

## The use of DNA barcoding to avoid adulteration in olive plant leaf products

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**Abstract.** Partovi R, Iranbakhsh A, Sheidai M, Ebadi M. 2020. The use of DNA barcoding to avoid adulteration in olive plant leaf products. *Asian J For* 5: 42-47. The leaves of olive plant species, either the cultivated (*Olea europaea*) and the wild (*O. europaea* var. *Cuspidata*) have been used for medicinal uses in Iran. The first species leaves have been used to control the blood pressure, while the leaves of wild olive have been used for abortion by locals. Our preliminary inspection of the medicinal plant market revealed that the leaves of these two olive species were sold mistakenly to the consumers and their health might be at risk. Therefore, we performed this investigation to produce DNA barcodes for correct identification of these two olive species and also identify the potential adulteration in our local market. We used Internal transcribed spacer (ITS) as well as plastid genome *trnL-F* intergenic spacer and ribosomal protein L16 (rpL16) sequences. These sequences after alignment and curation produced DNA barcodes that can differentiate the two olive species from each other. The phylogenetic trees constructed also separated the samples of these olive species and confirmed the potential use of these short DNA sequences for olive barcoding. The present study revealed that some of the local shops mistakenly sell the wild olive leaves instead of the cultivated olive leaf to be used for blood pressure. This mistake endangers the health of pregnant women consumers if they carry a child. We suggest using a combination of nuclear ITS and plastid intergenic spacer (*trnL-F* and rpL16) regions for DNA barcoding of olive plants to avoid leaf product adulteration.

**Keyword:** DNA barcoding, medicinal market, olive, rpL16, trnL-F

### INTRODUCTION

The olive plant (*Olea europaea* L.) complex occurs in the Canary Islands, the Mediterranean region, south-west Asia, Sino-Himalayan region, eastern as well as southern Africa (Green 2002). While African olive is a tropical wild olive, which is geographically isolated from its Mediterranean relatives (Zohary 1995). In the recent revision of *Olea* (Green 2002), the name *Olea europaea* subsp. *cuspidata* is proposed for the wild olive occurring in South to North-East Africa and southwest Asia.

In Iran, the olive plant occurs in both cultivated (*Olea europaea* subsp. *europaea*), and wild (*Olea europaea* subsp. *cuspidata* (Wall. & G. Don) Cif.). The wild *Olea europaea* subsp. *cuspidata* is naturally distributed in southern parts of Iran. Both cultivated and wild olive plants have edible and/or medicinal values. The cultivated olive (*O. europaea* subsp. *europaea*) is mainly used for olive oil, but also its leaves have been used to reduce blood pressure (Lockyer et al. 2017). Similarly, the wild olive (*O. cuspidata*) has medicinal properties like antibacterial, antifungal and antioxidant activity (Masoko and Makgapeetja 2015). The plant leaves are usually used as a remedy for eye infections, sore throat, urinary tract infections, kidney problems and backaches or headaches (Masoko and Makgapeetja 2015). The leaves are also recommended as hypotensive, emollient, febrifuge and stytic (Somova et al. 2003), and utilized for the treatment

of malaria in 1854 (Altinyay et al. 2011). The leaves of both cultivated olive *O. europaea*, as well as wild olive *Olea cuspidata* are important medicinal plant product in Iran that is used for several disorders. The leaves of *O. europaea* are used to reduce blood pressure, while leaves of *O. cuspidata* are used in the form of tea to avoid pregnancy among our local people (Abaza et al. 2015; Hashmi et al. 2015).

Our preliminary inspection of these plant products in local medicinal plant market noticed that both plant species products were sold with the same name, i.e. olive, and were given for blood pressure control. This urges for attention since it might cause unintended effects, particularly regarding the adulteration of medicinal plants. Adulteration of medicinal plant products is a dangerous act that can endanger the consumer's health. This might happen due to substitution or addition of another substance in the genuine medicinal product (El Beyrouthy and Abi-Rizk 2013), or due to careless plant collection, mislabelling or presence of high level of morphological similarity of the plant species in the field (El Beyrouthy and Abi-Rizk 2013). Irrespective of the reason for adulteration, this phenomenon seems to occur frequently in medicinal plant market of developing countries (Srirama et al. 2017). Medicinal plant product adulteration can result in various and extensive harm to the people using them. For example, cases of kidney failure were reported (Srirama et al. 2017), as a result of adulteration of the roots in *Stephania tetrandra* with that of

*Aristolochia fangchi*. Similarly, hepatotoxicity occurred due to mixing of the *Cinnamomum verum* bark with *C. cassia* and *C. malabattrum* (Srirama et al. 2017).

It is suggested to utilize integrative methods for authentication of the medicinal plants and investigating the cases for adulteration. A combination of various approaches and disciplines have been considered for such investigations such as physical and chemical methods like thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), external morphological features, DNA sequence similarity, and phylogenetic analyses using both tree-based criteria or character-based methods (Pasqualone et al. 2016; Ghorbani et al. 2017; Yu et al. 2018). Recently, determination of the genetic distance among the studied samples and with the correct plant specimens as well as BLAST approach and DNA barcoding have also been used (Sheidai et al. 2019). A DNA barcode is unique short DNA sequences that different plant species, cultivars or races from each other and the closely related plant taxa (Sheidai et al. 2019). It enables the researchers to correctly identify the target species. This approach has been utilized to identify the medicinal plants and distinguish the original products from adulterated species/DNA (Heubl 2010; Sheidai et al. 2019).

The present study was conducted with the following aims: (i) to use different DNA regions to differentiate wild versus cultivated olive plants; (ii) to provide proper barcodes for either species, (iii) to check the potential adulteration/ or misidentification of these plant products by the local medicinal plant markets based on different barcoding approaches. For these, we first used ITS (Internal Transcribed Spacer DNA), and the chloroplast intron sequences of *rpL16* and *trnL-F*, for distinguishing the two plant species of *O. europaea*, and *O. cuspidata*. We finally used *rpL16* sequences to identify the local market plants and investigate the potential adulteration or mistakes in these products.

## MATERIAL AND METHODS

### Plant materials

The wild olive (*Olea cuspidata*) leaf samples (four different trees) were collected in Southern Iran, where the local people collect the same plant leaves for consumption (Table 1). For ITS, six wild specimens were compared with four cultivated specimens (Table 2). For *trnL-F*, six wild specimens and five cultivated specimens gathered from NCBI were compared with each other (Table 3). The accession numbers of *Olea* species have been provided. For

*rp16*, we also applied two out-groups, six cultivated specimens collected from different shops in Tehran, Iran, and one wild specimen (Table 4).

### DNA extraction and PCR reactions

Fresh leaves were put to dry in silica gel powder. We used CTAB activated charcoal protocol (Krizman et al. 2006). The extracted DNA was examined in terms of quality and quantity by running on 0.8% agarose (Sheidai et al. 2013).

### ITS sequences investigation

The complete ITS region was amplified using forward ITS5 (5'- GGA AGT AAA AGTCGTAAC AAG G- 3') and reverse primers ITS4 (5'- TCC GCT TAT TGA TAT GC- 3') (White et al. 1990). The following program was used for amplification of nuclear region in a PCR reaction: 5 min initial denaturation step 94°C, followed by 40 cycles of 1 min at 94°C; 1 min at 53.5°C and 2 min at 72°C. The reaction was completed by a final extension step of 7 min at 72°C.

### Cp- DNA study

The intergenic spacer of chloroplast genome *rpL16* was amplified and sequenced with universal primers following the methodology of (Shaw et al. 2005; Timme et al. 2007). Each 20 µl of PCR tube contained 10 µl of 2x PCR buffer, 0.5 mM of each primer, 200 mM of each dNTP, 1 unit of Taq DNA polymerase (Bioron, Germany), and 1 µl of template genomic DNA at 20 ng µl<sup>-1</sup>.

The following program was used for amplification of nuclear region in a PCR reaction: 5 min initial denaturation step 94°C, followed by 35 cycles of 1 min at 94°C; 1 min at 54°C and 1 min at 72°C. The reaction was completed by a final extension step of 6 min at 72°C.

### Data analysis

DNA regions investigated were first aligned by MUSCLE program as implemented in MEGA 7 Program (Tamura et al. 2012). They were then cured and investigated for the nucleotide difference and also to find out those nucleotides which were unique for either wild or cultivated olive plants.

Different phylogenetic trees were constructed by using PAUP ver. 4. Followed by 1000 times bootstrapping (Swofford 2002). The TCS network and related sequence statistics were obtained by using POPART program (<http://popart.otago.ac.nz>).

**Table 1.** List of three specimens of *Olea europaea* subsp. *cuspidata* L. along with the information regarding altitude, latitude, longitude and herbarium number

Localities	Altitude	Latitude	Longitude	Voucher no.
Chaharmahal and Bakhtiari Province, Dehedz – Lordgan, Iran	1713	31°31'18"	50°28'26"	HSBU2018700
Kohgiluyeh and Boyer-Ahmad Province, Khersaan Road, Iran	1380	31°26'59"	50°28'57"	HSBU2018705
Chaharmahal and Bakhtiari Province, Lordgan, Monj, Gachahan, Iran	1151	31°26'48"	50°32'19"	HSBU2018711
Chaharmahal and Bakhtiari Province, Lordgan, Monj, Gachahan, Iran	1592	35°55'41"	57°41'53"	HSBU2018712

**Table 2.** The comparison between six wild specimens and four cultivated specimens applied for ITS

Species	Accession number
<i>Olea europaea</i>	KY313879.1
<i>Olea europaea</i>	AF231866.1
<i>Olea europaea</i>	KY313880.1
<i>Olea europaea</i> subsp. <i>cuspidata</i>	HQ117893
<i>Olea paniculata</i>	AF231867.1
<i>Olea europaea</i> subsp. <i>cuspidata</i>	AY040810.1
<i>Olea europaea</i> subsp. <i>cuspidata</i>	AM933049.1
<i>Olea europaea</i> subsp. <i>cuspidata</i>	AM933048.1
<i>Olea europaea</i> subsp. <i>cuspidata</i>	AM933047.1
<i>Olea europaea</i> subsp. <i>cuspidata</i>	AM933046.1

**Table 3.** The comparison between six wild specimens and five cultivated specimens applied for trnL-F

Species	Accession number
<i>Olea europaea</i> subsp. <i>cuspidata</i>	AY040810.1
<i>Olea europaea</i> subsp. <i>cuspidata</i>	AM933043.1
<i>Olea europaea</i> subsp. <i>cuspidata</i>	AM933045.1
<i>Olea europaea</i> subsp. <i>cuspidata</i>	AM933047.1
<i>Olea europaea</i> subsp. <i>cuspidata</i>	AM933048.1
<i>Olea europaea</i> subsp. <i>cuspidata</i>	AM933049.1
<i>Olea europaea</i>	AY040811.1
<i>Olea europaea</i>	FJ490798.1
<i>Olea europaea</i>	KY313879.1
<i>Olea europaea</i>	HQ117895.1
<i>Olea europaea</i>	AF231866.1

**Table 4.** Two out-groups applied for rpl16 sequencing

Species	Accession number
<i>Olea europaea</i> cultivar Frantolo	GU931818.1
<i>Olea europaea</i> cultivar Bianchera	NC 013707.2

## RESULTS AND DISCUSSION

ITS sequences obtained after curation produced 377 bp long sequences, which could differentiate wild and cultivated olives from each other. The preliminary statistics of the ITS sequences produced the nucleotide diversity ( $P_i$ ) = 0.26, the number of nucleotide differences between wild and cultivated olive samples was 174, and Tajima's D test ( $D$ ) = 2.82. Short sequences were identified which

were specific for either plant species which can be used as DNA barcodes (Figure 1).

Maximum likelihood (ML) phylogenetic tree based on ITS sequences (Figure 2) separated the studied plant samples of the two species in separate clades with 100% bootstrap value.

### CP-DNA trnL-F region

*TrnL-F* region sequences obtained in the wild and cultivated olive samples after curation produced 73 bp long sequences. The two olive species studied differed in 35 nucleotides, with nucleotide diversity ( $P_i$ ) = 0.27, and Tajima's D statistic:  $D = 1.48$  ( $p = 0.16$ ). Some of the short sequences of *TrnL-F* chloroplast inter-genic region also could differentiate the two plant species from each other and can act as barcodes (Figure 3). Maximum likelihood (ML) phylogenetic tree based on trnL-F sequences (Figure 4), separated the studied plant samples of the two species in separate clades with 100% bootstrap value.

### Chloroplast rpl16 sequences

Chloroplast rpl16 region sequences after curation produced a total length of 583 bp DNA segment with 229 polymorphic sites,  $P_i = 0.067$ . The two olive species differed in 218 nucleotides, few of which were unique in either species and can be used in olive species barcoding (Figure 5). The ML phylogenetic tree was obtained with dual purposes. First to differentiate the wild olive (denoted in Figure 6) from the cultivated samples studied (Samples A1), and second, to check the local medicinal plant products (Samples A2-A6, in Figure 6).

The result showed that wild olive is separated from the cultivated olive samples and is placed in a distant clade. Therefore, based on rpl16 sequences these two olive species can be differentiated. Out of 5 plants from the medicinal shops investigated, four samples were placed closer to the wild olive plant and only 2 samples (A2 and A6) were placed close to the cultivated olive plant. Therefore, it seems that misidentification of these two different plant species by the local shops can cause potential abortion problems for the consumers (Kadam and Gaykar 2018). As stated before, usually, the consumers purchase the cultivated olive leaves for controlling the blood pressure, but instead the leaves of wild olives have been sold to them which is used for abortion.

The result of the present study indicates that using a combination of molecular barcodes present in ITS, *trnL-F* and rpl16 sequences can help to differentiate the two olive species *O. europaea*, and *O. cuspidata*, and can improve the correct marketing of these medicinal plant taxa.





In conclusion, the present study emphasizes the use of modern molecular barcoding to improve olive leaf marketing in medicinal local shops. This approach can avoid misidentification of the closely related species of olive which have different medicinal properties. This, in turn, avoid harm to human health.

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