Pak. J. Bot., 54(2): 475-481, 2022. DOI: http://dx.doi.org/10.30848/PJB2022-2(35)

A COMPARATIVE STUDY OF *EUPHORBIA PEPLUS, EUPHORBIA HIRTA* AND *EUPHORBIA TIRUCALLI* BASED ON DNA BARCODING MARKERS

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Abstract

DNA regions (ITS, ITS2, *matK*, *rbcL* and *trnH*) have been examined for authentication and differentiation between *E. peplus, E. hirta* and *E. tirucalli* collected from Taif-KSA. The sequences of *Euphorbia* species were submitted in Genbank as new records within this research. ITS, ITS2 and *rbcL* provided good identification and clear resolution for the species, whereas, ITS2 and *rbcL* showed no or very little evolution. The highest sequence lengths were detected by *matK* and IT'S in *E. hirta*, *E. tirucalli* and then *E. peplus*. Our gathered results of Tajima relative evolutionary values revealed that ITS, *matK* and *trnH* showed rates of acceleration of evolution (*P*-values<0.05) within their sequences. The phylogenetic trees did not show any variability between the three *Euphorbia* species and the other retrieved GenBank accessions rejecting their endemism to flora of Saudi Arabia. The results support the hypothesis that *E. peplus, E. hirta* and *E. tirucalli* occur in the natural areas of Taif as naturalized species.

Key words: *Euphorbia,* Differentiation, ITS, *rbcl*, *matK*, *trnH*.

Introduction

Euphorbia L. with approximately to 2000 nearly species with remarkable structural variability and a global occurrence, is considered as the third largest genus of flowering plants (Frodin, 2004). Its habit ranges from small annual grasses to large trees, but *Euphorbia* is famous for its great diversity of xeromorphic growth forms characterized by a great variability in stem succulence (Rauh, 1998). The phylogenetic studies have recognized four major subgeneric clades; *Athymalus*, *Chamaesyce*, *Esula* and *Euphorbia,* that is considered as the largest subgenus in *Euphorbia* (>600 spp.) diverse mainly in tropics and subtropics (Dorsey *et al*., 2013). Its members are distinguishable by specialized inflorescences (cyathia) and their milky latex (Govaerts *et al*., 2000). They are well known for their utilities as ornamental and household plants such as *E. tirucalli* and owning latex that has contributed to the economic significance of some species such as *E. peplus*. The sap from this plant has been used as a treatment for warts, asthma, corns, and cancers of the skin, uterus, stomach and liver (Rizk, 1987; Berman, 2012). *E. hirta* also possesses antiasthmatic, antispasmodic, antifertility, antifungal, antibacterial, and antimalarial properties (Williamson, 2002).

For its climatic peculiarities, Saudi Arabia varies from other desert countries. Its varied factors give rise to environmental dynamics that have caused complexity in the structure and the diversity of vegetation cover in the country (Thomas *et al*., 2014). For that, there is always a need of applying a DNA based method such as DNA barcoding for authentication of those medicinal plants to ensure the safety in their extensive use. DNA barcoding, Because DNA is more stable and is found in all tissues, uses specific genes to find conserved sequences in the divergent plant species to produce an adequate reference genome library for identification, diversity and phylogenetic analyses. The DNA regions (*rbcL*, *matK*, *trnH*, ITS and ITS2) were universally investigated for identification and to discriminate many species of *Euphorbia* (Barres *et al*., 2011; Yang *et al*., 2012; Aubriot *et al*., 2013; Al-Hemaid *et al*., 2015; Moustafa *et al*., 2016). Horn *et al*., (2012) developed a universal phylogeny for *Euphorbia* and the other closely related genera in tribe Euphorbieae depending on the data of DNA sequence resulted from 10 molecular markers.

Based on DNA data, the previous studies suggested that the evolution of *Euphorbia* features as photosynthetic processes, growth and cyathial style were greatly homoplasious and this made *Euphorbia* genus possesses a complex biogeographic history that caused its distribution in almost all over the world. This highest distribution in the semi-arid, arid, tropical, and Mediterranean regions, associated with variable morphological patterns made it a typical model for the evolution and adaptation study of plant species to various environments (Park & Jansen, 2007; Bruyns *et al*., 2011).

Therefore our objectives are: (1) the amplification and the sequencing of ITS, ITS2, *matK*, *rbcL* and *trnH* sequences in three *Euphorbia* species, (2) comparing the genomic analyses among species to study these sequences in detail and (3) exploring the relationships between these species and others retrieved from Genbank. These objects will be useful in documenting and distinguishing the three species of *Euphorbia* and examining whether these species are endemic to Saudi Arabia or imported from abroad.

Materials and Methods

Plant materials: *E. peplus, E. hirta* and *E. tirucalli* (family Euphorbiaceae) were gathered from Taif highlands (Fig. 1), KSA. Identification of the species was done according to Chaudhary (1999).

Extraction of DNA and amplification process: The extraction of DNA from leaves of *Euphorbia* species was performed using CTAB method (Doyle & Doyle, 1987). Universal primers of ITS, ITS2, *matK*, *rbcL* and *trnH* were examined for the amplification process. They are mentioned in (Table 1).

Fig. 1. Photos of (A) *E. peplus*, (B) *E. hirta* (C) *E. tirucalli.*

Table 1. List of the investigated DNA barcoding primers.

locus	Primer name		Primer sequences (5'–3')
ITS	AB101	F	ACGAATTCATGGTCCGGTGAAGTGTTCG
	AB102	R	TAGAATTCCCCGGTTCGCTCGCCGTTAC
ITS2	ITS-S2F	F	ATGCGATACTTGGTGTGAAT
	ITS4	R	TCCTCCGCTTATTGATATGC
rbcL	rbcla	F	ATGTCACCACAAACAGAGACTAAAGC
	rbcla	R	GTAAAATCAAGTCCACCRCG
matK	matk-KIM1	F	ACCCAGTCCATCTGGAAATCTTGGTTC
	matk-KIM3	R	CGTACAGTACTTTTGTGTTTACGAG
trnH	psbAF	F	CGCGCATGGTGGATTCACAATCC
	trnH2	R	GTTATGCATGAACGTAATGCTC

Table 2. Accessions numbers of *Euphorbia* species sequences in GenBank.

Species	ITS	*matK*	ITS2	*rbcL rbcL*	*trnH*
E. hirta	LC434635	LC434636	LC434637	LC434638	LC434639
E. peplus	LC435036	LC435037	LC435038	LC435039	LC435040
E. tirucalli	LC435041	LC435042	-	-	-

The PCR sequencing: The PCR products of the five DNA barcodes of *Euphorbia* species were purified and then sequenced at Macrogen Inc., Republic of South Korea. The 12 sequences of *Euphorbia* plants were registered in GenBank. Their accessions numbers are mentioned in (Table 2).

The alignment of sequences and evolutionary relationships of species: ITS, ITS2, *matK*, *rbcL* and *trnH* sequences of *E. peplus, E. hirta* and *E. tirucalli* were subjected to BLAST of the GenBank to emphasize them from the other related accessions present in its database. By MEGA X software (Kumar *et al*., 2018), sequence alignments were achieved using MUSCLE algorithm. The evolutionary rate parameters (Tajima, 1993) among sequences of *E. peplus, E. hirta* and *E. tirucalli* were estimated through Tajima's test. The evolutionary history (Saitou & Nei, 1987) was concluded based on the Neighbor-Joining method. The best tree with the sum of branch length = 1.01600844 was revealed. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to conclude the phylogenetic tree. The evolutionary distances were estimated using the Maximum Composite Likelihood method (Tamura *et al*., 2004). This analysis contained 38 nucleotide sequences. The obscure positions for each sequence pair have been eliminated (pairwise deletion option). Finally, there were a total of 996 positions in the dataset. All analyses of evolution were performed using MEGA X software.

Results and Discussion

Plant invasions, occurred due to international trade and tourism, were thought to be one of the most main environmental matters especially in developing countries (Valladares-Padua, 2006). Characteristics of habitat as climate, domestic biodiversity and human activities, as well as the plants utilities, may lead to successful invasions and invasion patterns (Van der Wal *et al*., 2008). It was found that most of successful invasive species were introduced purposely as ornamental plants, medicine, or for forage and other targets (Mack, 2003). Tourism, domestic and international transportation and cross-border shipments of goods have been considered to be important carriers responsible for introducing and exchanging species (McNeely, 2000). A naturalized plant is known as an introduced (exotic, non-native) species, that can invariably reproduce and retain populations over many generations without direct human interference (Richardson *et al*., 2000). Creating a molecular database for the naturalized species in Taif, such as *Euphorbia* species, should be the first step towards an understanding and adequate knowledge of invasive plants.

Table 3. Statistics resulted from processes of sequencing, alignment and blasting.

Parameter	Species	ITS	*matK*	ITS2	*rbcL*	*trnH*
Sequence length	*E. hirta*	776	832	279	514	667
	E. peplus	606	590	284	523	363
	E. tirucalli	730	821	-	-	-
GC ratio	*E. hirta*	56.2	31.9	59.9	44.6	23.5
	E. peplus	61.1	31.2	70.4	45.7	22.6
	E. tirucalli	58.5	30.8	-	-	-
Number of the retrieved species	*E. hirta*	6	2	13	11	5
	E. peplus	9	8	8	13	3
	E. tirucalli	17	5	-	-	-
Variable sites	Among the three species	228	329	-	-	-
	between *hirta* and *peplus*	-	-	65	15	76

Species authentication and genetic variability: Extraction of DNA was performed successfully for all samples giving high quality of DNA and good yield. *E. peplus, E. hirta* and *E. tirucalli* were identified depending on the five tested loci by following approaches, similarity-based BLASTn and maximum likelihood bootstrap trees. Overall statistics of the five sites sequenced are summarized in Table 3.

Depending upon BLASTn approach (Table 3), the highest record of species was retrieved from the Genbank by ITS (6, 9 and 17), followed by *matK* (2, 8 and 5) for *E. hirta, E. peplus* and *E. tirucalli* respectively. When ITS2, *rbcL* and *trnH* of *E. hirta* and *E. peplus* were used as query sequences, ITS2 and *rbcL* retrieved the highest record of species, followed by *trnH* for *E. hirta* and *E. peplus.*

The highest sequence lengths (Table 3) were obtained by *matK* and ITS (832 & 776 bp) in *E. hirta*, followed by *E. tirucalli* and *E. peplus*. Variability was also detected in the variable sites for *matK* and ITS of them. GC ratio of the two loci were nearly identical in the three *Euphorbia* species. The sequence length of *trnH* was higher in *E. hirta* than that of *E. peplus*, whereas, ITS2 and *rbcL* sequence lengths and GC ratio were nearly identical in the two species (Table 3). The three loci detected variable sites that ranged from 76 to 15 between *E. hirta and E. peplus.*

Looking at Tajima relative evolutionary values (Table 4), *trnH* in *E. hirta*, ITS and *matK* in *E. peplus*, and *matK* in *E. tirucalli* revealed an acceleration in evolution ($p<0.05$), whereas, the rest of the genetic sites (ITS2 & *rbcL*) accept the null hypothesis of equal rates between lineages (no or very little evolution). In general, all barcodes in *E. hirta*, except *trnH* locus that displayed many differences between *E. hirta* from Taif and its related accessions studied from Asia, showed stability in the genetic makeup between *E. hirta* from Taif and accessions from other countries. In *E. peplus,* ITS and *matK* loci revealed considerable differences between sequence of *E. peplus* from Taif and those of the other countries, whereas, ITS locus in *E. tirucalli* showed stability in the genetic makeup between *E. tirucalli* (Taif) and accessions from other countries except India. Like in *E. peplus, matK* displayed numerous differences between *E. tirucalli* (Taif) and its related accessions studied from USA and France. These results were congruent with previous studies that *matK* is characterized by its rapid evolution (Fazekas *et al*., 2008; De Mattia *et al*., 2011).

By using the maximum likelihood tree method, the investigated species could be determined when it formed a monophyletic group with similar species. Based on the above, identification of our species was the highest at ITS for the three species (Fig. 2), while *rbcL* and ITS2 provided only clear resolution for *E. hirta* and *E. peplus* (Figs. 4 & 5).

Phylogenetic analysis based to ITS and matK sites: Although, PCR process was performed for all species, sequencing using ITS and *matK* loci was successful for the three species. To build the phylogenetic tree, sequences of barcode were compared with the publicly available DNA barcodes in GenBank to imitate a taxonomic assessment of the obtained molecular data. The phylogenetic tree of ITS (Fig. 2) demonstrated a monophyletic cluster for each species under study. Each of *Euphorbia* species was combined with its related accessions. Also, accessions from each country often met together demonstrating the taxonomic power of ITS. On the other hand, the *matK* tree (Fig. 3) revealed 4 clusters. Only, *E. hirta* (Taif) was combined with its related accessions from other countries. Whereas, *E. tirucalli* and *E. peplus* from Taif did not diverge and gathered in one cluster. This was due to the evolution occurred in sequences of *matK* of the two species and this was confirmed by the previous results of variable sites and Tajima relative evolutionary values. Despite its failure to distinguish between *E. tirucalli* and *E. peplus* collected from Taif, *matK* in addition to ITS still had a good discriminatory power as plant barcodes at low taxonomic levels, so they are recommended as desired options to reveal sequence variability for their suitable sequence length and clear interspecific divergence in many plant species (Stoeckle, 2003; Kress *et al.*, 2005; China Plant BOL Group, 2011).

Phylogenetic analysis based to *ITS2, rbcl and trnH* sites: Sequencing by ITS2, *rbcL* and *trnH* loci succeeded for only two species; *E. hirta* and *E. peplus*. A remarkable discrimination was achieved by ITS2 and *rbcL* phylogenetic trees than *trnH*. Trees of ITS2 and *rbcL* produced two distinct sets, one set for *E. hirta* and the other for *E. peplus* (Figs. 4 & 5). They also combined accessions from each country together demonstrating the taxonomic strength of them. Stoeckle (2003) mentioned that the universal barcode should have universality, short sequence and unique identifiers, thus we thought that the ITS2 and *rbcL* regions had suitable sequencing efficiency, good capacity for species identification and high discrimination between species belonging to genus *Euphorbia*. This conclusion harmonized well with those of Chiou *et al*., (2007), Song *et al*., (2012), Gu *et al*., (2013) and Maloukh *et al*., (2017).

Table 4. Tajima tests of barcodes of *E. hirta, E. peplus* and *E. tirucalli*.

Species	Locus	Outgroup	Test group		RI	RD	RA	RB	χ2	*P* value
			(A)	(B)						
E. hirta	ITS	India	Taif	China1	683	0	0	0	0.00	>0.05
		China1	Taif	India	683	0	0	6	6.00	<0.05
	ITS2	India	Taif	China1	256	0	0	0	0.00	>0.05
		China1	Taif	India	256	0	0	8	8.00	<0.05
		USA	Taif	UK1	272	0	1	0	1.00	>0.05
		UK1	Taif	USA	272	0	1	5	2.67	>0.05
	matK	China	Taif	Philippines1	832	0	0	0	0.00	>0.05
		Philippines1	Taif	China	832	0	0	0	0.00	>0.05
	rbcL	China1	Taif	Philippines1	312	0	0	0	0.00	>0.05
		Philippines1	Taif	China1	312	0	0	189	189	<0.05
		UAE	Taif	India1	511	0	0	0	0.00	>0.05
		India1	Taif	UAE	511	0	0	3	3.00	>0.05
		USA	Taif	Canada1	504	0	0	0	0.00	>0.05
		Canada1	Taif	USA	504	0	0	0	0.00	>0.05
	trnH	Philippines	Taif	China	317	1	247	0	247.0	<0.05
		China	Taif	Philippines	317	1	247	1	244.0	<0.05
		India1	Taif	Philippines	316	3	243	2	237.0	<0.05
		Philippines	Taif	India1	316	3	243	1	240	<0.05
E. peplus	ITS	KSA	Taif	China	374	7	80	11	52.32	<0.05
		China	Taif	KSA	374	7	80	24	30.15	<0.05
		Austria	Taif	Spain	401	0	99	1	96.04	<0.05
		Spain	Taif	Austria	401	0	99	1	96.04	<0.05
		USA1	Taif	USA5	332	0	49	0	49.00	<0.05
		USA5	Taif	USA1	332	0	48	0	49.00	<0.05
	ITS2	Spain	Taif	Austria	238	0	2	0	2.00	>0.05
		Austria	Taif	Spain	238	0	2	1	0.33	>0.05
		Canada	Taif	USA	212	1	1	1	0.00	>0.05
		USA	Taif	Canada	212	1	1	17	14.22	<0.05
	matK	Egypt	Taif	China	337	0	190	0	190.0	<0.05
		China	Taif	Egypt	337	0	190	0	190.0	<0.05
		UK1	Taif	Portugal	308	0	168	0	168.0	<0.05
		Portugal	Taif	UK1	308	0	168	0	168.0	<0.05
		Italy	Taif	Canada	242	0	130	0	130.0	<0.05
		Canada	Taif	Italy	242	0	130	1	127.0	<0.05
	rbcL	Egypt	Taif	China	520	0	0	0	0.00	>0.05
		China	Taif	Egypt	520	0	0	0	0.00	>0.05
		UK1	Taif	Italy1	514	0	0	0	0.00	>0.05
		Italy1	Taif	UK1	514	0	0	4	4.00	<0.05
		Canada1	Taif	USA	512	0	0	2	2.00	>0.05
		USA	Taif	Canada1	512	0	0	0	0.00	>0.05
	trnH	USA	Taif	Italy	134	2	2	93	87.17	<0.05
		Italy	Taif	USA	134	2	2	2	0.00	>0.05
E. tirucalli	ITS	France1	Taif	USA1	645	0	0	0	0.00	>0.05
		USA1	Taif	France1	645	0	0	1	1.00	>0.05
		India	Taif	S. Africa	609	0	1	2	0.33	>0.05
		S. Africa	Taif	India	609	0	1	9	6.40	<0.05
	matK	France1	Taif	USA1	406	1	267	1	264.0	<0.05
		USA1	Taif	France1	406	1	267	0	267.0	<0.05

RI: Identical sites within the three sequences
RD: Divergent sites within the three sequences
RA: Unique differences in the sequence A
RB: Unique differences in the sequence B
χ2 greater than 3.841 (P<0.05) : indicates acceleration in evolution
(P>0.05): No or not recognized evolution

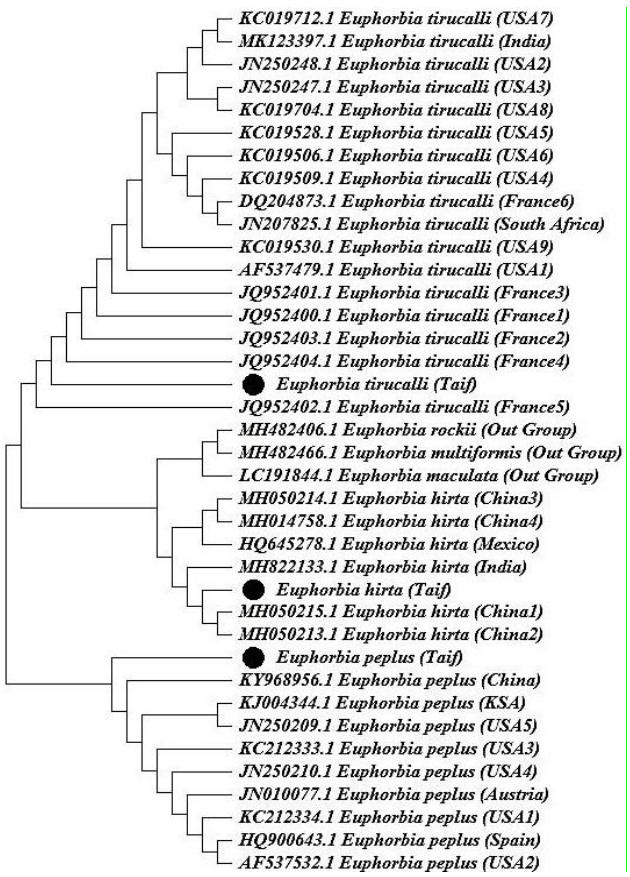

Fig. 2. Phylogeny tree of *E. hirta, E. peplus* and *E. tirucalli* based on ITS locus.

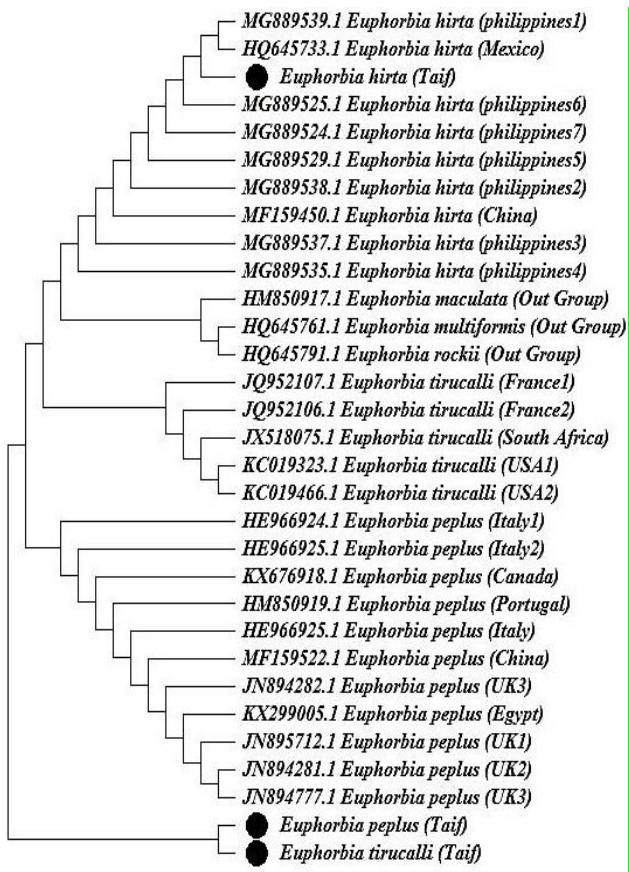

Fig. 3. Phylogeny tree of *E. hirta, E. peplus* and *E. tirucalli* based on *matK* locus.

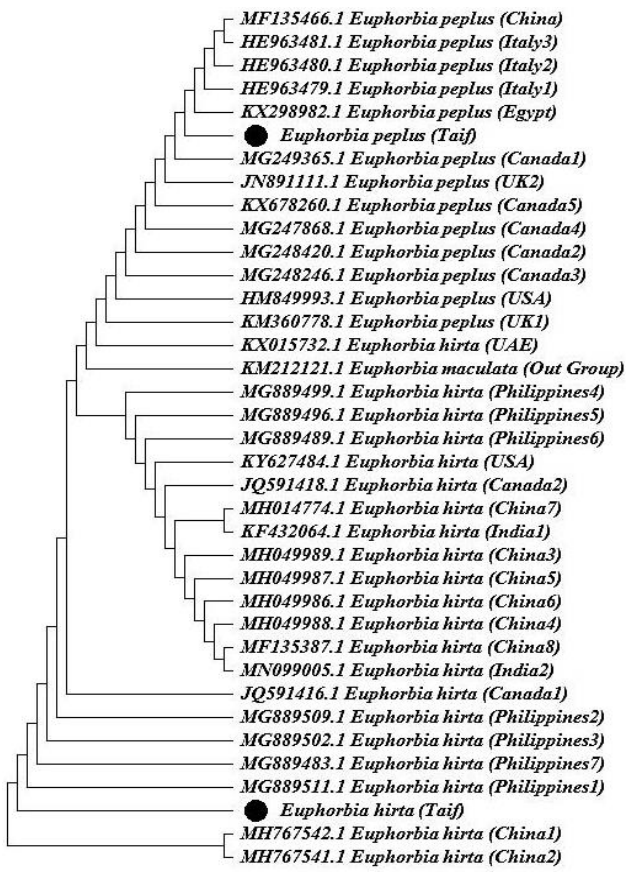

Fig. 4. Phylogeny tree of *E. hirta* and *E. peplus* based on *rbcL* locus.

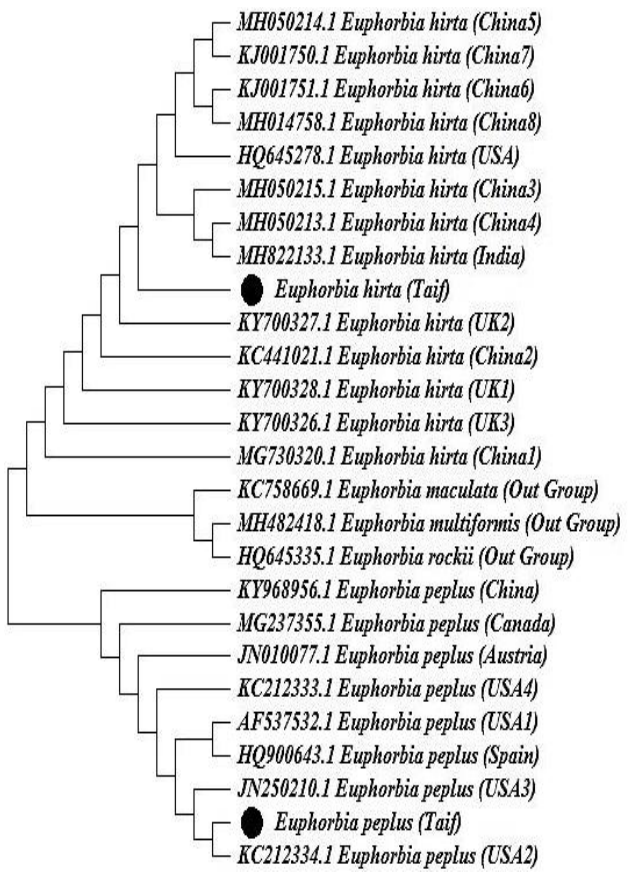

Fig. 5. Phylogeny trees of *E. hirta* and *E. peplus* based on ITS2 locus.

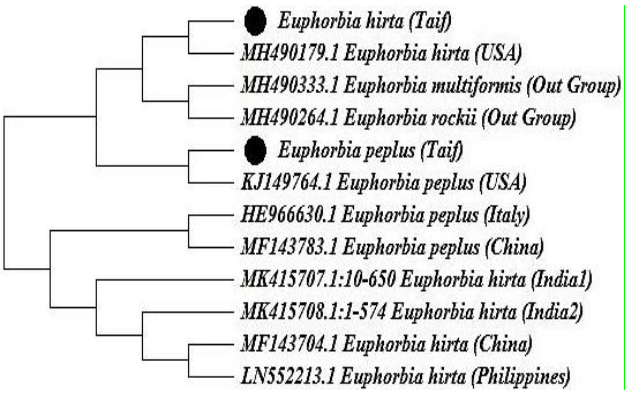

Fig. 6. Phylogeny tree of *E. hirta* and *E. peplus* based on *trnH* locus.

In contrast, tree of *trnH* could not discriminate between *E. hirta and E. peplus* completely (Fig. 6). Although this marker was accepted as barcode in some conditions for its discriminatory power (Ren *et al*., 2010; Pang *et al*., 2012), it provided little discrimination than the other plastid locus *rbcL* and this was in accordance with BOL Plant Working Group (2009) and Tripathi *et al*., (2013). On the basis of the results, it could be concluded that *rbcL* was the most universal and the easiest to amplify and showed a high efficacy in the discrimination of *E. hirta and E. peplus*. However, *trnH* was the most polymorphic and, therefore, may be suitable for the discrimination between other closely related species (Newmaster *et al*., 2008).

The genetic differences observed depending on the five barcode loci reflected the high efficacy of DNA barcoding approach in distinguishing between *Euphorbia* species. Whereas, the phylogenetic trees did not show any variability between the three *Euphorbia* species and the other retrieved GenBank species rejecting their endemism to flora of Saudi Arabia. This was compatible with Pahlevani (2017) who reported that *E. hirta and E. peplus* from section *Anisophyllum* belonging to subgenus *Chamaesyce* were no endemic species in southwestern Asia. The highest number of *Euphorbia* endemics were scored in Iran, Turkey, Yemen and Afghanistan, respectively. As a result of these observations, we can consider *E. peplus, E. hirta* and *E. tirucalli* as naturalized species that have previously entered through their original regions and now they occur in the natural areas of Saudi Arabia, threaten or try to replace the existence of the native floral elements. Mesic environment of western Saudi Arabia sheltered most of native species; however, the high biodiversity did not resist plant invasions in this area.

Conclusion

The DNA barcoding data were successful in validating and differentiating the *Euphorbia* species. Of the three plastid sites, *rbcL* displayed the highest level of universality in *Euphorbia* species under study, and *matK* and *trnH* performed lower. *matK* region has a high capability to show evolution in plant species. ITS and ITS2 as nuclear sites were recommended to reveal genetic variability, because they had a suitable sequence length and clear interspecific divergence within *Euphorbia* genome. *Euphorbia* species from Taif were in general similar to others from several countries retrieved from the GenBank. The three species could not be considered as endemic species in Kingdom of Saudi Arabia.

Acknowledgement

The work was funded by Taif University Researchers Supporting Project number (TURSP - 2020/141), Taif University, Taif, Saudi Arabia.

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(Received for publication 11 September 2020)