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Original article

Genetic markers for *Prunus avium* L: inheritance and linkage of isozyme loci

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Summary – The polymorphism of 10 enzyme systems in wild cherry (*Prunus avium* L.) was analysed using vertical polyacrylamide gel electrophoresis (AMY, GOT, ME) and isoelectrofocusing (ACP, IDH, LAP, MDH, PGM, SDH, TO) on 286 wild cherries. The products of around 41 loci could be distinguished in these systems, 13 of which displayed polymorphism. The genetics of 7 isozyme loci have been studied using 8 fullsib families: *acp1, lap1, sdh1 and mdh1* functioned as monomers, and *got1, idh1 and me1* were active as dimers. Two other isozymes (*pgm1* and *got2*) appeared to have simple inheritance, but it was not possible to verify this. Joint segregation of 13 locus pairs showed linkage between lap1 and got1 (r = 0.03 ± 0.02) and between *lap1* and *me1* (r = 0.05 ± 0.07). *Idh1, sdh1, acp1 and mdh1* are not linked to these loci. No linkage has been detected between *acp1* and *mdh1*.

***Prunus avium* L. / wild cherry / isozyme / variability / inheritance / linkage**

Résumé – Marqueurs génétiques pour* Prunus avium *L : déterminisme génétique et groupes de liaisons entre loci enzymatiques. *L'espèce* Prunus avium *comprend les cerisiers, variétés améliorées pour la production de fruits, et les merisiers, arbres forestiers de qualité sur lesquels portent aussi des programmes d'amélioration. Il serait utile pour ces programmes de disposer de marqueurs génétiques, pour les identifications clonales et interspecifiques, l'analyse de la variabilité naturelle et du système de reproduction et le contrôle des produits de croisements dirigés. Comme peu de marqueurs génétiques avaient été étudiés chez* P avium, *nos efforts ont porté sur la recherche et la caractérisation de loci enzymatiques. Le polymorphisme enzymatique a été étudié grâce à 286 merisiers provenant de France (216), d'Allemagne (14) et de Belgique (6). Le déterminisme et les liaisons génétiques ont été testés avec 8 descendances d'un demi-diallèle 14 × 14. Les extraits de bourgeons prélevés en hiver ont été analysés par electrophorèse sur gel d'acrylamide (AMY, GOT, ME) ou par isoélectrofocalisation (ACP, IDH, LAP, MDH, PGM, SDH, TO). Dans les zymogrammes obtenus, schématisés en figure 1, 13 loci enzymatiques polymorphes et 28 bandes monomorphes sont observés. Les hypothèses de déterminisme génétique (fig 1) ont été testées par un χ^2 dans les descendances (tableau II). Deux écarts significatifs (au niveau 5 %) aux proportions*

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génotypiques attendues par ségrégation mendélienne ont été notés, mais le test de χ^2 *global est non significatif. Le déterminisme de* acp*1*, got*1*, idh*1*, lap*1*, mdh*1*, me*1 *et* sdh*1 a ainsi été établi. Un seul type de test de déterminisme était possible pour* amy*1*, mdh*2*, got*2*, pgm*1 et* to*1, car les 14 parents du demi-diallèle ont les mêmes génotypes supposés (homozygotes pour les 4 derniers loci). Cela n'a pas permis d'établir avec certitude le déterminisme proposé. Cependant, en comparant le type de zymogramme obtenu par d'autres auteurs sur les mêmes enzymes, il semblerait que le déterminisme le plus probable pour* got*2 et* pgm*1 soit celui proposé. La coségrégation de 13 paires de loci a été testée par rapport à celle attendue en cas d'indépendance (table III), montrant une liaison significative entre* got*1 et* lap*1 (r = 0.03 ± 0.02), et entre* lap*1 et* me*1 (r = 0.05-0.07). Ce groupe de liaison est indépendant de* idh*1*, sdh*1*, acp*1 et* mdh*1 et aucune liaison ne semble exister entre ces 2 derniers loci. Toute autre relation entre loci n'a pu être testée avec les familles analysées.*

Prunus avium *L* / *merisier* / *isozyme* / *déterminisme génétique* / *liaison*

INTRODUCTION

The sweet cherry, *Prunus avium* L, is widely cultivated for its fruit crop, for which substantial improvements have been made for some time. The same species, named the wild cherry, growing naturally in Europe and West-Asia, produces a very valuable wood, justifying the forest breeding programmes which began recently.

Genetic markers would be useful for both fruit and wood breeding programmes. The identification of varieties, control of breeding material, and the assesment of specific purity would be possible with a series of environmental influence-free traits. Codominant, simply-inherited and mapped traits are useful for various purposes, such as the analysis of natural variability and of mating systems, the control of man-made crosses and of products of forestry clonal seed orchards.

Very few monogenic, simply-inherited morphological traits have been described for *Prunus avium*: fruit-juice colour and albinism, which are controlled by dominant-recessive pairs of alleles (Watkin and Brown, 1956).

The gametophytic incompatibility S locus is polymorphic with at least 6 alleles, among sweet cherry cultivars (Crane and Brown, 1937), as well as among wild cherry (Berger, 1963, Santi, unpublished data), but it is necessary to make crosses for the genotype determination.

Treutter and Feucht (1985) showed differences in phenolic composition among *P avium* clones. Phenolic compounds are potentially valuable genetic markers for breeding programmes and population genetic studies, since they may be linked directly to economically important traits (Doumanjou and Marigo, 1978; Friend, 1985) and involve regulator genes (Vernet *et al*, 1986). Unfortunately, phenolics are often affected by environmental factors and their inheritance is complex.

Such difficulties do not usually arise while using isozymes, the most widely used genetic markers, which have already proved useful for numerous plants as reviewed by Tanksley and Orton (1983). Some knowledge is now available on *P avium* enzyme variability. Feucht and Schmid (1985) showed protein and peroxidase banding pattern differences among *P avium* clones. Kaurisch *et al* (1988) pointed out variability for 3 isozyme loci (aconitase-2, 6 phosphoglucodeshydrogenase-1,

phosphoglucoisomerase-2). Nevertheless, the number of enzymes studied is still limited and no inheritance has been established. In addition no linkage map of *P avium* is available.

The purpose of this study therefore was to increase the number of isozyme loci available for *P avium* and to test their inheritance and linkage relationships.

MATERIAL AND METHODS

Plant material

Enzyme variability was studied with 286 wild cherries sampled throughout most of France (186) and in 4 populations in: Normandy (Northwest France, 61 trees), the Ardennes (Northern France 19 trees), Southern Germany (14 trees) and Southern Belgium (6 trees).

The inheritance and linkage analyses were made with 1 year-old plants of 8 fullsib families, which were chosen in a 14 × 14 half-diallel according to the availability of material and the variability of parent isozyme phenotypes: nr13 (clone 108 × clone 229), nr22 (109 × 208), nr27 (111 × 143), nr36 (111 × 229), nr71 (171 × 195), nr80 (195 × 226), nr81 (195 × 229), nr87 (208 × 226).

Buds were preferred to leaves for enzyme extraction. Buds were sampled during the 1987-1988 winter. The samplings were made in the original stands in Normandy and Bavaria on 15 to 100 year-old trees. The samplings of other wild cherries were made on 1-7 year-old vegetative copies in a clone bank in Olivet. The sampling period varied from November 1987 to March 1988. The buds were sampled from the lateral or apical position, on short or long shoots. The majority of the buds were vegetative, and exceptionally floral.

Sample preparation

Immediately after sampling, the buds were frozen in liquid nitrogen, freeze-dried and vacuum-stored until extraction. Lyophilization does not alter enzyme activity, since the extracts of fresh and lyophilized buds of 5 clones had similar electrophoretic patterns.

100 mg of scale-free buds were put into an aluminium bag, frozen in liquid nitrogen and crushed with a hammer. The powder was soaked for 1 h in 1.2 ml of extraction buffer (20 mM triscitric pH = 7.5 containing 12 mM 2β-mercaptoethanol, 5 mM dithiotreithol, 2 mM polyethylene glycol (MW = 6 000), 2% w/v PVP). When less than 100 mg of buds were available, the buffer volume was adjusted. The homogenate was centrifuged for 30 min at 14 000 *g*, and the supernatant, transferred into plastic vials, was stored at –60 °C until electrophoresis.

Electrophoretic procedure

The trisboric-EDTA polyacrylamide gel electrophoresis (Page) system (Dalet and Cornu, 1989) was performed for 3 enzymes. Glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1.) was run through a 10% acrylamide "running gel" and a 5% acrylamide "stacking gel". Malic enzyme (ME, EC 1.1.1.40) and amylase (AMY, EC 3.2.1.1) were run through 7% and 10% acrylamide "running gels", respectively. The power supply was set at 100 V for 1 h, then bromophenol blue and 15 µl (GOT) or 20 µl (ME and AMY) of extract were loaded into each slot. The voltage was then increased to 250 V and maintained for 5 h (AMY) or increased to 350 V and maintained for 6 h (GOT, ME). The temperature of the electrolytic buffer was kept at 4 to 6 °C using a cooler.

The other enzymes were run on 245 × 125 × 0.5 mm isoelectrofocusing (IEF) gels, containing acrylamide and bisacrylamide (concentration T = 5%, C = 4%), Servalyt carrier ampholytes (2%) w/v 3-10 pH gradient, 0.7% w/v 4-6 pH gradient; the latter was not added when running deshydrogenases, 1 µl/ml TEMED and 1.8 mM ammonium persulphate. The gels were run on 2 "Multiphor II" apparatus, with the cooling plates maintained at 2 °C. The electrolytes used were those described by Kinzkofer and Radola (1981). Six to eight µl of extract were loaded using a hand-made silicon applicator strip with 64 holes, lying across the gel. The power supply was set at a maximum of 1200 or 1500 V, 45 mA and

40 W for the 2 gels, and each run lasted about 2 h.

The staining procedures were those of Cardy *et al* (1983) for ME, GOT, isocitrate deshydrogenase (IDH, EC 1.1.1.42.), malate deshydrogenase (MDH, EC 1.1.1.37) and phosphoglucomutase (PGM, EC 2.7.5.1.). They were those of Roux and Roux (1981) for acid phosphatase (ACP, EC 3.1.3.2), and those of Beckman *et al* (1964) for leucine aminopeptidase (LAP). For AMY staining, gels were soaked 2:30 h in an acetate buffer 0.2 M pH = 4.5 with 2% w/v starch and 6% w/v $CaCl_2$, and then in the same buffer with 3% w/v KI and 0.3 w/v I_2. PAGE gels were fixed with 7% acetic acid, wrapped in plastic foil and stored at 4-6 °C. IEF gels were dried and stored at room temperature.

Checking zymogram stability

Zymogram stability was tested by varying the sampling conditions applied to the same clones as described in table I. No difference was noticed for 9 enzymes (ME stability was not tested), ensuring that the observed zymogram differences were independent of the tested sampling conditions.

Inheritance and linkage tests

Mendelian inheritance hypotheses were proposed after watching zymogram variability among the 286 wild cherries, except for ME, for which only the 14 parents of the half-diallel and 5 of their progenies were observed. Departure from or adequation to the expected segregation ratios in the observed families were tested using χ^2 tests.

RESULTS

Scored loci and inheritance hypotheses

All the observed zymograms of the 286 wild cherries are represented schematically in figure 1. Thirteen polymorphic isozyme loci and 28 monomorphic bands were scored.

Inheritance hypotheses (figure 1) are easy to propose for *acp1, got1, idh1, lap1, mdh1, me1* and *sdh1*, since at least 3 supposed genotypes (aa, bb, ab) appear directly from the observed phenotypes. The pattern of the bb zymogram (nr3 on figure 1) of the *got1* locus suggests that a monomorphic band is merging into the a-band. This band may be the product of a duplicated GOT-locus. For *mdh1*, one allele is thought to produce 2 bands. Con-

Table I. Varying factors tested for their influence on zymogram patterns

Clone	*Tree*			*Buds*		
	Location	*Age*	*Sampling date*	*Position*	*Branch*	*State*
109,198,208,	Orléans	4	Nov 27 1987	apical	long	vegetative
226,229	"	"	Jan 29 1988	"	"	"
	"	7	Mar 02 1988	"	"	"
	"	7	"	lateral	"	"
	"	1	"	"	"	"
224,816,817,	Normandie	old	Feb 19 1988	various	short	vegetative
834,843	"	"	"	"	"	floral
226	Orléans	7	Mar 02 1988	apical	long	vegetative
	"	"	"	apical	short	"
224	Orléans	4	Nov 27 1987	apical	long	vegetative
	Normandie	old	Feb 19 1988	variable	short	"

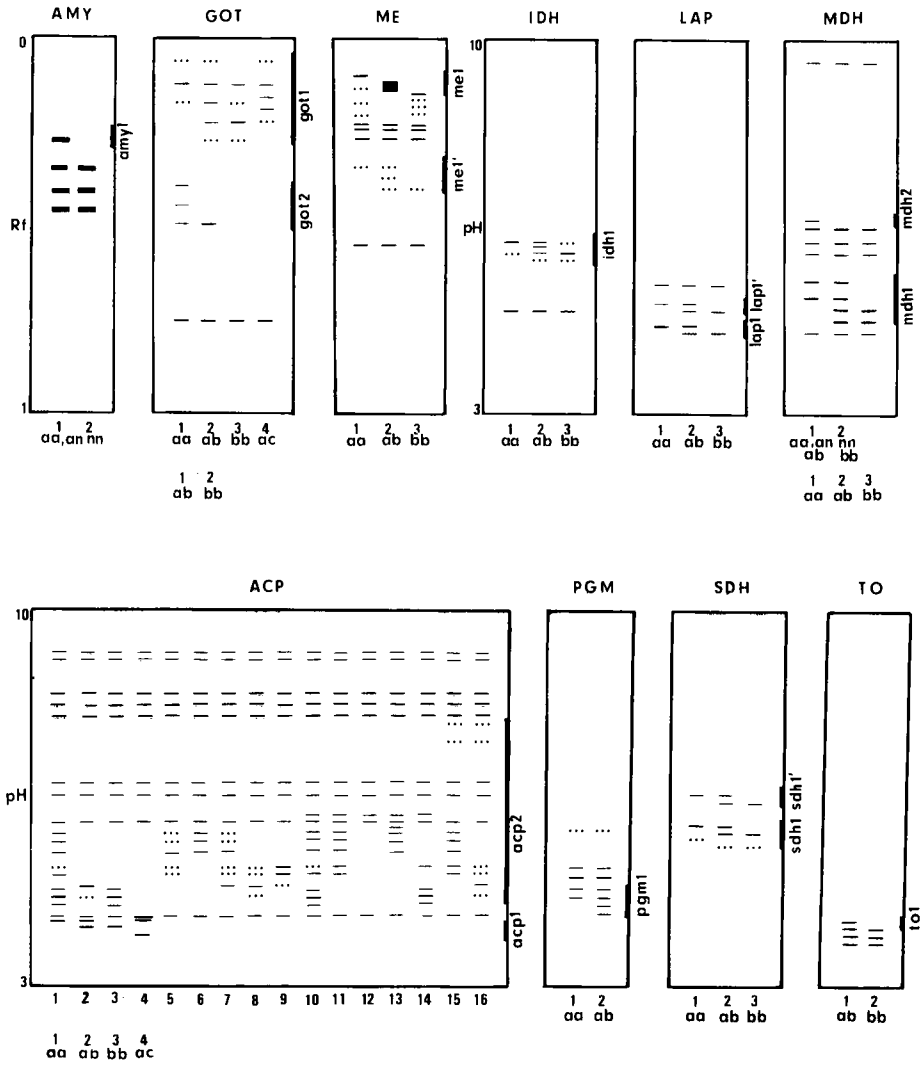

Fig 1. Observed zymograms of AMY, ME and GOT on PAGE gels, and of IDH, LAP, MDH, PGM, SDH, TO and ACP on IEF gels. Under each zymogram, the number of the phenotype and the proposed genotype are given for each polymorphic putative locus (with respect to the position of the loci from the top and the bottom of the zymogram). Two different genotypes are eventually proposed for a single phenotype. The "loci" which are supposed to be secondary products are noted with ′ and have no phenotype-numbers, a, b, c, are active alleles and n is a null allele.

versely, only 2 phenotypes of *amy1, got2, mdh2, pgm1* and *to1* were recorded in observed zymograms. Two hypotheses are proposed for *mdh2*: the product of the more frequent allele is thought to be merged into a monomorphic band; or a null allele is involved. The expected heterozygote was in general the least often observed phenotype: 1/285 for *got2*, 35/285 for *mdh2*, 9/285 for *pgm1*, 24/285 for *to1*, suggesting that the second homozygote was not recorded by chance. Further difficulties arise when interpreting *amy1* phenotypes, since the phenotype with the most numerous bands is the most frequent (241/285). A realistic hypothesis is to involve a null allele. *Sdh1'* and *lap1'* appear as secondary products and *me1'* is probably a heterodimer between *me1* and a monomorphic locus. No simple genetic hypothesis results from the examination of the *acp2* patterns.

Segregation at individual loci

The scored monomorphic bands among the 286 wild cherries were still monomorphic among the progenies.

All 14 parents of the half diallel were monomorphic for *amy1* (thought to be heterozygote with a null allele or homozygote with active alleles), and for *mdh2, got2, pgm1* and *to1* (thought to be homozygotes). The observed segregation ratios in 1 progeny of the 3 latter isozyme loci were those expected, and for *amy1* no evidence for a null allele appeared (table II).

Other loci were polymorphic among the 14 parents, allowing more diverse crossing combinations. The results in table II show 2 departures from Mendelian expectations, concerning the family n° 80 for the locus *sdh1* and the family n° 22 for the locus *acp1*. These departures are not significant for the total of 25 chi-square tests. Nevertheless, a linkage between the *acp1* locus and the incompatibility S-locus may provide such a result for the family 22, if the parents have 1 common S-allele, because the male parent is a heterozygote for the *acp1* locus.

Joint segregation at locus pairs

Over the 13 locus pairs tested for joint segregation 2 pairs had highly significant ($P < 0.01$) chi-square values for 2-locus segregation ratios. The loci involved are the pairs *got1/lap1* and *me1/lap1*. The recombination fraction between the loci is reduced in all families for these 2 pairs, although in the cross n° 27 the chi-square value is not significant for the *me1/lap1* pair.

The estimated recombination values show a strong linkage between *got1* and *lap1*, and between *me1* and *lap1*, but the precise respective position of the 3 loci is still unknown, since the linkage between *got1* and *me1* has not yet been tested. *Idh1, sdh1, acp1* and *mdh1* are not linked to this 3 locus group, according to 2, 4, 5 and 2 tests respectively. The *Acp1/mdh1* linkage relationship has only been tested among the *acp1, mdh1, idh1* and *sdh1* loci, and it involved only 14 sibs.

DISCUSSION

Inheritance

A limited number of sibs per family were analysed because buds were most often restricted in number and size. Nevertheless, we have determined that 7 isozymes detectable in *P*

Table II. Chi-square test for goodness-of-fit to expected Mendelian segregation ratios at individual loci

Locus	*Family*	*Parental phenotype*	*Progeny phenotype*			*Expected ratio*	*Chi-square test*
			A	*AB*	*B**		
acp1	71	AxA	38			1:0:0	–
	36	AxAB	8	12		1:1:0	NS**
	22	"	16	6		"	$P < 0.05$
	13	"	6	5		"	NS
	27	ABxAB	4	6	6	1:2:1	NS
got1	71	AxA	39			1:0:0	–
	27	AxAB	6	10		1:1:0	NS
	22	"	11	12		"	NS
	81	"	9	9		"	NS
	13	"	8	7		"	NS
	87	ABxAB	2	5	6	1:2:1	NS
	36	"	7	10	6	"	NS
idh1	71	AxA	39			1:0:0	–
	36	AxB		21		0:1:0	–
	27	"		15		"	–
	13	"		13		"	–
	22	AxAB	10	13		1:1:0	NS
lap1	22	AxAB	11	11		1:1:0	NS
	13	"	3	6		1:1:0	NS
	36	ABxAB	6	10	3	1:2:1	NS
	27	"	7	7	2	"	NS
	71	"	10	17	10	"	NS
mdh1	36	BxB			16	0:0:1	–
	71	"			21	"	–
	22	"			17	"	–
	27	ABxB		8	6	0:1:1	NS
me1	36	ABxB		5	3	0:1:1	NS
	27	"		7	4	"	NS
	13	"		3	6	"	NS
	71	ABxAB	9	14	6	1:2:1	NS
	22	BxB			18	0:0:1	–
sdh1	27	AxA	16			1:0:0	–
	22	AxB		23		0:1:0	–
	36	AxAB	13	10		1:1:0	NS
	71	ABxB		20	19	0:1:1	NS
	13	"		7	8	"	NS
	80	ABxAB	4	13	1	1:2:1	$P < 0.05$
amy1	36	AxA	13		0	1:0 or 3:1	–
pgm1	36	AxA	13			1:0:0	–
to1	36	BxB			13	0:0:1	–

* or N (nul phenotype) for *amyl* ** NS=not significant, even at 10% level

Table III. Chi-square test and recombination values between loci based on observed and expected 2 locus segregation ratios
* 1=aa, 2=ab, 3=bb, ** NS=not significant, even at 10% level, *** 95% confidence interval

Loci	*Family nr*	*2 locus genotype combinations*									*Expected ratio*	χ^2 *test*	$r \pm 2\sigma$***
		1/1	*1/2*	*1/3*	*2/1*	*2/2*	*2/3*	*3/1*	*3/2*	*3/3**			
got1/lap1	22	0	11	–	11	0	–	–	–	–	1:1:1:1	P < 0.01	0
	27	0	4	2	7	3	0	–	–	–	1:2:1:1:2:1	*P* < 0.01	0
	36	0	4	1	7	1	1	0	5	0	1:2:1:2:4:2:1:2:1	*P* < 0.01	0.088±0.090
got1/acp1	27	2	2	2	2	4	4	–	–	–	1:2:1:1:2:1	NS**	–
	36	3	3	–	3	7	–	2	3	–	1:1:2:2:1:1	NS	–
idh1/got1	22	6	4	–	8	5	–	–	–	–	1:1:1:1	NS	–
idh1/lap1	22	5	7	–	4	6	–	–	–	–	1:1:1:1	NS	–
acp1/lap1	36	1	5	2	5	5	1	–	–	–	1:2:1:1:2:1	NS	–
	27	2	1	1	2	3	1	3	3	0	1:2:1:2:4:2:1:2:1	NS	–
sdh1/got1	36	3	5	4	3	5	2	–	–	–	1:2:1:1:2:1	NS	–
sdh1/lap1	36	4	4	1	2	6	2	–	–	–	1:2:1:1:2:1	NS	–
	71	–	–	–	5	9	5	5	8	5	1:2:1:1:2:1	NS	–
sdh1/me1	71	–	–	–	4	7	1	4	1	4	1:2:1:1:2:1	NS	–
mdh1/lap1	27	–	–	–	5	2	1	2	3	1	1:2:1:1:2:1	NS	–
mdh1/acp1	27	–	–	–	4	2	2	0	3	3	1:2:1:1:2:1	NS	–
mdh1/me1	27	2	3	–	2	3	–	–	–	–	1:1:1:1	NS	–
me1/lap1	27	–	–	–	4	3	0	0	2	2	1:2:1:1:2:1	NS	–
	71	8	0	0	0	7	0	0	2	3	1:2:1:2:4:2:1:2:1	*P* < 0.01	0.052±0.068
me1/acp1	27	–	–	–	1	2	4	1	2	1	1:2:1:1:2:1	NS	–

avium buds (*acp1, got1, idh1, lap1, mdh1, me1, sdh1*) are inherited as single genes, since observed segregation ratios in several full-sib families are as expected. *Acp1, lap1, sdh1 and mdh1* seem to be monomers, while *got1, idh1* and *me1* have dimer-like patterns. These patterns are common in animals or plants (Tanksley and Orton, 1983, Pasteur *et al*, 1987), apart from MDH isozymes, which are usually dimers.

No more information will be forthcoming by analysing more families of the half diallel for *pgm1, to1, got2* and *mdh2*, since the 14 parents have the same homozygous genotype. New crossings, involving variable genotypes, will be necessary to obtain clear evidence of the allelic relationships among the observed bands of the putative loci. Nevertheless, one may compare the obtained phenotypes to those existing in other organisms. GOT isozymes are usually dimers, and PGM isozymes are usually monomers with duplicated bands (Pasteur *et al*, 1987), so the observed *pgm1* and *got2* phenotypes in *P avium* do not differ from the usual patterns, and therefore the proposed inheritance hypotheses are the most likely. TO isozymes are not usually monomers (Pasteur *et al*, 1987), so care should be taken with the proposed inheritance.

Conversely, identifying alleles of *amy1* and *mdh2* would be difficult, if the proposed inheritance is correct, since the supposed genotypes do not always result in different phenotypes. *Acp2* inheritance is not clear, though several progenies have been analysed. Out of the 13 isozyme loci, 9 may therefore be useful in population genetic studies. These are in addition to the 3 observed by Kaurisch *et al* (1988), who

proposed inheritance patterns, which although not tested, are the most probable.

Linkage relationships

P avium has 8 chromosome pairs. Among the 12 variable isozyme loci with known inheritance, 3 are on the same chromosome pair, 4 are not part of this linkage group, but are not necessarily on 4 different chromosomes, and the remaining 5 loci have unknown linkage relationships. New analyses will therefore be necessary to complete the linkage map.

The sizeable number of polymorphic isozyme loci in wild cherry will prove useful in our research programme, in the breeding programme as well as for population genetic studies.

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