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IDENTIFICATION AND PHYLOGENETIC STUDY OF *ARABIS ALPINA* L. FROM THE KINGDOM OF SAUDI ARABIA

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Abstract

The purpose of the current study was to determine the phylogenetic relationships between the *Arabis alpina* L. growing naturally in Afro-Alpina Mountains at south western regions of the Kingdom of Saudi Arabia (KSA) and its closely related species. A case study approach was applied to DNA barcode, secondary internal transcribed spacer (*ITS*2), chloroplast maturase-K (*mat*K), ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) for the identification and determination phylogenetic relationship. An analysis of barcode data was conducted using the basic local alignment search tool (BLAST), pairwise genetic distances and the maximum likelihood (ML) methods. The results showed the clear superiority of the nuclear *ITS*2 loci and chloroplast *mat*K gene with a 100% success rate found for DNA amplification, sequencing, and 100% species resolution. A maximum likelihood ML tree of *ITS*2 and *mat*K strongly supported the presence of variations between *A. alpina* of Saudi Arabia and specimens of *A. alpina* of different geographical origins. This study represented the first inspection to *A. alpina* in KSA, and is useful in species identification, conservation and evolutionary studies. More studies are needed to verify if the A. Alpina of the KSA can be considered a subspecies.

Key words: DNA, Barcode, *ITS*2, *mat*K, *rbc*L, Taxonomy.

Introduction

The Brassicaceae family includes 51 tribes, 321 genera, and roughly 4000 species, (Al-Shehbaz, 2012; Al-Shehbaz *et al.,* 2014; Nikolov *et al.,* 2019). The family has been a controversial and widely disputed subject within the field of taxonomy due to the large number of species belonging to it in contrast to the limited number of diagnostic features accepted, which has led to conflicts between studies of this family (Koch *et al.,* 2010). Recent developments in the field of DNA sequencing techniques have led to a renewed interest in understanding the ambiguity of Brassicaceae relationships.

DNA barcoding is a novel technique for identification and characterization of new plant species (Jamil *et al.,* 2014; Shinwari *et al.,* 2014; Khan *et al.,* 2015; Zahra *et al.,* 2016; Channa *et al.,* 2018; Shinwari *et al.,* 2018). Phylogenetic studies have contributed to the introduction of many acceptable interpretations and to the drawing of phylogenetic relations within the Brassicaceae family and between tribes and genera. Such studies have been applied using different chloroplast genes (Chase *et al.,* 1993; Steele & Vilgalys, 1994; Abdel-Khalik, 2002), and nuclear region (Heenan *et al.,* 2002: Warwick *et al.,* 2008). Tribal limits and relationships among and between tribes have been studied using chloroplast region *ndh*F (Beilstein *et al.,* 2006), *mat*K (Koch *et al.,* 2001) and the ITS region (Warwick *et al.,* 2008). The most recent phylogenetic study of Brassicaceae (Nikolov *et al.,* 2019) was based on sequence data of 1827 exons representing 63 species and 50 of the 52 recognized Brassicaceae tribes, using large set of herbarium material. Nonetheless, there remained doubts regarding the taxonomic status certain genera, which was not fully resolved.

Arabis is an important taxonomically complex genus in Brassicaceae with 70 species (Jordon-Thaden *et al.,* 2010) and (Al-Shehbaz *et al.,* 2005). It is characterized by the existence of branched trichomes, latiseptate siliques flattened parallel to the septum (German & Al-Shehbaz, 2008). *Arabis alpina* grows naturally in montane and alpine habitats and is widely distributed in Anatolia and adjacent regions (Ansell *et al.,* 2011). Assefa *et al.,* (2007) suggested the migration of *A. alpina* from the Middle East to the Arabian Peninsula, and then to eastern Africa via the mountains bordering the Red Sea.

Nuclear ribosomal *RNA* and chloroplast genes *trn*L-*trn*F have been tested to detect relationships of *Arabis* species of North America and eastern regions (Koch *et al.,* 2010). Koch & Grosser (2017) examined Phylogentic analysis of East Asian Arabis species, using chloroplast gene trnL-F and nuclear loci ITS1 and ITS2. Tracing of Ancestors was difficult to detect, because related species were either extinct or their relatives were distributed in Europe and Asia Minor. Furthermore, in a study complete chloroplast genomes of East Asian *Arabis* species were analyzed, it was inferred that multiple hybridization, and that the two organelles genomes were co-transferred (Kawabe *et al.,* 2018).

The adaption changes experienced by *Arabis alpina* species over the past decade in relation of the environment remain poorly understood. *Arabis alpina* serves as a good model for studying the impacts of harsh climatic conditions related speciation, genetic differentiation, and long-term adaptations to different geographical conditions (Karl *et al.,* 2012).

No phylogenetic molecular studies were conducted on *Arabis alpina* of the KSA, despite the many similarities, derived species, hybrids and taxonomic conflicts observed in the genus *Arabis*. The purpose of this study is to examine the relationship between *Arabis alpina* native to the southwestern highlands of the KSA and its closest relatives by measuring phylogenetic features using a molecular approach based on DNA barcodes *ITS*2, *rbc*L, and *mat*K.

Materials and Methods

Study area: Samples of *Arabis alpina* L. were collected from the "Reda Nature Reserve" located in the mountains of Sarawat roughly 20 km northwest of the city of "Abha". The Jarf Reda Reserve is located in the Afro-Alpina vegetation zone in the southwestern highlands of Saudi Arabia positioned up to 2500-3000 m above sea level. The area is characterized by slopes covered with dense vegetation (Authority of Saudi Wildlife, 2019).

Plant material: Samples were collected from the Reda Nature Reserve in 2015. Six specimens of fresh leaves (Table 1) were prepared for molecular experiments by drying the leaves in silica gel. Voucher specimens of the rest of the samples were prepared and stored in the herbarium of the Biology Department, Faculty of science, Umm Al-Qura University, Makkah, Saudi Arabia (UQU, proposed abbreviation). The plants were identified by experts in taxonomy based on their morphologies in reference to different sources (Collenette, 1999; Chaudhary, 2000, 2001; Thomas, 2017).

Table 1. Genbank accession numbers of *Arabis alpina* samples of KSA.

Samples Code	Gene Bank Accession No.		
	*ITS*2	*rbc*L	*mat*K
AP22A	MN227681	MN258858	MN276182
AP22B	MN227682	MN258859	MN276183
AP23A	MN227683	MN258860	MN276184
AP23B	MN227684	MN258861	MN276185
AP24A	MN227685	MN258862	MN276186
AP24B	MN227686	MN258863	MN276187

DNA extraction: DNA was isolated from the leaves using the DNeasy Plant Mini Kit extraction method (Qiagen/USA) following commercial protocols. The quantity of the extracted DNA was estimated using a NanoDrop (Thermo Scientific, Wilmington, USA). DNA was tested via 1% agarose gel electrophoresis and was imaged under ultraviolet light; the DNA samples were then stored at -20°C.

Primer sequences: Chloroplast *rbc*L and *mat*K regions were amplified with barcode primers (CBOL, 2009). The *ITS*2 region was amplified using the primer designed by (Chen *et al.,* 2010) (Table 2).

DNA amplification and sequencing: DNA regions were amplified according to the method described by (Maloukh *et al.,* 2017) using 25 µL reaction volumes containing 12.5 µL of Master Mix, 8.5 µL of Nano-pure water, 1 µL of each primer, and 2 µL of the DNA. The thermal cycle included an initial denaturation level of 94°C at 5 min, and then at 94°C until 45 s. Annulling temperatures dependent on the type of primers as shown in (Table 2) were applied for 45 s.Then 72°C applied at 1.5 min, and a final extension step held at 72°C for 10 min with 40 cycles, using Master cycler (Eppendorf Vapo Protect, NY, USA). The quality of PCR products was confirmed by electrophoresis on 1.5% agarose gel and purified with a 1.0% agarose gel using the QIAquick purification Kit (Qiagen, USA). Sequencing was performed using the Genetic Analyzer (Applied Biosystem, CA, USA).

Bioinformatic analysis: Sequences were assembled in (DNA Baser v. 4.16.0, 2013). The *ITS*2 locus was identified using a hidden Markov model to exclude5.8 S and 28 S sections of the *ITS*2 region (Keller *et al.,* 2009). Multiple sequence alignment was performed for each gene using the Muscle algorithm window in MEGA 7.0.27 (Kumar *et al.,* 2016). Then, sequences were manually adjusted by removing ambiguous regions.

The species resolution was evaluated for the three DNA loci (*ITS*2, *mat*K and *rbc*L) using the basic local alignment search tool against known specimens available through GenBank (BLAST). Query sequences were detected by selecting the highest maximum score and the lowest E-value (Gao *et al.,* 2011). Identification was performed at the family, genus and species levels following the methods described in (Meier *et al.,* 2006: Elansary *et al.,* 2017).

Pairwise genetic distances were calculated for the *ITS*2, *mat*K and *rbc*L loci at two levels: between *A. alpina* of the KSA and *A. alpina* of other countries (within species level) and between *A. alpina* of the KSA and the other *Arabis* species (between species level). This was performed using MEGA 7.0.27 software (Kumar *et al.,* 2016).

Available sequences of *Arabis* spp., for loci used in this study were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/). These were computed together with the sequences of *A. alpina* of Saudi Arabia, for the construction of the phylogeny trees. The evolutionary history was inferred by using the ML method in MEGA 7.0.27 software (Kumar *et al.,* 2016) by running 1,000 bootstrap replicates, using the Gamma distributed invariant site (G+1) nucleotide model. Number of discrete Gamma categories 5. Kimura–parameter model selection, and codon positions included were 1st+2nd+3rd+Noncoding. The tree of each gene was evaluated, based on the bootstrap supporting value or on the capacity for samples of one species to form a monophyletic group.

Table 2. Primer sequences used for the amplification DNA barcodes.

Region	Primer sequences F/R	Annealing temperatures °C
*ITS*2	*ITS*2-2F: 5′-ATGCGATACTTGGTGTGAAT-3′ *ITS*2-3R: 5′-GACGCTTCTCCAGACTACAAT-3′	56
*mat*K	*mat*K-KIM3F: 5'-CGTACAGTACTTTTGTGTTTACGAG3' *mat*K-KIM1R: 5' ACCCAGTCCATCTGGAAATCTTGGTTC-3'	55
*rbc*L	*rbc*La-F: 5'-ATGTCACCACAAACAGAGACTAAAGC-3' r*bc*La-R: 5'-GTAAAATCAAGTCCACCRCG-3'	

Table 3. Evaluation of three DNA barcoding regions used in the current study of *Arabis alpina* samples.

Variable	DNA region		
	ITS2	***mat*K**	***rbc*L**
Number of samples	6	6	6
Mean and range of GC content (%)	57.6(57-58.3)	31.6(31.2-31.9)	43.2 (42.8–43.8)
Efficiency of PCR amplification (%)	6(100%)	6(100%)	6(100%)
Sequencing success rate (%)	6(100%)	6(100%)	6(100%)
Amplified product length (bp)	~200	~800	~ 600
Mean and range of the sequenced length (bp)	250.2(240-260)	824.8(820-829)	646.3(652-637)

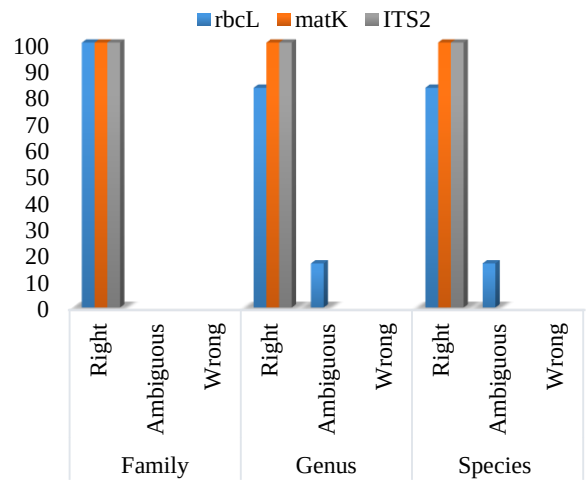

Fig. 1. Successful identification of *A. alpina* specimens of the KSA at three levels (family, genus and species) using blast and matching sequences drawn from the NBCI database. Values listed in the columns are estimated in percentages (%).

Results

This was designed to compare differences between *A. alpina* growing in the southwestern area of the Southwest Highlands of the KSA and its closely allied species.

PCR and sequencing: Genomic DNA isolation proved successful for all of the collected samples. The results obtained from the preliminary analysis of PCR amplifications of plant samples (6 specimens × 3 loci), which yielded a value of 100% for *rbc*L, *mat*K, and *ITS*2, are shown in Table 3. All specimens (100%) of them were successfully sequenced in the case of *rbc*L, *mat*K, and *ITS*2 loci. Sequence lengths ranged from 240 to 260 bp, 820 to 829 bp and 637 to 652 bp with means of 250, 824 and 646 bp for *ITS*2, *mat*K and *rbc*L, respectively. The GC % value ranged from 57 to 58.3 (average was 57.6) in *ITS*2 while in *mat*K, it ranged from 31.2 to 31.9 (average was 31.6). In *rbcL*, the value ranged from 42.8 to 43.8 with an average of 43.2.

Species resolution and barcode analysis: Figure 1 presents an overview of the specimen identification study based on the Basic Local Alignment Tool (BLAST) in NCBI. *ITS*2 and *mat*K sequences were correctly identified at 100% at the three levels. Regarding incorrect sequences, identification was 0% at the family, genus and species levels (Fig. 1). E-Values were valued at (0) for three genes.

The results as shown in Fig. 1 indicated that the *rbc*L successfully assigned values at the family level (100%) but less so at the lower levels (83%) at genus and species levels. Ambiguous detection was observed at the genus and species levels (16.7%).

Pairwise genetic distances: The data shown in Table 4 illustrate that the *ITS*2 locus presents the greatest differences in genetic distances between the *Arabis* species and among the *A. alpina* samples. On the other hand, the least significant genetic distances were found between species of *Arabis* in the *rbc*L locus.

The greatest genetic distance was observed for locus *ITS*2 between *A. alpina* of the KSA (0.127) and other species (*A. verna* from Italy), while the least significant differences were found between *A. alpina* of the KSA and other species (*A. ionocalyx* from Turkey, *A. montbretiana* from Afghanistan, *A. nepetifolia* from Iran, *A. aubrietioides* from Turkey, *A. deflexa* from Turkey, and *A. montbretiana* from Tajikistan). European samples? of *A. alpine* were found to be more similar with distance (0.004), and *A. alpina* of Saudi Arabia was found to be the least similar to *A. alpina* of Germany (0.013).

The genetic distance for *Arabis alpina* specimens of locus *mat*K remained unclear due to the lack of samples available for comparison. For the *Arabis alpina?* the least significant distance was found between *A. alpina* from Germany and *A. alpina* of Saudi Arabia. The greatest genetic distance was observed between *A. alpina* of the KSA and *A. lyliia* from Austria.

For locus *rbc*L, we found no genetic distance (GD= 0.000) among the *A. alpina* specimens. Genetic distances were not found to be significant between *A. alpina* samples of Saudi Arabia and other species of *Arabis* from Canada and Europe with GD values ranging from 0.006 to 0.008.

Phylogenetic tree: The phylogenetic trees shown in Figs. 2, 3, and 4 present relationships between *A.alpina* samples of KSA and its close relatives, based on data of chloroplast genes *mat*K , *rbc*L and of nrDNA *ITS*2 markers. As is shown in Figures 2, 3, and 4, the *Draba* species is a close relative of the *Arabis* species in both ML trees for loci *ITS*2, *mat*K and *rbc*L.

Interestingly, *A. alpina* specimens collected from Saudi Arabia were found to be clustered within same sub-branch (monophyletic) with a strong support node of up to 100% found in the phylogeny tree of the *mat*K and *ITS*2 genes. However, variation for *A. alpina* does not appear in the tree of the *rbc*L gene.

Figure 2 on the *ITS*2 locus presents the correlation between *A. alpina* and the nine species of *Arabis* (*A. aubrietoides, A. cypria, A. deflexa, A. ionocalyx, A. purpurea,* and *A. tianschanica, A. montbretiana, A. nepetifolia,* and *A. aubrietioides*), which are of the same sub-branch.

Table 4. Genetic divergence (GD) of *Arabis alpina* species based on pairwise genetic distances and three barcode loci using MEGA 7.0.27 software

Gene	Genetic distance levels	No. of Taxa	Minimum genetic distance (%)	Mean genetic distance (%)	Maximum genetic distance (%)	Mean standard deviation (%)
ITS2	Between *Arabis alpina* of the KSA and *Arabis alpina* of other countries	11	0.004	0.006	**0.013**	0.003
	Between *Arabi salpina* of the KSA and other species of *Arabis*	61	0.000	0.058	**0.164**	0.017
*mat*K	Between *Arabis alpina* of the KSA and *Arabis alpina* of other countries	7	0.000	0.002	**0.012**	0.002
	Between *Arabis alpina* of the KSA and other species of *Arabis*	13	0.012	0.056	**0.072**	0.007
*rbc*L	Between *Arabis alpina* of the KSA and *Arabis alpina* of other countries	12	0.000	0.000	**0.000**	0.000
	Between *Arabis alpina* of the KSA and other species of *Arabis*	23	0.006	0.004	**0.008**	0.004

Discussion

The main purpose of the current study is to identify and assess the phylogeny situation of *Arabis alpina* local to the southwestern region of the KSA from barcode DNA regions *ITS*2, *mat*K and *rbc*L.

We found the *rbc*L region showed high levels of PCR amplification (100%) and sequencing efficiency (100%); however, identification efficiency was measured at 83% at the genus and species levels. The *rbc*L locus also exhibited the least significant genetic distances among *Arabis* species, and no obvious differences were observed for A. *alpina*. Our results corroborate the findings numerous previous works (Elansary *Et al.,* 2017; El-Banhawy & Al-Juhani, 2019) noting the inadequacies of chloroplast gene *rbc*L for plant specimen identification. These results are likely to be related to the modality of inheritance of the plastid regions of plants.

Nuclear DNA region *ITS*2 and chloroplast *mat*K were more effective than counterpart gene *rbc*L. *ITS*2 and *mat*K exhibited the greatest ability to identify samples at the genus and species levels (100%). In addition, genetic distances within *A. alpina* and between *Arabis* species were the greatest in the *ITS*2 locus. These findings are consistent with the results of recent studies (Yu *et al.,* 2018; Al-Juhani, 2019) confirming the utility of *ITS*2 for species resolution and they support earlier observations made by (Hollingsworth, 2011) that *ITS*2 can be easily amplified with a high level of discrimination power. This study also demonstrates the capacity for *mat*K to resolve issues observed at different taxa levels, including at the species level, which is in agreement with other works (Steele & Vilgalys, 1994; Kron, 1997; Brochmann *et al.,* 1998) exhibiting the efficiency of the *mat*K gene. This may be the case because the chloroplast *mat*K gene evolved almost two to three times faster than *rbc*L as previous studies have demonstrated (Johnson and Soltis, 1994; Johnson & Soltis, 1995).

According to our results, there are no major differences between phylogenetic trees of nrDNA gene *ITS*2 and chloroplast barcode gene *mat*K. This also supports earlier observations showing no major in congruencies between results for cpDNA trnLF and nrDNA ITS and showing minor differences to likely be related to variations in modes of marker inheritance (Harris & Ngram, 1991; Karl *et al.,* 2012).

Results found for the phylogenetic tree nrDNA *ITS*2 locus support the presence of differences between *Arabis alpina* endemic of the southwestern highlands of Saudi Arabia; *Arabis alpina* specimens from Europe; and genera of *Arabis* from East Asia, Europe and Africa. Local *A. alpina* in the KSA form a monophyletic group with a high value of boots trapping.

Furthermore, the ML tree of *mat*K locus significantly supports the separation of *Arabis* of the KSA from the European *A. alpina and Arabs* species, which correspond with the phylogeny tree of the *ITS*2 gene. However, the great abundance of *ITS*2 sequences of *Arabis* genus available from the NCBI gene bank offer numerous means to draw comparisons.

Our results can be interpreted in several ways.Phylogeographic studies of *A. Alpin* using chloroplast DNA haplotypes (Koch *et al.,* 2006, 2017; Ansell *et al.,* 2011) have argued that *A. Alpina* originated from central Anatolia and underwent four different streams of migration:(1) migrations to central and northwestern Europe, (2) to Spain and North Africa, and (3) to East Asia, and (4) streams of migration toward the East African. However, other haplotype patterns dominate certain areas of the Mediterranean and overlap with main patterns observed for haplotypes dominating the Arabian Peninsula and the Caucasus/Iran (mountains of Lebanon), suggesting that these regions were independently colonized. It is likely that different patterns affected other regions (Ansell *et al.,* 2011).

Furthermore, high elevations of the southwestern regions of the KSA are characterized by fluctuating weather conditions in terms of temperature, sunlight, and oxygen ratios and may significantly affect the internal structures of plants, speciation, and polyploidization rates. This effect may be observed for the *Arabis* genus and for the closely related *Draba* species (Koch *et al.,* 2010). This study supports previous observations (Poncet *et al.,* 2010) used AFLP markers showing the existence of polymorphic loci related to variations in local environments of Alps populations.

The bootstrapped phylogenetic tree of the nrDNA *ITS*2 barcode presents nine species of *Arabis* that are very close and that overlap with *A. alpina*: *A. aubrietoides, A. cypria, A. deflexa, A. ionocalyx, A. purpurea,* in addition to *A. tianschanica, A. montbretiana, A. nepetifolia,* and *A. aubrietioides*. Similar observations was recorded by (Karl *et al.,* 2012) and these species are perennials distributed across the eastern Mediterranean, Anatolia and adjacent Levantine regions.

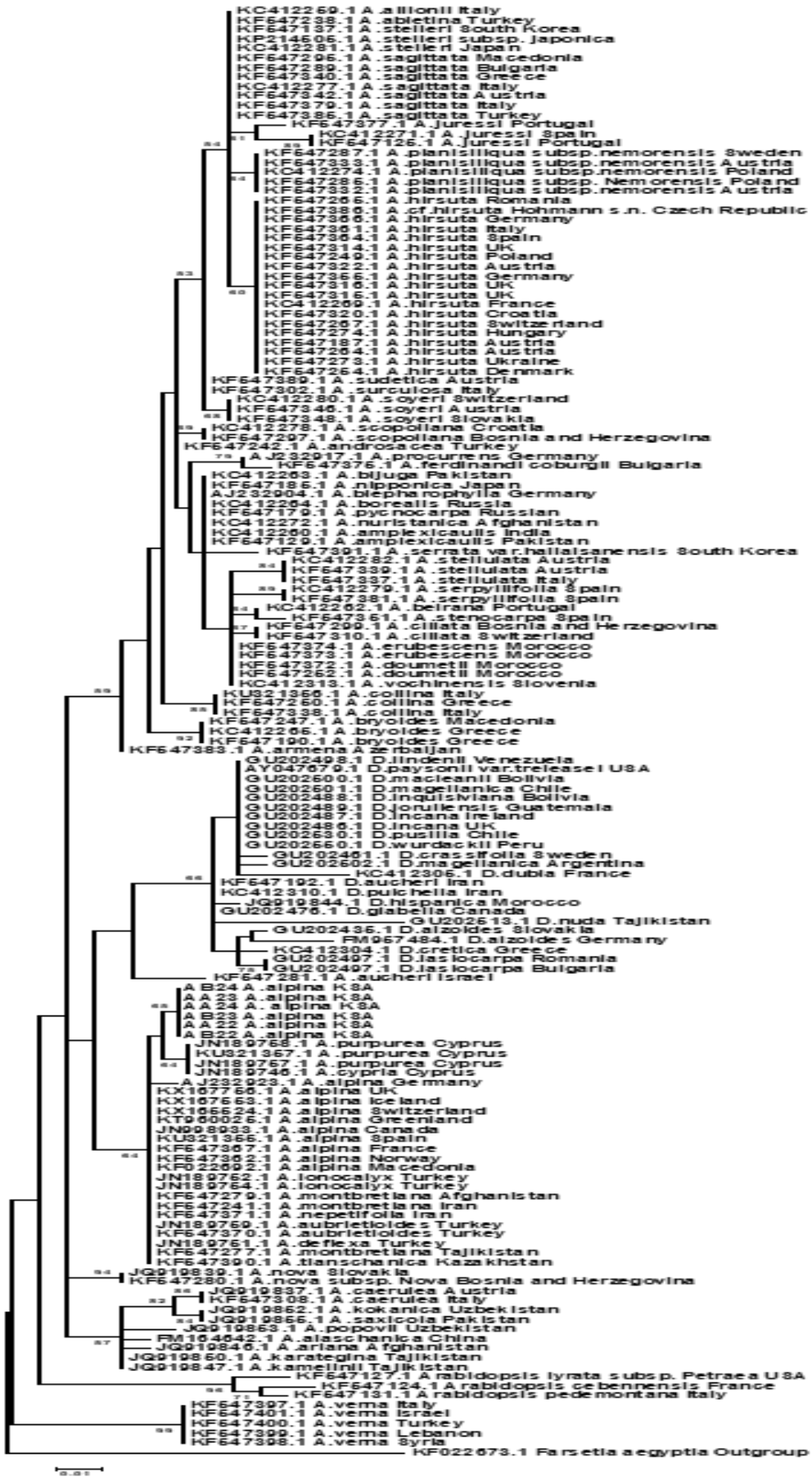

Fig. 2. Maximum Likelihood tree for *ITS*2 barcodes showing the relationship of *A. alpina* from KSA and of its closest relative species. Bootstrap 1000 replications. Less than 50% bootstrap value was hidden.

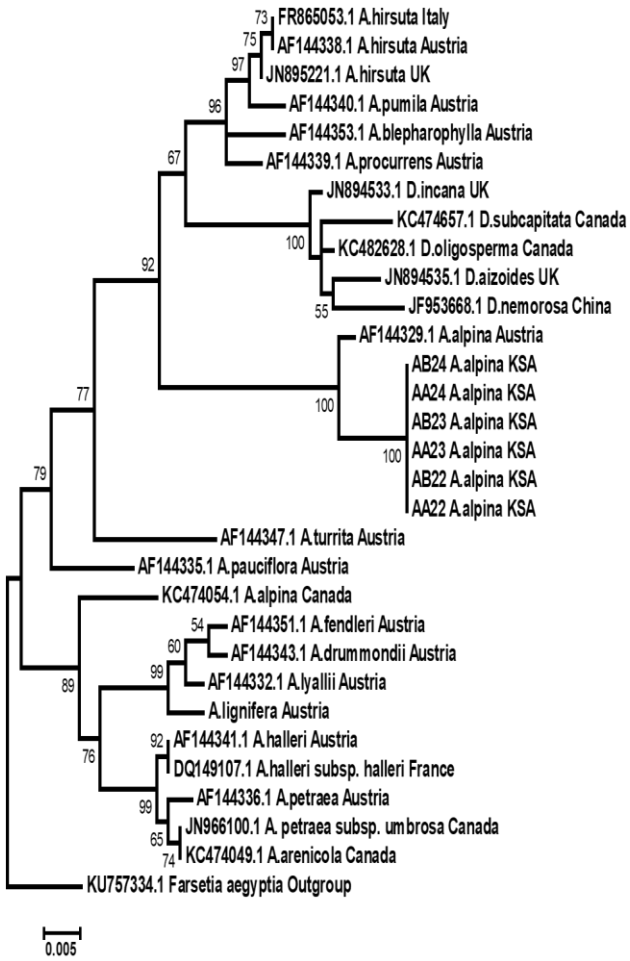

Fig. 3. Maximum Likelihood tree for *mat*K barcodes showing the relationship of *A. alpina* from KSA and of its closest relative species. Bootstrap 1000 replications. Less than 50% bootstrap value was hidden.

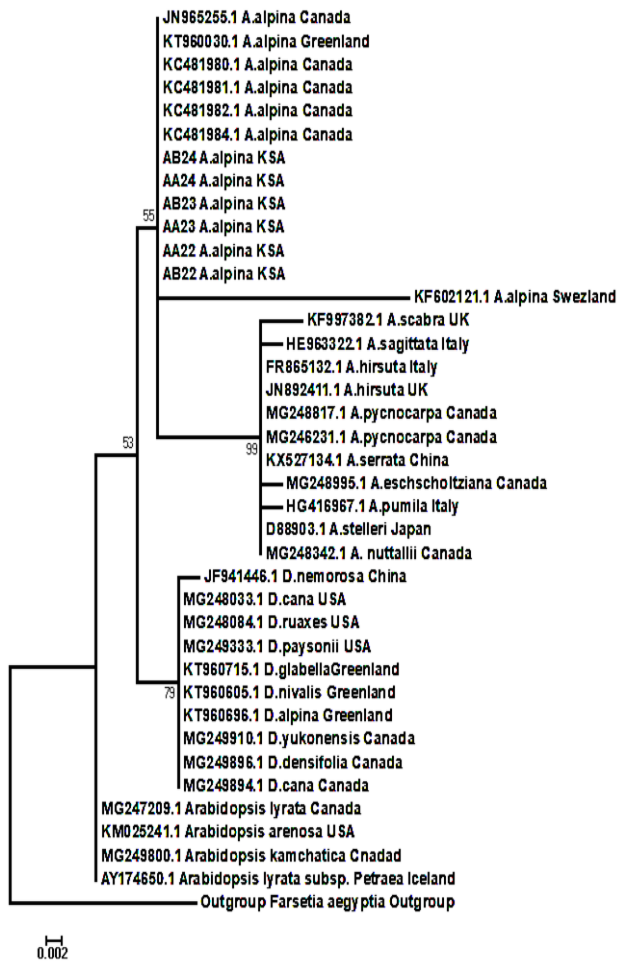

Fig. 4. Maximum Likelihood tree for *rbc*L barcodes showing the relationship of *A. alpina* from KSA and of its closest relative species. Bootstrap 1000 replications. Less than 50% bootstrap value was hidden.

Conclusions

This project was designed to evaluate the phylogenetic status of *A. alpina* growing in southwestern regions of the KSA and of its closest relative *Arabis*. The most obvious finding emerged from this study was the presence of clear variations between *Arabis* of the KSA and its closest relatives. The presence of highly supported phylogenetic trees created from nuclear *ITS*2 and chloroplast *mat*K genes also confirmed these findings.

Overall, this study supports the notion that DNA barcodes showing the existence of polymorphic loci could be related to variations in local environments of *A. alpina.* Considerably more work including morphology, is needed to determine whether *A. alpina* of the KSA can be considered as a subspecies.

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