

## Identification of olive pollen donor trees and pollinizers under controlled pollination environment using STR markers

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### Abstract

In the olive tree, the bag method is efficiently used to determine whether the pollen donor is compatible, and thus can be used as a pollinizer. Besides, paternity tests are claimed to enable identification of the pollen donor trees and thus pollinizers, via embryo genotype testing based on short tandem repeat (STR) markers. We examined here, on concrete data, gathered from studies in the literature, the advantages and drawbacks of both methods. We implemented the published data, reasoning in the frame of the sporophytic self-incompatibility model, by i) calculating the fruit set rate referred to 100 hermaphroditic flowers, ii) introducing the S-allele determinants in stigmata, iii) adding the S-pollen determinant(s) that coated pollen grain for each variety, when deciphered for each variety. Cross compatibility/incompatibility was deduced from theory and compared to recorded experimental fruit set. New conclusions revealed that when a variety failed as pollen donor, it was not always incompatible in theory. This fully changes previous conclusions. Thus, we suggest combining the bag method and STR protocol to answer most unsolved queries and to bring information dealing with fertilization by unwanted pollen in the host variety, and whether self-pollination may have occurred at the same time as some crosses. We showed that introduction of the sporophytic self-incompatibility model and attribution of S-alleles pairs to varieties, both efficiently improve the bag method and paternity tests on embryos harvested under the bags leading to a trustworthy identification of pollinizers for more varieties.

**Keywords:** *Olea europaea*; Paternity tests, Pollinizers; Self-incompatibility.

**Abbreviations:** STR: short tandem repeat; SSR: single sequence repeat;

### Introduction

To look for pollinizers in the olive tree two main ways are used based on our literature survey. The first method will be named 'wrapping bag method', it consists in controlling pollination: this means that the flowers on one branch were enclosed for isolation from airborne pollen in a paper bag, - verified to be pollen proof in the olive tree - two days before flowering -, and then, when half of the flowers are opened in the tree, the pollen from a known variety is introduced into the bag. Six to eight weeks later the fruit are counted.

In the olive tree, routinely, controlled pollination within a bag that wraps a branch of the host carrying inflorescences (further called 'the bag method') is used efficiently to determine whether the pollen donor is compatible, and thus, can be used as a pollinizer. Besides, from fruit harvested in commercial orchards on a known host variety, paternity tests are claimed to enable identification of the pollen donor trees and thus pollinizers, via embryo genotype testing based on short tandem repeat (STR) markers. However, substitution of the bag method by paternity tests has been suggested. The bag method has proved itself as able to release pertinent pollinizers, but cannot determine which embryos were fertilized by unwanted pollen or

whether self-pollination has occurred at the same time as crosses.

Recently, Breton et al. (2014) and Farinelli et al. (2015) have widely explained cautions that should be taken to calculate the rate for fruit setting under the bag based on 100 hermaphroditic flowers. S-alleles have been deciphered based on the sporophytic model inferring between them co-dominance in the stigma and style, whereas dominance relationships may exist between S-alleles leading to the expression of one or two determinants coating the pollen (Breton and Bervillé, 2012).

The second way will be named short tandem repeat (STR) protocol 'STR protocol', it is based on microsatellite (SSR for single sequence repeat) molecular markers that are used to identify the father of each embryo. In fact it consists for each embryo harvested in one host variety in reconstructing the profile in microsatellite markers to identify those markers brought from the pollen donor. One has to notice that the first protocol is based on a certainty: the pollen introduced into the bag has a known origin, whereas the second protocol is based on what has to be considered as a probability in the identification of the father. Whatever the software used to attribute the father, the probability of

identification is never a certainty. It is given as 'the most probable father', even if the level of confidence is higher than 99 %. It is necessary to explain further what 'the most probable father' means. However, under a bag, fruit setting may occur due to self-pollination and to crossing by foreign pollen. To bring clear and solid explanations, it is required to dissect most steps on bag method and STR protocol.

Recently, Arbeiter et al. (2014) asserted identification of pollen donors by analyzing embryos sampled on the variety "Istrska Belica" using microsatellite markers. Thirty-one embryo DNA samples, from fruit harvested in one tree in an orchard, were analyzed by STR markers, but only twelve were issued from an identified father and lead to identify seven different pollen donors. The Authors did not identify embryos issued by self-pollination of the variety. Authors concluded that i) the best pollinators are "Leccio del corno" as well as "Leccino" based on a few embryos, and ii) since self-pollination has not been observed, does this mean that self-pollination has not occurred or does it mean that self-pollination cannot occur? Are these conclusions solid and is the method universally applicable in the deciphering of pollinizers in this species?

To discuss efficiently and objectively the conclusions from the data, we introduced the self-incompatibility model drawn for the olive tree by Breton and Bervillé (2012) and confirmed in Breton et al. (2014) as in Farinelli et al. (2015). The model attributes two *S*-alleles to every olive variety. Based on Ugrinović and Štampar (1996) cross data, the *S*-allele pair remained unattributed to the variety "Istrska Belica". In Arbeiter et al. (2014) most of the varieties used in this study have been deciphered for the *S*-allele pair each carries. Consequently, attribution of the pair *R4R6* to "Istrska Belica" was suggested. Recently, the *S*-allele pair *R4R6* has been predicted to confer the highest self-fertility rate (Breton et al., 2016). This was sustained in "Moraiolo" known as highly self-fertile (Farinelli et al., 2015). "Moraiolo" genotype is *R4R6*, the determinants expressed in stigma and style are [*R4R6*], and the determinant expressed in the pollen is *R6*. Because embryos issued from self-pollination have not been detected by Arbeiter et al. (2014) using paternity tests; this asks us how far we can trust paternity tests in detecting self-pollination. Paternity tests have been used and are still used in forest trees to identify the species of pollen origin, not the pollen donor tree itself, because STR markers have been screened to be discriminant between two species in *Larix* (Wei et al., 2015), or in oaks (Gailing et al., 2014; Jensen et al., 2009). In forest tree, nobody tried identifying the father tree of an embryo of the same species. The bag method has been used widely in the olive tree, but most studies did not conclude with the identification of adequate pollinizers for host varieties. Thus, the main steps that could lead to biases in identification of pollen donors were highlighted, leading to possible errors in identifying pollinizers. It is of great importance not to release to olive-growers, inefficient so-called pollinizers that are planted for tens of years, because olive orchards are set up for decades. Instead of opposing the bag method and the paternity tests, the steps and protocols that lead to doubtful and wrong data and thus doubtful conclusions were stressed for the bag method and paternity tests. We elicited the reasons that lead to doubts on methods and errors in pollinizer identification.

Here, we did not carry out field experiments. We examined published data and we reasoned in the frame of the sporophytic self-incompatibility model, we added i) fruit set referring to 100 hermaphroditic flowers and not to one inflorescence only; ii) the *S*-allele pair for varieties already deciphered, which enabled us to predict *S*-determinants in stigma and *S*-determinants that coated pollen grains, and iii) Cross compatibility/incompatibility was deduced from the theory and compared to recorded experimental fruit set. Consequently, we provided new interpretations and conclusions from these data. We concluded in giving a new protocol to conciliate advantages of both methods to lead to a unique strict and trustworthy method to identify pollinizers in the olive.

## Results

### *Data collected from olive paternity test studies*

#### *Data collected from Ugrinović and Štampar (1996) experiments*

The partial diallel design included varieties "Istrska Belica", "Pendolino" and "Leccino". Based on fruit setting after self-pollination "Istrska Belica" displayed the highest rate (0.21 fruit / inflorescence, F/Inf), "Pendolino" (0.03 F/Inf) and "Leccino" (0.14 F/Inf). Inter-crosses showed the highest rates for "Leccino" x "Istrska Belica" (5.45 F/Inf) and "Leccino" x "Pendolino" (5.75 F/Inf).

In the reverse direction "Istrska Belica" x "Leccino" appeared compatible (3.79 F/Inf). "Pendolino" and "Istrska Belica" in both directions remained undetermined. Based on these data and the Breton's et al. model (Breton et al., 2014) the pairs *R1R2* and *R1R5* have been attributed to "Pendolino" and "Leccino" (Farinelli et al., 2015), respectively, but no conclusion was drawn for "Istrska Belica" because we did not know both the number of flowers per inflorescence and the percentage of hermaphroditic flowers.

#### *Data collected from Arbeiter et al. (2014) experiments*

Table 1 shows the number of embryos assigned to putative pollen donors in "Istrska Belica". Based on these data and the Breton's et al. model (Breton et al., 2014) enables us to assert that the pollen donors "Ascolana tenera", "Leccino", and "Picholine" carrying the *S*-allele pairs *R2R4*, *R1R5*, and *R1R3*, respectively, are father of some embryos (Table 1). However, all varieties carrying one of these pairs were not assigned as father (Table 2). Consequently, failure of "Moraiolo" as pollen donor to "Istrska Belica" cannot be attributed to incompatibility between the two varieties.

#### *Data collected from Marchese et al. (2016) experiments*

Basically, self-fertility and cross-compatibility in "Arbequina" and "Koroneiki" have been determined in a high density orchard. Because cross efficiency had appeared low to Marchese et al. (2016), gene flow through open-pollination was determined from other varieties grown in the vicinity. Their data are summarized in Table 3, but we have added some knowledge, since we know the *S*-allele pairs in "Arbequina" and "Koroneiki", we can declare that the 2 varieties are compatible in both directions of the crosses.

**Table 1.** Number of embryos assigned to putative pollen donors in “Istrska Belica.” rewritten from Ugrinović and Štampar (1996) with the attributed S-allele pairs to each variety.

Pollen donors	Father assigned	S-allele pair	Eliminated S-alleles in Istrska Belica
“Ascolana tenera”	1	R2R4	R2
“Buga”	1	nd	
“Črnica”	1	nd	
“Grignan”	1	nd	
“Leccino”	3	R1R5	R1, R5
“Leccio del corno”	3	nd	
“Picholine”	2	R1R3	R1, R3
Total number of embryos	12		
			Putative S-alleles in Istrska Belica
			R4R6

S-allele pair: R1R2; S-determinants expressed in stigma [R1R2]; S-determinant expressed in pollen: R2.

**Table 2.** Putative compatible pollen donors in “Istrska Belica.” without fruit setting rewritten from Arbeiter et al. (2014) with the attributed S-allele pairs and pollen S-determinant to each variety ICO: inter-compatible; IIC: inter-incompatible.

Pollen donors	Number of embryos	Pollen determinants	Expected
“Pendolino”	0	R2	ICO
“Arbequina”	0	R1R3	ICO
“Itrana”	0	R2	ICO
“Santa Catherina”	0	R2	ICO
“Athena”	0	R2	ICO
“Coratina”	0	R2	ICO
“Maurino”	0	R2	ICO
“Frantoio”	0	R5	ICO
“Moraiole”	0	R6	IIC

**Table 3.** Percentage of self-fertilization of “Arbequina” and “Koroneiki” in self-pollination test and open pollination test rewritten from Marchese et al. (2016) with the attributed S-allele pairs and pollen determinant to each variety “Arbequina” host.

Pollen donor	Stigma	Pollen	Number of embryos	% of self-pollination	Expected
“Arbequina”	[R1R3]	R1R3		0	SI
“Arbosana”	[R4R5]	R5	2	0	ICO
“Biancolilla”	[R4R5]	R5	1		ICO
“Brandofino”	nd	R2R5R6*	1		ICO
“Erbano”	[R4R5]	R5	2		ICO
“Giarraffa”	[R2R3]	R2	1		ICO
“Minuta”	[R4R5]	R5	2		ICO
“Nocellara de Belice”	nd	R2R5R6*	2		ICO
“Nocellara Messinese”	[R4R5]	R5	2		ICO
“Name lacking”	nd		2		

“Koroneiki” host

Pollen donor	Stigma	Pollen	Number of embryos	% of self-pollination	Expected
“Koroneiki”	[R2R6]	R6		70	SF
“Aitana”	[R4R5]	R5	2	0	ICO
“Arbosana”	[R4R5]	R5	1		ICO
“Erbano”	[R4R5]	R5	1		ICO
“Indemoniata”	[R4R5]	R5	2		ICO

**Table 4.** Number of pollen tubes (n # 60) in ovules of host varieties rewritten from Seifi et al. (2015) with the attributed S-allele pairs and pollen determinants to each variety. IPI: index of pollination; S-d: S-determinant; SF: self-fertile; IIC inter-incompatible; ICO: inter-compatible.

Year	Host variety	S-d stigma	Pollen donor	Pollen tube in ovule	IPI	S-d pollen
2004	“Frantoio” self R4R5	[R4R5]	“Frantoio”	0	0	R5
			0			
			“Barnea”	1		R6
			“Mission”	9		R3
			“Koroneiki”	0		R6
2004	Open “Koroneiki” self	[R2R6]	“Koroneiki”	22		
			0	0	R6	
			“Barnea”	0	0	R3
			“Mission”	6	0.43	R3
			“Frantoio”	3	0.21	R5
2004	Open “Kalamata” self	[R2R4]	“Kalamata”	14		
			0	0	R2	
			“Barnea”	3	1.08	R3
			“Mission”	0	0	R3
			“Frantoio”	0	0.15	R5
2005	Open “Kalamata” self	[R2R4]	“Kalamata”	1		
			0	0.18	R2	
			“Barnea”	1	1.36	R3
			“Mission”	0	0.36	R3
			“Frantoio”	2	0.64	R5
	Open			0		

Moreover, with "Arbequina" [R1R3] the varieties "Arbosana", "Biancolilla", "Erbano", "Minuta", and "Nocellara Messinese" are compatible, all producing pollen carrying the R5 determinant (symbolized R5), plus "Giarraffa" producing pollen R2. With "Koroneiki" [R2R6] compatible varieties are "Aitana", "Arbosana", "Erbano", "Indemoniata" all producing pollen R5. Data collected from Seifi et al. (2011, 2012) experiments "Kalamata" (as a host) with "Barnea", "Benito", and "Katsourela" (six "Kalamata" embryos assigned in each) gave embryos, but none with "Arbequina", "Azapa", and "Picual" (zero "Kalamata" embryos assigned in each). By the introduction of the S-allele pair for each variety in Table 4, it is possible to check whether the number of embryos is correlated with incompatibility (0 or low number of embryos) and cross compatibility (high number), for each pairwise combination of the varieties. Data cross results match prediction for "Barnea", but not for "Arbequina" and "Picual". Based on pollen germination data, considering pollen tubes that reached the ovule all experimental data matched prediction.

## Discussion

### **New conclusion from Arbeiter et al. (2014) experimental data**

Since "Istrska Belica" has produced embryos with seven pollen donors, that is to say: "Ascolana tenera" - which harbors the S-allele pair *R2R4*-*"Leccino"* (*R1R5*), and "Picholine" (*R1R3*), then, consequently, "Istrska Belica" cannot harbor either *R1*, *R2*, *R3* or *R5*. "Istrska Belica" should harbor *R4R6* S-alleles. However, it might carry *R4R4*, but, this S-allele pair has never been found in any olive variety yet, and this pair has been predicted to lead to self-incompatibility (Breton et al., 2016). *R6R6* cannot exist because *R6* is the most dominant S-allele in the olive tree. The only way to check whether "Istrska Belica" carries *R4R6* is to verify that crosses failed between "Istrska Belica" and varieties carrying either *R1R6*, *R2R6*, *R3R6*, *R4R6* or *R5R6*, since the pollen of all these individuals is R6. However, in Table 4 among varieties that carry *R2R4* only "Ascolana Tenera" is declared father to one embryo, whereas "Athena", "Coratina", and "Maurino" also harboring *R2R4*, thus R2, did not produce embryo in this experiment. The reason remains unknown to the authors. Based on Seifi et al. (2011) data, the pollen from these varieties has, probably, in this grove, less chance to land on the pistil of the host tree "Istrska Belica", because of the location of the trees in the orchard. The STR method does not allow concluding on negative results. In contrast, under bags the absence of embryos after the supplying of Rx pollen infers that the host varieties should carry *Rx*. However, with RyRz pollen the host varieties should carry *Ry*, *Rz* or *RyRz*. Further crosses are required to determine which S-allele(s) is (or are) present.

Olive fruit carry one pit with most of the time two embryos, 60 to 80 % of pits depending on the variety and the pollinizer (Farinelli et al., 2012). If researchers used seedlings after germination of pits, the DNA from the two embryos were not mixed as in Díaz et al. (2006) and de la Rosa et al. (2004). If researchers used the kernel to prepare DNA, the two embryos have to be separated before DNA extraction. The separation of the two embryos - when they are two - has not been done by Marchese et al. (2016), but probably this was

not done either (it is unclear) by Seifi et al. (2011) and by Arbeiter et al. (2014). In this situation, the number of embryos analyzed must be 1.6 more than the corresponding number of pits that infers one embryo per pit. Consequently, the SSR profile from each of these preparations is the sum of two profiles, and may therefore reveal 3 or 4 SSR alleles instead of the two expected. This causes concerns on the rigor of these studies. When seeds have been used to obtain seedlings, the DNA profile obtained from each embryo is correct. The average heterozygosity in the olive tree is between 75 to 85 %. One STR profile of one embryo should contain 2 SSR alleles by locus. When one embryo profile displays one STR allele at one locus, the reason is that the host tree and the pollen donor share this SSR allele. If one embryo profile displays 3 or 4 SSR alleles as reported by Marchese et al. (2016), thus, it is due to the mixture of two DNAs from two embryos in the preparation. Because each embryo has its own father, the combined profiles with 3 or 4 SSR alleles is due to one or two different fathers. Depending on the SSR alleles, the choice could or could not be done. Marchese et al. (2016) attributed a LOD score of 99% when the profile of one DNA preparation displayed only two SSR alleles at each locus and a LOD score of 80 % for the other profiles. If the two embryos have different fathers the calculation of LOD 80 % is strictly wrong.

### **Parameters that may affect fruit set within bags**

When enclosed in a bag, flowers from the host tree display several stages. The receptivity of the stigma is between 4 to 7 days depending on the varieties (Villemur et al., 1984). Thus, 5-10 bags should wrap at least 200 hermaphroditic flowers to get significant fruit set enabling comparison between bags from one pollen donor. The number of hermaphroditic flowers has to be counted (the best) or estimated (a stopgap) in each bag. However, each bag should undergo calculation separately because unwanted pollen may unequally affect fruit set in every bag. Pollen from a donor tree should not be stored overnight. Pollen is fragile and rapidly undergoes drying out. No study has been able to fix a time threshold for pollen keeping its ability to fertilize ovule. The presence of unwanted pollen has to be determined on male sterile varieties (Breton et al., 2014). Frozen pollen that has been proved successful in olive tree was not used in these studies (Villemur et al., 1984). Each series of bags - are, in fact, never repeats of an assay because of airborne pollen and separate operations in the opening of each bag. In practice, when most bags in the series are empty, one bag with fruit is suspect, and fruit set may be due to airborne pollen. In contrast, when most bags in the series display fruit, empty bags should not be included in calculation, because failure of fruit set is probably due to pollen suffering during transport or any other reasons.

### **Open-pollination rate**

Some branches have to be marked to determine the higher open-pollination rates (HO-PR) for each varieties (Farinelli et al., 2015). This rate is a specificity of each variety and is essential to calculate the inter-compatibility index (ICOI) and the index for self-incompatibility (ISI). Branches have to be chosen at different cardinal points and different levels of

the canopy of trees to take into account variation of pollen diffusion by wind, which is irregular at each tree level.

### ***Paternity tests***

#### ***To detect pollen donor***

Each team has listed varieties that have been analyzed with STR markers showing that all are differentiated. Thus, STR allele frequencies may differ between studies, and thus discriminant power of alleles at each locus may vary a bit depending on teams. However, this list may be quite different from the list of varieties considered as potential pollen donors in the 70 ha around the mother tree of the embryo. Thus, this calculation is essential.

In each area where fruit were harvested to submit embryos to STR analyses, it is required to analyze all present varieties, and most of feral and abandoned trees producing pollen. Relationships between varieties in the data bank and the genotypes in the area of potential pollen donors should be carefully determined, otherwise the risk of confusion between a potential pollen donor and the variety in the data bank increases. The 'most probable father' infers that putative fathers are clearly different. Yet, if some trees in the area of 70 ha are progenies from other trees, their distinction cannot be done, and thus the confidence in identifying the true pollen donor decreases.

Further controls should be made based on the bag method to verify whether the pollen of the most probable father is actually compatible with the mother. In any case, if the bag method is not used before paternity tests, it has to be used after, to verify at least, that the most probable father is compatible. Thus, it is better to start experiments by using the bag method, thus paternity tests will be more trustworthy because carried out on a known father, and thus the paternity test can be applied confidently.

However, for each haploid profile deduced from one embryo, no Author provided results on how many fathers it may have, because it is sure that several varieties are possible, and thus it is important to know all of them. If feral trees progenies of the 'most probable father' are present in the origin area of the embryo, then, the set of markers cannot discriminate among fathers. All authors have given 'the most probable father', which is insufficient to conclude which is surely the father of this embryo. Because several embryos are analyzed, it is likely that all of them do not always have, as father 'the more probable varieties'. It is thus important not to conclude on 'the most probable father' only.

#### ***To detect self-pollination***

The presence of one STR allele as marker is informative. But here, the whole set of markers is considered to identify a variety, that means between seven to ten loci examined by each team. The absence of one allele only means that through the meiosis process it has not been retained and transmitted to the gamete either ovule or pollen. Thus, no conclusion on its absence is valid. Because of the heterozygosity rate higher than 75 % in the olive tree, most markers at each locus, are absent in almost half of the gametes.

To assert self-pollination after paternity tests, no marker from a pollen donor has to be detected in one embryo

harvested in the host tree. Consequently, when the mother and the father share one S-allele, the diagnostic is uncertain. Whether self-pollination can occur in the presence of foreign compatible pollen is not well documented for the olive tree, either under the bag, or in an orchard. Self-pollination occurs in varieties that carry one dominant S-allele (Breton et al., 2016). Competition studies between the self- and foreign pollen have not been carried out yet. Under bags a self-compatible variety always yields less than half of the fruit it would yield with a pollinizer. The yield with adequate pollinizers may be above 3-6 times more than the yield by self-pollination (Taslimpour et al., 2008). Erroneous diagnostic for self-pollination should come when the pollen donor has given markers that already exist in the host tree. In the case of a male sterile variety such as "Lucques" and "Olivière" in the absence of compatible pollen, fruit set fails, but if a low contamination rate was observed, and because some embryos lack STR markers from pollen donors, some embryos could be declared due to self-pollination. Embryos are diploid and cannot be due to self-pollination. In any case, to detect self-pollination, all loci should be examined and at all loci, no SSR allele from a putative pollen donor can be present. It is thus clear that SSR markers cannot allow to give diagnostic for most embryos. In all studies quoted here, about half of the embryos remain without possible diagnostic. Comparison of profiles from embryos harvested on the same host tree, should theoretically reveal cross events as well as embryos issued from self-pollination. However, statistics are weak, thus no conclusion is possible, and more than half of the embryos remain without attributed father. Under a bag, when no foreign pollen is introduced, homogeneous fruit setting between bags suggests self-pollination, unless fruit set is due to unwanted pollen. In this case fruit set should appear erratic between bags due to variation in contamination rate between bags.

#### ***Setting up a novel protocol conciliating bag method and STR protocol***

We therefore suggested to researchers to combine both approaches. The bag method is paramount. All analyzed embryos should be produced under a bag. This will make acceptance or strict rejection possible whether the father matches the SSR profile. However, when fruit setting are low under bags, self-fertility does not match the sporophytic model applied to the olive tree, other molecular controls based on STR, could be performed on the embryos obtained under a bag. Consequently, after crosses, STR protocol should check : 1) the cross is well due to the pollen introduced into the bag; 2) whether self-pollination may have occurred in mixture with cross; 3) low fruit set or failure of fruit setting is due to unwanted pollen or to absence of compatible pollen.

### **Materials and methods**

#### ***Plant materials***

Several sets of olive varieties have been chosen by different teams to check self-fertility and inter-crossing. They included international varieties grown in several countries. All varieties are listed in Tables 1 to 4.

### **Experiment design**

The bag method is controlled pollination within a bag that wraps a branch carrying inflorescences just two days before flowers will open: i) to determine whether the variety may self-pollinate and ii) after another branch with pollen is introduced into the bag, whether the pollen introduced in the bag will lead to fruit set. Each team has used its own protocol with many variants in the way to bring pollen, to transfer it into bags, to count flowers either hermaphroditic or male, and to refer fruit setting under the bag, but details on all protocols are lacking in Materials and Methods section of each article. Thus, several concerns are raised all along the experiments. Then, we propose to go on step by step to reveal drawbacks and eventually faults during protocols for both bag method and STR protocol.

### **Experimental design and conduction**

Because some local varieties have difficulties to lead to fruit, researchers try to find pollinisers from other countries.

### **Common protocol used by the teams**

Since data were collected from literature, we summarized the protocols used by the teams. Trees have been chosen in collection or commercial orchards. For most of them the verification whether each matches the denomination based on STR markers, has not been carried out. Moreover, in many commercial mono varietal orchards, all individuals are not identical because of errors along the multiplication of clones and further plantation phases.

### **Traits measured**

The number of fruit within bags was recorded 6 weeks after flowering. The number of flowers per inflorescence and the percentage of hermaphroditic flowers have not been given. Thus, the number of fruit refers either to one inflorescence or to 100 hermaphroditic flowers.

### **Paternity tests**

For paternity tests, embryos were harvested from seeds of an identified mother, the host, but the origin of the pollen is unknown, and is determined by genotyping each embryo. However, because in Oleaceae one seed may carry two embryos, some teams have waited and extracted DNA from seedlings, which ensure the use of one offspring only. Some teams have broken seeds and scraped the content of the seed without verification for one or two offsprings. Moreover, the set of STR markers used in paternity tests is specific to each team. Each set was screened for its efficiency to discriminate varieties. Thus, the same variety used in different teams displays several profiles for different SSR loci. The programs used to identify the father reveal 'the most probable father' for each embryo, but no further verification has been done, to see whether 'the most probable father' - based on bag method - is compatible with the host variety. Several studies have been published, that provided many data on different studies of paternity tests in the olive tree. Studies based on paternity tests examined here are from

Arbeiter et al. (2014), Marchese et al. (2016), Seifi et al. (2011), Díaz et al. (2006); Mookerjee et al. (2005), De la Rosa et al. (2006), and Wu et al. (2002). These studies covered most concerns that it is possible to meet in the olive tree. We are limited by published studies on paternity tests, although many unpublished studies are known, but are still kept confidential. After self-pollination, the STR protocol can check: 1) fruit set is due to self-pollination; 2) fruit set is due to unwanted pollen.

In any case, combination of the two protocols should enhance the ways to decipher S-alleles in varieties and to confidently identify pollinizers for each variety.

### **Statistical analysis**

Each series of bags – are, in fact, never repeats of an assay because of airborne pollen and separate operations to opening on each bag. When fruit set occurs after self-pollination, it could be difficult to interpret correctly fruit set data after crosses, because the foreign pollen may compete with the self-pollen. Fruit set is not the sum of fruit set after self-pollination added to fruit set after cross pollination. Also, variations in fruit setting between bags may impede clear conclusions

### **Cautions**

#### **For used varieties**

All trees used, female (host tree) as well as male (pollen donor) in such experiments, have to be controlled by microsatellite markers to check each matches the correct standard of the denomination. Airborne pollen diffused by each orchard grove is supposed to move to a maximum of 1.5 km, which infers the area to sample for feral and abandoned olive trees – potential donors of pollen - around each host tree is a circle of 3 km in diameter that covers about 70 ha. The set of varieties in the data bank is a specificity for each team. Unidentified trees in each area around sampled trees for fruit from which the embryos were pulled out, should be sampled and analyzed with the same set of STR markers. The discriminant ability of the STR markers has to be calculated again on all trees sampled in the 70 ha, it could be different from the calculation in the data base of varieties.

#### **For molecular markers**

For paternity tests in forest trees the set of SSR has been screened for those discriminant between species of pollen donors and host species (Wei et al., 2015; Gailing et al., 2014; Jensen et al., 2009). In the olive the set of markers is limited in loci and no screening for discriminant markers was performed, beside the fact that these markers discriminate the set of varieties. Differentiation of clonal varieties is much more difficult than differentiation of species as in conifers and in oak species.

### **Conclusion**

The only method which allows attributing a pollinizer with certainty to an olive tree host is the bag method. Here, we showed that the bag method permits to identify pollinizers

with certainty, because data are solid, reproducible, and numerous. It remains to verify the coincidence for blossoming between the host and the pollen donor in commercial orchards. Plenty of studies are based on this method. The STR protocol allows identifying the most probable father variety that was deduced from the haploid profile, enabling to look for the father. ‘The most probable father’ does not warrant it is the true father. Thus, the STR protocol has to be used as a complement to the bag method to verify whether the low fruit set are due to unwanted pollen or to abnormalities in the self-incompatibility mechanism. Data from the STR method are never numerous and most embryos remain without any diagnostic, which increases the cost of the method. Controlled pollination based on the bag method and STR protocol have not to be opposed.

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### References

- Arbeiter AB, Jakše J, Bandelj D (2014) Paternity analysis of the olive variety “Istrska Belica” and identification of pollen donors by microsatellite markers. *Scientific World J.* Article ID 208590.
- Breton CM, Bervillé AJ (2012) New hypothesis elucidates self-incompatibility in the olive tree regarding S-alleles dominance relationships as in the sporophytic model. *CR Biologies* 335: 563-572.
- Breton CM, Farinelli D, Koubouris G, Bervillé AJ (2016) A model based on S-allele dominance relationships to explain pseudo self-fertility of varieties in the olive tree. *Euphytica* 210: 105-117.
- Breton CM, Farinelli D, Shafiq S, Heslop-Harrison JS, Sedgley M, Bervillé AJ (2014) The self-incompatibility mating system of the olive (*Olea europaea* L.) functions with dominance between S-alleles. *Tree Genetics & Genomes* 10:1055-1067.
- De la Rosa R, James CM, Tobutt KR (2004) Using microsatellites for paternity testing in olive progenies. *HortScience* 39: 351-354.
- Díaz A, Martín A, Rallo P, Barranco D, De la Rosa R (2006) Self-incompatibility of ‘Arbequina’ and ‘Picual’ olive assessed by SSR markers. *J Am Soc Hort Sci.* 131:250– 255.
- Farinelli D, Pierantozzi P, Palese AM (2012) Pollinizer and cultivar influence seed number and fruit characteristics in *Olea europaea* L. *Hortscience* 47(10): 1430-1437.
- Farinelli D, Breton CM, Famiani F, Bervillé AJ (2015) Specific features in the model of olive self-incompatibility system: method to decipher S-allele pairs for varieties based on fruit setting. *Scientia Hort.* 181: 62-75.
- Gailing O, Curtu AL (2014) Interspecific gene flow and maintenance of species integrity in oaks. *Ann For Res.* 57(1): 5-18.
- Jensen J, Larsen A, Nielsen LR, Cottrell J (2009) Hybridization between *Quercus robur* and *Q. petraea* in a mixed oak stand in Denmark. *Ann For Sci.* 66(7): 10.1051/forest/2009058
- Marchese A, Marra FP, Costa F, Quartararo A, Fretto S, Caruso T (2016) An investigation of the self- and inter-incompatibility of the olive cultivars ‘Arbequina’ and ‘Koroneiki’ in the Mediterranean climate of Sicily. *AJCS.* 10(1): 88-93.
- Mookerjee S, Guerin J., Collins G, Ford C, Sedgley M (2005) Paternity analysis using microsatellite markers to identify pollen donors in an olive grove. *Theor Appl Genet.* 111: 1174-1182.
- Seifi E, Guerin J, Kaiser B, Sedgley M (2011) Sexual compatibility and floral biology of some olive cultivars. *New Zeal J Crop Hort.* 39: 141-151.
- Seifi E, Guerin J, Kaiser B, Sedgley M (2012) Sexual compatibility of the olive cultivar ‘Kalamata’ assessed by paternity analysis. *Spanish J Agric Res.* 10(3): 731-740.
- Seifi E, Guerin J, Kaiser B, Sedgley M (2015) Flowering and fruit set in olive: a review. *Iranian J Plant Physiol.* 5(2): 1263-1272.
- Taslimpour M, Bonyampour A, Rahemi R (2008) Determining the best polliniser of olive (*Olea europaea* L.) (‘Dezfoul’) in Fars Province. *Amer-Euras J Agric Environ Sci.* 4: 682-686.
- Ugrinović K, Štampar F (1996) Fertilization of olive (*Olea europaea* L.) cultivars “Istrska Belica”, “Pendolino” and “Leccino” by different pollinators. *Acta Horticulturae* 474: 767-770.
- Villemur P, Musho U-S, Delmas JM, Maamar M, Ouksili A (1984) Contribution à l’étude de la biologie florale de l’olivier (*Olea europaea* L.): stérilité mâle, flux pollinique et période effective de pollinisation. *Fruits* 39: 467-473.
- Wei Z, Qu Z, Hou C, Liu Y, Zhang L, Yang C, Wei H (2015) Genetic diversity and paternal analysis of open-pollinated progenies of *Larix olgensis* seed orchard. *J Nat Sci.* 1(1) e19.
- Wu S, Collins SG, Sedgley M (2004) A molecular linkage map of olive (*Olea europaea* L.) based on RAPD, microsatellite and SCAR markers. *Genome* 47: 26-35.