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Morphological and molecular evidence for hybridization in *Grewia tenax* complex

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Grewia tenax (Forsk.) Fiori widely distributed in Pakistan. *Grewia tenax* (Forsk.) Fiori and *Grewia erythraea* Schweinf., found in complex and an intermediate population also reported from the southern part of Pakistan. *Grewia tenax* is a polymorphic taxon some authors recognized *Grewia erythraea* and *Grewia tenax* as separate species but others considered a single taxon. This has led to confusion.To confirm the hybridization all three species were investigated by means of morphological and molecular (Random Amplified Polymorphic DNA) studies. The taxonomic relationship of all three taxa was also explored using the SPSS (Ver. 20.0) cluster analysis and UPGMA cluster analysis using the NTSYS PC. Ver. 2.01. The results suggest that both the taxa are separate species. Comparison of micro and macro morphological characters and RAPD analysis authenticate, the *Grewia tenax* and *Grewia erythraea* are completely different species and both the taxa are sympatric in distribution. While intermediate population is interspecific hybrid and hybridization between two taxa take place on a limited scale.

Keywords: Hybridization; *Grewia tenax; Grewia erythraea*; Intermediate; RAPD; Morphology; Molecular studies.

INTRODUCTION

Genus *Grewia* belongs to subfamily Grewioideae of the family Malvaceae, comprising 280 species, mainly distributed in the tropics of the old world (Mabberley, 2008). In Pakistan genus *Grewia* L. represented by 10 species. *G. erythraea* Schweinf., also reported by Stewart (1972) from Pakistan. While in the flora of Pakistan Ghafoor (1974) treated *G. tenax* and *G.erythraea* as a single taxon and used *G.erythraea* as synonym of *G. tenax.* Miller & Morris (1988) also treated both the taxa with different species. Sebsebe (1999) in "Flora of Somalia" also treated both taxa separately. Abedin et al., (1999) in "Flora Kingdom Saudi Arab" also treated both taxa as independent species.

Hybrid plant may be product of grafting between specifically or genetically different twigs or it may be the result of any natural or experimental matting or crossing between two varieties. Many biologists believe hybridization as an important and remarkable evolutionary process (Stebbins 1950, Stace 1975, Grant 1983, Rieseberg 1993, Arnold 1997). While, Wagner (1970) considered it evolutionary noise. Hybridization may lead to the important evolutionary changes and also lead origin and transfer of new adaptation and formation of new species (Stebbins 1950, Arnold 1995). Hybridization may also increase the extinction of rare species. Presently role of hybridization in evolution persists (Levin et al., 1996).

Morphological concepts used and play an important role in all major fields of plant biology such as ecology, physiology, evolutionary biology, genetics and molecular biology (Rutishauser & Sattler, 1997). Usually, it was believed the

appearance of morphological and physical character of hybrid plants intermediate between parents because hybrid combines alleles from their parental species (Arnold and Hodges, 1995). Yakandawala et al., (2017) confirmed the hybridization between exotic and native species of *Nymphaea* by using morphological data and observed intermediate characters of hybrid plants. While in hybrid expression of character also shows variety of pattern (Johnston et al., 2001). Genetic basis and expressions of molecular characters are more reliable and predictable as compare to morphological character (Rieseberg & Ellstrand, 1993). To assess the genetic variation and in clarification of genetic relationships at the specific and the intraspecific level molecular characters are powerful tools (Chakravarthi and Naravaneni, 2006). Molecular studies are important evidence in the evaluation of genetic variation and the clarification of genetic relationship within and between species and also useful in the genetic study of hybrid (Joshi et al*.,* 2000). Chen & Mii (2012) investigate the interspecific hybridization in *Begonia semperflorens* and *Begonia Pearcei* by RAPD analysis. Tovar-Sánchez and Oyama (2004), studied hybridization in *Quercus* by using morphology and molecular markers.Carine et al., (2007) analyzed the morphological and molecular data for the confirmation of hybridization between two species of *Convolvulus*.

The present study was conducted for detailed information on the systematic and distinguish two taxa *Grewia erythraea* and *Grewia tenax* in *Grewia tenax* complex and also investigating the hybridization between *G. erythraea* and *G. tenax* using morphological and molecular data.

MATERIALS AND METHODS

Morphological studies

Fresh material was collected from different localities of Karachi, Hawke's Bay, Kirthar, Tikko Baran and Thana Bulla khan between 2015-2016. Herbarium sheets were prepared and deposited in the Karachi University Herbarium, Centre for Plant Conservation, University of Karachi. Morphological observations were made with the help of light and stereo microscope. Qualitative and quantitative, vegetative and floral characters were studied by stereo and scanning electron microscope (JSM- 6820). Stigma and leaves trichomes morphology were studied by scanning electron microscope in the SEM laboratory of the Centre for Plant Conservation, University of Karachi.

DNA Extraction and RAPD Analysis

Leaf DNA was extracted from the fresh samples with modifying cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) with some modification. The Nano Photometer ™ P-360 (Implen, Germany) was used to check the concentration of extracting DNA in each sample and the quality of the DNA was analyzed by horizontal agarose gel electrophoresis (Cleaver Scientific HU10, UK). At -20 ° the DNA was stored till further utilize.

In the present study total 45 random primers of operon series (OPA, OPB, OPC, OPL, OPH) were used. Amplification reaction of 20 µl contained 1X PCR buffer, 4mM $MgCl_2$, 0.6mM dNTPs, 0.6µM each primer, 1 unit *Taq* polymerase, 25ng genomic DNA and the appropriate volume of PCR grade water. For DNA amplification thermal cycler conditions was set as follows: initial denaturation of DNA at 94 °C for 1 min, 45 cycles of denaturation of DNA at 94°C for 1 min, annealing of primer at 36°C for 1 min, primer extension at 72°C for 2 min and final extension at 72°C for 10 min. The amplified products were run on agarose gel electrophoresis (Cleaver Scientific HU10, UK) on 1.5% agarose (MOLEQULE-ON, New Zealand) gel and visualized on Gel Documentation System (UVI Tech, UK).

Data Analysis

Total 40 quantitative and qualitative characters were taken in constructing dendrogram. Each character state was converted into numeric data (1-9) and data matrix was arranged. The cluster analysis of morphological characters was conducted by using the Ward linkage method with SPSS (Ver. 20.0) software. The Euclidean distance was used as a dissimilarity coefficient in cluster analysis of morphological data and constructs a dendrogram.

For RAPD data, binary data matrix was composed by scoring the presence (+) or absence (-) of amplified bands for each gel and data were analyzed by means of the Nei's similarity index (Nei& Li, 1979), according to the equation,

$S= 2 Nab/ (Na + Nb)$

With the help of similarity matrix data, dendrogram was constructed by applying UPGMA cluster analysis using the NTSYSPC. Ver. 2.01

RESULTS

Morphological study

Morphological variability was high within *Grewia erythraea, Grewia tenax* and hybrids between them. Out of 40 characters evaluate in this study 16 characters were similar in all three taxa studied in *Grewia tenax* complex and 15 characters in all three taxa are different with each other (Table 1).

Table 1. Distinguish Morphological character of *Grewia tenax* complex.

Morphological Character	*Grewia erythraea*	*Grewia tenax*	Hybrid
Plant height (m)	0.8-1	1.5-3	0.6-0.8
Leaf apex	Obtuse	Acute	Acute to obtuse
Leaf shape	Obovate	Ovate to orbicular	Elliptic to obovate
Trichomes type	Stellate, cushioned	Multi radiate tufted	Dendritic and simple
Leaf length (cm)	2-3.5	3.5-7	3-4.5
Leaf breadth (cm)	1-3	2-6	2-3.5
Petiole length (mm)	1-3	4-10	2-5
Flower diameter (mm)	1.5-3	4-6	3-5
Pedicel size (mm)	1-2.5	4-8	3-4.5
Sepal length	8-12	12-22	10-16
Petal length	6-8	8-14	5-7
No. of stamens	50-65	65-80	55-70
Shape of stigma	2 lobed	8-12 lobed	4-8 lobed
Fruit size (mm)	4-6	4-7	3-6
Fruit lobes	2-4	1-4	2-6

Figure 1: Scanning electron micrograph of Trichomes of a. *Grewia erythraea* b. *Grewia tenax* c. Hybrid

***Grewia erythraea* Schweinf.**

Under shrub, semi erect, height 0.8-1m. Woody stem ascendant, glabrous, grey brown in color. Leaves sparsely pubescent, stellate, cushioned trichomes (Figure 1a) obovate, 2-3.5 cm long, 1-3 cm broad, dentate, cuneat base, obtuse apex; Petiole 1-3 mm long. Flower solitary, 1.5-3cm in diameter, off white in color. Pedicel 1-2.5 mm. Sepals oblong 8-12mm long, obtuse, hairy. Petals elliptic 6-8mm long, bilobed at apex, densely hairy at the base with nectarines gland. Stamens 50-65, anther basifixed. Ovary 4 lobed densely hairy, style equal to or slightly longer than stamen, stigma 2 lobed (Figure 2a). Drupe 4-lobed, orange, densely hairy. Each lobe 3-5mm in diameter.

***Grewia tenax* (Forssk.) Fiori**

Shrub, erect, height 1.5-3 m. Woody stem erect to caulescent, stellate hair, brown in color, stipulate. Leaves pubescent, ovate to orbicular, 3.5-7 cm long, 2-6 cm broad, dentate, simple and multiradiate tufted trichomes (Figure 1b), obtuse to truncate at base, acute apex; Petiole 4-10 mm long. Flower solitary or paired, 4-6 cm in diameter, off white in color. Pedicel 4-8mm. Sepals oblong 12-22mm, obtuse, hairy. Petals elliptic 8-14mm long, bilobed at apex, densely hairy at the base with necteries gland. Stamen 65-80, equal to or slightly longer than petals, anther basifixed.Ovary 4-lobed, glabrous, style slightly longer than stamen, stigma 8-12 lobed (Figure 2b).Drupe 1-4 lobed, orange, shiny, glabrous, each lobe 4-7mm

in diameter.

Hybrid Plant

Under shrub, semi erect, height 0.6-0.8m. Woody stem ascendant, glabrous, grey brown in color. Leaves sparsely pubescent, dendritic and simple trichomes (Figure 1c), elliptic to obovate, 3-4.5 cm long, 2-3.5 cm broad, dentate, cuneat at base, acute to obtuse apex; Petiole 2-6cm long. Flower solitary or rarely paired, 3-5cm in diameter, off white in color. Pedicel 3-4.5 mm. Sepals oblong to linear 10-16mm, obtuse, hairy. Petals elliptic 7-10mm long, bilobed at apex, densely hairy at the base with nectarines gland. Stamens 55-70, anther basifixed. Ovary 4 lobed densely hairy, style equal to or slightly longer than stamen, stigma 6-8 lobed (Figure 2c).Drupe 4-6lobed, orange, densely hairy. Each lobe 3-6mm in diameter.

Molecular analysis

In the present study, 45 core set of primers applies on two morphologically distinct species *Grewia tenax* and *Grewia erythraea* and one hybrid, which shares the intermediate morphological traits of both parental species. The result showed out of 45 primers only five primers OPA 7, OPA 8, OPA 11, OPA 12, OPA 18 produced 76 bands for the *Grewia tenax* complex (Figure 3). The size of bands generated by OPA7, OPA8, OPA11, OPA12 and OPA18 ranged from 450bp to 2400bp and 82.89% polymorphism was generated for all primers (Table 2). Consequently the RAPD profile of *Grewia tenax* complex from our study strongly supports that RAPD can be proved very useful evidence for recognition of hybrid from their parents.

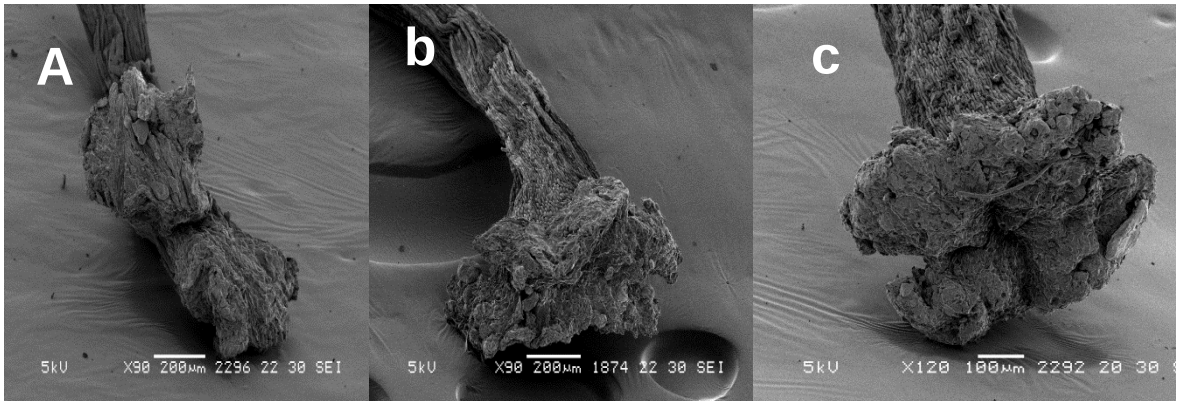

Figure: 2 Scanning electron micrograph of shape of stigma; a. *Grewia erythraea.* b. *Grewia tenax.* c. Intermediate.

Table 2: Total numbers of amplified and polymorphic fragments generated by PCR using RAPD primers

Primer name	Base sequence	Total No. of bands (y)	Total No. of Polymorphic bands (x)	Total No. of monomorphic bands	Polymorphism (%) x / y * 100	Size range of amplified products (bp)
OPA7	5' GAAACGGGTG 3'	13	7	6	53.84	550-1550
OPA8	5' GTGACGTAGG 3'	16	15	1	93.75	450-2400
OPA11	5' CAATCGCCGT 3'	22	20	2	90.9	900-2100
OPA12	5' TCGGCGATAG 3'	14	11	3	78.57	800-2000
OPA18	5' AGGTGACCGT 3'	11	10	1	90.9	950-1900
	Total	76	63	13	82.89	450-2400

Table 3; Similarity matrix generated using the Nei's estimate of similarity

Name of taxa	1	2	3
Grewia erythraea	1.00		
Grewia tenax	0.648	1.00	
Hybrid	0.88	0.528	1.00

Statistical analysis

Cluster analysis by using Ward linkage with SPSS of *Grewia tenax* complex shows the existence of two main groups *viz.*, group A and group B (Figure 4). Group "A" comprises of only *Grewia tenax* this species does not cluster to other two taxa. Group B includes *Grewia erythraea* and intermediate have the same point of origin.

The dendrogram of *Grewia tenax* complex also constructed by using UPGMA cluster analysis**.** Similarity matrix generated by using the Nei's estimate of similarity (Table 3.). Dendrogram showed *Grewia erythraea* and hybrid plants clustered in a same group while *Grewia tenax* making its own separate group (Figure 5). These two groups were joined together at about 0.59 genetic distance level.

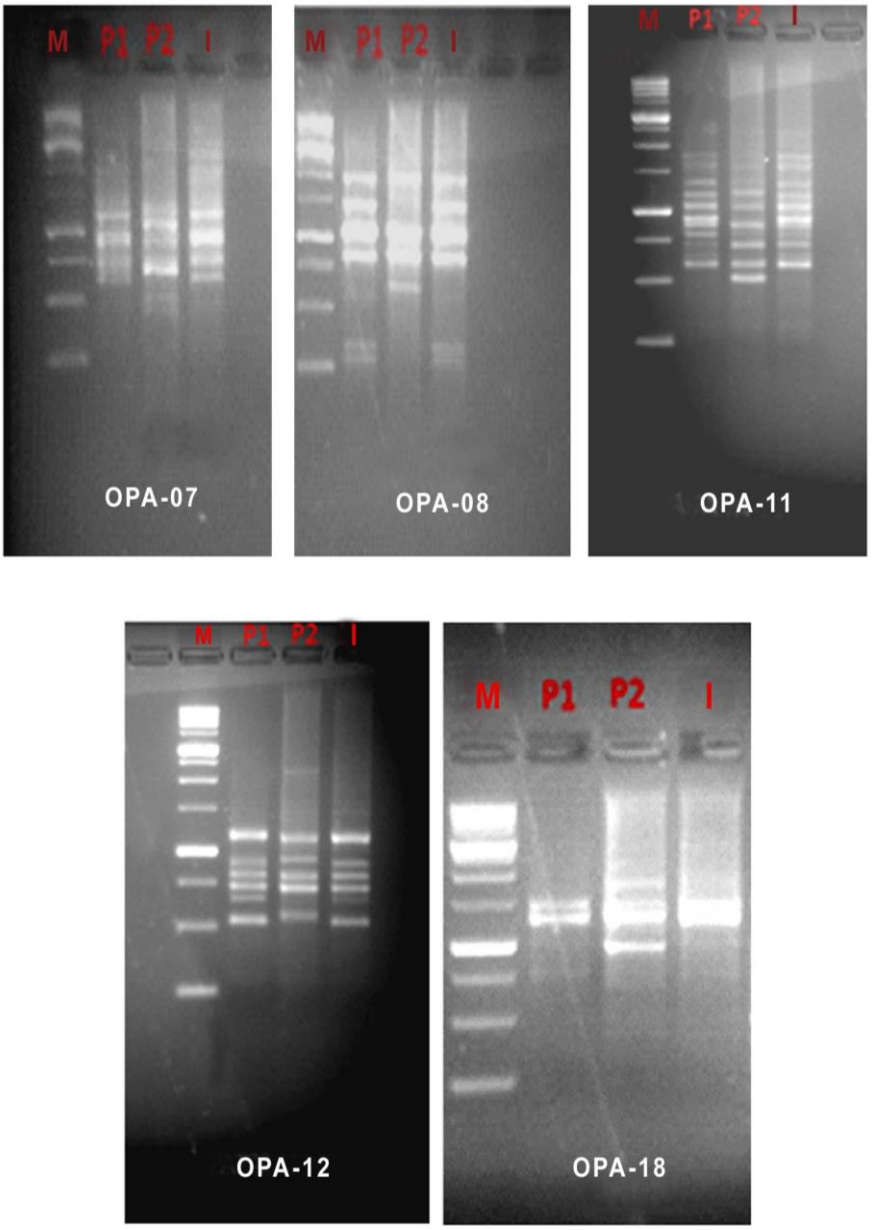

Figure 3: RAPD profile of *Grewia tenax complex* (Lane M is a marker 1kb ladder, P1=*Grewia erythraea*, P2= *Grewia tenax*, I=Hybrid)

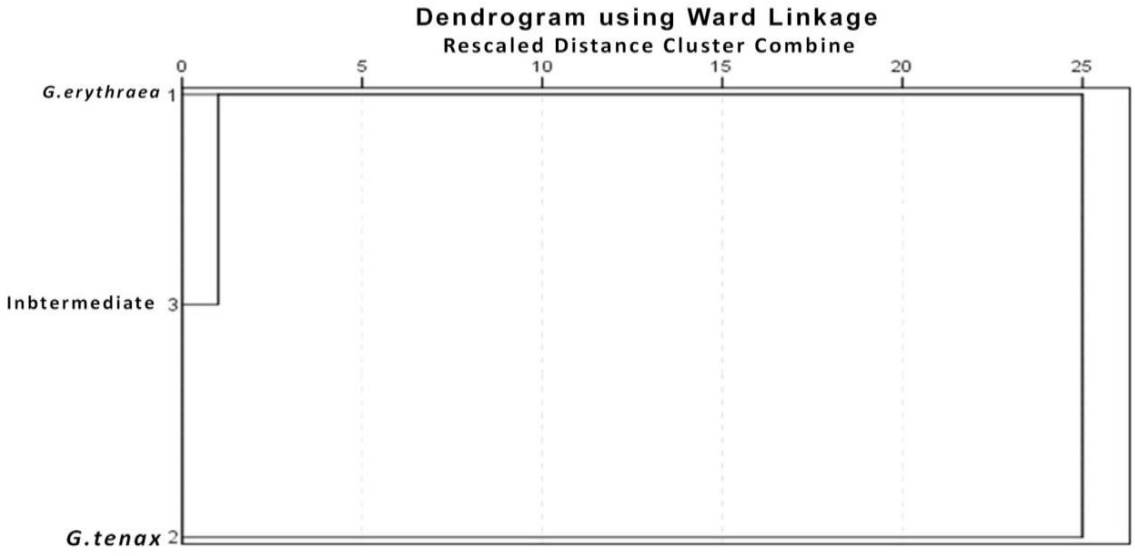

Figure 4; Dendrogram showing the relationship of *Grewia* species in *Grewia tenax* complex

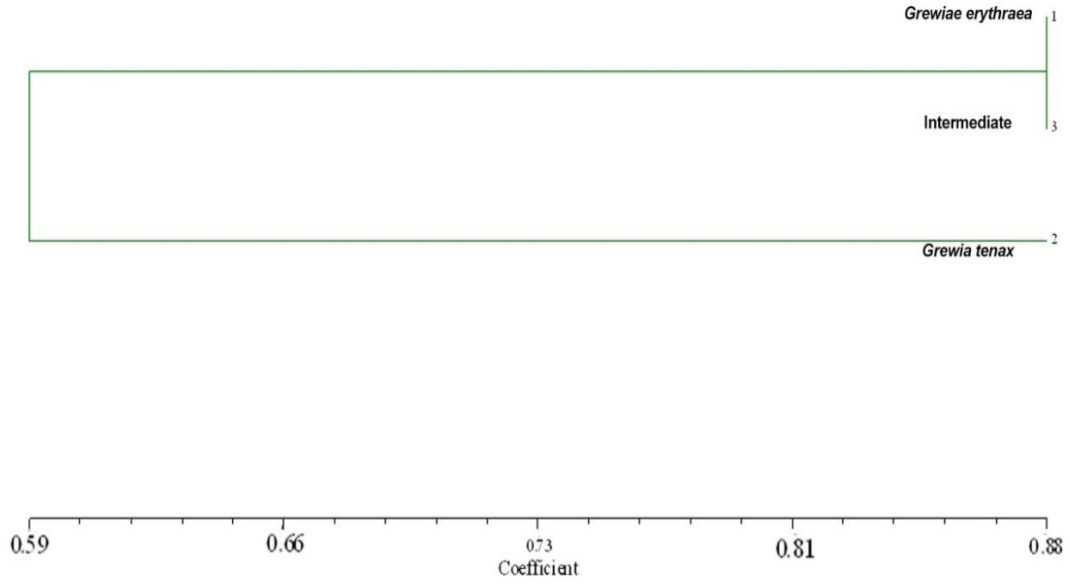

Figure 5: Dendrogram of three species of *Grewia tenax* complex constructed using Nei and Lei's similarity index.

DISCUSSION

Our studies convincingly demonstrate the *Grewia tenax* complex containing two species, *Grewia tenax* and *Grewia erythraea* and hybridization between them on a limited scale. The experimental results support the hybridization in *Grewia tenax* complex. Ghafoor (1974) used *Grewia erythraea* as synonyms of *Grewia tenax* and both taxa as a single taxon.WhileStewart (1972) in "Annotated catalogue of the vascular plants of W. Pakistan and Kashmir" treated both taxa *Grewia tenax* and *Grewia erythraea* separately.

Morphologically the hybrid plants show highest resemblance with *Grewia erythraea*. All three taxa found in the *Grewia tenax* complex can be differentiated by plant height, leaf size, apex, shape, leaves trichomes, petiole size, flower diameter, pedicel size, ovary surface, stigma shape and fruit surface. *Grewia tenax* has greater in size as compare to *Grewia erythraea* and hybrid plant. Leaf shape also differentiated in all three taxa *Grewia erythraea* leaves was obovate in shape with obtuse apex, leaves of *Grewia tenax* was ovate to orbicular with acute apex while in hybrid leaves were elliptic to obovate. Leaf size can also differentiate. In *Grewia tenax* leaves were large in size while in *Grewia erythraea* leaves are small. Leaves trichomes also observed as distinguish character in *Grewia tenax* complex *Grewia erythraea* has stellate, cushioned trichomes, *Grewia tenax* showed multiradiate tufted trichomes while hybrid showed dendritic and simple trichomes. Stigma shape can also differentiate in all three taxa. *Grewia erythraea* showed 2 lobed stigma, 8-12 lobed stigma was observed in *Grewia tenax* while 4-8 lobed stigma was present in hybrid plants.

It has been considered that hybrid sometimes shows extreme or novel characters, but now these have been reviewed to be unimportant and unusual from an evolutionary point of view (Stebbins, 1974). Although, Rieseberg and Ellstrand (1993), studied morphological character expression in hybrid and found that over 10% of characters measure in 1st generation hybrid were extreme. Although, Yorkston (2005), studied hybridization between *Sida fallax and sida rhombifolia* and compiled a list of 17 morphological character expressions in hybrid and parental plants and found that out of 17 morphological characters 4 were intermediate, 3 traits were dominant, one trait was extreme relative to both parents and 9 traits did not statically differ among parents and hybrid.

RAPD analysis is an important tool to investigate the hybrid origin by comparing the banding patterns with expected parental species**.** Like morphological characters RAPD analysis showed that *Grewia erythraea* and *Grewia tenax* both are completely isolated parental species and hybrid is closely related to *Grewia erythraea.*

In the present study, 45 core set of primers applies on two morphologically distinct species *Grewia tenax* and *Grewia erythraea* and one hybrid, which shares the intermediate morphological traits of both parental species. 76 bands were generated by the five primers for the *Grewia tenax* complex. OPA7 generated 13 bands, OPA8 generated 16 bands, OPA11 generated 22 bands, OPA12 generated 14 bands and OPA18 generated 11 bands (Figure 3). The percentage of polymorphism generated for all primer was 82.89%. The size of bands generated by OPA7, OPA8. OPA11, OPA12 and OPA18 ranged from 450bp to 2400bp. The results indicate that all samples had different banding patterns.

Dendrogram prepared by SPSS cluster analysis by using morphological characters shows two main groups. *Grewia tenax* does not cluster it to other, but its form a cluster of its own. The delimiting characters are shrub with brown, pubescent, erect to caulescent stem, obtuse to truncate leaf base and glabrous ovary and fruit while *Grewia erythraea* and hybrid plant produce single groupdue to similarities in their semi erect habit, with glabrous, ascendant, gray, brown stem, cuneate leaf base, ovary and fruits hairy.

Dendrogram produced by UPGMA cluster analysis by using molecular character also showed two groups *Grewia erythraea* and hybrid plants were linked together 0.88 genetic distance level. Range of genetic distance levels of *Grewia tenax* complex 0.59 to 0.88. The similarity indices show the relationships of individual with each other. Lower genetic similarity indices of two species indicate farther genetic relation between species, while higher similarity indices show individual have closer genetic relation among them.

Our studies confirmed earlier studies (Stewart 1972; Hashmi and Qaiser 1990; Sebsebe 1999; Abedin et al., 1999) reporting *Grewia erythraea* and *Grewia tenax* are two distinct entities. Morphological and molecular studies also support that hybridization between them on a limited scale and hybrid plants have the intermediate character of both taxa.

CONCLUSION

Morphological and molecular studies convincingly demonstrate the *Grewia tenax complex* containing two species *Grewia tenax* and *Grewia erythraea* and hybridization between them on a limited scale. The experimental results support the hybridization in *Grewia tenax* complex Hybrid plants in complex exhibit intermediate morphological characters to their parent forms. Comparisons of morphological characteristics such as plant height, leaf size, leaf shape, leaf base, ovary surface, fruit surface, leaves trichomes type and stigma surface, molecular studies and statistical analysis revealed that *Grewia tenax* and *Grewia erythraea* are two distinct entities and hybrid plants have the intermediate character of both taxa.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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