plant species identification as both also have high rate of amplification and sequencing success, thus can be recommended for higher identification success and useful for the interpretation of plant evolutionary relationships at genus and family level as represented by their molecular as well as phylogenetic near relative data on Nearest Neighbour Analysis. Alvarez on the other hand, recommended the possible use of *trnH-psbA* as a DNA barcode marker, on the basis of its 96% identification success rate. They believed it as a good candidate and a potential barcode for the identification of plant species (Alvarez & Wendel, 2003).

Neighbour joining cluster analysis: Neighbour Joining Phylogenetic trees were formulated with the help of aligned consensus barcode sequences of *rbcL*, *matK* and *trnH-psbA* individually as well as in combination (Figs. 2-6). In these trees genome of the plant species determines the clustering of species at their corresponding neighbours. According to Kuzmina *et al*., (2012) and Saarela *et al*., (2013), neighbour joining method was used in many floristic studies. Thus the species possessing individual cluster with more than 50% bootstrap value would be considered as discriminated species (Felsenstein, 1985). Whereas Berry & Gascuel (1996) state that 500 replicates of bootstrap did not illustrate the correctness of the tree either it helps to assess information about the barcode sequence to evaluate the tree topology (branching order). All aligned sequences were subjected to Kimura 2-parameter (K2P) for the construction of phylogenetic trees (Kimura, 1980). In this research study three trees were constructed on the basis of independent plant barcode marker, i.e *rbcL*, *matK* and *trnH-psbA*, while two different trees were also constructed by using two barcode markers and all barcode markers in combination such as*rbcL+ matK* and*rbcL+matK+trnH-psbA*. According to Neighbour Joining Cluster Analysis formed on the basis of individual *rbcL* tree the topology of order Rosales was quite different from the cladogram formed on the basis of morphological characters and DNA sequence data proposed by Zhang in 2011 (Fig. 1). It was noticed that family Moraceae formed separate clade while Rhamnaceae, Cannabaceae, Rosaceae and Urticaceae were sister to each other on the other clades (Figs. 2, 4, 5, 6). On the other hand, it was also observed that in the tree formulated on the basis of *matK* gene marker (Fig. 3) the placement of all five families was almost similar to the cladogram formed by the study of Zhang 2011 (Fig. 1). As family Rosaceae rested in separate clade while Cannabaceae, Urticaceae and Moraceae were sister to each other on separate clade from Rosaceae. This situation was totally altered in the trees formed by Neighbour Joining method on the basis of *rbcL*, *matK*, *trnH-psbA*, *rbcL+matK* and *rbcL +matK+trnH-psbA*. As family Moraceae was present at a separate clade while rest of all families were sister to each other on separate clades. On the basis of the number of nodes and bootstrap values these trees were also good for providing species identification except in *rbcL* tree only where two nodes had less than 50% while rest of all nodes had more than 80% bootstrap value (Fig. 2). In *matK* tree the bootstrap value of all the species was 80% to 100% (Fig. 3) and similarly in case of *trnH-psbA* tree of three highlighted species, the bootstrap values were 51%, 83% and 84% in rest of the plant species were 100% (Fig. 4).

It is pertinent to record that the performance of the molecular markers, *rbcL*, *matK* and *trnH-psbA* was found good and reliable when they were employed individually or even in combination in the discrimination of the species of their respective families, in proving their taxonomic identity as well as the convincing role in the cluster analysis thus providing valuable data on the primitive or advanced status of the taxa involved in order Rosales (Figs. 5-6). The formation of phylogenetic tree was criticized by Fazekas *et al*., (2008) and Saarela *et al*., (2013) because they considered those trees were not capable enough to utilize low divergence though capable for differentiating groups but not best enough in the construction of phylogenetic trees.

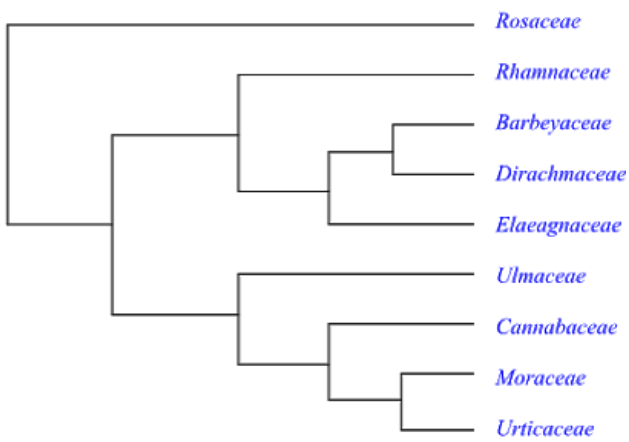


Fig. 1. Cladogram of Order Rosales based on DNA sequence data by Zhang *et al*., 2011.

Table 2. GPS co-ordinates of all collected plant species.

Family	Plant species	Latitude	Longitude
Cannabaceae	1. *Cannabis sativa* L.	31.412808°	74.141808°
Rhamnaceae	1. *Sageretia thea* (osbeck) M.C. Johnst.	31.566459°	74.484461°
	2. *Ziziphus mauritiana* Lam.	31.567161°	74.484475°
	3. *Zizyphus nummularia* (Burm.f.) Wight & Arn.	31.566651°	74.485150°
Rosaceae	1. *Potentilla supina* L.	31.567789°	74.485870°
	2. *Spirea cantoniensis* Lour.	31.557144°	74.327927°
Moraceae	1. *Artocarpus integrifolia* L.	31.557085°	74.327319°
	2. *Artocarpus lacucha* Buch.Ham.	31.557126°	74.327174°
	3. *Ficus elastica* Roxb.ex Hornem	31.557169°	74.327195°
	4. *Ficus lyrata* Warb.	31.557163°	74.327077°
	5. *Ficus nerrifolia* Sm.	31.557094°	74.327234°
	6. *Ficus benghalensis* L.	31.557286°	74.326876°
	7. *Morus macroura* Miq., Pl. Jungh.	31.602165º	74.387882º
Urticaceae	1. *Boehmeria rugulosa* Wedd.	31.405045°	74.135218°
	2. *Parietaria judaica* L.	31.235041º	74.153281º

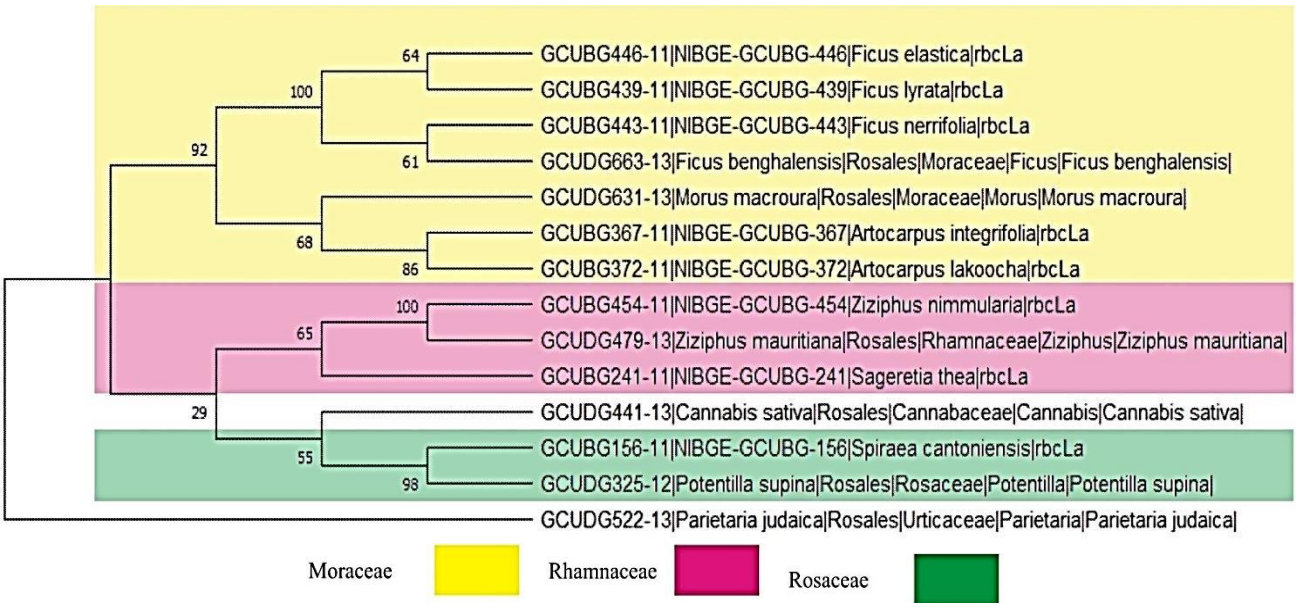


Fig. 2. Cladogram of Order Rosales based on *rbcL* sequence.

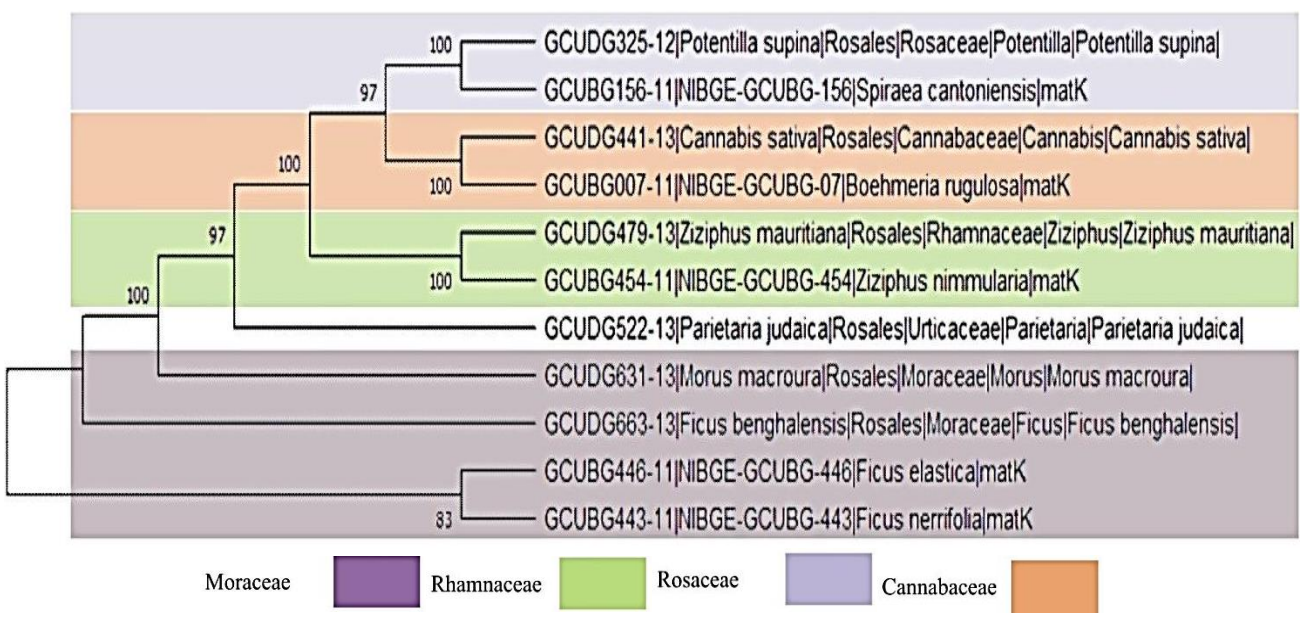


Fig. 3. Cladogram of Order Rosales based on *matK* sequence.

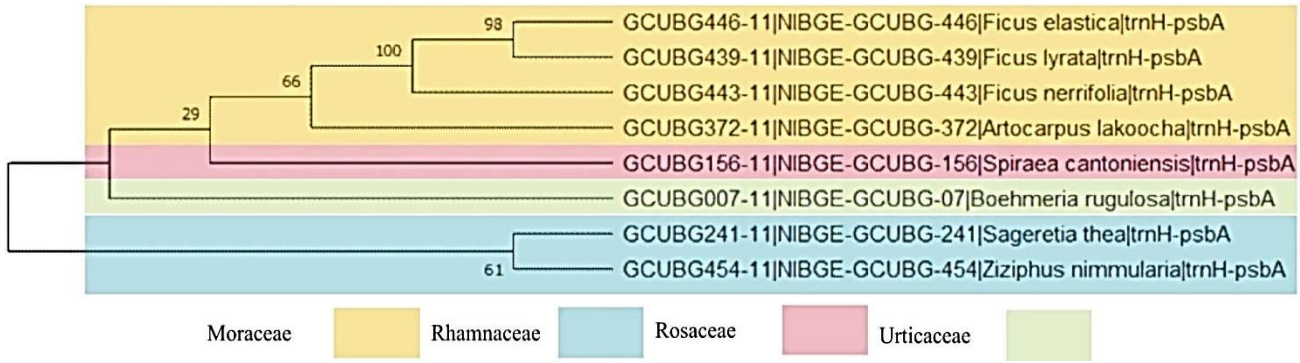


Fig. 4. Cladogram of Order Rosales based on *trnH-psbA* sequence.

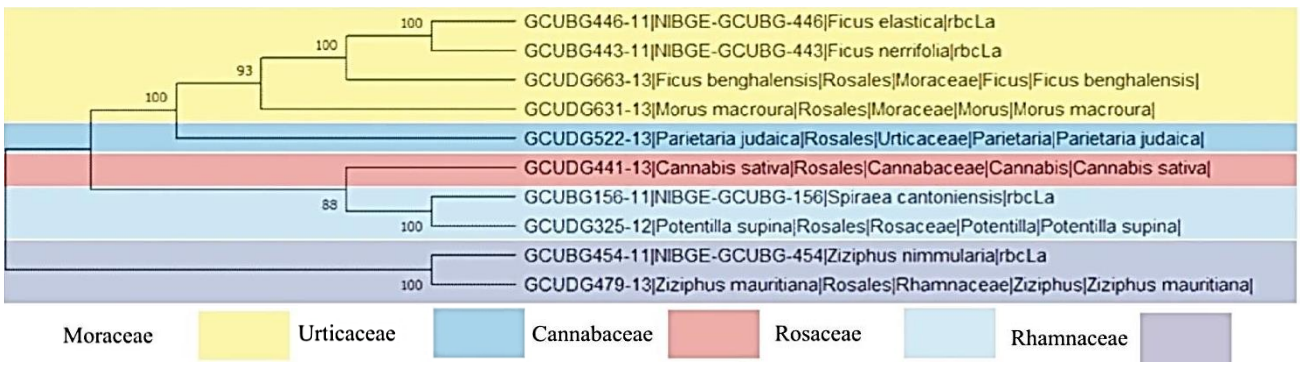


Fig. 5. Cladogram of Order Rosales based on *rbcL+matK* sequence.

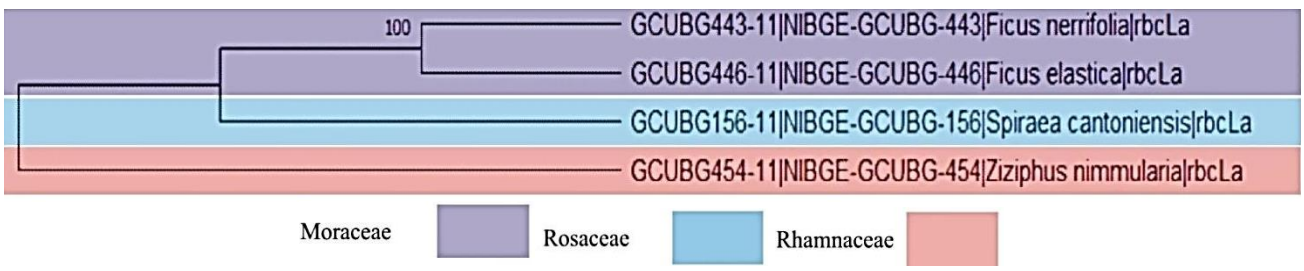


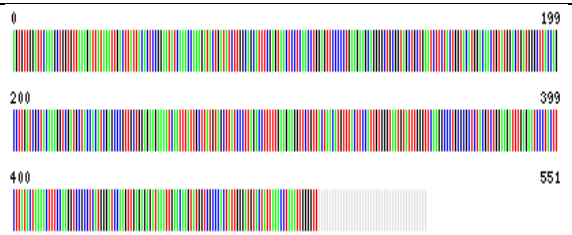
Fig. 6. Cladogram of Order Rosales based on *rbcL+matK+trnH-psbA* sequence.

Discussion

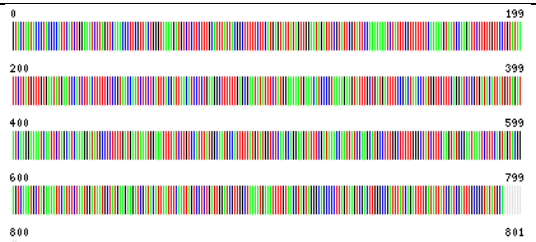
The present study led to the establishment of molecular data inventory of all plant species included in order Rosales with their images, barcode sequence data, voucher numbers and GPS coordinates (Fig. 7). The findings in the present studies rules the efficacy of all three barcode markers used *rbcL*, *matK* and *trnH-psbA*. It was also observed that all barcode markers were good and in line with the study of Zhang *et al*., (2012). According to Zhang the possible topology of order Rosales was [Rosaceae+ [[Rhamnaceae + [Elaeagnaceae+ [Barbeyaceae + Dirachmaceae]] + [Ulmaceae+ [Cannabaceae + [Moraceae + Urticaceae]]]]]. The present study does not agree with the position of Rhamnaceae, as it is not sister to Rosaceae either it is sister to family Cannabaceae. While other rest of families are same described by morphological position. So this study again confirmed the phylogenetic topology with molecular evidence.

The successful and fruitful use of DNA Barcoding was previously explained by many researchers such as Newmaster & Ragupathy (2009) and Kelly *et al*., (2010) and especially in plants by Kress and Erickson (2010) by using nuclear genome as well as plastid genome. They supported the view that DNA Barcoding could be used for effective discrimination of plant species and also in different plant groups, but on the contrary some workers like Chase & Fay (2009), Felsenstein (1985) and Zhang *et al*., (2012) considered DNA Barcoding was not good or reliable in genetically complicated plants. Certain studies in literature revealed the fact that DNA Barcode worked well for a specific plant group but at the same time failed in another plant group as reported by Liu *et al*., (2011). The performance of barcode markers was also tested by number of researchers by using them individually and also in combination. Yang concluded that same set of barcode marker behaved differently in same plant group as studied by Yang *et al*., (2012). The findings in the present study revealed that the taxonomic performance of all the molecular markers, i.e., *rbcL*, *matK* and *trnH-psbA* was good both when used individually as well as in combination, in the re-affirmation of the identity of 15 plant species of 5 families of order Rosales from district Lahore, Punjab Pakistan as well as in constructing phylogenetic relationships/ trees among the different taxa involved.

Cannabis sativa
Family. Cannabaceae



rbcL



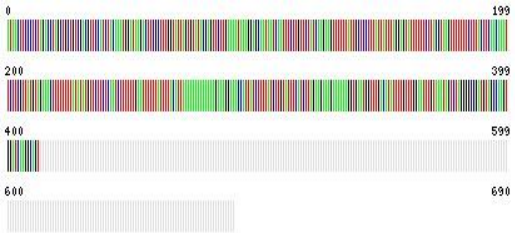
matK

trnH-psbA

Sageretia thea
Family. Rhamnaceae

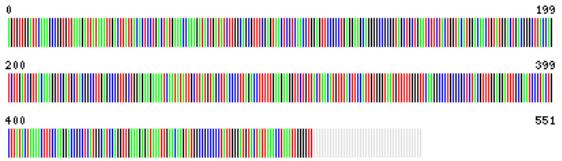
rbcL

matK

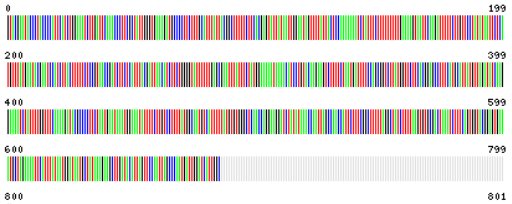


trnH-psbA

Ziziphus mauritiana
Family. Rhamnaceae



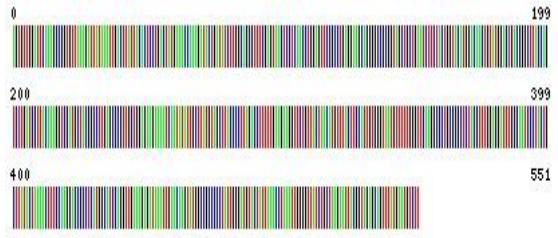
rbcL



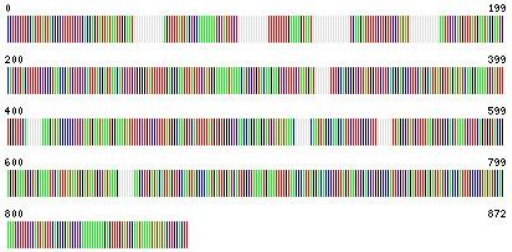
matK

trnH-psbA

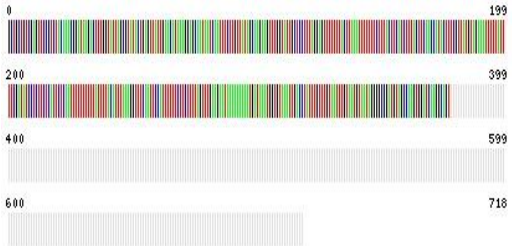
Ziziphus nimmularia
Family. Rhamnaceae



rbcL



matK

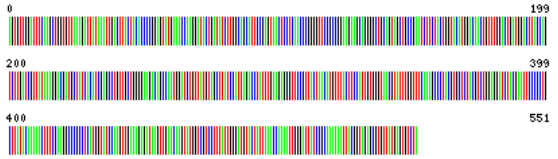


trnH-psbA

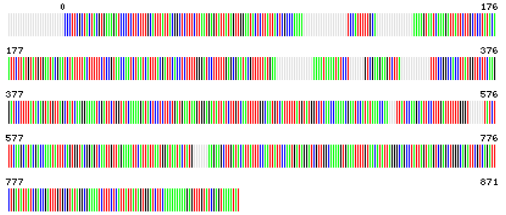
Fig. 7. Plants of study site with their barcode markers.

Fig. 7 (Cont'd.).

Potentilla supina
Family. Rosaceae



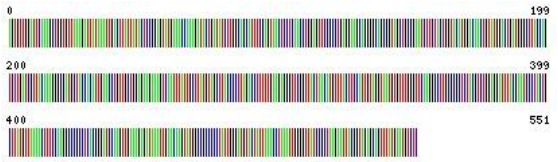
rbcL



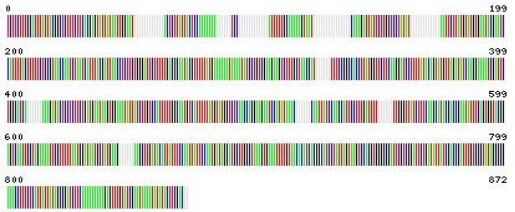
matK

trnH-psbA

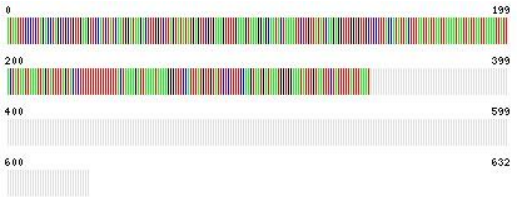
Spirea cantoniensis
FamilyRosaceae



rbcL

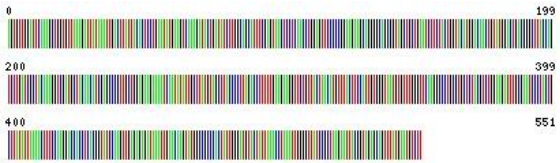


matK



trnH-psbA

Artocarpus integrifolia
Family. Moraceae

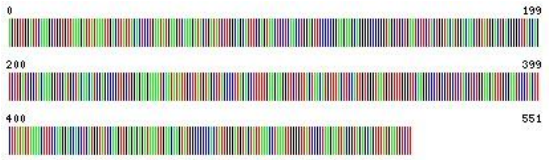


rbcL

matK

trnH-psbA

Artocarpus lacucha
Family. Moraceae



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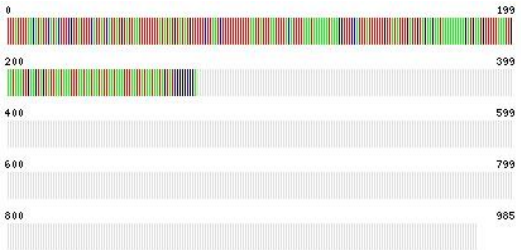
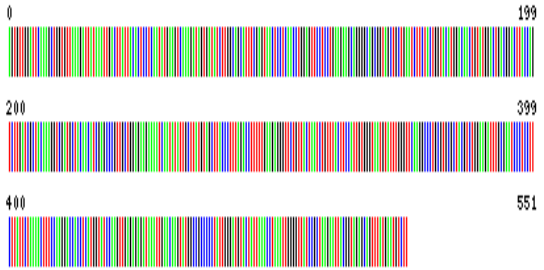
matK



trnH-psbA

Fig. 7 (Cont'd.).

Ficus elastica
Family: Moraceae



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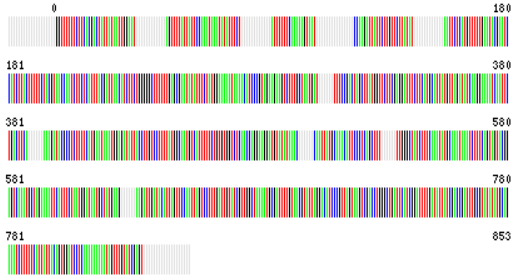
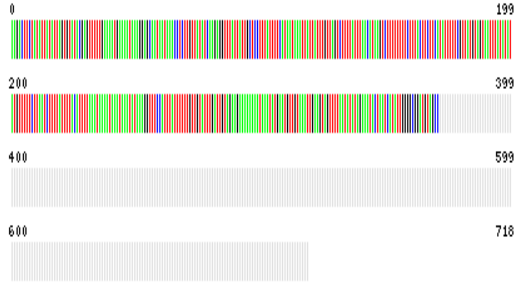
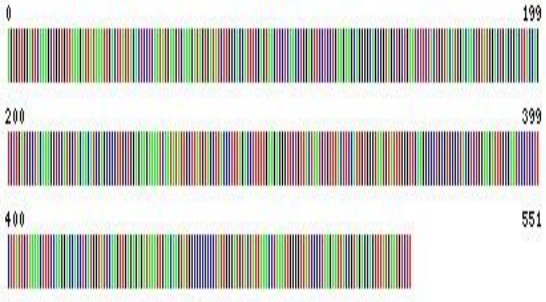


matK



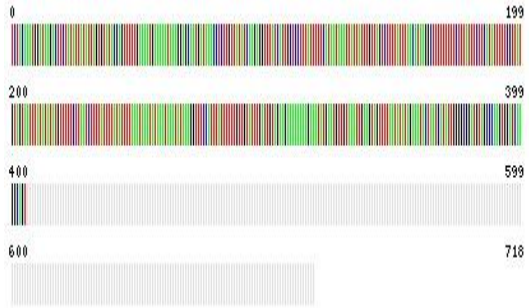
trnH-psbA

Ficus lyrata
Family. Moraceae



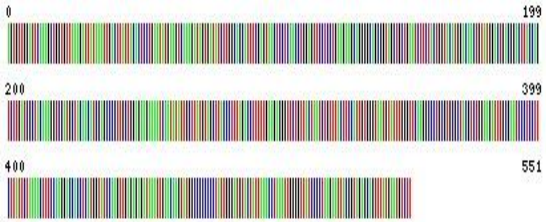
rbcL

matK



trnH-psbA

Ficus nerrifolia
Family. Moraceae



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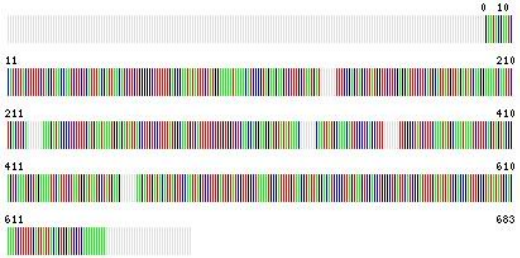
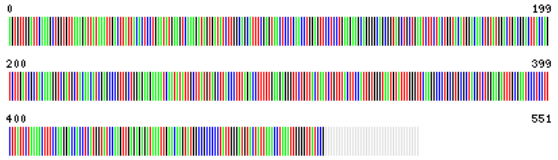


matK

trnH-psbA

Fig. 7 (Cont'd.).

Ficus Benghalensis
Family. Moraceae



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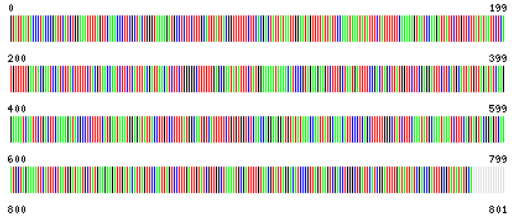
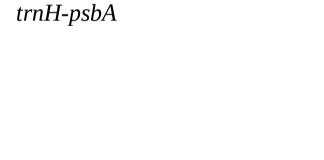
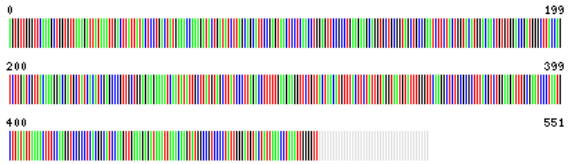


matK



trnH-psbA

Morus macroura
Family: Moraceae



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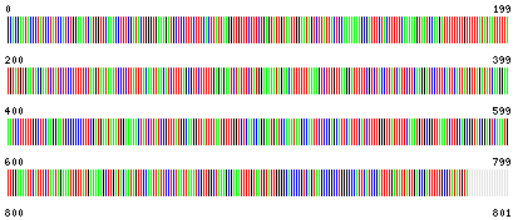
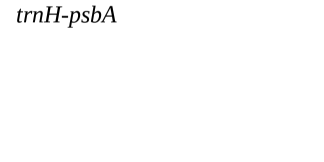


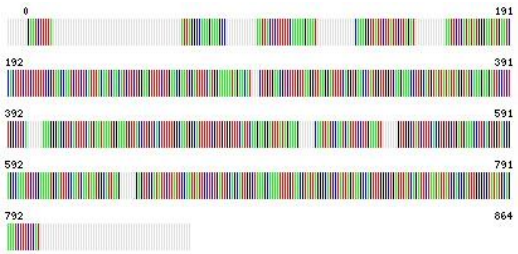
matK



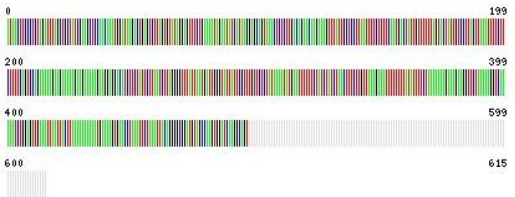
trnH-psbA

Boehmeria rugulosa
Family: Urticaceae

rbcL

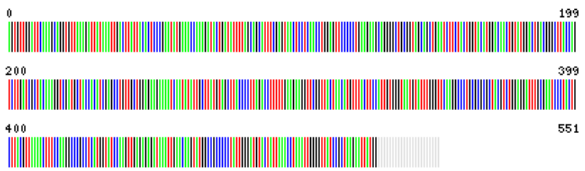


matK

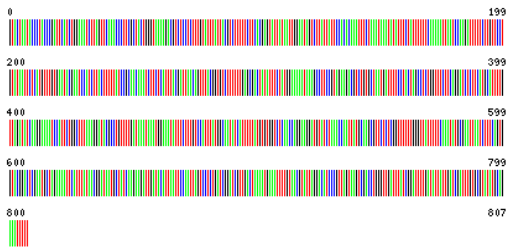


trnH-psbA

Parietaria judaica
Family: Urticaceae



rbcL



matK

trnH-psbA

Conclusion

Coupling DNA Barcoding with morphology provides important insights into species delimitations for several taxa in the field of plant taxonomy. With the consideration of discriminatory power, cost-efficiency and effort, the barcode combination of *rbcL+trnH-psbA* seems to be the best choice for barcoding of order Rosales. Thus the three barcode markers, *matK*, *rbcL*, and *trnH-psbA* worked reasonably well in the identification of flowering plant species of order Rosales of district Lahore, up to genus level and as well as in the phylogenetic topology with molecular evidence, in the form of Neighbour Joining Tree. The utilization of DNA Barcoding for the species identification strongly depends upon the availability of an accurate and complete molecular database, which was provided by BOLD, Guelph University, Canada.

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