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Gene flow in an almond orchard

J.F. Jackson* and G.R. Clarke

Department of Horticulture, Viticulture, and Oenology, Waite Agricultural Research Institute, University of Adelaide, South Australia 5064, Australia

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Summary. Gene flow by pollen between trees is essential for nut set in commercial almond orchards, due to the self-incompatibility of almond cultivars used. A study of gene flow has been carried out in an orchard composed of single rows of a "pollinating" cultivar between every double row of the most commercially desirable cultivar, Nonpareil. This "two-to-one" planting pattern was repeated throughout the orchard, and several "pollinating" cultivars were used in various parts of the orchard in an attempt to provide flowers for cross-pollination with Nonpareil at all stages of flowering of the latter. Using isozyme markers GPI-2, LAP-1, AAT-1, PGM-1, and PGM-2 and three newly-defined isozyme markers for almond – IDH, G6PD, and SDH – it has been shown that the gene flow resulting in nut set is quite restricted, taking place most strongly between neighboring halves of cross-compatibile pairs of trees. Even that half of a tree facing away from the "pollinating" tree has significantly less gene flow to it, while the next tree further on has few nuts set by fertilization from the "pollinating" tree under consideration. This result is surprising considering the comparatively large distances that the honeybee brought into the orchard in large numbers must travel within the orchard. To explain this apparent paradox and the observation that in most cases only a small proportion (<20%) of flowers set nuts, it is suggested that the honeybee predominantly visits only one cultivar, flying along the row of the cultivar to do so, and that cross-pollination results from accidental or rare visits involving two or more compatible cultivars.

Key words: Almond orchard – Gene flow – Pollen – Honeybee

* To whom offprint requests should be addressed

Introduction

Almond (*Prunus dulcis* D. A. Webb syn. *P. amygdalus* Batsch) is essentially self-incompatible as grown in commercial orchards in California and Australia (Kester and Griggs 1959; Baker and Gathercole 1977). Therefore, cross-pollination between different almond cultivars is necessary for adequate fertilization and nut set, and the orchardist organizes his orchard so as to have several cross-compatible almond cultivars within it. This means that pollen must move about the orchard to achieve cross-pollination; in other words, there must be gene flow. This gene flow is brought about by the introduction of bees into the orchard (Hill et al.1985). The very fact that bees are the pollen vectors has contributed to the uncertainty of actual gene flow patterns that lead to nut set in almond orchards. It is known that at least 30–50% of flowers on almond trees is capable of setting nuts, yet in commercial orchards with a high density of bees, the set varies from 5–30% of flowers and varies enormously from year to year (Hill et al. 1985). Additionally, the best juxtaposition of pollinating trees relative to the commercially desirable almond cultivar, Nonpareil, is still debated, largely because gene flow patterns are not understood. Observations on flight patterns of individual bees have been made and some conclusions have been drawn (Thorpe 1979); however, this approach requires enormous numbers of observations and even then it cannot be deduced that any one particular encounter actually leads to fertilization and nut set, or which pollen actually fertilizes particular flowers. More than one almond pollinator cultivar is used in commercial orchards, the reason put forward being that it is necessary to span the whole flowering period of Nonpareil, since the various "pollinator" cultivars flower at different times, some earlier and some later than Nonpareil. The net result is

that at least one "pollinator" cultivar is in full flower at each of the various early, middle, and late flowering phases of Nonpareil, and it is presumed that gene flow is effective over sufficiently large distances within the orchard (at least across several rows) to set nuts efficiently on Nonpareil at all of these phases.

To test these and other assumptions, we have carried out a study of gene flow in a commercial almond orchard at Angle Vale, South Australia. To achieve this, we identified three polymorphic isozyme markers (IDH, G6PD, and SDH) which, together with five isozyme markers identified by Hauagge et al. (1987a, b), were used to detect pollen genes in embryos of mature nuts set on individual trees within the orchard. An hypothesis was developed from the results, which explained the observed gene flow in terms of the behavior of the honeybee.

Materials and methods

Orchard

The almond orchard used for this investigation is at Angle Vale, South Australia. It is sited near Adelaide, has a Mediterranean climate with average rainfall of 460 mm, and the trees are irrigated. The orchard has been the subject of an earlier study by Hill et al. (1985). Bees were brought into the orchard just before flowering, there were four hives/hectare, arranged in groups of four to ten hives at several points throughout the orchard, and each group was at least 20 rows (trees) from the nearest group.

Plant materials

Leaf samples were obtained from fresh new growth on known almond cultivars in the Angle Vale orchard. Pollen was collected from the same source. Flowers were taken at the point of opening, and anthers were excised and allowed to reach anthesis in the laboratory. This took approximately 24 h. Extraneous anther tissue was removed with a sieve (Jackson 1989) and the pollen was used immediately for isozyme analysis. Nuts for molecular marker (isozyme) analysis of embryos were collected from under each tree after they were shaken to harvest. In general, 100 nuts were sampled at random from under each half of each tree, the diving line for each half being taken through the trunk of the tree and parallel to the line of the various cultivar-rows in the orchard (see Fig. 2). For this study, a line of trees was selected running perpendicular to the rows of like cultivars, so that gene flow emanating out from the rows could be estimated by isozyme analysis of the nuts produced by the pollen flow, assuming segregation of isozyme markers in the embryo according to Mendelian ratios (see below). The embryo of each mature nut (Hawker and Buttrose 1980) was excised for isozyme analysis.

Isozyme analysis

Isozymes investigated. Isozymes utilized were glucose phosphate isomerase at the second locus (*GPI-2*), leucine aminopeptidase at the first locus (*LAP-1*), aspartate amino transferase at the first locus (*AAT-1*), and phosphoglucomutase at the first and second loci (*PGM-1* and *PGM-2*) (Hauagge et al. 1987a, b). We also utilized polymorphism exhibited by isocitrate dehydrogenase (IDH), glucose-6-phosphate-dehydrogenase (G6PD), and shikimic acid dehydrogenase (SDH).

Extraction of plant materials. Leaf and pollen was extracted and subjected to isozyme analysis to check that the almond cultivars included in this study were the same isozyme patterns as reported by Hauagge et al. (1987a, b) for these cultivars in California. Leaf material (0.3 g) was ground with 0.15 g polyvinylpolypyrrolidone and 2.0 ml of extraction buffer containing 0.05 *M* TRIS-0.15% citric acid-0.12% cysteine HCl-0.1% ascorbic acid, pH 8.0. Grinding was carried out with a pestle and mortar, the mixture was centrifuged at $3{,}000 \times g$ in an Eppendorf 5414 S centrifuge, and the supernatant was used for gel electrophoresis. Pollen was extracted in the same way, except that 2 mg was extracted with 0.25 ml extraction buffer, no polyvinylpolypyrrolidone being used in this case. Embryos were extracted as described above for leaf material, except that 0.4 g embryo was used. In all cases, gel electrophoresis of the supernatant for isozyme analysis was carried out within 1 h of extraction.

Gel electrophoresis. Unlike previously reported isozyme studies on almond leaves and embryos (Hauagge et al. 1987a, b) and almond pollen (Cerezo et al. 1989), we used Cellogel (Chemtron, Italy) as the medium for electrophoresis of extracts instead of starch gel. We found Cellogel to be more predictable in its behavior from day to day and, in our hands, it gave sharper bands than starch gel. Cellogel electrophoresis was carried out as described by Richardson et al. (1986). Running buffers for electrophoresis were 0.05 *M* TRIS-maleate (pH 7.8) for GPI, LAP, AAT, IDH, and SDH; 0.025 *M* TRIS-glycine (pH 8.5) for PGM; and 0.02 *M* sodium phosphate (pH 7.0) for G6PD. Approximately 1 µl of supernatant was used for each sample, and electrophoresis was carried out at 200 V (constant voltage, varying current), 2 °C, for approximately 1.75 h. Following the run, staining for GPI, AAT, LAP, PGM, IDH, and G6PD was carried out as described by Richardson et al. (1986). SKDH staining utilized a solution made up immediately prior to use by adding 6 mg shikimic acid, 0.1 ml 25 m*M* NADP, 0.1 ml 14.5 m*M* MTT; and 0.1 ml 6.5 m*M* PMS to 2.0 ml 0.1 *M* TRIS (pH 8.5). Stained gels were incubated in an oven at 37 °C and photocopies were made as the isozyme patterns developed. Scoring the resulting patterns was carried out according to Richardson et al. (1986) for the *GPI-2*, *LAP-1*, *AAT-1*, *PGM-1*, *PGM-2* loci described by Hauagge et al. (1987a, b), using the nomencla-

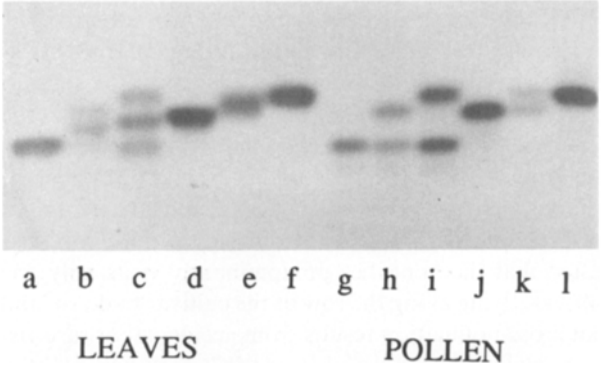

Fig. 1. Isocitrate dehydrogenase (IDH) isozyme patterns in extracts from almond leaves and pollen. Leaf extracts correspond to *lanes a–f*, pollen extracts *g–l*. *Lane a* shows Thompson cultivar, genotype *aa*; *lane b* Chellaston, *ab*; *lane c* Ne Plus Ultra, *ac*; *lane d* Peerless, *bb*; *lane e* Grant, *bc*; *lane f* Pethick Wonder, *cc*; *lane g* Thompson pollen; *lane h* Chellaston pollen; *lane i* Ne Plus Ultra pollen; *lane j* Peerless pollen; *lane k* Grant pollen; *lane l* Pethick Wonder pollen

ture adopted for the genotypes of these isozymes by the latter authors. Isozyme patterns and genotype nomenclature for IDH, SDH, and G6PD are described in the Results section.

Results

Isozyme variation in almond cultivars

Isocitrate dehydrogenase (IDH). Variability was found in IDH by Cerezo et al. (1989), who investigated isozymes in almond pollen. Like these authors, we found faint anodal and strong cathodal bands; the latter were designated the *IDH-2* locus. By examining several cultivars and carrying out electrophoresis with diploid as well as haploid material, we were able to go further and assign genotype to three alleles at the *IDH-2* locus. As shown in Fig. 1, almond cultivars Thompson, Peerless, and the South Australian cultivar Pethick Wonder were homozygous for alleles *a*, *b*, and *c*, respectively, while examples of heterozygotes at the *IDH-2* locus were the South Australian cultivar Chellaston (*ab*), and Ne Plus Ultra (*ac*) and Grant (*bc*). The heterozygotes, as expected, gave different patterns in leaf extracts as compared to pollen extracts. *IDH-2* has proven to be a very useful molecular marker for our gene flow studies.

Shikimic acid dehydrogenase (SDH) and glucose-6-phosphate-dehydrogenase (G6PD). We have extended the range of useful isozymes for almond to SDH and G6PD, neither being described by previous investigators. Both loci have been assigned genotypes with two alleles.

Isozyme genotype in almond cultivars

Using the three isozyme markers (IDH, SDH, and G6PD) delineated here, together with the five described for almond by Hauagge et al. (1987a, b), we now had sufficient molecular markers to allow a gene flow study to be carried out in the large section of the orchard that was composed of Nonpareil, Peerless, Ne Plus Ultra, Fritz, and the South Australian cultivar Keanes. Genotypes for these five almond cultivars are listed in Table 1.

It should be noted that careful examination of the progeny of controlled cross-pollinations between these cultivars gave the expected Mendelian segregation patterns, as also noted by Hauagge et al. (1987a, b). These authors drew attention to the null allele at the *LAP-1* locus in the Mission cultivar, which was assigned an nc genotype in this cultivar. We have found the null allele at the *LAP-1* locus in Fritz also, and assign this cultivar with genotype nb (Table 1). It is of interest that Fritz is thought to be an offspring of Mission (Hauagge et al. 1987b), therefore such an occurrence of a null allele at this locus is not altogether unexpected. More surprising was our finding that Peerless has the genotype nn at the *AAT-1* locus. Segregation patterns for Peerless were not reported by Hauagge et al. (1987a, b). We find Fritz has the genotype *na* at the *AAT-1* locus.

Gene flow in the almond orchard

Pollen parents were deduced from isozyme patterns in the embryos for reconstruction of gene flow patterns, using the markers given in Table 1. For example, the Fritz pollen contribution could be identified readily by the fact that the *GPI-2* locus is a heterozygous *ab* and is the only source of the *b* allele at that locus in this study. Add to this the fact that the Fritz parent is the only cultivar in this study with *na* alleles at the *AAT-1* locus and that at *IDH-2* it is a heterozygous *ab*, etc., and it can be readily perceived that the pollen contribution from Fritz to the embryo genotype can easily be identified and quantified. Pollen contribution from the other "pollinating" cultivars were similarly deduced, the *IDH-2* locus being particularly useful with its three possible alleles.

As can be see from Fig. 2, gene flow from the pollinator varieties in the various parts of the orchard is quite restricted. Regardless of whether the source of pollen is Peerless, Ne Plus Ultra, Fritz, or Keanes, by far the highest recipient of pollen genes leading to successful nut set is to be found in that half of the tree next to the source of pollen. Even the other side of the tree facing away from the pollen source has far less flow of that pollen to it, while the second tree in line going away from the source has very little gene flow to it at all. And as nuts are examined much further away still, e.g., in other areas of the orchard many rows from the source under consideration, gene flow from that source has ceased altogether; instead, pollen genes from the adjacent pollinator variety to the tree being looked at are present in the nuts pro-

Table 1. Isozyme genotypes for eight polymorphic isozymes in the five almond cultivars used in the gene flow study

Cultivar	Isozyme							
	GPI-2	LAP-1	AAT-1	PGM-1	PGM-2	IDH	G6PD	SDH
Nonpareil	*aa*	*bc*	*ab*	*ab*	*ab*	*ab*	*ab*	*bb*
Peerless	*aa*	*bb*	*nn*	*ab*	*ab*	*bb*	*ab*	*bb*
Ne Plus Ultra	*aa*	*bc*	*ab*	*bb*	*ab*	*ac*	*ab*	*bb*
Fritz	*ab*	*nb*	*na*	*ab*	*bb*	*ab*	*ab*	*bb*
Keanes	*aa*	*cc*	*ab*	*ab*	*bb*	*ac*	*bb*	*ab*

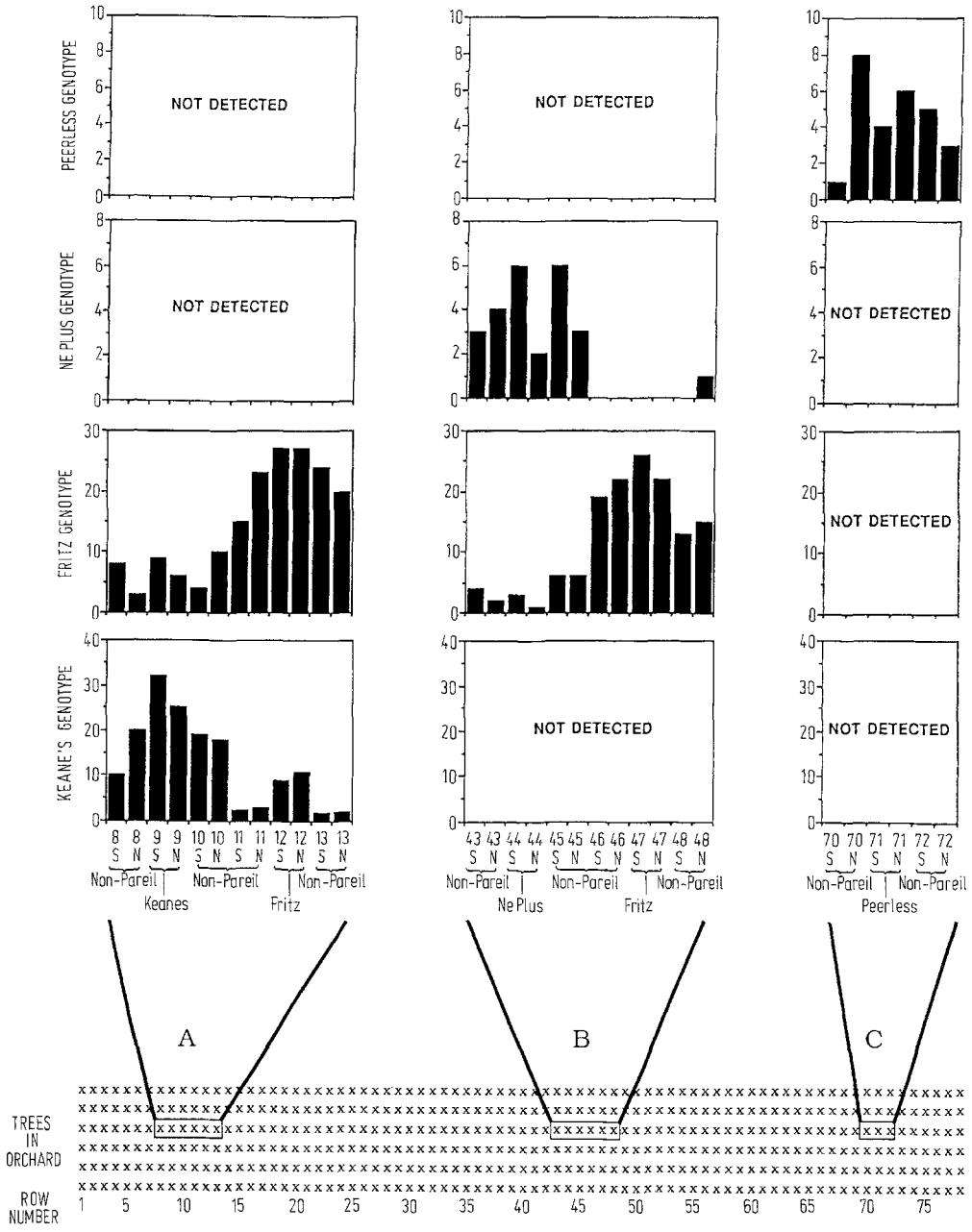

Fig. 2. Gene flow in an Australian almond orchard at Angle Vale. This major section of orchard is composed of 78 rows of trees, with two rows of Nonpareil cultivar for every one row of pollinator variety. Gene flow was investigated in trees running at right angles to the line of rows of cultivars, so that pollen (gene) flow emanating out from each row of particular cultivars and resulting in nut set could be estimated. Three areas in the orchard where different pollinators are used were investigated in this way, so as to follow gene flow between different combinations of almond cultivars. Because it was anticipated that there could be significant differences between each half of each tree, depending on whether it was facing towards or away from a particular source of pollen genes, nuts were collected and analyzed for pollen genes from two sides of each tree. Results are presented in terms of numbers of nuts with stated genotype (numbers of nuts analyzed was 50 per half tree). Each half of each tree is designated by row number and whether it is the northern (N) or southern (S) side of each tree. The northern end of the orchard is to the *right* of diagram (row 78)

duced. In short, gene flow is so restricted in the commercial orchard examined that the planting of different "pollinator" cultivars which flower at different times, in order to supply pollen at all of the flowering time of Nonpareil, is rendered a futile exercise; the pollen gene source is predominantly from the neighboring tree.

Discussion

Hauagge et al. (1987b) showed that the five isozyme markers GPI-2, LAP-1, AAT-1, PGM-1, and PGM-2 are all polymorphic, and inheritance studies indicated segregation of these markers in typical Mendelian ratios. We

have identified three further isozyme markers – IDH, G6PD, and SDH – which are also polymorphic in almond. Each of these appeared to be composed of a dimer with two subunits, G6PD and SDH, with two alleles each. We assigned three alleles to IDH. The latter was found to be polymorphic in almond pollen by Cerezo et al. (1989), however, these latter authors were not able to assign alleles to IDH in almond. Inheritance studies by the present authors showed segregation according to Mendelian ratios for IDH, G6PD, and SDH, as well as for GPI-2, LAP-1, AAT-1, PGM-1, and PGM-2, based on observations with each isozyme on young leaves for each parent tree and on isozyme patterns in embryos obtained by controlled crosses between these parent trees. The eight loci described above were sufficient to enable adequate discrimination between the genotypes for gene flow studies.

As shown in Fig. 2, gene flow as studied in all three parts of the orchard was much more restricted than expected, particularly with regard to the distances between the different pollinator cultivars. Thus, in all cases the "pollinator" genes were found in highest concentration in that half of the Nonpareil tree facing that particular pollinator tree. The proportion of nuts set from the particular pollinator fell off dramatically on that half of the tree facing away from the "pollinator", while it fell to even lower levels in the next tree. In this second tree, again that half facing the pollinator showed a higher proportion of pollen genes from the "pollinator" than that facing the other way. The third tree had very low proportion of the "pollinator" genes under consideration. By ten trees or more away (76 m or more), the amounts of the particular "pollinator" genes had fallen to zero in the orchard under study. Gene flow in this almond orchard in the 1988 flowering season was thus very restricted and took place predominantly between neighboring compatible trees. While this supports the practice of some orchardists in using a one-to-one planting of Nonpareil with a compatible cultivar, it does not support the notion (and widespread practice) of using several pollinators, where they are planted out in separate rows, many rows (trees) apart.

Gene flow by pollen has been estimated for several other plant populations and considerable flow was found to take place over longer distances than reported here. Thus, for *Pseudotsuga menziesii*, Neale (1983) found 20–27% gene flow at 161 m distant and 4–15% over 2,000 m. Ellstrand et al. (1989) reported 8.6% gene flow over 1,000 m for populations of wild radish. The pollen vectors in these cases were likely to have been syrphid flies and honeybees. It must be remembered, however, that we only have the means, by using paternity analysis by isozyme, of measuring a "net, apparent" gene flow. The honeybee responsible for cross-pollination in the orchard may well fly hundreds or even thousands of meters in all if we hypothesize that each bee visits only one cultivar by flying along the rows of that cultivar before it visits another cultivar (e.g., the tree in the next row), perhaps accidently. That visit to an alternative cultivar, which may result in fertilization and nut set, would appear as a "net" gene flow to the neighboring tree in the next row, a result found for the almond orchard under study. Observation of the behavior of some bees by the present authors suggests that the above hypothesis for cross-fertilization is a plausible one. The observations of Thorpe (1979) are of a similar nature. We will attempt to test this hypothesis further by developing methods to determine from the pollen which almond cultivars are visited by individual bees (Stanley and Linskens 1974).

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