Molecular phylogeny of *Acer monspessulanum* L. subspecies from Iran inferred using the ITS region of nuclear ribosomal DNA

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Abstract. *Khademi H, Mehregan I, Assadi M, Nejadsatari T, Zarre S. 2015. Molecular phylogeny of* Acer monspessulanum *L. subspecies from Iran inferred using the ITS region of nuclear ribosomal DNA. Biodiversitas 17: 16-23.* This study was carried out on the *Acer monspessulanum* complex growing wild in Iran. Internal transcribed spacer (ITS) sequences for 75 samples representing five different subspecies of *Acer monspessulanum* were analyzed. Beside this, 86 previously published ITS sequences from GenBank were used to test the monophyly of the complex worldwide. Phylogenetic analyses were conducted using Bayesian inference and maximum parsimony. The results indicate that most samples of *A. monspessulanum* species from Iran were part of a monophyletic clade with 8 samples of *A. ibericum* from Georgia, *A. hyrcanum* from Iran and one of *A. sempervirens* from Greece (PP= 1; BS= 79%). Our results indicate that use of morphological characteristics coupled with molecular data will be most effective.

Keywords: Biogeography, maple, phylogeny, Sapindaceae

INTRODUCTION

The genus *Acer* L. is a member of Sapindaceae that mainly grows in tropical and subtropical regions. This genus is one of the most diverse tree genera in the Northern Hemisphere with approximately 129 species and is the largest tree genus of the northern hemisphere besides *Quercus* (Fang 1966; Grimm et al. 2006). A number of characteristics are shared among all members of *Acer*. The arrangement of the two winged pericarps (samaras) in the fruits ranges from subparallel to diverging at about a right angle.

A number of characters that are beneficial for identification of species in the field may have evolved independently (lobe shape, margin of lobes). These traits may be highly variable within species like pubescence of lower leaf surface (Grimm et al. 2007). Several different florescence types, including racemes, panicles, corymbs and spikes, occur in this genus. These variations make infrageneric divisions very difficult. Species delimitation and phylogenic relationships within the genus *Acer* are also very controversial (Kholie 1967; Judd et al. 2002). Fang (1966) proposed a different system in which the genus was divided into two subgenera, mainly on the basis of simple versus compound leaves (Koidzumi 1911). In Ogata's system (Ogata 1967), the genus was classified into 26 sections (Momotani 1962). In 1970, Murray published his monograph of the Aceraceae with 7 subgenera, 24 sections and 35 series within *Acer* (Murray 1970). Ogata's system was essentially followed by Xu (1966), with some additions and amendments. More recently de Jong (1994) recognized only 19 series in 16 sections, providing a quite different arrangement from those of other authors (Pax

1902; Xu 1966, 1998; Ogata 1967; Xu et al. 2008). Some researchers discussed the infrageneric phylogenetic relationships in the genus by analyzing gross morphology, seed proteins, fossils and geographic distributions, but the conclusions were not in consensus (Momotani 1962; Rechinger 1969; Pax 1985, 1986; Wolfe and Yanai 1987; Thorne 1992).

Acer monspessulanum is a medium-sized deciduous tree or densely branched shrub that grows to a height of 10-15 m (rarely to 20 m) (Fontaine 2011).The trunk is up to 75 cm diameter, with smooth, dark grey bark on young trees, becoming finely fissured on old trees. Among similar maples is most easily distinguished by its small three-lobed leaves, 3-6 cm long and 3-7 cm wide, glossy dark green, sometimes a bit leathery, and with a smooth margin, with a 2-5 cm petiole. The leaves fall very late in autumn, typically in November. The flowers are produced in spring, in pendulous, yellow to white corymbs 2-3 cm long. The samaras are 2-3 cm long with rounded nutlets (Rushforth 1999; van Gelderen and van Gelderen 1999).

Acer monspessulanum from *Acer* section (van Gelderen et al. 1994) has distinct small, 3-lobed leaves, while its close relatives *A. hyrcanum* and *A. opalus* normally have 5-lobed leaves. *Acer ibericum* displays dimorphic leaves that are 5-lobed in juvenile plants and sucker shoots, and 3-lobed in older plants (Grimm et al. 2007). *Acer monspessulanum* fossils are fairly common in Late Miocene and Pliocene floras from southern Europe and south-western Asia (Kvacek et al. 2002; Sachse 2004).

The ITS is highly variable nuclear region suitable for phylogenetic reconstruction of closely related taxa. The utility of this marker has already been investigated in other

plant groups including trees, e.g. *Acer* (Tian et al. 2002; Grimm et al. 2006; Grimm et al. 2007) and *Crataegus* (Zarrei et al. 2014, 2015) and bulbs (Zarrei et al. 2009).

The aim of this study is to clarify taxonomy and to delimit *Acer monspessulanum* subspecies that grow in Iran using ITS marker and comparing results with morphological traits.

MATERIALS AND METHODS

Plant samples

The ITS sequencing was performed on 75 individuals from 15 populations of *Acer monspessulanum* distributed in Iran. Population name, localities, altitude, and herbarium number for each population are shown in Table 1. The plant specimens were identified in the Department of Biology, Science and Research Branch of Islamic Azad University in Tehran, by the aid of local and regional Floras, and voucher specimens of the plants with numbers 14821-14835 were deposited in the IAUH (Table 1). The specimens were collected during July and December 2014.

Wherever possible, five trees from at least 50 m distant from each other were sampled randomly from each population. Fresh leaves were collected and kept in 50 CC falcon tubes, filled with Silica Gel, for the purpose of drying them (Chase and Hill 1991). The leaves were then used as a DNA extraction source.

DNA extractions and ITS amplification

Total DNA was extracted following a modified CTAB protocol of Doyle and Doyle (1990) using the DNeasy Plant Mini kit (Qiagen, Germany). We amplified the Internal Transcribed Spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA using primer combinations 18S (forward primer 5'-CCT TMT CAT YTA GAG GAA GGA G-3') and 28S (reverse primer 5'-CCG CTT ATT KAT ATG CTT AAA-3'). The PCR protocol for ITS region included: 34 cycles of 18 seconds denaturation (94°C), 30 seconds annealing (53°C), and 60 seconds elongation (72°C), with two additional minutes elongation (Gaskin and Schaal 2003). The quality of PCR products was checked by electrophoresis on a 1.0% agarose gel and then visualized under UV light.

Table 1. List of Acer monspessulanum subspecies investigated in our analysis and their morphological characters and locality in Iran (small= up to 2×2 cm, large=2-3 × 3.5-4 cm)

Taxon	Locality with herbarium numbers and GenBank accession numbers	Major features of morphological traits (Rechinger 1969)
A. monspessulanum ssp. *turcomanicum* (Pojark.) Rech. f.	Iran: Khorasan Shomali, 45 km N of Shirvan, Golul-Sarani, 2302 m, Basiri 14823 (IAUH)	Leaves: large Loculus inside: hairy Loculus outside: sparsely hairy
A. monspessulanum ssp. *ibericum* (M.B.) Yaltirik *A. monspessulanum* ssp. *ibericum* (M.B.) Yaltirik *A. monspessulanum* ssp. *Ibericum* (M.B.) Yaltirik *A. monspessulanum* ssp. *ibericum* (M.B.) Yaltirik	Iran: Azarbayejan Sharghi, Kaleybar, Arasbaran forest, Venigh, 1070 m, Masoud, 14821 (IAUH), KT587662 Iran: Azarbayejansharghi, Kaleybar, Arasbaran forest, Tuali, 850 m, Masoud, 14822 (IAUH), KT587663 Iran: Golestan, Gorgan, Golestan National Park, 677 m, Khademi, 14833 (IAUH), KT587665 Iran: Mazandaran, Amol, Haraz road, Chelav, 737 m, Khademi, 14834 (IAUH), KT587661	Leaves: large Loculus inside: hairy Loculus outside: glabrous Lower surface midrib: glabrous
A. monspessulanum ssp. *assyriacum* (Pojark.) Rech. *A. monspessulanum* ssp. *assyriacum* (Pojark.) Rech. *A. monspessulanum* ssp. *assyriacum* (Pojark.) Rech.	Iran: Kordestan, Mariwan, Mohhamadeh village toward Benavechele, 1550 m, Khademi, 14828 (IAUH), KT587655 Iran: Kordestan, Mariwan, Mohhamadeh village toward Benavechele, 1510 m, Khademi, 14829 (IAUH), KT587653 Iran: Kermanshah, Jawanroud toward Salas, 1585 m, Khademi, 14832 (IAUH), KT587654	Leaves: large Loculus inside: hairy Loculus outside: glabrous Lower surface midrib: sparsely hairy
A. monspessulanum ssp. *cinerascens* (Boiss.) Yaltirik *A. monspessulanum* ssp. *cinerascens* (Boiss.) Yaltirik *A. monspessulanum* ssp. *cinerascens* (Boiss.) Yaltirik *A. monspessulanum* ssp. *cinerascens* (Boiss.) Yaltirik *A. monspessulanum* ssp. *cinerascens* (Boiss.) Yaltirik	Iran: Fars, Marwdasht, Jahanabad village, 1756 m, Khademi, 14824 (IAUH), KT587656 Iran: Fars, Marwdasht, Bizjan village, Dorodzan Dam, 1715 m, Khademi, 14825 (IAUH), KT587657 Iran: Fars, Marwdasht, Chav road, 1823 m, Khademi, 14826 (IAUH). KT587658 Iran: Fars, Bayza. Tang Tir forest, 1632 m, Khademi, 14827 (IAUH), KT587659 Iran: Kohgiloye-va-Boir Ahmad, Gachsaran, Gachsaran, 15 km to Choram, After Abrigoon, Deel neck, 1600 m, Mehrgan, 14835 (IAUH)	Leaves: small Loculus inside: densely hairy Loculus outside: glabrous
A. monspessulanum ssp. *persicum* (Pojark.) Rech. *A. monspessulanum* ssp. *persicum* (Pojark.) Rech.	Iran: Kerman, 25 km from Dalfard toward Jiroft, 980 m, Meyjani, 14830 (IAUH) Iran: Kerman, Meyjan, 1218 m, Meyjani, 14831 (IAUH), KT587664	Leaves: small Loculus inside: glabrous Loculus outside: sparsely hairy

Table 2. List of taxa used in our analysis with their GenBank accession numbers.

Taxon	Region	GenBank accession numbers
A. hyrcanum ssp. *hyrcanum*	Iran	AY605305
A. hyrcanum ssp. *hyrcanum*	Iran	AY605306
A. hyrcanum ssp. *hyrcanum*	Iran	DQ366129
A. hyrcanum ssp. *hyrcanum*	Iran	DQ366130
A. ibericum	Georgia	AM238352
A. ibericum	Georgia	AM238353
A. ibericum	Georgia	AM238354
A. ibericum	Georgia	AY605307
A. ibericum	Georgia	AY605308
A. ibericum	Georgia	AY605309
A. ibericum	Georgia	AY605310
A. ibericum	Georgia	AY605311
A. ibericum	Georgia	AY605312
A. ibericum	Georgia	AY605313
A. ibericum	Georgia	AY605314
A. monspessulanum	France	AM238407
A. monspessulanum	France	AM238408
A. monspessulanum	France	AM238409
A. monspessulanum	France	AM238410
A. monspessulanum	France	AM238411
A. monspessulanum	France	AM238412
A. monspessulanum	France	AM238413
A. monspessulanum	France	AM238414
A. monspessulanum	France	AM238415
A. monspessulanum	France	AM238416
A. monspessulanum	Bulgaria	AM238423
A. monspessulanum	Bulgaria	AM238424
A. monspessulanum	Bulgaria	AM238425
A. monspessulanum	Bulgaria	AM238426
A. monspessulanum ssp. *monspessulanum*	France	AM238355
A. monspessulanum ssp. *monspessulanum*	France	AM238357
A. monspessulanum ssp. *monspessulanum*	France	AM238358
A. monspessulanum ssp. *monspessulanum*	France	AM238359
A. monspessulanum ssp. *monspessulanum*	France	AM238361
A. monspessulanum ssp. *monspessulanum*	France	AM238362
A. monspessulanum ssp. *monspessulanum*	France	AM238363
A. monspessulanum ssp. *monspessulanum*	France	AM238364
A. monspessulanum ssp. *monspessulanum*	France	AM238365
A. monspessulanum ssp. *monspessulanum*	France	AM238366
A. monspessulanum ssp. *monspessulanum*	France	AM238367
A. monspessulanum ssp. *monspessulanum*	France	AM238368
A. monspessulanum ssp. *monspessulanum*	France	AM238369
A. monspessulanum ssp. *monspessulanum*	France	AM238370
A. monspessulanum ssp. *monspessulanum*	France	AM238371
A. monspessulanum ssp. *monspessulanum*	France	AM238373
A. monspessulanum ssp. *monspessulanum*	Spain	AM238374
A. monspessulanum ssp. *monspessulanum*	Spain	AM238375
A. monspessulanum ssp. *monspessulanum*	Spain	AM238376
A. monspessulanum ssp. *monspessulanum*	France	AM238377
A. monspessulanum ssp. *monspessulanum*	France	AM238378
A. monspessulanum ssp. *monspessulanum*	France	AM238379
A. monspessulanum ssp. *monspessulanum*	France	AM238380
A. monspessulanum ssp. *monspessulanum*	France	AM238381
A. monspessulanum ssp. *monspessulanum*	France	AM238382
A. monspessulanum ssp. *monspessulanum*	France	AM238383
A. monspessulanum ssp. *monspessulanum*	France	AM238384
A. monspessulanum ssp. *monspessulanum*	France	AM238385
A. monspessulanum ssp. *monspessulanum*	France	AM238386
A. monspessulanum ssp. *monspessulanum*	France	AM238387
A. monspessulanum ssp. *monspessulanum*	France	AM238388
A. monspessulanum ssp. *monspessulanum*	France	AM238391
A. monspessulanum ssp. *monspessulanum*	France	AM238393
A. monspessulanum ssp. *monspessulanum*	France	AM238394
A. monspessulanum ssp. *monspessulanum*	France	AM238395
A. monspessulanum ssp. *monspessulanum*	France	AM238396
A. monspessulanum ssp. *monspessulanum*	France	AM238397
A. monspessulanum ssp. *monspessulanum*	France	AM238398
A. monspessulanum ssp. *monspessulanum*	France	AM238399
A. monspessulanum ssp. *monspessulanum*	Germany	AM238401
A. monspessulanum ssp. *monspessulanum*	Germany	AM238402
A. monspessulanum ssp. *monspessulanum*	Spain	AY605315
A. monspessulanum ssp. *monspessulanum*	Spain	AY605316
A. monspessulanum ssp. *monspessulanum*	Spain	AY605317
A. monspessulanum ssp. *monspessulanum*	France	AY605318
A. monspessulanum ssp. *monspessulanum*	France	AY605319
A. monspessulanum ssp. *monspessulanum*	France	AY605320
A. monspessulanum ssp. *monspessulanum*	France	AY605321
A. monspessulanum ssp. *monspessulanum*	France	DQ366124
A. monspessulanum ssp. *monspessulanum*	France	DQ366125
A. monspessulanum ssp. *monspessulanum*	France	DQ366126
A. monspessulanum ssp. *monspessulanum*	France	DQ366127
A. monspessulanum ssp. *monspessulanum*	France	DQ366128
A. obtusifolium	Cyprus	AM238327
A. obtusifolium	Cyprus	AM238328
A. obtusifolium	Cyprus	AM238329
A. obtusifolium	Cyprus	AM238330
A. obtusifolium	Syria	AM238331
A. obtusifolium	Syria	AM238332
A. opalus	France	AM238417
A. opalus	France	AM238418
A. opalus	France	AM238419
A. opalus	France	AM238420
A. opalus	France	AM238421
A. opalus	France	AM238422
A. sempervirens	Greece	AM238334
A. sempervirens	Greece	AM238335
A. sempervirens	Greece	AM238336
A. sempervirens	Greece	AM238337
A. sempervirens	Greece	AM238340
A. sempervirens	Greece	AM238341
A. sempervirens	Greece	AM238342
A. sempervirens	Greece	AM238343
A. sempervirens	Greece	AM238344
A. sempervirens	Greece	AM238348
A. sempervirens	Greece	AM238349
A. sempervirens	Greece	AM238350
A. sempervirens	Greece	AM238351
A. sempervirens	Greece	AY605349
A. sempervirens	Greece	AY605350
A. sempervirens	Greece	AY605351
A. sempervirens	Greece	AY605352
A. sempervirens	Greece	AY605353
A. sempervirens	Greece	DQ366122
A. sempervirens	Greece	DQ366123
A. velutinum	Iran	AY605361

Note: ssp. = subsp. = sub species

Phylogenetic analyses

Phylogenetic reconstructions were performed with 15 samples from each of 75 accessions (15 populations) belonging to five subspecies of *Acer monspessulanum* from Iran (Table 1). In addition, we used the ITS sequence of 86 accessions of *Acer* from GenBank. List of non-Iranian taxa used in our analysis with GenBank accession numbers are shown in Table 2. We used ITS sequences of *Acer velutinum* Boiss. from GenBank as the outgroup based on the earlier studies including Grimm et al. (2006) (Table 2).

The 3' region of the 18S rDNA, the 5' region of the 26S rDNA, and the whole ITS1-5.8S rDNA-ITS2 region were sequenced for all the taxa, and these were compared to sequences produced for other maples. Forward and reverse sequences were visually compared and edited, and then aligned using Sequencher 4 software (Gene Codes Corporation, Ann Arbor, MI, USA). In addition to our sequences, 86 ITS sequences from other taxa were taken from GenBank (Table 2). All ITS sequences were assembled and aligned using MacClade 4 (Maddison and Maddison 2005). The parsimony analyses were performed using PAUP*4.0b10 (Swofford 2002), with the following options: heuristic search with 1,000 random-addition-sequence replicates; tree bisection-reconnection (TBR) branch swapping; saving all most parsimonious trees. Character state changes were treated as equally weighted. Relative clade support was estimated using 1,000 bootstrap replicates in PAUP* via full heuristic searches and simple taxon addition. Clades with a bootstrap value of 50% or more were considered as robustly supported nodes. The consistency index (CI) and retention index (RI) were calculated to assess the amount of homoplasy present in the data. The best-fitting substitution model (TrN+I) was determined under the Akaike Information Criterion (AIC; Akaike 1974) using Modeltest 3.7 (Posada and Crandall 1998). The Bayesian analysis (BA) of the ITS datasets were performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). TrN+I is a transitional model with six rates. For the ITS dataset, the TrN+I model was chosen. The amount of proportion of invariable sites (I) was 0.6732.

RESULTS AND DISCUSSION

The data set of the ITS region included 675 characters, 43 of them parsimony informative. Strict consensus tree (length of 138 steps, consistency index (CI) = 0.703, retention index (RI) = 0.904) is shown in Figure 1. Figure 2 shows tree from Bayesian analysis using MrBayes. All sampled species of Iranian *Acer* were part of a monophyletic clade with Posterior Probability (PP) = 1 and Bootstrap Support (BS) = 79% (Clade N; Figure 1). Since Iranian *A. monspessulanum* origin are from Mediterranean (Rechinger 1969), we compare other studies that have involved North Africa samples, and found that European, North Africa and Asia Minor samples are in one clade as Acer core clade with BS 75% and PP 0.96 (Grimm et al. 2007).

Our maximum parsimony results (Figure 1) indicate that Iranian *A. monspessulanum* subspecies (13 populations) in clade N are closely related to eight *A. ibericum* samples from Georgia, one sample of *A. hyrcanum* from Iran and

two samples of *A. sempervirens* from Greece (pp= 1, BS= 79). This agrees with results reported by Grimm et al. (2007) where different taxa of one of one group fall into three lineages. In their results, *Acer monspessulanum* and *A. ibericum* + *A. hyrcanum* group together in the graph and long proximal edges indicate that they are most closely related. Clones of *A. monspessulanum* are distinct and placed near the center of the graph (Grimm et al. 2007).

Clade O that includes six specimens of *A. ibericum* from Georgia with one specimen of *A. monspessulanum* subsp. *turcomanicum* from Iran and one specimen of *A. sempervirens* from Greece (PP= 0.79; BS= 62%) has proved Rechinger results about Iranian *Acer* origin. Clade M comprises 4 species of *A. monspessulanum* from Bulgaria with PP= 0.8 and BS= 77%. Clades M and N together are in clade C (PP= 0.95). This close relation between Iranian and Bulgarian *Acer monspessulanum* species samples support this notion that they have an origin in Mediterranean region (Rechinger 1969). Clade L has two species from France, one *A. monspessulanum* and *A. monspessulanum* ssp. *monspessulanum.* Clade K include clade L with another *A. monspessulanum* ssp. *monspessulanum* from France (PP= 0.84). Clade J includes clades K and L with four taxa of *A. monspessulanum* ssp. *monspessulanum* from France and Spain. Clade I have three *A. monspessulanum* ssp. *monspessulanum* from France with PP= 0.91. Clade H include 17 *A. monspessulanum* ssp. *monspessulanum* with *A. opalus*, all from France (PP= 0.97). Each clades of G and F has two *A. monspessulanum* ssp. *monspessulanum* from France with PP= 1 and BS= 80%. Clade E comprise clades F, G, H, I, J, K and L from France and Spain. Clade D has only one species *A. monspessulanum* from France that with clade E are in clade B with BS= 50%. Clade P that has one *A. velutinum* from Iran, consider as out-group in our analysis. Data analysis indicates that the classification of species according presence or absence of hairs in inner or outer surface of loculus is a true morphological characteristic for delimitation of subspecies in *Acer monspessulanum*.

The observed polytomies in clades E and N indicated that these taxa are taxonomically closely related and there were not enough time passed since divergence from their ancestral taxa (Zarrei et al. 2009). More divergent markers, i.e. low-copy nuclear genes, could potentially resolve these branches.

Based on our results using Bayesian analysis, some well resolved clades were present (Figure 2). Clade A comprise two subclades, E and F. Clade E includes four *A. monspessulanum* specimens from Bulgaria and clade F includes 27 taxa (PP= 1), that 13 of them are *A. monspessulanum* subspecies from Iran and others are *A. ibericum* (from Georgia), *A. sempervirens* (from Greece) and *A. hyrcanum* subsp. *hyrcanum* (from Iran), this clade proved Mediterranean origin of Iranian *Acer* (clade F; Figure 2). One of our taxa (*A. monspessulanum* subsp. *ibericum*) placed in clade L with five *A. ibericum* from Georgia and an *Acer sempervirens* from Greece with high support (PP= 0.79). This placement indicates that *A. monspessulanum* and *A. sempervirens* are closely related together (Grimm et al. 2007).
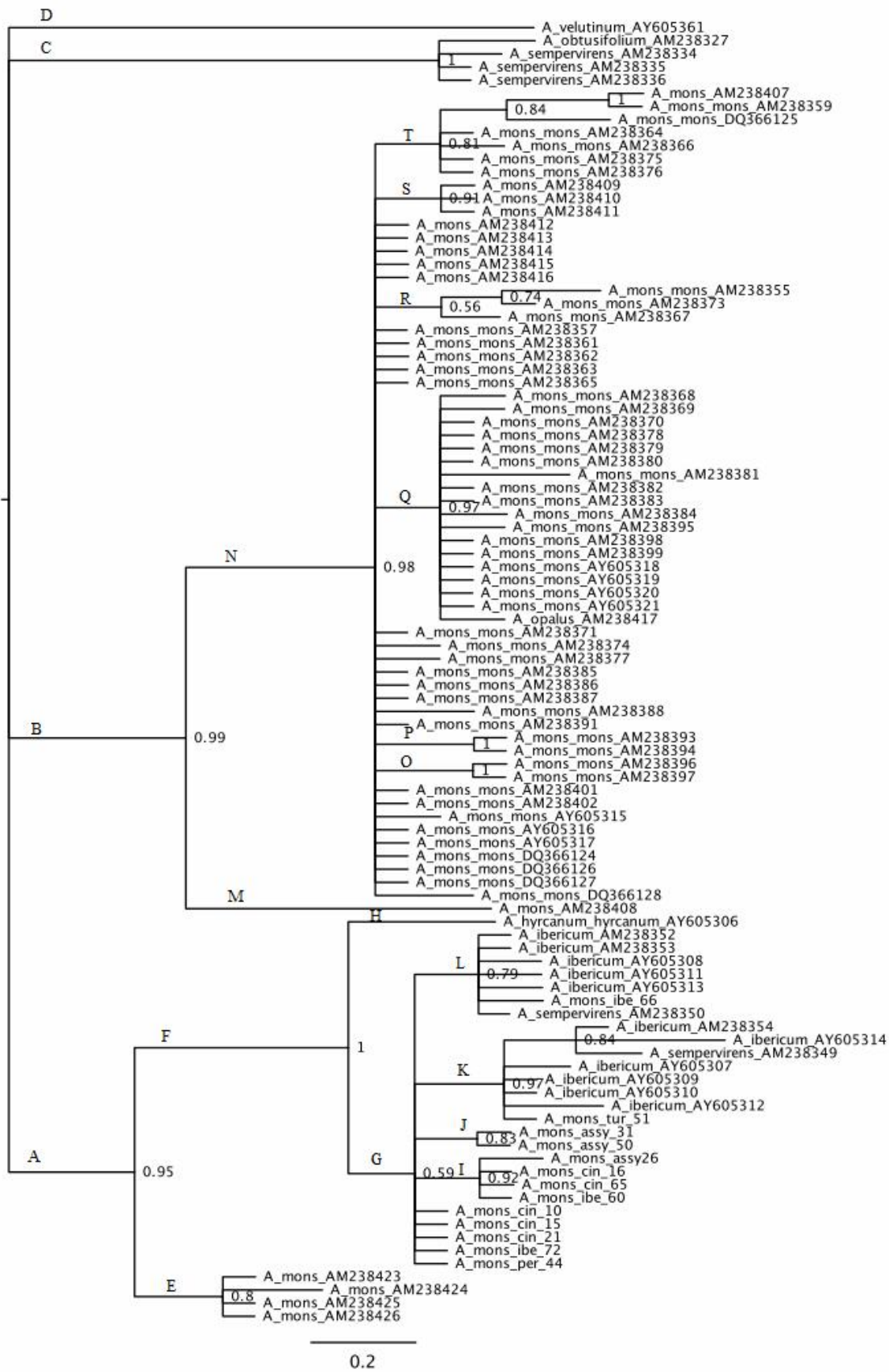
Figure 1. Phylogenetic relationships of 13 samples of *Acer* from Iran, on the basis of the analysis of internal transcribed spacer (ITS) sequences. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are bootstrap values

Figure 2. Bayesian tree. Numbers on nodes represent posterior probability values

Clade B (Figure 2) have two subclades M and N (PP= 0.99) that include European *Acer monspessulanum* (from France, Spain and Germany). Clades G and N show polytomies since comprise species their morphology are closely related together.

Clade C (Figure 2) have four taxa, three of them are *A. sempervirens* from Greece and one *A. obtusifolium* from Cyprus (PP= 1) that in Grimm et al. (2007) study In Europe, their distribution ranges from northern, cool-temperate latitudes (southern Sweden, *A. pseudoplatanus* L., naturalized) throughout central, western, and south-eastern Europe (*A. hyrcanum* Fischer & Meyer, *A. opalus* P. Miller, *A. pseudoplatanus*) to the subtropical Mediterranean (*A. monspessulanum* L., *A. opalus*, *A. heldreichii* Orphanides ex Boissier, *A. sempervirens* L., and *A. obtusifolium* Sibthorp & Smith), and with an eastward expansion to Asia Minor, the Caucasus, and Iran (*A. trautvetteri* Medvedev, *A. hyrcanum*, *A. ibericum* Bieberstein ex Willdenow, *A. monspessulanum*, *A. pseudoplatanus*, and *A. velutinum* Boissier) (Grimm et al. 2007).

Subspecies geographical distribution show that *assyriacum* subspecies from Kordestan and Kermanshah provinces are near together and support with Bayesian analysis (PP= 0.82). *A. monspessulanum* subsp. *ibericum* collected from Arasbaran forest (Azarbayejansharghi province) placed in clade L (Figure 2) with *A. ibericum* from Georgia (PP = 0.79) that from geographical distribution approach is justifiable. All Iranian *Acer monspessulanum* in the present survey made a clade with two *A. sempervirens* from Greece and eleven *A. ibericum* from Georgia and one *A. hyrcanum* subsp. *hyrcanum* from Iran with high Bayesian support (PP= 1).

Because ITS results couldn't delimitate on subspecies level we used from morphological traits. Some morphological features are important for identification of subspecies *A. monspessulanum*, such as size of the leaves, lower surface midrib hair and loculus inside and outside base on presence or absence of hair. One of the most important characteristic traits for distinguish between some subspecies of *A. monspessulanum*, is presence or absence of hair inside and outside their loculus. Based on the size of the leaves we have two groups, (i) small (up to 2×2 cm) that has two subspecies (ssp. *persicum* and ssp. *cinarescens*) and (ii) large (2-3×3.5-4 cm) with three subspecies (ssp. *turcomanicum*, ssp. *assyriacum* and ssp. *ibericum*). Lower surface midrib hair separate only two subspecies of *A. monspessulanum*, one of them is glabrous (ssp. *ibericum*) and the other is sparsely hairy (ssp. *assyriacum*), so this trait is not a proper discriminative factor for other three subspecies. Loculus outside hair has two state, (i) glabrous (ssp. *turcomanicum*, ssp. *persicum*) and (ii) sparsely hairy (ssp. *ibericum*, ssp. *assyriacum*, ssp. *cinarescens*). We have three group base on loculus inside hair, (i) hairy (ssp. *turcomanicum*, ssp. *ibericum*, ssp. *assyriacum*), (ii) densely hairy (ssp. *cinarescens*) and (iii) glabrous (ssp. *persicum*) (Wolfe and Yanai 1987), so the most important and discriminative character to detect

subsp. *persicum* from subsp. *cinarescens* is loculus inside hair.

The internal transcribed spacer of the nuclear region (ITS) is a widely used molecular marker for reconstruction of evolutionary patterns in plant kingdom. It has been used both in the higher taxonomic level (i.e. family) as well as lower even below the species rank (Zarrei et al. 2014). Our results indicate that this marker could be potentially valuable in delineating subspecies boundaries in maple species. The limiting factor is that this marker is not well diverged in some groups. A more divergent molecular marker such as low copy nuclear genes and intergenic nuclear spacers could potentially be helpful. Based on our experiences working on *Acer* and results of others studies on tree taxa (Zarrei et al. 2014); we suggest combining our ITS DNA sequences with additionally markers to increase the power of our phylogenetic analysis and improve resolution of unresolved clades. Such strategies have been applied before (see Zarrei et al. 2015). The implication of next generation sequencing data has been already proven in revolving systematic problem with closely related species (Liston et al. 2015).

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