Ann Sci For (1990) 47, 219-227

Original article

Genetic markers for *Prunus avium* L. 2. Clonal identifications and discrimination from *P cerasus* and *P cerasus* x *P avium*

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(Received 1 March 1989; accepted 9 October 1989)

Summary – The polymorphism of 9 enzyme systems (ACP = EC 3.1.3.2., AMY = EC 3.2.1.1., GOT = EC 1.1.1.37., IDH = EC 1.1.1.42., LAP = EC 3.4.11.1, MDH = EC 1.1.1.37., PGM = EC 2.7.5.1., SDH = EC 1.1.1.25., TO = EC 1.15.1.1.) was studied in 198 wild cherry, "plus-trees" selected mostly in France. The variability at 8 loci allowed the positive characterization of most of them (72%). Among the 45 "plus-tree" clones supplied to French nurseries in 1988, 2 pairs remain indistinguishable. Keys for distinguishing wild cherries from sour or duke cherries were found in 3 enzyme systems (ACP, LAP, SDH): 3–10 additional bands were found in 33 sour or duke cherry cultivars of various origins, compared to 286 wild cherries. But these isozymes are probably insufficient to allow detection of minor introgressions of sour cherry in wild cherries.

Isozyme / *Prunus* / wild cherry / sour cherry / duke cherry / identification

Résumé – Marqueurs génétiques pour *P avium* L. 2. Identification clonale et differenciation entre *P avium, P cerasus* et leurs hybrides. *Des clefs d'identification clonale seraient utiles pour les programmes d'amélioration fruitère ou forestière de* P avium *(cerisiers et merisiers). Le polymorphisme de 9 systèmes enzymatiques (ACP = EC 3.1.3.2., AMY = EC 3.2.1.1., GOT = EC 1.1.1.37., IDH = EC 1.1.1.42., LAP = EC 3.4.11.1, MDH = EC 1.1.1.37., PGM = EC 2.7.5.1., SDH = EC 1.1.1.25., TO = EC 1.15.1.1.) a été observé par électrophorèse sur gel d'acrylamide et par isoélectrofocalisation. Ces données ont permis d'identifier individuellement 142 merisiers, soit 71,7% sur un total de 198 «arbres-plus» de la population d'amélioration forestière rassemblée à l'INRA d'Orléans, France. Les autres «arbres-plus» sont répartis dans 25 groupes composés de 2, 3 ou 4 clones (fig3). Le cas du clone 108 reste indéfini, en raison d'une erreur d'étiquetage détectée au cours des analyses électrophorétiques. Parmi les 45 clones fournis aux pépiniéristes, 2 fois 2 clones (112 + 171 et 164 + 165) n'ont pu être différenciés; en conséquence, il s'avère nécessaire d'augmenter le nombre de marqueurs génétiques.* P avium *se croise facilement avec* P cerasus *(cerisier acide) ou avec* P cerasus *(cerisier anglais, voir fig 1) et les descendants sont parfois peu faciles à distinguer morphologiquement de* P avium. *Aussi, pour chacune des 9 enzymes, les zymogrammes de 5 variétés de cerisiers acides ou anglais prélevés et analysés en mars ou août 1988 ont été comparés avec les zymogrammes de 286 merisiers originaires de*

France, d'Allemagne et de Belgique. Trois systèmes enzymatiques (ACP, LAP, SDH) permettaient de caractériser les cerisiers acides ou anglais par rapport aux merisiers. Leur analyse a ensuite porté sur 29 variétés clonales de cerisiers acides et anglais originaires de plusieurs pays européens et échantillonnés en février 1989. Les résultats ont été confirmés: 3 à 10 bandes supplémentaires ont été notées parmi ces variétés (fig 2 et tableau I) par comparaison aux zymogrammes des merisiers. La fiabilité de ces marqueurs pour différencier P avium *de* P cerasus *et de leurs hybrides sera cependant mieux établie en analysant un échantillon plus représentatif de la variabilité de* P avium *dans toute l'aire naturelle.*

Isozyme* / Prunus / *cerisier acide* / *cerisier anglais* / *merisier* / *identification

INTRODUCTION

Keys for the clonal identification of *P avium* (sweet or wild cherry) would be useful for several reasons in breeding programmes. First of all, the control of clonal banks, of cuttings and of *in vitro* propagated plants used for breeding procedures, would be of great interest. On the other hand, a control of commercial plants would be possible through clone labelling in clonal seed orchards (which supply seeds for forestry plantations) and of clonal varieties (for forestry or fruit production).

The 3rd interest of genetic markers would be to attest the specific purity of collected material. For instance, *P avium* and *P avium* x *P cerasus* are very similar, especially in winter (Feucht, personnal communication).

P avium, P fruticosa (ground cherry) and *P cerasus* (sour cherry) make up the *Eucerasus* section of the *Cerasus* subgenus. Morphological and biochemical clues exist (Olden and Nybom, 1968) for the hybrid origin of the tetraploid *P cerasus* (4*x*, *x* = 8 is the basis chromosomic number of *Prunus*), the parent species being *P fruticosa* (4*x*) and *P avium* (2*x*). The latter species may produce diploid gametes, thus allowing the production of a fertile tetraploid (Olden and Nybom, 1968). Similarly, crossing *P avium* with *P cerasus* may produce fertile tetraploid plants, named duke cherries (fig 1). Sour or duke cherries crossed with sweet cherry may produce many plants according to the results obtained by Crane and Brown (1937): 22.6%, 20% and 15.1% of fruits

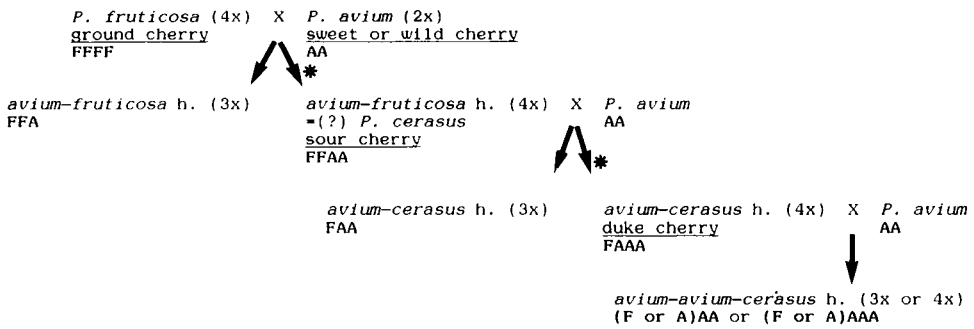


Fig 1. Hybridizations involving *P avium* h.: hybrid A,F: symbolize the chromosomes of *P avium* and *P fruticosa* *: *P avium* is supposed to produce diploid gametes sometimes (see text).

were obtained from hand-pollinated flowers in controlled crossings of sweet cherry with compatible sweet cherry, sour cherry and duke cherry, respectively.

Tri or tetraploid hybrids may therefore occur naturally wherever *P avium* and *P cerasus* stand together: mostly in the central and eastern area of the natural range of *P avium* (Europe and West-Asia), and wherever man spreads sour and duke cherry varieties near sweet or wild cherries. As a consequence, by collecting supposed *P avium* material (seeds, or branches...), hybrids can be collected.

Cytological analyses may reveal an introgression of *P cerasus* in supposed *P avium* (excepted if it is limited to chromosomic inversion), but these analyses are far less easy to make than some biochemical analyses. Furthermore, biochemical analyses are made for additional objectives, as intraspecific identifications or population genetic studies.

Phenolic compounds may contribute to intra and interspecific characterizations, as shown by Treutter and Feucht (1985), but difficulties may occur in comparing material from different origins, since the accumulation of these compounds is widely dependent on environmental conditions.

Such problems are usually avoided by using isozymes, therefore numerous authors have already used them to identify clones (Wendel and Parks, 1983) or species (Plessas and Strauss, 1986). Kaurisch *et al* (1988) showed zymogram differences for several enzyme systems among *P avium* clones. *P avium* and *P cerasus* may be distinguished according to peroxidase and protein banding patterns (Feucht and Schmid, 1985) and malate dehydrogenase zymograms (Hancock and Iezzoni, 1988). Only a limited number of clones were involved in these studies.

In this work, using the genetic markers described earlier (Santi and Lemoine, 1990), a new key for distinguishing *P avium* from *P cerasus* or from *P avium* x *P cerasus* products, and for the characterization of *P avium* clones is proposed, on the basis of a great number of analysed plants.

MATERIAL AND METHODS

Plant material

We analysed 286 wild cherries, sampled throughout France (186) and in 4 populations in Northwest France (61 trees), North France (19 trees), in Bavaria (14 trees) and in Belgium (6 trees). Among the wild cherries sampled in France, 198 were part of the forestry breeding population ("plus-trees" phenotypically selected) gathered at INRA-Orléans, France. Among them, 45 were supplied in 1988 to nurseries for vegetative propagation and commercialization.

Far less sour or duke cherries were sampled: 33 clonal varieties, mostly gathered in the Fruit-tree Breeding Station of Bordeaux, France (only 3 were sampled in Olivet gardens, France). These clones were native to France and various European countries, as specified in table I. The sampled area is larger than those of the wild cherries. Two varieties (Montmorency2, Cerise Anglaise) were sampled and analysed last March 1988, 3 (Montmorency1, Delkarsun, "x") in August 1988 and 29 (including 1 of the previously sampled: Montmorency1) in February 1989.

Electrophoretic procedures

Bud enzyme systems were analysed by vertical polyacrylamide gel electrophoresis: amylase (EC3.2.1.1), glutamate oxaloacetate transaminase (EC 2.6.1.1), and isoelectric focusing: acid phosphatase (EC 3.1.3.2.), isocitrate dehydrogenase (EC 1.1.1.42), leucine aminopeptidase (EC 3.4.11.1), malate

Table I. Enzyme analyses of sour and duke cherries. +, –, ?: present, absent, undetermined additional bands in zymograms. 1 to 5: band numbers indicated in figure 2.
* B=Bordeaux, O=Olivet ** S=sour cherry, D=duke cherry, ? sour or duke cherry.

*Code and sample location**		*Name and species***		*Origin*	*Scored bands*									+
					ACP					*LAP*		*SDH*		
					1	*2*	*3*	*4*	*5*	*1*	*1*	*2*	*3*	*4*
Analysed in 1988														
V1280	B	Montmorency	S	France	+	+	+	+	+	+	–	–	+	–
V2397	B	Delkarsun	D	France	+	+	–	+	+	+	–	–	+	–
–	O	Montmorency2	S?	?	+	+	+	+	+	+	+	–	+	–
–	O	Cerisier anglais	D?	?	+	+	+	+	+	+	+	–	+	–
–	O	?	?	?	+	+	+	+	+	+	–	–	+	–
Analysed in 1989														+
V603	B	Ferracida	S	France	+	+	+	?	+	+	+	+	+	+
V1280	B	Montmorency	S	France	+	+	+	?	+	+	+	+	+	+
V1711	B	Montmorency	S	France	+	+	+	?	+	+	+	+	+	+
V1745	B	Montm de Sauvigny	S	France	+	+	+	?	+	+	+	+	+	+
V1818	B	Cerasus haut-rhin	S	France	+	+	–	?	+	+	+	+	+	+
V1828	B	Cerasus haut-rhin	S	France	+	+	+	?	+	+	+	+	+	+
V1837	B	Cerasus haut-rhin	S	France	+	+	–	?	+	+	+	+	+	+
V2023	B	North Star	S	US	+	+	+	?	+	+	+	+	+	+
V2037	B	Layat	S	Cent Europe	+	+	+	?	+	+	+	+	+	+
V2117	B	Crisana2	S	Rumania	+	+	+	?	–	+	+	+	+	+
V2118	B	Marculesti	S	Rumania	+	+	+	?	+	+	+	+	+	+
V2119	B	Nana	S	Rumania	+	+	+	?	+	+	+	+	+	+
V2152	B	Katirli	S	Turkey	+	+	–	?	+	–	+	+	+	+
V2153	B	Kutamya	S	Turkey	+	+	–	?	+	–	+	+	+	+
V2154	B	Macar	S	Turkey	+	+	+	?	+	+	+	+	+	+
V2197	B	Crisana	S	Rumania	+	+	+	?	+	+	+	+	+	+
V2198	B	Grossa gamba	S	Rumania	+	+	–	?	+	–	+	+	+	+
V2204	B	Meteor korai	D	Hungary	+	+	–	?	+	+	+	+	+	+
V2207	B	Ciganymeggy 7	S	Hungary	+	+	+	?	+	+	+	+	+	+
V2208	B	Erdi nagygyu molscu	?	Hungary	+	+	+	?	+	+	+	+	+	+
V2209	B	Erdi botermo	D	Hungary	+	+	–	?	+	+	+	+	+	+
V2210	B	Ujfhertoï furtos	D	Hungary	+	+	+	?	+	+	+	+	+	+
V2212	B	Hartay meggy	S	Hungary	+	+	+	?	+	+	+	+	+	+
V2288	B	Oblacinskaia	S	Rumania	+	+	–	?	+	+	+	+	+	+
V2322	B	Mascara	S	Denmark	+	+	+	?	+	+	+	+	+	+
V2324	B	Kelleris 16	S	Denmark	+	+	+	?	+	+	+	+	+	+
V2325	B	Rubin	S	Denmark	+	+	–	?	+	+	+	+	+	+
V2326	B	Fanal	S	Denmark	+	+	+	?	+	+	+	+	+	+
V2327	B	Steinbär	S	Denmark	+	+	+	?	+	+	+	+	+	+

dehydrogenase (EC 1.1.1.37), phosphoglucomutase (EC 2.7.5.1), shikimate dehydrogenase (EC 1.1.1.25), and tetrazolium oxidase (EC 1.15.1.1).

The extraction procedure, gel and buffer composition and staining procedures have been detailed previously (Santi and Lemoine, 1990). For the latest sampled cultivars (February 1989), the following modifications were made:

– Doubled quantities of βmercaptoethanol (25 mM) and polyethylene glycol (2% w/v)

were used in the extraction buffer, in order to improve the protection of proteins,
– 4–6 pH gradient carier ampholytes were not added in the isoelectric focusing gels used for ACP and LAP. Therefore less bands were distinguishable in ACP zymograms.

Eleven polymorphic loci from 9 enzyme systems were found among the 198 "plus-trees". The observed phenotypes, and the genetic control of allozyme variation at *acp1, got1, idh1, lap1, mdh1, pgm1* and *sdh1* were described before (Santi and Lemoine, 1990). For the latter loci, phenotypes numbered 1, 2 and 3 are genotypes aa, ab, and bb, a and b being 2 alleles. The *acp2* polymorphism also seems to be under genetic control, with regard to unpublished data concerning segregation in several crosses. As direct evidence for the genetic basis of *amy1, mdh2* and *to1* variations is lacking, it cannot be excluded that the observed polymorphism is due to environmental impacts. As a consequence, only phonotypic variations for the former 8 loci were used for the identification key.

The supposed specific bands of sour or duke cherries were those which were either never or exceptionally observed in zymograms of the 286 wild cherries analysed (described in Santi and Lemoine, 1990).

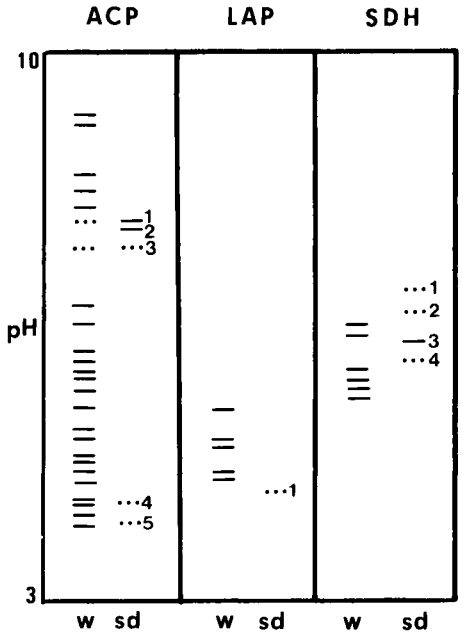


Fig 2. Observed zymograms of cherries. W: all bands scored in wild cherry zymograms (...: exceptionally scored bands) SD: additional bands, always (___) or occasionally (...) scored in sour or duke cherry zymograms.

RESULTS

Interspecific identification

A preliminary survey of sour or duke cherry variability was performed for the 9 enzyme systems and 5 sour or duke cherry varieties. The observed zymograms, compared with wild cherry zymograms, showed additional bands (table 1, fig 2) for only 3 enzyme systems: ACP, LAP and SDH. Other sour and duke cherry electrophoretic analyses were therefore performed with only these 3 enzyme systems.

On a total of 10 additional bands recorded in sour or duke cherry zymograms (fig 2 and table I), variable occurrence was recorded:

– 3 (ACP bands nr 1,2, SDH band nr 3) were noticed in all observed patterns,
– 1 (ACP band no 4) was present in the 1 five first varieties analysed, but was not distinguishable in the others since the 4–6 pH gradient Servalyt, which improves banding separation, was omitted in IEF gels,
– 3 (ACP bands no 3,5, LAP band no 1) were lacking among 10, 1 and 3 clones, respectively,
– 3 SDH bands (nos 1,2,4) were recorded in zymograms of individuals sampled in February, but not always in zymograms of individuals sampled in March or August. The cultivar Montmorency1 had all SDH bands when

sampled in February 1989 and only 1 when sampled in August 1988, suggesting that the expression of the corresponding isozymes is influenced by physiological state.

Intraspecific identification

Phenotypes at 8 loci for each "plus-tree" are presented in figure 3, as an identification key. Loci varied in their degree of variability: 16 phenotypes were scored for *acp2* whereas 3 were scored for *acp1, got1, idh1, lap1, mdh1* and *sdh1* and only 2 (1 of which was far less frequent) were detected for *pgm1*. A total of 23 328 combinations are possible. In the key, loci were used successively according to phenotypic diversity (number of phenotypes and size of the least frequent phenotype).

The great majority of "plus-trees" (142/198 = 71.7%) had a single 8-locus combination, and 56 of them were divided into 25 groups of 2, 3 or 4 trees. Among them, the "plus-trees" 164 and 165, and the "plus-trees" 135 and 136 were close enough (5 m and 100–200 m) so that suckering may be the explanation for their likeness. But for trees 135 and 136, the estimation of occurrence probability of the 8-locus phenotype is relatively high (fig 3), and their amy1 phenotypes seem different. On the other hand, the "plus-trees" 164 and 165 gave different results in clonal tests. Therefore no evidence of very similar trees appears amongst our "plus-trees" collection.

Several zymograms were made with mislabelled vegetative copies of the clone 108 and it was therefore impossible to identify this clone. The mislabelling error has been exhibited by using isozyme. Among the 45 clones supplied to nurseries, 2 pairs of clones are still indistinguishable: clones 112 + 171 and clones 164 + 165, and the identify of clone 108 is unknown.

Variable patterns of ACP, LAP and SDH were noticed among the 33 sour or duke cherries analysed, allowing them to be partially discriminated (15 groups of 1–6 clones, data not shown).

DISCUSSION

Interspecific identification

It may be supposed that the additional isozymes found in sour and duke cherry zymograms can be encoded by *P fruticosa* loci, but their precise genetic control is unknown. These loci may even be homologous loci such as those of *P avium*, whose allels are different through speciation phenomena. Similarly, the *avium*-like isozymes of *P cerasus* may be encoded by homologous loci of *P avium* and *P fruticosa*. This knowledge is lacking since no *P fruticosa* has been analysed, and therefore allelic frequencies cannot be estimated accurately in our *P cerasus* sample.

We are looking for genetic markers which would characterize the *P fruticosa* genome *versus* the *P avium* genome positively, *i e*, we need genetic markers never found in *P avium*, and fixed or often present in *P fruticosa* and *P cerasus* genome. Are the isozymes found specifically in our sour and duke cherry sample examples of such markers?

The 286 wild cherries sampled were limited to the western area of the natural range of *P avium*. According to the hypothesis of the hybrid origin of *P cerasus*, hybridization occurred in eastern and central Europe and western Asia,

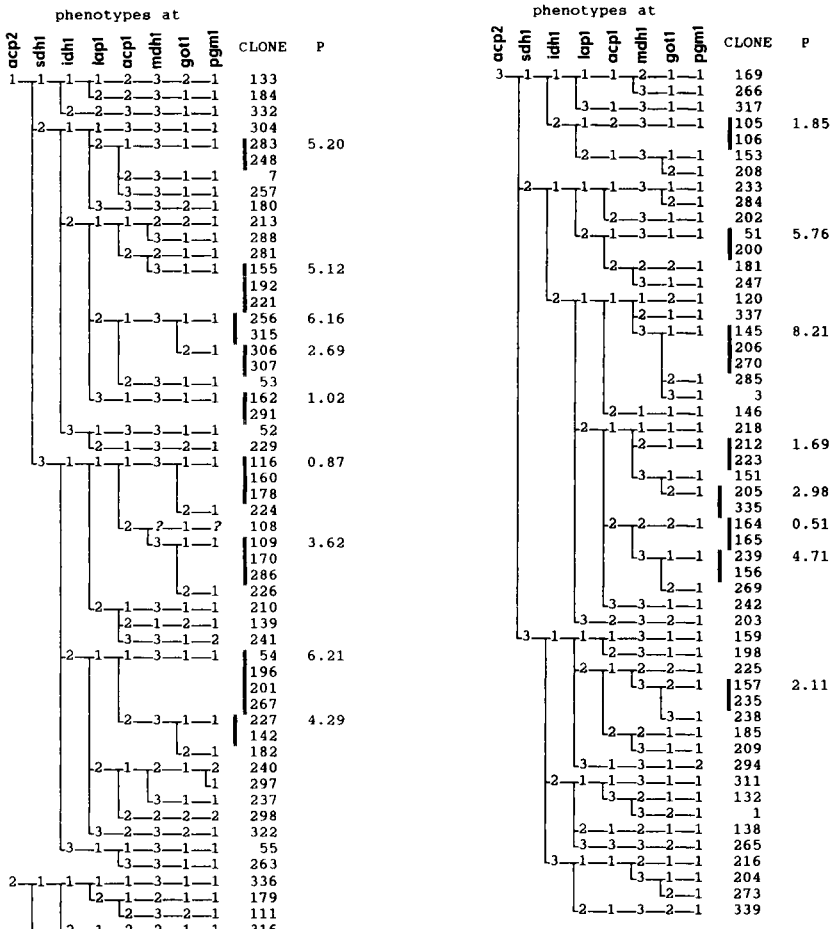
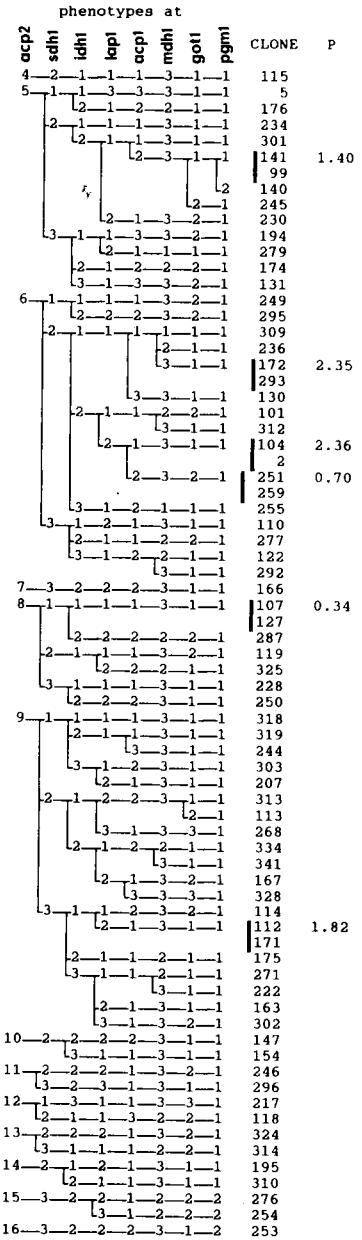


Fig 3. 11-locus phenotypes used as an identification key for 198 wild cherries P: occurrence probability (x1000) of the phenotype, computed by multiplication of the occurrence probability of the isozyme phenotypes.



where the *P avium and P fruticosa* ranges overlap. So perhaps, some bands, scored "additional" with respect to this wild cherry sample, are not additional according to the variability in the wild cherry range.

A problem is raised for 3 wild cherries (clones 253, 254, 276) of the 286 analysed: their ACP zymograms (*acp2* phenotypes nos 15 and 16 in Santi and Lemoine, 1990) faintly contain the bands nos 1 and 3, which are always (no 1) or most often (no 3) present in sour or duke cherry zymograms. This may simply indicate that these bands do not characterize sour or duke cherries. The difference in the proportion of zymograms of wild cherries and of sour or duke cherries containing these bands may be due to differences of allelic frequencies in the prospected area (France or close to France for wild cherries, Europe for sour or duke cherries).

However, this may also indicate a slight (the 3 clones are morphologically *P avium*-like) introgression of *P cerasus* in these 3 *P avium* accessions. As no additional molecular information exists, cytological studies are necessary since these clones, part of the Forestry Breeding Population, may be involved in controlled crossings.

The validity of the proposed markers would be better if wild cherries growing in the common range of *P cerasus, P avium* and *P fruticosa* did not contain them. Nevertheless, it would be interesting to detect introgressions, even minor ones, in *P avium*-like accessions, in order to control the input material in the breeding population. For such a purpose, isozyme polymorphism seems insufficient, even through MDH (Hancock and Lezzoni, 1988), proteins or peroxidases (Feucht and Schmid, 1985) and untested enzyme systems

may provide other discrimination keys. RFLP, which allows a far better sampling of the genome, would provide a more sensitive tool.

Intraspecific identification

The 8 isozyme loci used have less discriminating power than the 15 isozyme loci involved for *Camellia japonica* in a similar study (Wendel and Parks, 1983): 72% and 95% of clones were uniquely characterized, for a total of 198 and 173 clones, respectively. Other genetic markers are necessary for the completion of identification, to allow control of the varieties. If more information is to be obtained for the genetic control of variations at *amy1, mdh2* and *to1* loci, identification might be completed (11% more "plus-trees" might be identified in our sample). Three isozyme loci (Kaurisch *et al*, 1988) are variable among several sweet cherry varieties and therefore provide other genetic markers. Phenolic compounds may also provide additional keys, if necessary (Treutter and Feucht, 1985).

More genetic markers are thus available for cherry breeders, for identification purposes, as well as for other purposes. For instance, population genetic studies have been conducted, and various points concerning the reproductive system have already been taken up (Santi, 1988).

ACKNOWLEDGMENTS

The author thanks Pr Feucht for helpful discussions. Special thanks go to Mr Saunier for assistance in obtaining collections of sour and duke cherries.

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