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IDENTIFICATION OF CLONES IN *CYPRIPEDIUM CALCEOLUS* L. (ORCHIDACEAE)

Principles of clone identification

Vegetative reproduction plays a remarkable role in higher plants and that is why the identification of clones is an extremely important problem in plant population biology.

The clones of different species grow at different rates and may live for hundreds and thousands of years (Oinonen, 1967*a*, *b*, 1969, 1971; Cook, 1983; Shirreffs, Bell, 1984; Jerling et al., 1985).

Gene drift, natural selection, ontogenetic variability, population regulation and competition are important ecological and evolutionary problems, affected by the precise clonal identity of ramets and the physiological relations among them (Cook, 1983). One of the methods in identifying clones is to dig up the plants and to establish whether the shoots are connected or not. This method is too troublesome in studying big populations and it cannot be approved of from the standpoint of nature conservation either, especially in the species under protection. On the other hand, the studies of their population biology are extremely important. Besides, using the digging method it is not possible to identify clones already fragmented.

What are the alternative possibilities? On the basis of some morphological character or characters (phenes) clones have been identified. The mistakes in applying this method may be quite remarkable when the morphological variability in a population is small and sexual reproduction occurs abundantly (Oinonen, 1971).

A promising method in identifying genets is electrophoresis of isoenzymes (Harper, 1978; Cook, 1983). During the last decade clones have been identified using this method in several species: *Agrostis stolonifera* (Wu et al., 1975), *Populus tremuloides* (Cheliak, Dancik, 1982), *Anemone nemorosa* (Shirreffs, Bell, 1984), *Spartina patens* (Silander, 1979), *Picea engelmannii*, *Abies lasiocarpa* (Shea, Grant, 1986), and others.

The ramets of the same clone have identical genotypes. If two ramets have even a slight difference in any locus, they are undoubtedly different genets. In order to prove that two ramets belong to the same clone it is not enough to show that they are identical in some few loci. The more loci are examined and found to be identical, the greater is the probability that the ramets are really genetically identical (Shea, Grant, 1986).

Lin Wu et al. (1975) consider simplicity and precision the advantages of the isoenzyme method. Its shortcoming is considered to be the limited amount of variation. Because of this possibly not all the clones existing in a population could be found out.

The present paper deals with the results of a polyacrylamide gel electrophoretic study of several isoenzyme systems on clonal isolates from natural populations of *C. calceolus* in Estonia.

Materials and methods

Material for studying isoenzymes was collected from 214 ramets in five different populations of *Cypripedium calceolus* in the Estonian SSR in the summers of 1986 and 1987. The top of the second leaf (from ground) of a flowering shoot was taken for the analysis.

To study the growth and branching of the rhizome, three smaller clones were dug up. As the plant is under nature conservation in Estonia, it was not possible to carry out more work of this kind.

Enzyme extracts were prepared by crushing small cutlings of a leaf tissue in 0.2 ml aliquots of a cold buffer containing 0.05 M tris-hydroxymethyl-aminomethane (tris), 0.01 M EDTA and 5 mM cysteine hydrochloride. After removal of cell debris, 20—50 mg aliquots of a sucrose — Sephadex G-200 4 : 1 mixture were added to the enzyme extracts to increase their viscosity. The extracts were immediately subjected to electrophoresis in a polyacrylamide gel. After electrophoresis, the gels were incubated and stained in histochemical enzyme reaction mixtures (Jaaska, 1981; Jaaska, Jaaska, 1986; Яаска, Яаска, 1977).

The variability of the following isoenzymes was analyzed: alcohol dehydrogenase (ADH, EC 1.1.1.1), superoxide dismutase (SOD, EC 1.15.1.1), aspartate aminotransferase (AAT, EC 2.6.1.1), peroxidase (PRX, EC 1. 11.1.7), esterase (EST, EC 3.1.1.1) and 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44).

Results and discussion

The zigzag rhizome of *Cypripedium calceolus* is situated at a depth of 10—15 cm. The length of yearly increments is 0.5—1.1 cm (the mean is 0.8 cm) in Estonia and the angle between them is approximately 130 degrees (Kull, 1987). However, the yearly increment is noted to be only 0.4—0.6 cm in Siberia (Амельченко et al., 1986). The horizontal growth of a rhizome need not take place during the first years. The older parts of rhizomes studied were vertically orientated.

On the rhizomes studied about 13 to 16 years' increments could be counted. The age of a rhizome of this species has been stated to be 25—30 years (Серебряков, 1952).

E. Oinonen (1969) has shown that in many cases the diameter of a clone is in good correlation with its age. Consequently, if we take 8 mm as an average yearly increment of a rhizome, the age of a clone may be estimated.

In some populations studied by us the clonal character of *Cypripedium calceolus* is clearly visible (Muhu, Ussisoo, Tooma), while the horizontal distribution of shoots is much more homogeneous in others (Hiiumaa, Õisu). In populations with homogeneous spacial distribution sexual reproduction is presumed to be considerable. This is indicated by an abundant appearance of juvenile plants (juvenility was established by digging up some of these small plants).

To study the possibility of identification of clones using the method of isoenzyme analysis, samples were taken from five different sites in Estonia, and the variability of six isoenzymes (SOD, AAT, PRX, ADH, EST and 6PGDH) was determined. The variation of AAT proved to be the greatest: all the samples were different. Only two samples were different in SOD and ADH, the rest of the isoenzymes studied did not show any variability.

One minor and two major isoenzyme zones of AAT of independent banding variation among the samples of different clones can be distinguished in Fig. 1. The AAT enzymograms and their variation pattern

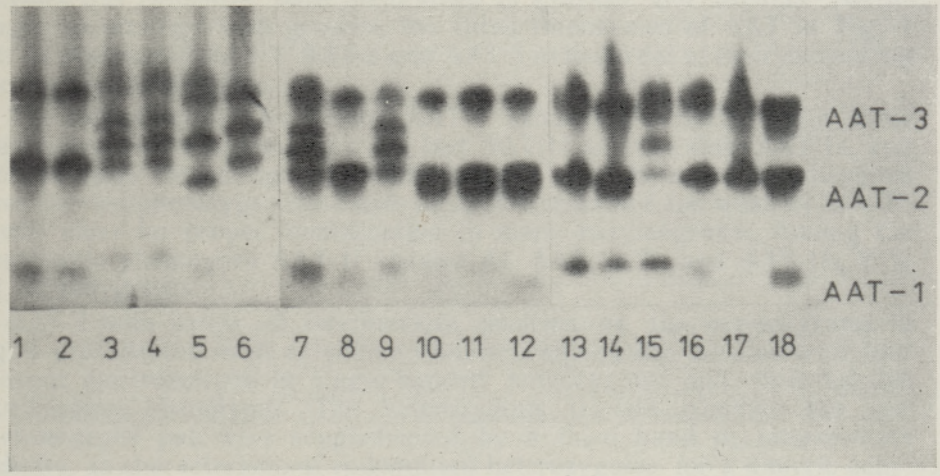

Fig. 1. Variation of aspartate aminotransferase (AAT) in *Cypripedium calceolus*. *1—12* enzymograms of ramets from different clones in Ussisoo and *13—18* on Muhu Island.

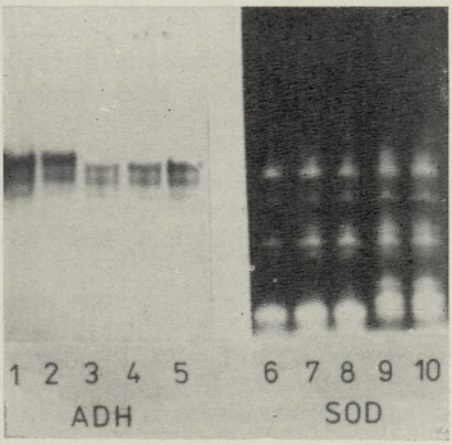

Fig. 2. Two slow (*1—2*) and three fast (*3—5*) electromorphs of alcohol dehydrogenase (ADH) in different clones on Hiiumaa Island and the variation of superoxide dismutase (SOD) on Muhu Island *6—10*.

observed in *Cypripedium calceolus* are very similar to those described for grasses of the wheat group (Jaaska, 1976, 1981, 1983, a. o.). In grasses three dimeric AAT isoenzymes with three-banded (triplet) electrophoretic phenotypes in heterozygotes and singlebanded (singlet) phenotypes in homozygotes have been specified (Hart, 1975; Jaaska, 1976, 1981, and others). The same variation pattern of AAT isoenzymes may be seen in Fig. 1 for *C. calceolus*, suggesting that the genetic homology of AAT isoenzymes has evidently been maintained in grasses and orchids. Accordingly, in analogy with grasses, the three isoenzymes of AAT in Fig. 1 will be designated AAT-1, AAT-2 and AAT-3 in the order of their decreasing electrophoretic mobility.

The fastest minor isoenzyme AAT-1 exhibits a variation with three phenotypes: two electromorphs, the fast AAT-1f and the slow AAT-1s, and a broad band combining both electromorphs in a presumed heterozygous three-banded phenotype. The bands of AAT-1 remained too weak or insufficiently clear for proper identification in many enzymograms. Among the identifiable phenotypes AAT-1f showed 44, AAT-1s 26 and AAT-1fs 29% out of 102 genets.

AAT-2 with bands of intermediate position on the enzymograms in Fig. 1 exhibits the most extensive variation among the isolates with four frequent electromorphs in single-banded homozygous and three-banded heterozygous phenotypes. The heterozygous triplets combine the four electromorphs pair-wise supplemented by a third band of intermediate mobility, as characteristic of codominant heterozygotes for a dimeric enzyme. The four frequent electromorphs are labelled AAT-2a, 2b, 2c and 2d in order of their decreasing mobility. Among the *C. calceolus* isolates analyzed, the following phenotypes could be found: 2aa — 6, 2ab — 16, 2ac — 14, 2ad — 8, 2bb — 16, 2bc — 8, 2bd — 12, 2cc — 1, 2cd — 12 and 2dd — 12%.

The slowest isoenzyme AAT-3 exhibits a variation with three phenotypes: two electromorphs, fast AAT-3f and slow AAT-3s, and a broad band of hybrid isoenzyme of intermediate mobility.

In Fig. 2 some electrophoretic phenotypes of ADH and SOD can be seen: fast and slow variants in ADH, and the same in SOD-1.

To make sure that the isoenzyme phenotypes of ramets of the same clone are identical, two or three analyses were taken from the same clones, and the identity of AAT phenotypes was confirmed. Two or three samples of the same ramet were taken during the vegetation period and no differences were noted.

The analysis of ramets from three clones on Muhu Island indicated that the clones were different in the variation of AAT, ADH and SOD; the ramets of the same clone were identical. In Ussisoo the analysis of three ramets from two clones did not show any difference in ADH, SOD and PRX. The clones were different in AAT enzymograms, while the ramets of the same clone were identical.

To prove the common clonal origin of two clusters, the identity of two or three isoenzymes is presumably insufficient. Only supposition can be made on the common origin of different clusters on the basis of these analyses. The distance between two ramets analysed was 99 cm in Õisu, but they were identical in all loci of AAT and PRX. The samples taken in Ussisoo were studied for the variation of AAT and SOD. Two pairs of ramets were found that did not differ in any locus. The distances between ramets were 30 and 150 cm, respectively. The age (A) of a clone may be calculated as follows:

$$A = \frac{d}{n} + k,$$

where d is the distance between two farthest ramets (diameter of a clone), n is the mean yearly increment (0.8 cm in our case) and k is the age of the rhizome before the horizontal growth begins (it was considered to be five years in our case).

In our samples the maximum age of a clone should be 129 years in Oisu, but 42 and 192 years, respectively, in Ussisoo according to the formula used.

Quite frequent are the clones with a diameter of 20 to 24 cm. Their age, according to the above formula, should be about 30—35 years. As the older part of the rhizome decays, it can only be the minimal age.

In conclusion it may be stated that the isoenzyme method can be used successfully for the identification of clones of *Cypripedium calceolus*.

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KLOONIDE ERISTAMINE KULDKINGAL *CYPRIPEDIUM CALCEOLUS* L. (ORCHIDACEAE)

Kloonide eristamine on möödapääsmatu, kuid raske ülesanne vegetatiivselt paljunevate taimede populatsioonibioloogiliste uuringute puhul. Peamisteks meetoditeks kloonide eristamisel on: 1) taimede maa-aluse osa üleskaevamine; 2) rametite määramine morfoloogiliste tunnuste alusel; 3) isoensüümide kasutamine. Viimast tuleb pidada kõige efektiivsemaks. See leidis kinnitust ka kuldkinga uuringute puhul. Suurima varieeruvusega ensüümiks viiest uuritust osutus aspartaat-aminotransferaas. Mõningal määral on kasutatavad ka superoksiidi dismutaas ja alkoholi dehüdrogenaas.

Тийу КУЛЛЬ

ИДЕНТИФИКАЦИЯ КЛОНОВ У БАШМАЧКА НАСТОЯЩЕГО *CYPRIPEDIUM CALCEOLUS* L. (ORCHIDACEAE)

Различение и идентификация клонов является необходимой, однако трудной задачей при популяционно-биологических исследованиях вегетативно размножающихся растений. Основными методами идентификации клонов являются: 1) извлечение корневища с ветвями, 2) идентификация побегов по морфологическим признакам (фенам), 3) метод изоферментов. Последний является наиболее надежным, что подтвердилось и в наших исследованиях. Из пяти изученных ферментов наиболее вариабельными оказались аспартат-аминотрансфераза (ААТ), супероксиддисмутаза (СОД) и алкогольдегидрогеназа (АДГ).