



## Article

# Morphological, Molecular and Genomic Characterization of Two Inter-Subspecific Hybrids between Olive Cultivars and Olive Subspecies

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**Abstract:** Two inter-subspecific F<sub>1</sub> hybrids have been obtained by crossing olive cultivars ('Frantoio' and 'Coratina') with pollen donors from olive subspecies (*Olea europaea* subsp. *cuspidata*) to enrich the germplasm of cultivated olive in southern China. This study aimed to investigate the characterization of morphological traits and molecular markers in the two hybrids and their parents of crosses. The morphological study showed a significant difference between genotypes according to the main discriminative parameters on qualitative and quantitative traits of leaf, fruit, and endocarp. A set of six co-dominant polymorphic simple sequence repeats (SSRs) were used for molecular identification, and SSR analysis confirmed that two progenies were the offspring of their cited parents based on the presence of parental specific SSR alleles. Three single-copy nuclear loci (SCNL) primer pairs were used for amplification of single-copy genes in the two progenies and their parents and after then PCR products were sequenced. Sequence alignment analysis on the effective data showed a total of 15 different base sites between two progenies, which were confirmed as true inter-specific hybrids between olive cultivars and subsp. *cuspidata*.

**Keywords:** inter-subspecific hybrid; morphological characterization; SSR; SCNL; *Olea europaea* subsp. *europaea*; *Olea europaea* subsp. *cuspidata*



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## 1. Introduction

Native to the Mediterranean region, tropical and central Asia, and various parts of Africa, the olive tree (*Olea europaea* L.) is demonstrated to have originated from Levant in the Middle East, after which several differentiations occurred, the most cited of which was from the East to the West Mediterranean and spread to the surrounding areas from there several millennia ago [1–5]. Cultivated olive (*O. europaea* L. subsp. *europaea* var. *europaea*; Oleaceae) is the most iconic tree of the Mediterranean Basin (MB) as an economically significant species and a keystone of traditional Mediterranean agriculture [6–8]. Production of olive is mainly concentrated around the MB, where about 90% of the total olive production in the world occurs. In more modern times, the olive tree has continued to spread outside the MB, and today it is farmed in places as far removed from its origins as southern Africa, Australia, Japan, and China (IOC). A huge number of olive cultivars in all growing areas were yielded from empiric local selection within olives and crosses between previously selected or introduced cultivars and other local cultivars or wild olives [9]. The traditional olive cultivars that are still widely used are selections from the local autochthonous cultivars, which are well adapted to their natural environment. Breeding of new cultivars are now devoted towards developing and selection of new varieties more suitable to modern olive agriculture [10]. The recurrent selection for high production in the original local region filtered potentially valuable genetic variants and associated phenotypes out of olive

cultivars, ranging from disease and insect resistance to drought tolerance. Wild olive germplasm (*O. europaea* subsp. *europaea* var. *sylvestris*) and olive subspecies (*O. europaea* subsp. *cuspidata*) have also been identified as a possible source of tolerance to abiotic stress [11–13]. A wide genetic diversity has been reported for wild olives, which could be particularly interesting for the introgression of some agronomic traits and resistance to biotic and abiotic stresses in breeding programs [14–18]. Crossbreeding between olive cultivars and wild olive with specific characters might represent a useful strategy to exploit the enormous gene pool represented by the wild olive and olive subspecies for plantations in traditional and new olive-growing countries [9,15,19–22].

In China, the olive tree has been introduced and cultivated for more than 50 years in sub-tropical areas with a non-Mediterranean climate. The estimated production of olive oil in China was only 6000 tons, almost 9 times smaller than its total olive oil consumption in 2019–2020 (IOC). It is expected to obtain more productivity of olive trees in suitable growing areas in China, especially in the western provinces of China, such as Gansu, Sichuan, Shaanxi, and Yunnan. Although a few olive clones or cultivars domesticated in the regions are well adapted to their environment, they have often lacked other desired agronomic characteristics and have failed in many cases to respond enough to new cultivation methods. The requirement for new diverse olive cultivars has increased markedly during the last three decades due to the trend of establishing olive industries with environmental adaptation to such adverse agroecological conditions. However, the potential cultivar improvement due to clonal selection is relatively limited as the genetic composition of those selections is basically unchanged or only slightly modified from that of the original cultivar [20]. Exploring the genetic diversity of the large number of well-defined olive cultivars is crucial due to their natural and basic source for creating new cultivars. The potential use of wild olive germplasm and olive subspecies also known as oleasters as a source of genetic variability for important agronomic traits has been suggested, particularly regarding resistance to specific adverse biotic and abiotic environmental conditions [17,18,21]. Olive subspecies *O. europaea* subsp. *cuspidata* is native to a widespread area from southeast Europe and northeast Africa through southwest Asia to the drier parts of Yunnan and Sichuan in China [22]. It is a plastic species adapted to different climates [22], ranging from semi-arid to meso-humid ones, and shows resistance to fungal diseases [11,23]. In Sichuan and Guangxi in China, the grafted olive trees on the rootstock of *Olea europaea* subsp. *cuspidata* showed conspicuous resistance to *Pseudomonas solanacearum* E. F. and had better suitability to the clay acidic soil in the subtropical area [24]. The crosses between cultivated olive with wild olives and olive subspecies have been recently reported [15,19,23], with the intention to enlarge the basis of genetic variability for olive breeding [9]. It is confirmed that the two subspecies are crossable and that subsp. *cuspidata* can be used as genetic resources for the olive and vice versa [11]. Furthermore, comparison and pre-breeding evaluation of the wild olive and olive subspecies progenies obtained by controlled crosses and open pollinations revealed genetic diversity [12,15,25]. In the last few decades, *O. europaea* subsp. *cuspidata* has been used for pollen donors in controlled crossing with well-defined olive cultivars as an alternative approach to increase the diversity sources in southern China [26,27]. Progenies have already been obtained by crosses and open-pollinations of cultivars, and their evaluation has been also carried out under field conditions [28,29].

The experimental crosses between several olive cultivars and the wild related subspecies *cuspidata* as pollen donors were undertaken in Yunnan and Sichuan, Southern China in the last two decades. The hybrids of two parental combinations were obtained and tested in the field trial. On account of the preceding information on crossing and evaluation, the aim of this study was to investigate the characterization of morphological traits and molecular markers in the hybrids of crosses between olive cultivars and olive subspecies, *O. europaea* subsp. *cuspidata*, representing a wide range of genetic variability and the relationships between them, and to compare and identify genotypes being potentially used as genetic resources.

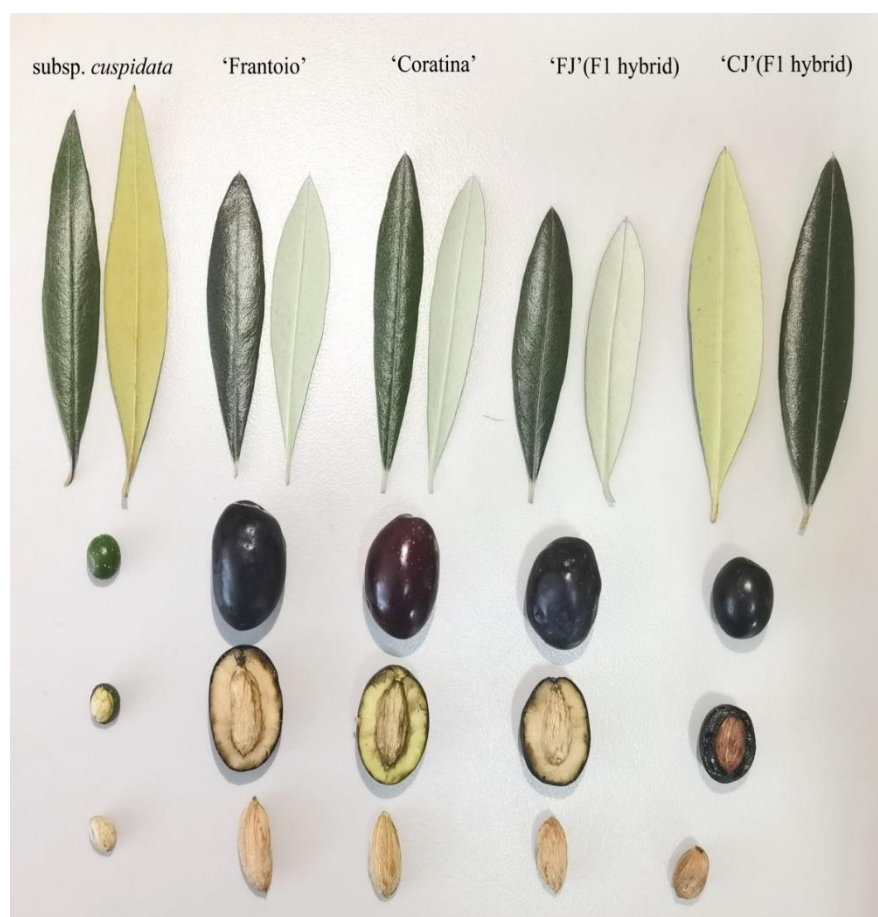
## 2. Materials and Methods

### 2.1. Plant Materials

The experimental material used in the present study comprised two F<sub>1</sub> hybrids (hereafter named 'FJ' and 'CJ'), obtained through the genetic controlled crossing of two olive cultivars with olive subspecies (*O. europaea* subsp. *cuspidata*), and their parents (Table 1 and Figure 1). The two F<sub>1</sub> hybrids were supposed to be the result of crossing combinations between 'Frantio' and 'Coratina' as the mother parents with pollen from the olive subspecies (*O. europaea* subsp. *cuspidata*) collected in Southern China.

**Table 1.** Two F<sub>1</sub> hybrids of olive inter-subspecies and their parents.

F <sub>1</sub> Hybrid	Crossing	Collecting Region
'FJ'	'Frantio' × subsp. <i>cuspidata</i>	Yunnan Province, Southern China
'CJ'	'Coratina' × subsp. <i>cuspidata</i>	Sichuan Province, Southern China



**Figure 1.** Morphological characters of leaf, fruit and endocarp for *O. europaea* subsp. *cuspidata*, 'Frantio', 'Coratina', 'FJ' and 'CJ' from left to right in the line.

### 2.2. Morphological Characterization

Morphological description was performed according to the methodology for primary characterization on leaf, fruit and endocarp in the World Catalogue of Olive Varieties published by IOC [30], including four leaf characteristics (length, width, shape and layer color of scales below), five fruit characteristics (length, width, shape, apex and base), and seven endocarp characteristics (length, width, shape, apex, base, number of grooves and tip of apex) (Table 2).

**Table 2.** Ten qualitative traits of morphological characteristics in two olive hybrid progenies and their parents.

Qualitative Trait		<i>Subsp. cuspidata</i> (♂)	'Frantoio' (♀)	'Coratina' (♀)	'FJ' (F <sub>1</sub> Hybrid)	'CJ' (F <sub>1</sub> Hybrid)
Leaf	Underside color	Brown	Silvery-grey	Silvery-grey	Silvery-grey	Light brown
	Shape	Lanceolate	Elliptic	Elliptic-lanceolate	Elliptic-lanceolate	Elliptic-lanceolate
Fruit	Shape	Spherical	Ovoid	Elongated	Ovoid	Ovoid
	Apex	Rounded	Rounded	Rounded	Rounded	Rounded
	Base	Rounded	Truncated	Rounded	Truncated	Rounded
Endocarp	Shape	Ovoid	Elliptic	Elongated	Elliptic	Ovoid
	Apex	Rounded	Pointed	Pointed	Rounded	Rounded
	Base	Rounded	Pointed	Pointed	Rounded	Rounded
	Number of grooves	High	Medium	Medium	High	High
	Tip of apex	Without mucro	With mucro	With mucro	Without mucro	Without mucro

The morphological investigation included 6 quantitative traits and 10 qualitative traits of morphological characters (Table 3). All the measurements were evaluated for random samples of 40 leaves and fruits from each tree at the height of shoulder from southern side of the canopy. Endocarps were removed and were subject to characterization.

**Table 3.** Mean and range of six quantitative traits of morphological characteristics in two F<sub>1</sub> hybrids and their parents.

Quantitative Trait		<i>Subsp. cuspidata</i> (♂)	'Frantoio' (♀)	'Coratina' (♀)	'FJ' (F <sub>1</sub> Hybrid)	'CJ' (F <sub>1</sub> Hybrid)
Leaf	Length/cm	7.62 (6.66–8.63)	6.43 (5–8.9)	6.27 (5.1–7.1)	5.29 (4.57–6.12)	6.41 (4.8–8.1)
	Width/cm	1.21 (0.87–1.51)	1.69 (1.28–2.09)	1.32 (1.05–1.6)	1.15 (0.97–1.42)	1.23 (0.9–1.5)
	Shape index	6.30 (4.41–10.65)	3.86 (3.03–5.26)	4.83 (3.27–6.38)	4.6 (3.22–6.31)	5.21 (3.2–9)
Fruit	Length/cm	1.17	1.64 (1.5–1.9)	1.41 (1.3–1.5)	1.55 (1.39–1.7)	1.54 (1.37–1.72)
	Width/cm	0.96	2.33 (2.1–2.9)	2.2 (2–2.4)	1.13 (1.03–1.25)	1.21 (1.07–1.34)
	Shape index	1.22	1.42 (1.11–1.93)	1.56 (1.5–1.62)	1.37 (1.24–1.37)	1.28 (1.18–1.45)
Endocarp	Length/mm	7–8	17.5 (16–19)	18.3 (17–20)	12.82 (11.65–14.18)	12.64 (11.45–13.9)
	Width/mm	5–6	8.36 (7.85–9.78)	7.81 (7.58–8)	7.09 (6.54–7.77)	7.48 (6.82–8.24)
	Shape index	1.17–1.6	2.1 (1.84–2.27)	2.34 (2.23–2.57)	1.81 (1.67–1.93)	1.69 (1.58–1.83)

### 2.3. DNA Extraction and Microsatellite Amplification

Total genomic DNA was extracted from leaves according to the CTAB method with slight modifications [31]. DNA concentration and purity were evaluated by NanoDrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

A set of six microsatellite (SSR) primer pairs was used for fingerprinting the progenies and their parents (Table 4). The choice was made based on literature data and the degree of polymorphism, as well as on the clearness and reproducibility of amplified DNA fragments. Three SSRs (DCA3, DCA11 and DCA18) have been developed by Sefc et al. (2000) [32], two (UDO99-11 and UDO99-19) by Cipriani et al. (2002) [33] and one (EMO90) by de la Rosa et al. (2013) [34]. PCR amplifications were carried out in a final volume of 20 µL containing 5 ng of DNA, 1.5 mM MgCl<sub>2</sub>, 60 µM dNTPs, 1.25 units of Taq DNA polymerase (TaKaRa), 1 µM of forward primer labeled with a fluorescent dye (FAM or HEX) and 1 µM of reverse primer. SSR amplifications were performed on a thermal cycler (Biometra®) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s (denaturation), T<sub>m</sub> for 30 s (annealing) and 72 °C for 30 s (extension), and a final extension at 72 °C for 7 min.

**Table 4.** Primer sequences of six microsatellite loci used in molecular analysis.

Source	SSR	Primer Sequence (5′–3′)	Repeat Motif
Sefc et al., 2000 [32]	DCA3	F- CCCAAGCGGAGGTGTATATTGTTAC	(GA)19
		R- TGCTTTTGTCTGTTTGGAGATGTTG	
	DCA11	F- GATCAAACACTACTGCACGAGAGAG R-TTGTCTAGTGAACCCCTTAAACC	(GA)26(GGGA)4
Cipriani et al., 2002 [33]	DCA18	F- AAGAAAGAAAAAGGCAGAATTAAGC	(CA)4CT(CA)3 (GA)19
		R- GTTTTCGTCTCTACATAAGTGAC	
	UDO99-11	F-TGACTCCCTTTAAACTCATCAGG R-TGCGCATGTAGATGTGAATATG	(CT)7(CA)10(CT)2(CA)2 CT(CA)2CT(CA)9
De la Rosa et al., 2013 [34]	UDO99-19	F-TCCCTGTAGCCTCGTCTTG R-GGCCTGATCATCGATACTC	(GT)20(AT)5
		F-CATCCGGATTTCTTGCTTTT R-AGCGAATGTAGCTTTGCATGT	
	EMO90		(CA)10

After successful amplification, evaluated by electrophoresis in 1.5% agarose gel, PCR products were combined with Liz 500 internal size standards and were separated using an automatic capillary sequencer (ABI 3730 Genetic Analyser Applied Biosystems). The fragments were visualized as peaks with the respective size and intensities using Peak Scanner v.1.0 and GeneMapper v.4.0 softwares from Applied Biosystems (Foster City, CA, USA).

#### 2.4. Parentage Analysis Using SSR Markers

According to the microsatellite profile, the offspring of each cross was classified as true hybrid or foreign origin. Parentage was analyzed based on the inheritance of one allele at a single SSR locus transmitted from the parent to the offspring. If one maternal and one paternal allele were present for each locus, the progeny was considered a ‘true hybrid’. In the case where the parentage was different from that expected, the real parent was estimated [7,35].

#### 2.5. Genomic DNA Isolation and DNA Sequencing of SCNL

Total genomic DNA was isolated and purified from leaf tissue (100 mg) using the DNeasy Plant Mini Kit (Tiangen Biotech Beijing, Beijing, China) following the manufacturer’s instructions. The DNA precipitate was dissolved in 50 µL TE solution and stored at –20 °C until use.

A set of primer pairs for single-copy nuclear loci (SCNL) have been developed and screened for the identification of olive cultivars and subsp. *cuspidata* by Shao (2019) [36]. Three SCNLs (SWH1, SWH5 and SWH6) were used for amplification of single-copy genes in the progenies and their parents (Table 5). PCR amplifications were carried out in a final volume of 25 µL containing 5 ng of DNA, 12.5 µL PCR mix (2.5µL 10× Buffer solution, 2.5 µL dNTPs, 1.25 units of Taq DNA polymerase), 1 µM of primer and 1 µM of reverse primer. PCR amplifications were performed on a thermal cycler (ABI Veriti96) under the following conditions: initial denaturation at 94 °C for 4 min, followed by 10 cycles at 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing) and 72 °C for 2 min (extension), and 26 cycles at 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing) and 72 °C for 2 min (extension), and a final extension at 72 °C for 10 min. After successful amplification, evaluated by electrophoresis in 1.5% agarose gel, PCR reaction products were analyzed using an automatic imager G-Box (Chemi XX6, Syngene, Karnataka, India). The PCR products were purified and sequenced using Sanger sequencing by TsingKe Company (Beijing, China) in Beijing.

**Table 5.** Characteristics of single-copy nuclear loci (SCNL) developed for *O. europaea* L. used in sequencing analysis.

Locus	Primer Sequence (5'–3')	Tm (°C)	GenBank Accession No.
SWH1	F-ACTTCATTTTACACCCATTTT R-CACTTGATGCTTTTGTTCCTTT	50	MG725069–MG725089
SWH5	F-CAAGAAAAACAAAGAAAGAGCA R-GTAAACTACCTGGGAACAAACC	52	MG725153–MG725173
SWH6	F-GGCAGAGGATTACAGGACAGG R-AGAGGGAGAAAGGGGTAGCAG	58	MG725174–MG725194

### 2.6. Data Analysis

After capillary electrophoresis of SSR primers were considered for alleles and allelic data obtained from GeneMapper V4.0 analysis, peaks in allelic profiles were analyzed using SSR markers for the variation between two F<sub>1</sub> hybrids and their parents. Contig Express software was used to analyze the peak maps of genome sequences and cut off the 50~60 bp low-quality sequence sites at both ends of the sequences, and the overlapped sequences were manually collated. Then, BioEdit was used to perform sequence alignment analysis on the effective data.

## 3. Results and Discussions

### 3.1. Morphological Characterization of Two Inter-Subspecific Progenies and Their Parents

The morphological criteria of olive description presented in the World Catalog of Olive Varieties [30] were used to describe two progenies and their parents on a total of sixteen recorded traits, including in quantitative and qualitative characters of leaf, fruit and endocarp. Morphological characteristics of five samples are presented in Table 2 and shown in Figure 1. The results of morphological characterization showed the specific differences between two olive cultivars, 'Frantoio', 'Coratina', and subsp. *cuspidata* on leaf, fruit and endocarp. The upper surface of the leaves of subsp. *cuspidata* was less varnished than subsp. *europaea*, and the lower face was a golden green (sometimes reddish) and not silvered as for subsp. *europaea* [11]. The endocarp of subsp. *cuspidata* had an ovoid shape, while that of 'Frantoio' and 'Coratina' were represented as being elliptic and elongated [37].

Between two olive cultivars as mother parents of two F<sub>1</sub> hybrids, 'Frantoio' was significantly different from 'Coratina' in the shapes of leaf, fruit and endocarp. Two F<sub>1</sub> hybrids, 'FJ' and 'CJ', were very similar in phenotypic traits with the difference for the shapes of fruit base and kernel. Fruit shape, base and endocarp shape of 'FJ' were longated, truncated and elliptic, while 'CJ' had ovoid fruit shape, rounded fruit base and ovoid endocarp shape. Comparison of the two hybrids with their mother parents showed that both 'FJ' and its mother parent, 'Frantoio', were similar in fruit base and endocarp shape. In terms of the difference in the shapes of leaf, fruit, endocarp and fruit base, both 'FJ' and 'CJ' showed similarity with their father parent, subsp. *cuspidata*, for other traits. In particular, the hybrid 'CJ' was similar with its father parent, subsp. *cuspidata*, but different for the shapes of leaf and fruit. Moreover, the undersides of the leaves of the mother parent, 'Frantoio' and 'Coratina', and the hybrid 'FJ' were silvery-grey in color with pointed tips. In contrast to this, the hybrid 'CJ' and its father parent, subsp. *cuspidata*, showed brown undersides of the leaves with hooked tips.

The mean and range of leaf, fruit and endocarp in size were shown in Table 3. There were the differences in traits between two hybrids and their parents. Except for leaf length and width, all traits of father parent. *O. europaea* subsp. *cuspidata*, were lower than those of mother parents and two hybrids. Between two mother parent olive cultivars, each quantitative trait of 'Frantoio' was higher than that of 'Coratina', with the exception of endocarp length. Fruit and endocarp of two F<sub>1</sub> hybrids were between mother parent and father parent in size. Fruit length and endocarp length of 'FJ' were higher than those of parents and another F<sub>1</sub> hybrid, 'CJ'. The comparison between two F<sub>1</sub> hybrids showed that leaf length and width, fruit width and endocarp width of 'FJ' were lower than those of 'CJ'.

In general, morphological description of olive cultivars involves characteristics of the tree, fruit, leaf and inflorescences, among others. Of these, endocarp characteristics are considered as the most discriminating and stable ones of strict genetic control, while other characteristics, such as those of the fruit, are more influenced by environmental conditions [37]. Moreover, endocarps may also be conserved for a long time, and they are easily exchanged among collections. For these reasons, the description of the endocarp has been frequently used to catalog olive cultivars [30,38–40].

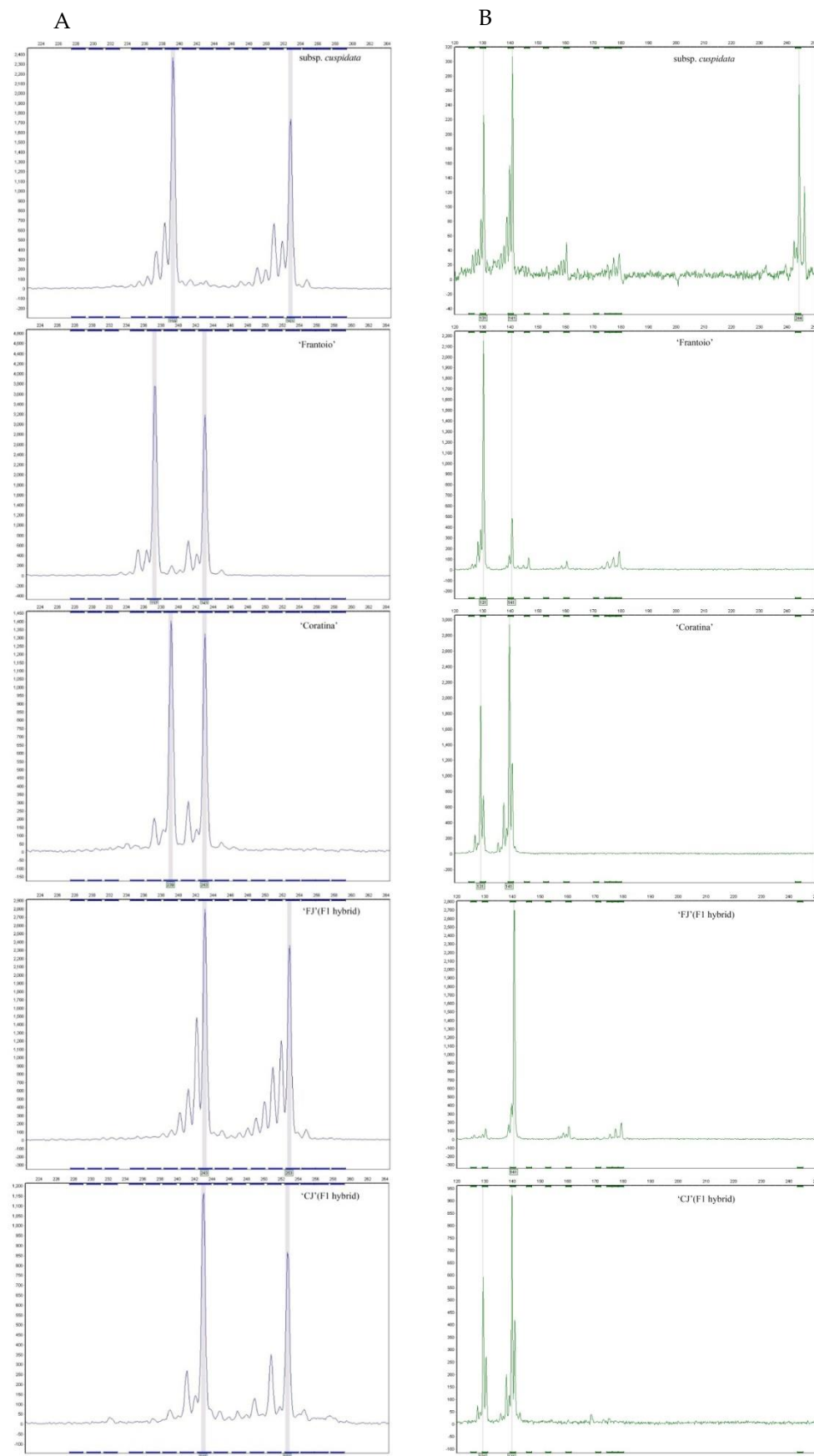
### 3.2. Parentage Analysis and Progeny Discrimination by SSR

The six microsatellites appertaining to the series DCA [24], UDO [25] and EMO [26] were successfully amplified in all the studied five genotypes, two hybrids and their parents (Table 6). Five SSR loci showed polymorphisms between mother parents, ‘Frantoio’ and ‘Coratina’. The microsatellite allelic profiles of two F<sub>1</sub> hybrids were compared with those of their related parental lines because alleles at all SSR loci of offsprings were inherited from their parents. The inheritance of SSR alleles for ‘FJ’ and its parents revealed that those of 243bp and 253bp at DCA3 (Figure 2A), 141bp at DCA11 (Figure 2B) were inherited from ‘Frantoio’ and subsp. *cuspidata*, respectively. Similarly, the 172bp and 174bp at DCA18, 114bp and 120bp at UDO11, 130bp and 166bp at UDO19 were also inherited from ‘Frantoio’ and subsp. *cuspidata*, respectively. In the same way, another F<sub>1</sub> hybrid ‘CJ’ inherited SSR alleles from its parents, ‘Coratina’ and subsp. *cuspidata*. Two F<sub>1</sub> hybrids (‘FJ’ and ‘CJ’) were confirmed as the offspring of their cited parents because their patterns of SSR alleles were successfully matched to their mother and father on the basis of the presence of parental-specific SSR alleles.

**Table 6.** SSR molecular markers of two F<sub>1</sub> hybrids and their parents.

Hybrid/Cultivars	SSR Locus					
	DCA3	DCA11	DCA18	UDO11	UDO19	EMO90
<i>subsp.cuspidata</i> (♂)	239/253	131/141/244	172/196	112/114	130/130	182/186
‘Frantoio’ (♀)	237/243	131/141	174/176	110/120	130/166	186/192
‘Coratina’ (♀)	239/243	131/141	174/178	110/126	112/112	186/192
‘FJ’ (F <sub>1</sub> hybrid)	243/253	141/141	172/174	114/120	130/166	186/186
‘CJ’ (F <sub>1</sub> hybrid)	243/253	131/141	172/178	112/126	112/112	182/186

The SSR markers in this study have been previously used in the genotyping of olive cultivars ‘Frantoio’ and ‘Cortina’ [35,39]. In theory, SSR markers should produce the same profile regardless of the analysis system, the conditions or the equipment used, so that the data are comparable. Nevertheless, it has been shown that SSR profiles may differ between different laboratories and that these differences may be equally attributable to the equipment and/or the technical ability of the worker [41]. In this study, olive cultivars ‘Frantoio’ and ‘Coratina’ were detected by the alleles of 174/176 bp and 174/178 bp in DCA 18. There was a 3 bp allele difference between the alleles in DCA 18, which produced 177/179 bp and 177/181 bp of ‘Frantoio’ and ‘Coratina’ in the previously published data [5,39]. These discrepancies may be explained by experimental errors or sizing discrepancies, as has been previously observed in similar studies of olive cultivar identification for the standardization of a set of microsatellite markers [41].

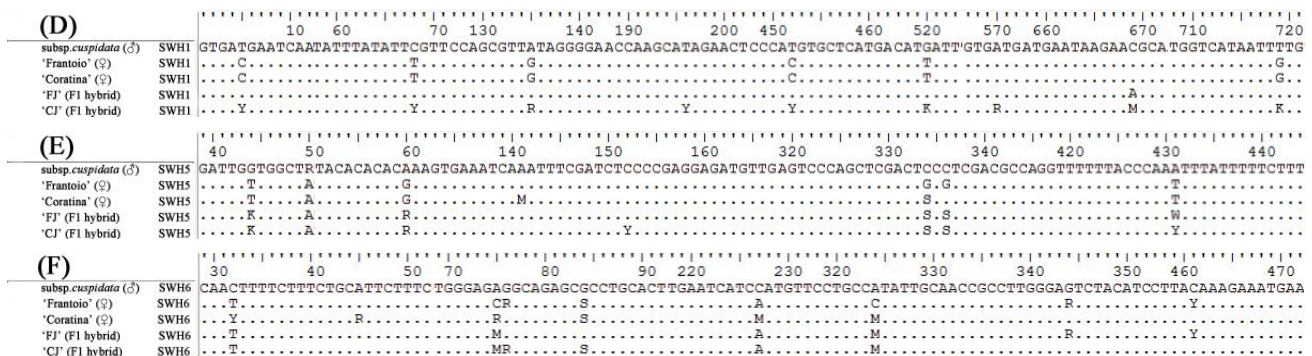


**Figure 2.** Allelic profiles at SSR locus DCA3 (A) and DCA 11(B) of DNAs from *O. europaea* subsp. *cuspidata*, 'Frantoio', 'Coratina' and two F<sub>1</sub> hybrids, 'FJ' and 'CJ' in the line.



### 3.3. DNA Sequence Alignment of Progenies and Their Parents by SCNL

Besnard and El Bakkali (2014) investigated the sequence variation at five nuclear single-copy genes in 41 native and invasive accessions of the Mediterranean and African olive subspecies, including in the wild individuals from the native range (13 *cuspidata* and 17 *europaea*) and 11 trees from the invasive range [42]. Their data confirm that four invasive individuals are early-generation hybrids, which indicate that sequences of single-copy genes are powerful enough to detect introgression from a subspecies to another. In the study, three SCNLs (SWH1, SWH5 and SWH6) [29] were used for amplification of single-copy genes in the two progenies and their parents. After that, PCR products were sequenced and performed alignment analysis on the effective data (Figure 3). Results of the sequence comparison showed that there were a total of 15 different base sites between two progenies, 'FJ' and 'CJ', among which there were the number of different base sites, nine in SWH1 (Figure 3D), two in SWH5 (Figure 3E) and four in SWH6 (Figure 3F) amplified sequences. In addition, the 5th, 68th, 135th, 452nd, 520th and 719th sites in SWH1 amplified sequence (Figure 3D) and the superposed 44th and 60th sites in SWH5 amplification sequence (Figure 3E) confirmed that 'CJ' was a true hybrid of 'Coratina' and *subsp. cuspidata*. Similarly, the superposed bases at the 44th, 60th, 334th, 336th and 431st base sites in the SWH5 sequence (Figure 3E) and the 75<sup>th</sup> and 324th in SWH6 sequence (Figure 3F) indicated that 'FJ' was the true hybrid between 'Frantoio' and *subsp. cuspidata*.



**Figure 3.** DNA sequence alignment of the PCR product amplified by SCNLs, SWH1 (D), SWH5 (E) and SWH6 (F) with parents (*subsp. cuspidata*, 'Frantoio', 'Coratina') and two F<sub>1</sub> hybrids ('FJ' and 'CJ').

A genomic approach based on genotyping by sequencing (GBS) is promising and may soon allow the investigation of patterns of differentiation and admixture over the whole olive complex [42]. In fact, the utility of single-copy gene sequences in olive population genomic and phylogenetic studies would provide more integrated and adequate information for phylogenetic and population-based studies, improving efficient discrimination during species identification and resolving the phylogenetic relationships and cultivar identification.

## 4. Conclusions

Five samples composed of two olive cultivars introduced from Italy, *subsp. cuspidata* from southern China and two F<sub>1</sub> hybrids obtained from two genetic combinations were investigated morphologically, measuring both qualitative and quantitative traits of leaf, fruit and endocarp and at the molecular level by SSR markers and SCNLs. A significant difference in morphological traits was found between two progenies and their parents, olive cultivars as the mother and *subsp. cuspidata* as the father. The hybrid 'CJ' and its father parent, *subsp. cuspidata*, showed brown undersides of the leaves with hooked tips. Fruit and endocarp two hybrids were between the mother parent and father parent in size. Two F<sub>1</sub> hybrids were confirmed as offspring of their cited parents because their patterns of SSR alleles were successfully matched to their mother and father on the basis of the presence of parental-specific SSR alleles. Results of the sequence alignment with three

SCNLS showed that there were a total of 15 different base sites between two progenies, and their amplified sequences indicated that two progenies were the true hybrid between olive cultivars and subsp. *cuspidata*.

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