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Full Length Research Paper

Outcrossing rate in olive assessed by microsatellite and inter simple sequence repeat (ISSR) markers

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Olive is known to be an allogamous species. The aim of this study was to estimate the magnitude of cross-pollination rate using microsatellite simple sequence repeats (SSR) and inter simple sequence repeats (ISSR) molecular markers in olive genotypes. The DNA from maternal plants and 23 progenies of two different accessions, Ascolano USA and MGS GRAP541 were extracted and screened with two microsatellite and ten ISSR markers. The outcrossing rate and other related parameters were analyzed using the MLTR application. The set of estimates, individually and collectively, support the hypothesis of frequent allogamy in both olive genotypes evaluated, with high rates of outcrossing in both markers.

Key words: *Olea europaea* L., outcrossing rate, zygotic embryos, mating system, molecular markers.

INTRODUCTION

The cultivated olive (*Olea europaea* subsp. *europaea*) is a diploid species (2n = 2x = 46 chromosomes) (Besnard et al., 2008). Some reports show a varied degree of self-incompatibility in olive (Lavee et al., 2002; Díaz et al., 2006), which depends on the genetic background, climatic conditions and location, thereby favouring outcrossing. Días et al. (2006) also pointed out the possibility to increase the fruit production by growing different varieties in the same field. Olive pollen is mainly dispersed by wind and therefore, it might reach large distances. The updrafts are capable of raising the pollen to thousands of meters (Cabral, 2009). Besides wind, insects, including bees, may help in pollination by collecting pollen grain (Lavee, 1985; Free, 1993); although olive flowers do not produce nectar (Lavee, 1985). Thus, it is important to evaluate the outcrossing rate in specific environmental conditions since this event does not only depend on the compatibility between varieties (Mekuria et al., 2002).

Brazilian olive production has increased exponentially over the past decade. One strategy adopted to develop new varieties adapted to local environment is by allowing the outcrossing of adult plants in an open orchard and then screening selected progenies in specific conditions to verify yield, industrial and gastronomic qualities, adaptability and stability (Val et al., 2012). To assess outcrossing rates, as well as, to know how many pollen donors are involved in a half sibling olive family, a powerful approach is the use of molecular markers (Ferreira et al., 2010); among those, an optimal choice is the use of microsatellite (SSR) and ISSR markers. Besides the Mendelian inheritance, the codominant and multi-allelic profiling, broad genome coverage and reproducibility are the best advantages of SSR (Aradhya et al., 2003). Although ISSR markers present dominant nature, not allowing the identification of heterozygote profiles, they also offer a multilocus pattern, broad

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genome coverage, reasonable level of reproducibility and relatively low cost (Reddy et al., 2002) encouraging their use. Therefore, the aim of this study was to evaluate the magnitude of cross-pollination in two olive genotypes by using microsatellite and ISSR markers in zygotic embryos cultured *in vitro* and in their maternal plants.

MATERIALS AND METHODS

Plant material and DNA extraction

The olive genotypes evaluated were MGS GRAP541, a Brazilian variety, and Ascolano USA, a North American variety. These varieties were chosen for this study because they have a high level of genetic divergence as indicated by Val et al. (2012) and because the physiological fruit ripening occur at the same time of the year. Young leaves and ripe fruits were collected from olive accessions belonging to the germplasm collection of the Agricultural Research Agency of Minas Gerais (EPAMIG) located in Maria da Fé, Brazil (latitude 22° 18' 28"; longitude 45° 22' 30"; altitude 1258 m; climate type Cwb). In this germplasm, blooming trees of more than 60 different accessions of *Olea europaea* are preserved in very close neighboring sites, ensuring an adequate supply of pollen. After embryo isolation from the fruit endocarp, the embryos were germinated in MS medium (Murashige and Skoog, 1962) containing 30 g L^{-1} sucrose and 7 g L^{-1} agar, pH 5.8 and incubated in growth chamber with temperature 25±1°C and a photoperiod of 16 h light with irradiance of 36 μmol m^{-2} s^{-1}. Genomic DNA was extracted from leaf tissues of each maternal plant and from *in vitro* seedlings (progenies) using the CTAB method described by Doyle and Doyle (1990).

Microsatellite markers

We used two microsatellite markers previously described as polymorphic for the genus *Olea*, denoted as GAPU 12 (Carriero et al., 2002) and GAPU 71 B (Muzzalupo et al., 2009). The amplification reactions were performed at a final volume of 30 μL containing 50 ηg DNA, 6 μL of 5× reaction buffer, 1.5 μL of $MgCl_2$ (1.5 mM), 0.5 μL of dNTPs (200 μM each), 0.6 μM of each primer (Sigma, USA) and 0.75 U of Taq DNA polymerase (Go Taq Flexi, Promega, USA). The reactions were programmed for an initial denaturation step of 5 min at 94°C followed by 37 cycles of denaturation at 94°C for 50 s, annealing of the primers for 50 s (variable temperature) and extension of the primers at 72°C for 1 min. The amplifications were performed using a "Touchdown" system in which the primer annealing temperature was decreased by 1°C per cycle during the first 5 amplification cycles, resulting in annealing temperatures that ranged from 62 to 58°C. From the sixth cycle on, the annealing temperature was set at 57°C. The reaction products were subjected to electrophoresis on a 6% denaturing polyacrylamide gel at 60 W and then stained with silver nitrate according to the method described by Creste et al. (2001). Afterwards, we constructed a matrix in which each allele at every locus was numerically designated from 1 to the maximum number of alleles identified per locus.

Inter simple sequence repeats (ISSR) markers

We used 10 ISSR markers denoted as UBC 807, UBC 809, UBC 810, UBC 817, UBC 818, UBC 823, UBC 834, UBC 846, UBC 849, and UBC 818 described by Martins-Lopes et al. (2007) and Terzopoulos et al. (2005). The amplification reactions were performed in a final volume of 25 μL, containing: 50 ηg DNA, 5 μL 5× reaction buffer, 1.5 μL $MgCl_2$ (1.5 mM), 0.5 μL dNTPs (200 μM each), 1.5 μL of primer (0.6 μM, Sigma, USA) and 0.75 U of Taq DNA polymerase (Go Taq Flexi, Promega, USA). The reactions were programmed for an initial denaturation step of 2 min at 95°C followed by 40 cycles of denaturation at 95°C for 45 s, annealing temperature of primers 50°C for 1 min and extension of primers at 72°C for 2 min, followed by a final extension step at 72°C for 5 min. Samples were subjected to electrophoresis on 1.5% agarose gel immersed in TBE buffer [90 mM Tris-borate (pH 8.0) 10 mM EDTA] at 110 V voltage. Afterwards, they were subsequently stained with ethidium bromide (0.2 mg/mL) and photographed. The data were plotted into a binary matrix where 0 was "missing data", 1 was "allele presence" and 2 was "allele absence".

Data analysis

The mating system was analyzed based on the model of crossing mix of Ritland and Jain (1981), with the aid of Multilocus MLTR software version 3.4 (Ritland, 2002) available at http://www.genetics.forestry.ubc.ca/ritland/programs.html. The genetic parameters estimated were: (a) Multilocus outcrossing rate of the population (t_m) by the method of Expectation-Maximization (EM); (b) The average population single locus (unilocus) outcrossing rate (t_s); (c) Correlated paternity (r_p) and; (d) The multilocus inbreeding coefficient wright or fixation indices of maternal generation (F_m). The variances of the estimates were obtained by bootstrapping (1000 bootstraps). The estimation method was based on the maximum likelihood equations (Ritland, 1983). This estimation process was used according to the following conditions: the genotypes of the female parents are known and the gene frequencies in the pollen pool are unknown. For the microsatellite data, it was also estimated as the observed and expected heterozygosity (Levene, 1949), using the software POPGENE version1.32 (Yeh and Boyle, 1997).

RESULTS AND DISCUSSION

The reproducible and doubtless scorable bands obtained by co-dominant microsatellite markers (Figure 1) and dominant ISSR (Figure 2) showed an average rate of multilocus outcrossing; t_m = 1.1335 and 1.200, respectively, proving the allogamous nature of these two olive genotypes. The single locus outcrossing (t_s) for SSR and ISSR were: 1.1605 and 1.1105, respectively, showing similar values with t_m. However, the estimates were slightly higher than 1, reaching values above the theoretical superior limit. According to the MLTR manual, generally, the maximum likelihood approach has some drawbacks, especially if the amount of sampled individuals is relatively small. Therefore in this case, the estimates of outcrossing might be biased upwards, although quite similar outcrossing values, higher than 1 were also reported by Gaspar et al. (2009) in a study carried out with *Pinus pinaster*. These authors genotyped only 25 offspring per family. Other study performed by Ferreira et al. (2010) also mentioned estimates of t_s and t_m higher than 1. They sampled 20 individuals per

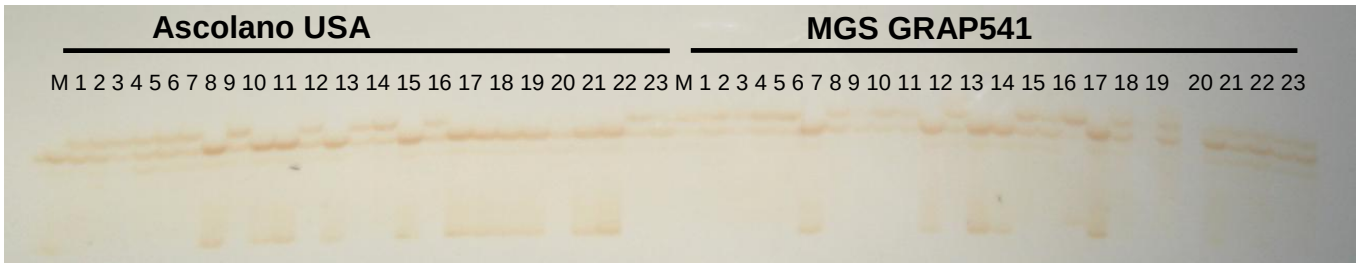

Figure 1. Microsatellite marker GAPU-12 used in Ascolano USA and MGS GRAP541, where "M" are the maternal plants and individuals 1 to 23 are their respective progenies.

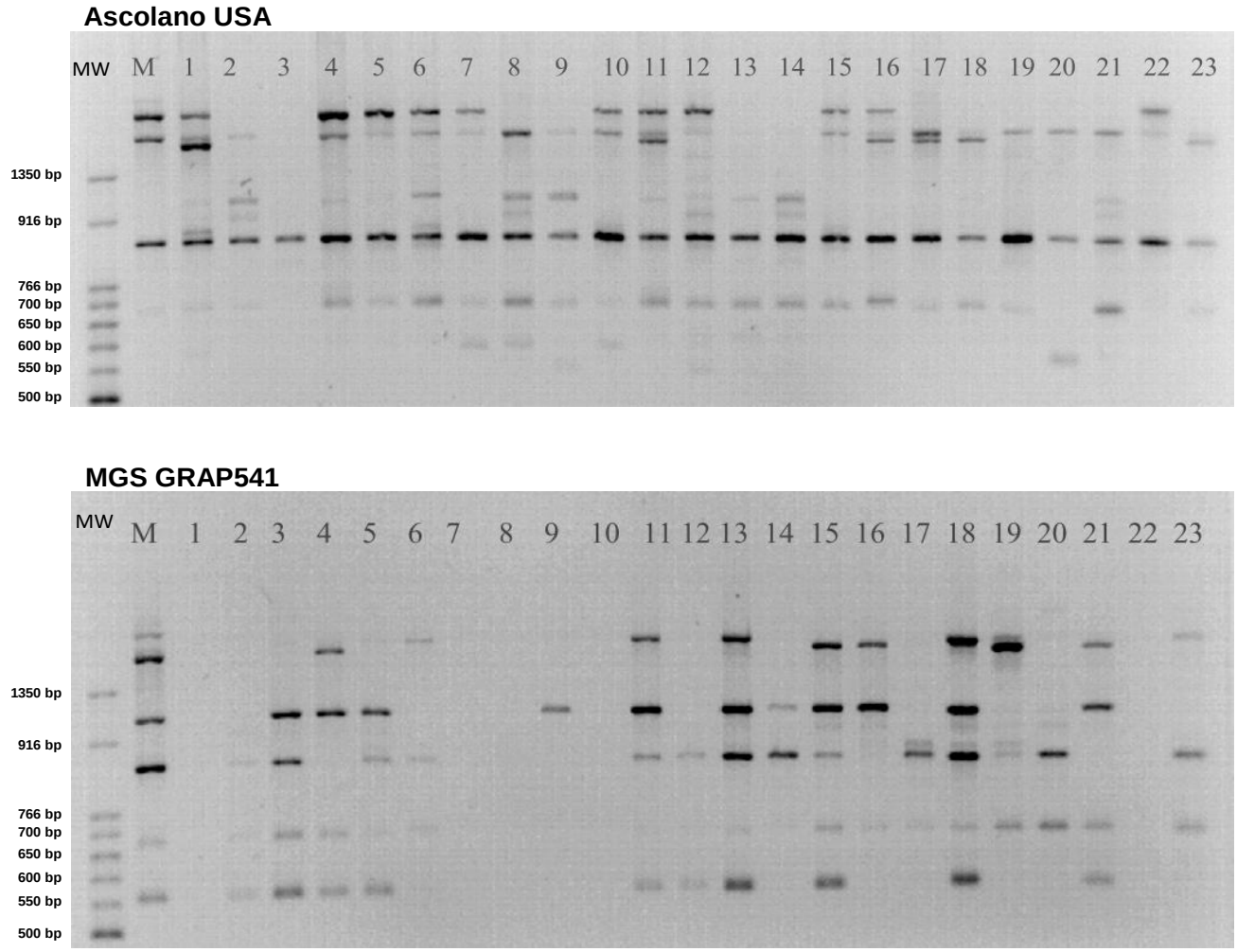

Figure 2. ISSR marker UBC 823 in Ascolano USA and MGS GRAP541, where "M" are maternal plants and individuals 1 to 23 are the progenies. MW = molecular weight 50 bp DNA ladder.

progeny, a similar number used in this work. The results were also consistent when analyzing both markers (t_m mean = 1.078 and t_s mean = 1.066) (Table 1). The values of t_m and t_s in the absence of endogamy are expected to be high and similar (Ferreira et al., 2010) as observed in this study. Despite the small number of seedlings (only

Table 1. Estimates of multilocus outcrossing rate (t_m), single locus outcrossing (t_s), correlated paternity (r_p), and fixation index of maternal generation based on SSR, ISSR and both markers (F_m) followed by the standard deviations.

Parameter	ISSR	SSR	Both marker
Ascolano USA			
t_m	1.200±0.000	1.067±0.003	1.200±0.000
t_s	1.108±0.000	1.121±0.003	1.080±0.000
r_p	-0.230±0.179	-0.181±0.000	-0.091±0.000
F_m	-0.200±0.024	-0.128±0.001	-0.200±0.001
MGS GRAP541			
t_m	1.200±0.000	1.200±0.000	0.956±0.000
t_s	1.113±0.000	1.200±0.000	1.052±0.000
r_p	-0.145±0.000	-0.123±0.000	-0.999±0.000
F_m	- 0.200±0.000	-0.200±0.001	-0.200±0.001

twenty three) evaluated per genotype, the results of t_s were quite similar for both genotypes: 1.080 (Ascolano USA) and 1.052 (MGS GRAP541).

Olive trees are mainly pollinated by wind and some varieties are considered as self-incompatible. However, climatic conditions can strongly affect the self-incompatibility magnitude. The flowers of olive trees are small and have a couple of stamens and carpels (Fabbri et al., 2004) and could be either perfect or staminate (Seifi, 2008). Some olive varieties may produce up to half million flowers per plant in one season. According to Collani et al. (2009) the self-incompatibility system in olive is characterized more as a gametophytic mechanism than a sporophytic mechanism, although no experimental data are now available and information still scanty. The high values of estimated t_m and t_s indicate no self-pollination, corroborating with the hypothesis of self-incompatibility. These results are congruent with those obtained for other tree species such as *Pinus pinaster* (Gaspar et al., 2009), an allogamous plant. Comparison of the difference between the average values of outcrossing rate in multilocus and single locus outcrossing progenies (t_m-t_s) for microsatellite markers (-0.025) and ISSR markers (0.0895) indicate a low proportion of biparental inbreeding; although this result does not influence the coefficient of correlated paternity. The correlated paternity (r_p), which is the probability that the two siblings are outcrossed full-sibs, showed close values between microsatellite and ISSR markers in the progenies of Ascolano USA (-0.230 and -0.181) and MGS GRAP541 (-0.145 and -0.123) as indicated in Table 1. The ratio $1/r_p$ estimates the most probable number of effective pollen donors. Thus, the r_p provides further support for the occurrence of outcrossing in these progenies, with the additional estimation of the number of potential male parents involved in the progeny array. In this study the number of pollen donors, considering the two progenies and the two individual markers, ranged around 4 to 5 (Ascolano USA) and 6 to 8 (MGS GRAP 541). This parameter indicates the crossing compatibilities of these genotypes with other accessions belonging to the olive orchard. The high density of potential donors in the same field associated with the anemophilous pollination nature optimizes rates of cross-pollination (Van-Treuren et al., 1993; Ferreira et al., 2010).

Another way to detect the outcrossing rate is by using the fixation indices of maternal generation (F_m) as suggested by Parzies et al. (2008). In this study, the average of the two progenies F_m was: -0.164 and -0.200 for SSR and ISSR markers, respectively, indicating the absence of inbreeding in the maternal generation. Therefore, it is other additional support for the high outcrossing nature of these two divergent varieties of *O. europaea*. The observed (Ho) and expected heterozygosity (He) were quite similar in both genotypes. Ascolano USA generated values of Ho = 0.7391 and He = 0.5925 while MGS GRAP541 generated values of Ho = 0.7332 and He = 0.5680. These estimates from SSR marker, which have codominant nature, evidenced the allogamy in the genetic profile composition of those individuals. The estimates of observed heterozigosity in this study are higher than the values showed by other studies with different olive varieties (Sefc et al., 2000; Rekik et al., 2008), especially in our case, where the evaluated individuals are half-siblings and therefore, tend to share some alleles.

Conclusion

The *O. europaea* genotypes Ascolano USA and MGS GRAP541 were proved to be predominantly cross-pollinated varieties. The individual data of microsatellite

and ISSR markers, as well as the evaluation of combined data were effective to show the occurrence of outcrossing in both olive genotypes evaluated.

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