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INVESTIGATING HYBRIDIZATION AND VARIABILITY BETWEEN *FICUS* SPECIES IN SAUDI ARABIA THROUGH DNA BARCODING APPROACH AND MORPHOLOGICAL CHARACTERS

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Abstract

Recently, research interest has spread through the DNA barcoding for economically important species of plants. In this work, the morphological characterization and DNA barcoding were investigated to discriminate between *Ficus carica, Ficus cordata* and *Ficuspalmata* inhabiting Taif highlands to show the possibility of hybridization between them. *F. carica* L. (cultivated tree) and *F. palmata* Forssk. (wild tree) showed great morphological similarities that reflected a probable interspecific hybridization between them. The observed hybrids were of low commercial importance. The obtained results of DNA barcodes (ITS, matK, rbcL and trnH) exposed different evolutionary events between the four regions, allowing each other to complement the discrimination between *Ficus*species. The phylogenetic trees revealed excess genetic diversity and distinguished *Ficus* species inhabiting Taif from other species retrieved from the GenBank proving their endemism to flora of Saudi Arabia. Transition/transversion bias (R) and rate of Tajima evolution for rbcL, trnH-psbA and ITS showed that the rate of evolution of *Ficus* species relatively accelerates and enhances hybridization between them. Entomorphology seemed to play an important role in hybridization and causing notable genetic diversity between *F. carica* and *F. palmata* in Taif. ITS was suggested as a plant barcode for its discriminatory power at low taxonomic levels than plastid barcodes.

Key words: *Ficus,* Hybridization, ITS, rbcL, matK, trnH-psbA.

Introduction

DNA barcoding has proved to be an extensively used and effective tool for rapid and accurate species identification and discrimination leading to a wide range of targets such as ecological genomic studies (Valentini *et al.*, 2009), revealing the unknown species and biodiversity surveys (Thompson & Newmaster, 2014), support for the intellectual property rights (Stewart, 2005), food safety (Galimberti *et al.*, 2012) and wildlife forensic analyses (Deguilloux *et al*., 2002). The three plastid loci: rbcL, matK and trnH-psbA and the internal transcribed spacer (ITS) have been considered to be the most efficient barcoding loci for displaying high amount of variation among complex plant groups (Laiou *et al*., 2013). Many previous studies employed these regionsfor authentication and taxonomic purposes (Shinwari*et al.,* 1994a; Shinwari *et al.,* 1994b; Shinwari, 2002; Shinwari & Shinwari, 2010, Shinwari *et al*., 2014 and Zahra *et al.,* 2016). Recognition of tree species through DNA barcodes is difficult due to low mutation rate of the chloroplast DNA, hybridization and the great similarity between them (Petit & Hampe, 2006). Nevertheless, microsatellite markers and DNA barcoding was utilized in several previous studies of tree taxa as in *Ficuscarica* (Saddoud *et al*., 2005; Guasmi *et al*., 2006; Achtak *et al*., 2009; Baraket *et al*., 2009, 2011).

With nearly 800 species, *Ficus*is considered to be the largest genus in the Moraceae (Berg & Corner, 2005). The common edible fig (*F. carica*) is a temperate species native to theMediterraneanregion and southwestAsia where wild and cultivated trees are found (Zukovskij, 1950; Storey, 1975; Ferguson *et al*., 1990). *F. carica, F. cordata*and *F. palmata*are three species of eight growing in Saudi Arabia (Chaudhary, 1999). Two subspecies of *F. carica* L. are known, *F. carica* L. subsp. *sativa* (common fig) and *F. carica* L. subsp. *caprificus* (caprifig or wild type) (Edmond *et al*., 1975; Weiblen, 2000). Fig fruits have mild laxative activity (Baraket *et al*., 2009) and their extracts are widely used to treat several diseases such as inflammation, jaundice , epilepsy, influenza, whooping cough, bronchitis, tonsillitis, enteritis, bruises, toothache and bacillary dysentery (Alqasoumi *et al*., 2014).

Ficus species are distinguished by their unique inflorescenceand special pollination condition, which depend on wasp species for pollination. Each *Ficus*species is pollinated by one or a few specialized wasp species, without this process fig trees could not reproduce by seed (Valizadch *et al.*, 1987). Pollination mechanism is one of the highest correlations with the genetic variables. Thus, the specific identification may be difficult, but they can be recognized as a group. Moreover, figs are subjected to adaptive radiation (the process in which species diversify rapidly)in variousbiogeographicregions, leading to very high levels ofgenetic diversity (Molbo *et al*., 2003; Yang *et al*., 2015).

In this study, four barcodes; rbcL, matK, trnH-psbA and ITS were applied to the three species; *F. carica, F. cordata* and *F. palmata* inhabiting Taif, Saudi Arabia to assess the efficiency of each marker for accurate species discrimination by identifying any previously unknown genetic diversity and to highlight possible hybridization occurring among the three species.

Materials and Methods

Plant materials: *F. carica* (1), *F. cordata* (2) and *F. palmata* (3) belonging to family Moraceae were collected from Taif highlands (Alshafa and Elhada), Saudi Arabia. Species identification was done according to Chaudhary (1999). Leaf and fruit characters of the studied species were measured.

DNA extraction and amplification process: The genomic DNA was extracted from leaves of the three Moraceae species using CTAB method (Doyle & Doyle, 1987). The amplification of the purified DNA was achieved using universal primers as mentioned in Table 1. PCR process started with DNA denaturation at 94°C for 5 min. Then 35 cycles were adjusted including denaturation at 94°C for 1 min, then annealing at 52°C for 30 sec and finished by extension at 72°C for 2 min. A final extension was done at 72°C for 8 min.

The PCR sequencing: The PCR products of the three Moraceae species for the four DNA barcodes were purified and sequenced at Macrogen Inc., South Korea. All sequences of *Ficus* species generated in this study were deposited in GenBank (accession numbers are listed in Table 2).

The sequences alignment and phylogenetic trees: The sequences of ITS, matK, rbcL and trnH-psbA of *F. carica, F. cordata* and *F. palmata* were subjected to BLAST of the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to distinguish them from the other related *Ficus* species found in this database. Alignments of sequence were achieved by MUSCLE algorithm (Edgar, 2004; Tamura *et al*., 2013). The evolutionary rate parameters between sequences of *F. carica, F. cordata*and *F. palmata*were calculated by Tajima's relative rate test (Tajima, 1993). Transition/transversion bias (R) and Nucleotide substitution rates were calculated using Maximum Likelihood method. MEGA6 Software was used to build the phylogenic trees by the Maximum likelihood bootstrap (MLB) analysis depending on a total of 1000 bootstrap replicates (Tamura *et al*., 2013).

Results and Discussion

Traditionally, degree of genetic diversity is detected by morphological markers. In spite of their expression was influenced by agronomic strategies and environmental conditions, the morphological description should be made before more in-depth molecular studies are performed (Hoogendijk & Williams, 2001).

Morphology features: Although, *F. carica* L. (cultivated tree) and *F. palmata* Forssk. (wild tree) are taxonomically differentiated by leaf size (*F. palmata* has smaller leaves up to 16 cm, while *F. carica* has large leaves up to 30 cm or more) and fruit size (*F. palmata* has smaller fruit 1-2 cm, while *F. carica* has large fruit 3-5 cm), they showed great morphological similarities; leaves were ovate-cordate, simple-lobed (2-5 lobes), leaf margin serrate, leaf apex acute-obtuse, base of lamina cordate-trunicate and the fruits were syconium of oblate-globose shape, purple-brown-black colour, with edible or non-edible taste (Fig. 1). This strong relation between the two species appeared in taxonomy as section *Ficus* subsect. *Ficus* that includes only *F. carica* and *F. palmata* (Rønsted *et al*., 2008). The previous similarities reflected a probable interspecific hybridization between them. The observed hybrids were of low commercial importance (having non-edible fruits) and leaf heterophylly was highly expressed. Hybridization between *F. carica* and *F. palmata* could be done due to cross pollination by wasps and growing in the same area as noted in the two sites; Alshafa and Elhada under study. These observations were similar to those of Condit (1950) and Storey (1975). On the other hand, *F. cordata* was morphologically distinct from *F. carica* and *F. palmata* by its lanceolate narrow leaves (less than 4 cm), small green non-edible syconium and growing in different habitat (rocky slopes) (Fig. 1-F).

Molecular features: Specific DNA barcodes have the ability to distinguish closely related species. We, therefore, used ITS, trnH-psbA, matK and rbcL loci (Fig. 2) to study the genetic divergence among *F. carica, F. cordata*and *F. palmata*that may lead to the detection of any hybridization event during their lifetime.

***matK* marker:** In genome of *Ficus*species, we found that sequence length (≥ 811) and GC ratio (32) of matK were so far identical in the three species of *Ficus* (Table 3). It was worthy to note that transversions were found to be more than transitions. Moreover, matK did not reveal any evolutionary rate of *Ficus*species from Taif, because results of transition/transversion bias (R=0) and Tajima relative evolutionary rate (*P*-values were higher than 0.05) accepted the null hypothesis of equal evolution rates between *Ficus*species (Table 4). A phylogenetic tree that was reconstructed depending on *Ficus* species from Taif and 10 related species that retrieved from the GenBank produced two distinct groups (Fig. 3). Group (A) included species belonging to *F. carica*. The second group (B) diverge the three *Ficus* species from Taif into two subgroups. The first group had *F. carica* and *F. palmata* together, whereas the other consisted of *F. cordata* and *F. cordata* subsp. *salicifolia* from GenBank. Depending upon previous studies, matK was recommended as the first option to reveal sequence variability, because it had a high evolutionary rate, suitable sequence length, low transition/transversion rate and clear interspecific divergence (Selvaraj *et al*., 2008; Anon., 2009). Our findings demonstrated that matK marker was effective in the discrimination at the species level and having low rate of transition/transversion, but it failed to show any evolution within the genome of *Ficus*.

Table 1. List of the investigated DNA barcoding primers.

Locus	Primer name		Primer sequences (5'–3')	Ann. Temp.	Reference
ITS	AB101	F	ACGAATTCATGGTCCGGTGAAGTGTTCG	52°C	Sun *et al.,* (1994)
	AB102	R	TAGAATTCCCCGGTTCGCTCGCCGTTAC		
rbcL	rbcLa	F	ATGTCACCACAAACAGAGACTAAAGC	52°C	Levin *et al.,* (2003)
	rbcLa	R	GTAAAATCAAGTCCACCRCG		Kress & Erickson (2007)
matK	matK-KIM1	F	ACCCAGTCCATCTGGAAATCTTGGTTC	52°C	Fazekas *et al.,* (2012)
	matK-KIM3	R	CGTACAGTACTTTTGTGTTTACGAG		
trnH	psbAF	F	CGCGCATGGTGGATTCACAATCC	52°C	Sang *et al.,* (1997)
	trnH2	R	GTTATGCATGAACGTAATGCTC		Tate & Simpson (2003)

Table 2. Accession numbers in GenBank of sequences of *Ficus* species generated in this study.

Species	ITS	matK	rbcL	trnH-psbA
F. carica	LC375796	LC375797	LC375798	LC375799
F. cordata	LC375800	LC375801	LC375802	LC375803
F. palmata	LC375804	LC375805	LC375806	LC375807

Table 3. Statistics derived from the sequencing, alignment and BLAST processes.

Parameter	Species	ITS	matK	rbcL	trnH-psbA
Sequence length	*F. carica*	830	820	534	270
	F. cordata	850	820	531	429
	F. palmata	808	811	528	450
GC ratio	*F. carica*	61	32	43	17
	F. cordata	61	32	43	29
	F. palmata	60	32	43	30
Number of theretrieved species from the GenBank	*F. carica*	8	9	10	10
	F. cordata	8	1	2	0
	F. palmata	1	0	5	1

Table 4. Mean nucleotide substitution rates and transition/transversion bias (R) in loci for *F. carica, F. cordata* and *F. palmate* calculated by Maximum Likelihood method.

Locus	Transition				Transversion				(R)
	A→G	G→A	T→C	C→T	A or G→T	T or C→A	A or G→C	T or C→G	
ITS	20.35	12.41	13.21	8.92	4.72	4.11	6.99	6.73	1.15
matK	0.00	0.00	0.00	0.00	19.01	14.92	8.28	7.79	0.00
rbcL	7.00	10.06	22.19	27.78	4.60	4.84	3.67	3.37	2.00
trnH-psbA	3.54	10.25	8.39	27.34	8.34	10.66	2.56	3.68	0.69

Fig. 1. Variations in leaf shape in *Ficus* species. (A) *F. palmata* (with edible fruit), (B) *F. palmata* (heterophylly), (C) *F. palamta* (hybrid), (D) *F. palmata* (with non-edible fruit), (E) *F. carica*and (F) *F. cordata*.

***rbcL* marker:** rbcL is widely used in phylogenetic studies due to the easiness of amplification, sequencing, alignment and in DNA barcoding identification at the family and generic levels for most land plants. It has straight forward recovery of the gene sequence and produce large amount of easily accessible data (Hollingsworth *et al*., 2011). As in matK, sequence length and GC ratio of rbcL were almost identical in the three species of *Ficus* (Table 3). An accelerated evolution was observed within *Ficus* genome through the high transition/transversion bias (R=2) and Tajima relative evolutionary rates (*P*-values <0.05) (Tables 4 and 5). The phylogenetic tree of rbcL (Fig. 4) demonstrated 3 monophyletic clusters. Clusters A and C grouped all retrieved species of *F. carica* and *F. palmata*, respectively. whereas, *Ficus* species from Taif did not diverge and gathered in one cluster (B). rbcL marker recorded the lowest divergence of plastid genes and this could be explained by the symmetry in *rbcL*sequence within the *Ficus* genome. This result supported the point of view of Renner, (1999), Salazar *et al*., (2003), Kress *et al.*, (2005), Fazekas *et al.*, (2008), Anon., (2009) and Zahra *et al*., 2016 that rbcL had little discriminatory power made it unsuitable at the species level.

***trnH-psbA* marker:** Recently, the non-coding intergenic sequence trnH-psbA is widely used plastid barcode that exhibits high sequence divergence. It has also considerable rates of nucleotide substitution and complicated molecular evolution that leads to ambiguous alignment. In this study, trnH-psbA recorded the lowest sequence length that ranged from 450 to 270 bp and with variable GC ratios as shown in Table (3). An obvious evolution was also detected between *F. carica*and the two other species through Tajima relative evolutionary values (68.61, 78.68) that displayed an accelerated rates of evolution (*P*-values <0.05) (Table 5). The phylogenetic tree of the three *Ficus* species and their related species from GenBank (11) produced two groups as shown in Figure 5. Group (A) included all species belonging to *F. carica*with separation of *F. carica* collected from Taif in an independent clade. On the other hand, *F. cordata* was combined with *F. palmata* in the second group (B). In contrast, trnH-psbA provided better discrimination than the plastid gene rbcL and this was in accordance with those of Ren *et al*., (2010) in *Alnus*& Pang *et al*., (2012) in more trees of angiosperms.

***ITS* marker:** The ITS locus is a potent phylogenetic marker at the species level revealing high levels of the interspecific divergence. The highest sequence length was recorded in *F. cordata*, whereas, GC ratios were equal in the three species (Table 3). Transitions were found to be more than transversions within sequences of *Ficus* species (Table 4). Transition/transversion bias (R= 1.15) indicated an evolution within *Ficus*genome. Tajima relative evolutionary rate also supported an accelerated rates of evolution (*P*-values<0.05) among the three species (Table 5). Phylogenetic tree reconstructed using all sequences (Fig. 6) divided species into three distinctive groups. Group (A) included species belonging to *F. cordata,* however *F. cordata* from Taif also occurred in a separate clade. The second group (B) consisted of the retrieved species of *F. carica.* The third (C) grouped *F. carica* and *F. palmata* collected from Taif together. ITS was suggested as a plant barcode for its discriminatory power at low taxonomic levels than plastid barcodes (Stoeckle, 2003; Kress *et al.*, 2005; Anon., 2011).

Table 5. Tajima relative rate tests of loci for *F. carica, F. cordata* and *F. palmata*.

Locus	Outgroup	Testing group		RI	RD	RA	RB	χ2	*P* value
		(A)	(B)						
ITS	*F. palmata*	*F. carica*	*F. cordata*	414	2	8	227	204.1	<0.05
	F. cordata	*F. carica*	*F. palmata*	414	2	8	8	0.00	>0.05
	F. carica	*F. cordata*	*F. palmata*	414	2	227	8	204.1	<0.05
matK	*F. palmata*	*F. carica*	*F. cordata*	809	0	0	1	1.00	>0.05
	F. cordata	*F. carica*	*F. palmata*	809	0	0	1	1.00	>0.05
	F. carica	*F. cordata*	*F. palmata*	809	0	1	1	0.00	>0.05
rbcL	*F. palmata*	*F. carica*	*F. cordata*	517	1	4	0	4.00	<0.05
	F. cordata	*F. carica*	*F. palmata*	517	1	4	0	4.00	<0.05
	F. carica	*F. cordata*	*F. palmata*	517	1	0	0	0.00	>0.05
trnH-psbA	*F. palmata*	*F. carica*	*F. cordata*	109	6	90	8	68.61	<0.05
	F. cordata	*F. carica*	*F. palmata*	109	6	90	4	78.68	<0.05
	F. carica	*F. cordata*	*F. palmata*	109	6	8	4	1.33	>0.05

The Tajima relative rate test was used to examine the equality of evolutionary rate for *F. carica, F. cordata* and *F. palmata*
RI is the identical sites in all three sequences
RD is the divergent sites in all three sequences
RA is the number of unique differences in the sequence A
RB is the number of unique differences in the sequence B
χ2test statistic more than 3.841 (p<0.05) indicates accelerated evolution
P value greater than 0.05 is often used to accept the null hypothesis of equal rates between lineages

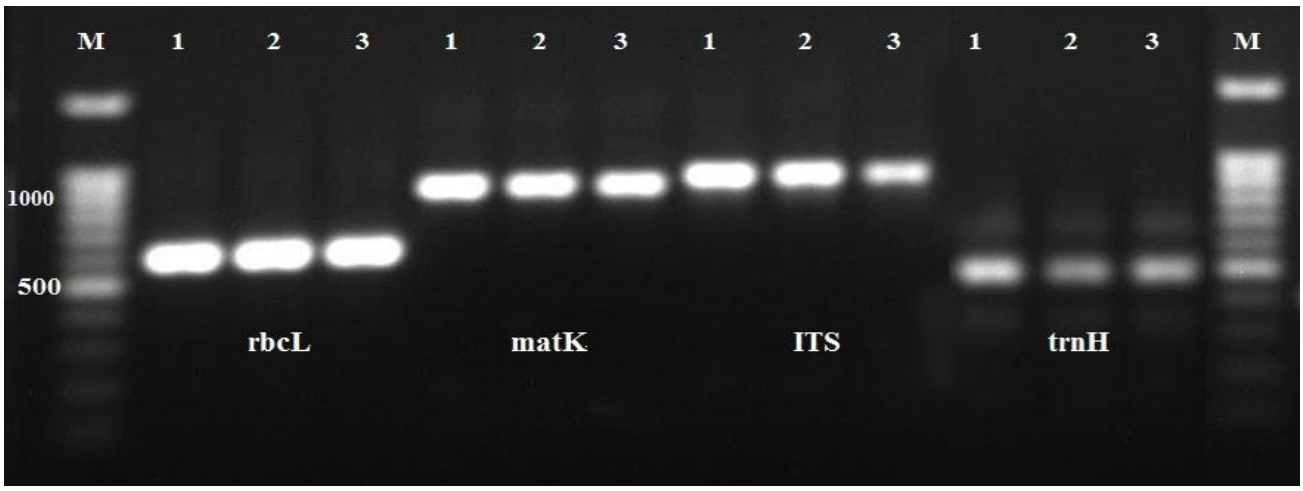

Fig. 2. Electrophoretic DNA bands generated for the four loci of *Ficus* species. (1) *F. carica*, (2) *F. cordata*and (3) *F. palmata*.

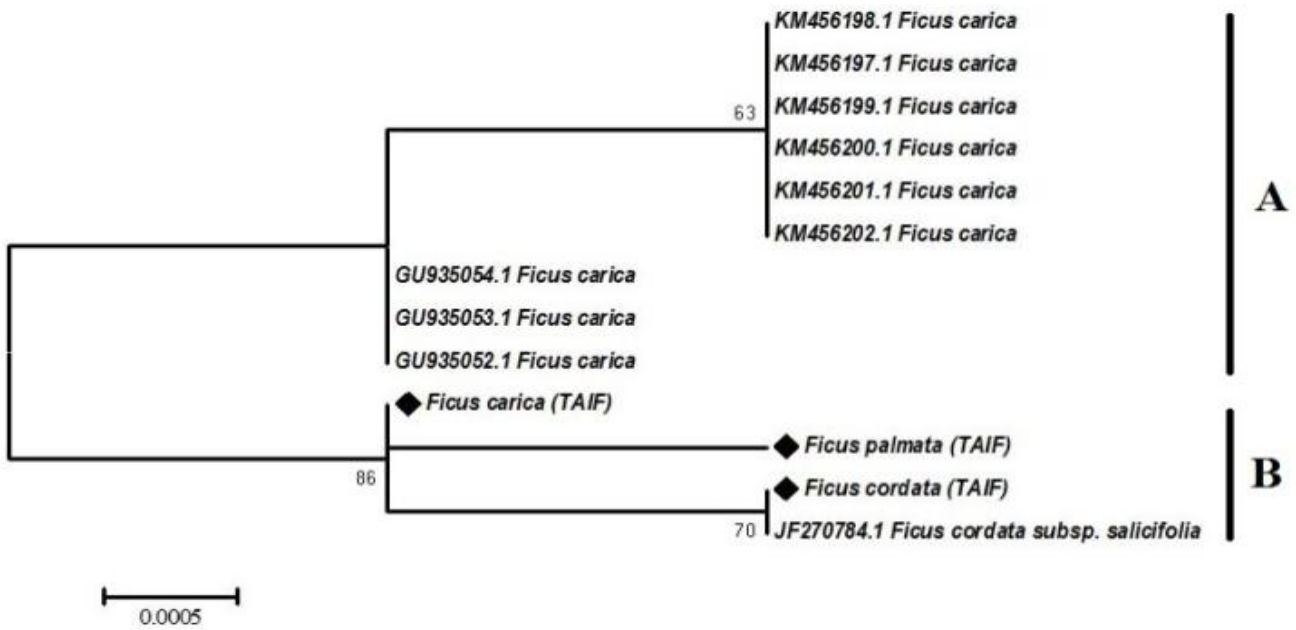

Fig. 3. Phylogeny tree of *Ficus*species based on matK locus.

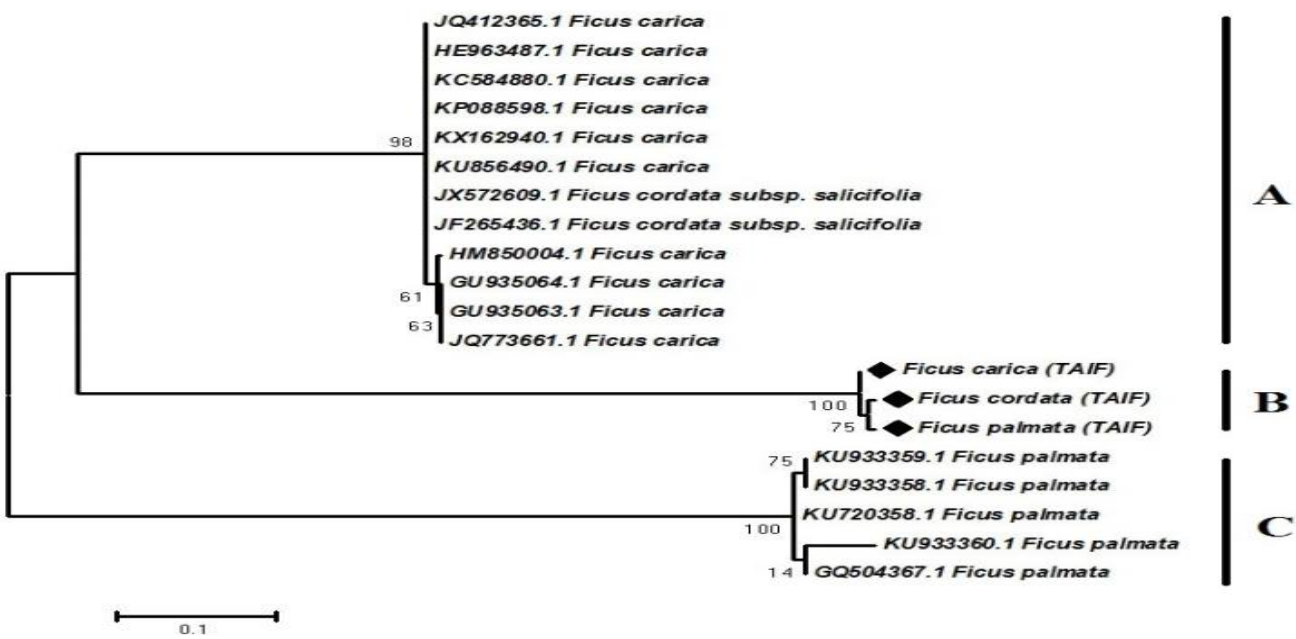

Fig. 4. Phylogeny tree of *Ficus* species based on rbcL locus.

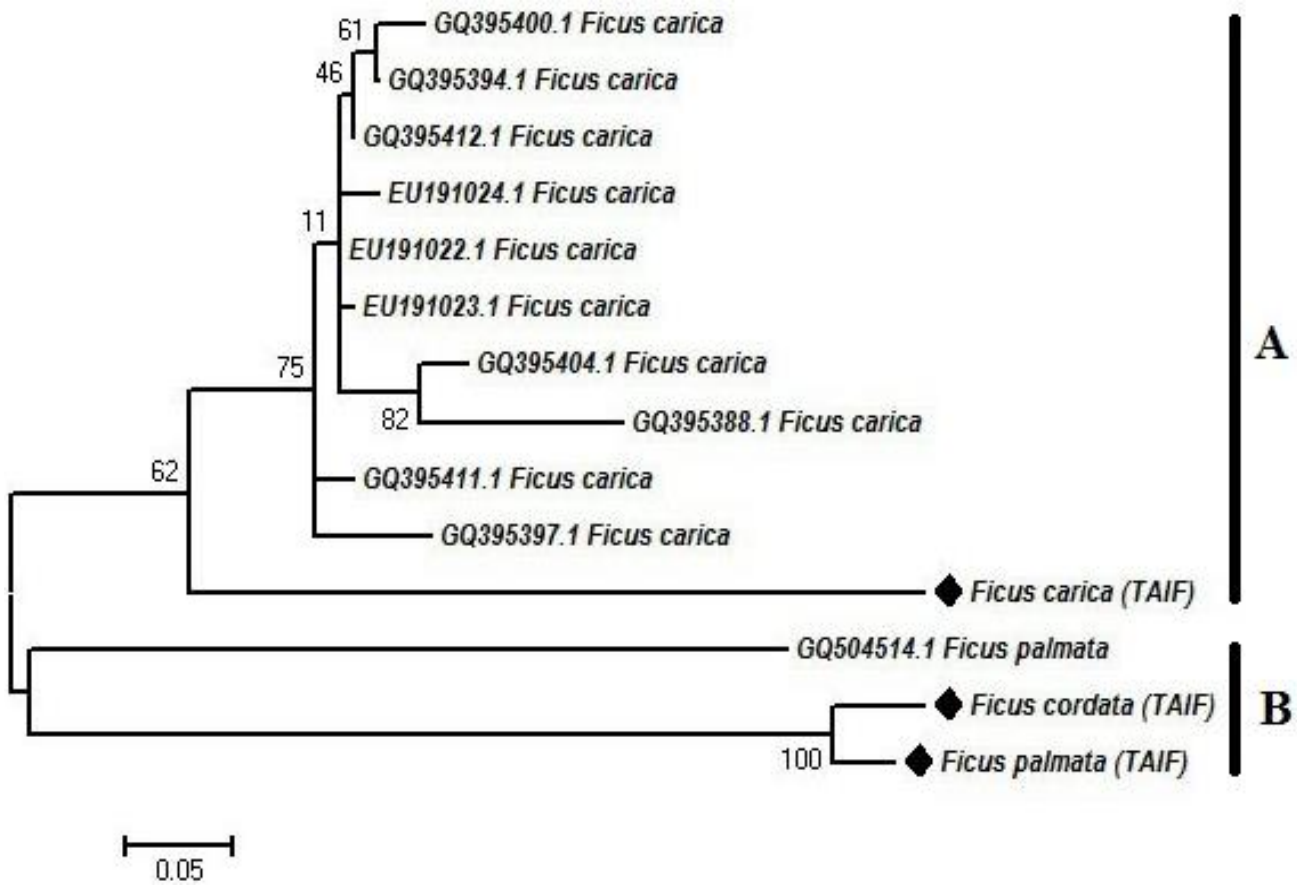

Fig. 5. Phylogeny tree of *Ficus*species based on trnH-psbA locus.

Fig. 6. Phylogeny tree of *Ficus*species based on ITS locus.

The statistical differences observed between ITS and plastid loci, as well as among the three plastid loci themselves indicated the presence of different evolutionary events between the four regions, allowing each other to complement the discrimination between *Ficus* species. The phylogenetic divergence of the three *Ficus* species from other retrieved GenBank species contribute in different ways to revealing excess genetic diversity and distinguishing the fig species inhabiting Taif proving their endemism to flora of Saudi Arabia. This could be explained by the fact that the polymorphisms of the queried sequences have different origins from a molecular level viewpoint causing variability in genetic diversity and relationships between plant species. Furthermore, results of nucleotide substitution rates (transitions higher than transversions), transition/ transversion bias (R) and rate of Tajima evolution for rbcL, trnH-psbA and ITS in addition to the obvious relationship between *F. carica* and *F. palmata* that was exhibited by the phylogenetic trees of matK, rbcL and ITS showed that the rate of evolution of *Ficus*species relatively accelerates and this enhances hybridization between them. These findings were in harmony with those of Li *et al*., (2011) and Castro *et al*., (2015) who suggested that the inconsistencies in the phylogenies of the investigated barcoding loci for closely related varieties of *F. carica* reflected hybridization, polymorphisms and the different mutation rate between them. Thus fig hybrids occurring in Taif highlands have been found in quite good number, becoming a naturally occurring taxon and having great fitness like their parents. This could be explained by the coevolution of *Ficus* and wasps through the specificity of *Ficus* as a host and the tendency of the wasp species to visit hosts different to that of their original *Ficus* plant, thus increasing the probability of hybrids and new species in the end (Ma *et al*., 2009).Therefore, we think that hybridization has a role in the evolution of fig lineages. The results of morphological evaluation confirmed the usefulness of phenotypic markers as a preliminary step for plant genetic resources characterization that should be accompanied with molecular evaluation which is highly important to determine the genetic correlations among these resources.

Conclusion

The data demonstrated that *Ficus* species from Taif were distinguished by little genetic diversity from others retrieved from the GenBank. Despite the modest genetic variation levels between *Ficus* species, the four analyzed loci presented their notable weaknesses and strengths for species discrimination in *Ficus* at the interspecific taxonomic level, which should be accounted before using them as DNA barcodes for authentication of plant trees. On the other hand, it was obvious that the insect pollinators seemed to play an important role in this genetic diversity of *Ficus* species and in allowing widespread hybridization that could have a positive impact on them. Eventually, we suggest combining morphology and DNA barcoding approach, especially if we want to authenticate, maintain and develop our valuable species.

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