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Studies on *Ranunculus* Population: Isozymic Pattern

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ABSTRACT

Species of *Ranunculus* is small herb grows at quite high altitudes, ranging from several hundreds to more than a thousand meter above sea level. They can occupy a variety of habitats such as moist soils or can even grow sub merged or floating in stream. A few numbers of species from different populations have been recorded to have morphological complexity, which could cause a problem for the work of taxonomists in making decisions. In order to support taxonomists in solving their problem, a new experimental method using SDS-PAGE will be used to explore the isozyme data. The main purpose of this research was to investigate whether or not isozyme data can be used to clarify the morphological complexity of *Ranunculus* species. In this study, nine species of *Ranunculus* from different populations were used. Five to twenty plants were sampled for electrophoresis studies. Four enzyme systems: peroxidase, esterase, malate dehydrogenase and acid phosphatase were chosen. The results showed that every enzyme gave its specific pattern in each species and common band always found in nine species tested. This experiment proved that genetic data (isozyme) quite promoting to be applied in higher plant taxonomy in solving the morphological complexity problem.

Key words: isozyme, PER, EST, MDH, ACP, *Ranunculus*.

INTRODUCTION

The genus *Ranunculus* consists of about 500 species, and is recorded as the large genus within the family Ranunculaceae. The *Ranunculus* species usually grow at quite high altitudes, ranging from several hundred to more than a thousand meters above sea level. They can occupy a variety of habitats, in wet or moist soils and muddy water, or can even grow submerged or floating in steams.

Species are widely distributed around the world and the number in different countries varies. About 146 species have been recorded in Russia (Komarov and Schischkin, 1963). On the other hand, in Java (Indonesia) only 3 species so far have been recorded (Eichler in Backer and Bakhuizen van den Brink, 1963). Japan, India and Taiwan each have 20-30 species, and in New Zealand approximately 50 are recorded. This confirms they are typical plants of cool climate regions. In tropical countries such as Indonesia they can only grow in the mountains. Table 1. shows the distribution of *Ranunculus* species used in this study.

Enzyme variations

The term "isozyme" has been used by Brown (1990) for "the multiple zones of enzyme activity that are observed when electrophoresis gels are subject to histochemical stains to detect the presence of more or less specific enzymes". In addition, he stated that isozymes might precisely or correctly is applied for multiple bands coded by more than one structural gene locus for particular enzymes. Alternatively the allozyme term is used if the occurrence of multiple bands arises from the alleles which are segregating at a single locus.

The use of isozyme in plant taxonomy

In recent years, uses of isozyme data for plant taxonomic purposes have increased quite rapidly. This method offers a very powerful tool in studying lower hierarchies of plant taxa such as the species, sub-species or population level (Reisenberg *et. al*., 1988; Burden *et. al.*, 1980; Brown, 1990).

Isozyme data is particularly useful if the morphological characters of species appear to overlap. At the present time, there is no doubt that isozyme data provides a powerful tool in the era of molecular taxonomy. It is likely that by the next decade the use of isozyme data in the work of taxonomist will be intensively adopted. Molecular biology techniques will use data both from DNA sequence and isozyme analysis for research purposes in plant taxonomy.

DNA sequencing needs quite expensive funds to run it, but it provides the best data, and has wider applications for all levels of taxa. On the other hand, "isozyme analysis" is less expensive (even affordable) and relatively easy and rapid to perform. It also has the ability to handle quite a large numbers of samples simultaneously (Brown, 1990). From the undoubted advantages of both the above approaches, financial considerations frequently determine which experimental taxonomic research will be adopted. In this project, the second approach is the alternative to be adopted.

Studies of enzyme patterns in higher plant taxonomy have been carded on for more than two decades (Mitra *et. al.*, 1970; Conklin and Smith, 1971; Reisenberg and Soltis, 1987; Burden at al., 1980; Moran *et. al.*, 1990; Brown, 1990). These studies of plant enzymes involved both alloenzyme variations (Crawford *et. al.*, 1988, 1985; James *et. al.*, 1983), and isozyme variations (Reisenberg, l987). The principle methodology in studying isozyme and allozymes in plant is electrophoresis. Electrophoresis examines the movement of the protein and enzymes in buffered gel when they are subjected to an electric current.

Band forms on electrophoresis gels which reveal the activity of enzymes using specific enzymic detection methods, at least implies functional similarity and analogy of the proteins being compared (Mitra *et. al.*, 1970). Protein variations revealed by electrophoresis have been considered as a powerful approach in measuring the genetic diversity in populations (Brown, 1978; Brown *et. al.*, 1978). In order to ascertain the level of generic variation within and/or between populations, isozymes and allozymes can be used as main sources of data, because an enzyme marker which is separated by electrophoresis furnished a simple means for rapid partitioning of the variability within and between populations at the gene level.

Although studies of enzyme variations by means of electrophoresis in *Ranunculus* species is here being conducted for the first time, this method has been extensively employed for other species, for example; *Pisum sativum* (Bowling and Crowden, 1970); *Datura* species (Conklin and Smith, 1971); *Nicotiana* species (Smith *et. al.* 1970; Bredemeijer, l984); *Dubautia agyroxiphium* and *Wilkesia* (Witter, 1988); *Glycine canescent* and *G. aryrea* (Brown, 1990); *Glycine tomentella* (Doyle *et. al.*, 1986); *Chenovidium santae-clarae* (Crawford *et. al.*, 1988); *Eucalyptus* and *Acacia* (Moran *et. al.*, 1990); *Thuja plicata, Eremaea* species (Coates and Hnatiuk, 1990); *Emex australis* (Panetta, l990).

Since multiform enzymes are presumably the direct product of multiform (alleles) of genes, they may serve as molecular markers which are useful for analyzing genetic dissimilarity among species (Conklin and Smith, 1971). "Undoubtedly allozymes will continue to be used in biosystematics and phylogenetic applications, and the importance of isozyme data will be strengthened if they are treated carefully like any other group of taxonomic characters, with criteria for category assignments clearly stated and the possibility of selective interactions considered" (Johnson, 1973). Although the use of isozyme data in the studies of populations is increasing, it is better that a group of enzymes be studied rather than a single system.

In this present study four enzyme systems were chosen, namely peroxidase, esterase, malate dehydrogenase and acid phosphatase.

Peroxidase is an easy enzyme to detect in gel electrophoresis, but interpretation of isoperoxidase data requires can, as has been reported in their studies of *Shorea loprosula* and *Xerospermum* species. Pattern may change during physiological development. Conklin and Smith (1971) have reported an increased number of bands in *Datura* species as leaves approach maturity. In addition, they recorded no difference in band patterns from

plants from the green house or from field grown plants.

Like peroxidase, the esterase has numerous isozymes, and more in herbaceous plants than in woody plants. Smith *et al.* (1970) reported band number of esterase in *Nicotiana* species to range from 3-15. Even though there are more esterase band present than peroxidase, this enzyme system may not be particularly useful in discriminating between species, as Mitra *et al.* (1970) recorded in *Hordeum* species.

Table 1. *Ranunculus* species and population sources used for electrophoresis.

Population	Species	Plant numbers
Liawenee	*R. triplodontus*	16
Nive River	*R. triplodontus*	20
Rat Castle	*R. triplodontus*	15
Clarence Weir	*R. triplodontus*	20
Ouse River	*R. triplodontus*	13
Projection Bluff	*R. triplodontus*	10
Wild Dog Plains	*R. triplodontus*	12
Black Mary Plains	*R. pimpinellifolius*	9
Pine Lake	*R. gunnianus*	6
Lake Augusta	*R. gunnianus*	5
Projection Bluff	*R. decurvus*	10
Rat Castle	*R. decurvus*	10
Projection Bluff	*R. collinus*	10
Rat Castle	*R. collinus*	10
Wild Dog Plains	*R. collinus*	10
Liawenee	*R. pascuinus*	10
Wild Dog Plains	*R. amphitricus*	10
Green View	*R. lappaceus*	9
Wild Dog Plains	*R. nanus*	20
Camerons Lagoon	*R. nanus*	20
Saint Patrict Plains	*R. nanus*	20
Ouse River	*R. nanus*	20
Clarence Weir	*R. nanus*	20

Unlike esterase, malate dehydrogenase isozymes are fewer in number, i.e. only about 3 or 4 as recorded by Gottlieb (1982). Moran and Hooper (1983), again showed for malate dehydrogenase that there are more isozymes bands in herbaceous than in woody species.

Acid phosphatase isozymes are likely to give many bands since this enzyme may be dimeric. This enzymic marker gives good resolution where polyploid species are studied, for example in hexaploid bread wheat (Gottileb, 1982). This enzyme system may give significant results since several *Ranunculus* have several polyploid species. Recent studies of isozyme variations in plant taxonomy have made substantial contributions in examining inter relationships between species or species differences, provided then data are integrated with a proper analysis. The value of using isozyme data in association with morphological characters in resolving problems species complexes is potentially great.

To examine any possible effect of environmental change on the isozyme patterns in *Ranunculus*, both field and transplanted leaf samples were examined Field harvested leaves were of uncertain age, which may account for some quantitative differences observed within population sample. Leaves examined after four months transplanting were of uniform age and at an equivalent stage of physiological development, and therefore would be expected to give a uniform pattern unless genetic variability existed within the population.

MATERIALS AND METHODS

Gel Preparation

Acrylamide gel electrophoresis was employed. The gel buffer was tris-citric buffer pH 8.4 (Stock Solution A). Stock Solution A: 4.5 grams of TRI (Hydroxymethyl) Methylamine (PURISS), 0.51 grams of citric acid and 500 ml of deionized water. Stock Solution B: 30 grams of acrylamide, 0.80 grams of N N'-methylene-bis-acrylamide and 100 ml of deionized water.

The gel was made by mixing 20 ml of solution B and 40 ml of solution A. This mixture was deaerated on a Buchi rotary evaporator for 5 minutes after which 0.04 ml of N, N, N', N'-tetramethyl-ethylenediamine was added and with carefully mixed To polymerize the gel, 0.06 grams of ammonium persulphate was added and mixed carefully immediately before pouring the solution into the gel mould (BIO-RAD Model 360). Using this model, at least 4 thin gels each with 10-14 slots can be cast simultaneously.

Extraction and loading the samples

Laminas and petioles were examined separately. Material from each plant was ground individually in a staining dish using 0.15-0.35 ml of protein extracting solution for

laminas and 0.1-0.15 ml for petioles. Despite the voluminous literature on extraction methodology which suggests the need to use frozen plant material (liquid nitrogen), it was found unnecessary for the systems studied in this project to use other than an ice cool buffer and to hold plant material and extracts in an ice bath. The extracts were transferred to a small glass vial, 2 mm diameter, 3 cm long, and centrifuged at 3500 rpm for 15 minutes. The supernatants were then applied in the gel slots. The amount of sample loaded in each slot was, for peroxidase about 10-15 ul, while for the other enzymes about 15-24 ul.

The protein extracting solution consisted of 0.018 grams of cysteine, 0.021 grams of ascorbic acid, 5 grams of sucrose, diluted in 20 ml of borax buffer pH 8.4 (tank buffer).

Electrophoresis

The electrophoresis chamber used in this project was a mini vertical slab cell manufactured by BIO-RAD, USA, model 360. This model has advantages in allowing use of very small amounts of samples, as well as allowing a short running time.

Electrophoresis was conducted at a constant current of 5 mA for peroxidase (PER) and 7 mA for esterase (EST), malate dehydrogenase (MDH), and acid phosphatase (AP), at room temperature far about 60 minutes including a pre-electrophoresis time of approximately 10 minutes. Electrophoresis was stopped when the bromophenol blue marker dye had traveled about 56 mm from the slot toward the anode.

Staining Procedures

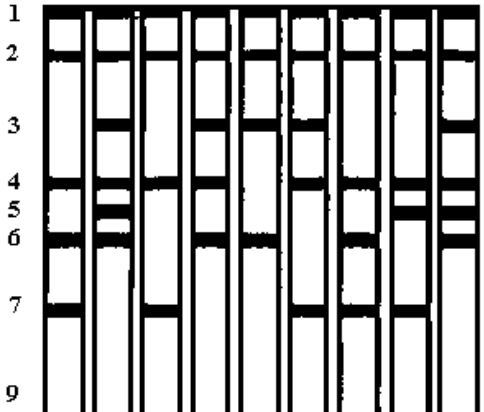
Four enzyme stains were used routinely.

Peroxidase

0.0125 grams of o-Dianisidine, dissolved in 2.5 ml of acetone, then add 50 ml of 0.2 id acetate buffer pH 4.5 and 2 drops of H_2O_2.

Esterase

0.0125 grams of 1-naphthyl acetate dissolved in 2.5 ml acetone, then add 50 ml of 0.2 M phosphate buffer pH 6.5 and 0.0125 grams of Fast Blue BE Salt



Malate Dehydrogenase

15 ml of 0.I M Tris-HCl pH 8 was mixed in 125 ml of deionized water. Then 10 ml of 0.2 M Sodium Malate pH 7.5, 0.020 grams of MTT (2,5-Diphenyl tetrazolium Bromide) and 0.005 grams of PMS (Phenazine Methosulphate) was added.

Incubate gel for 30-40 minutes in the dark, and then transfer to a fresh solution containing in addition 0.020 grams of NAD (Nicotilamide Adenine Dinucleotide).

Acid Phosphatase

0.0125 grams of l-naphthyl phosphate dissolved in 2.5 ml of acetone then add 75 ml of 0.2 M acetate buffer pH 4.5, 0.025 grams of Fast Black K Salt and 0.025 grams of Fast Garnet GBC Salt.

A number of other enzyme systems, namely Glucose-6-Phosphate Dehydrogenase. 6 Phospogluconate Dehydrogenase, Alcohol Dehydrogenase, Isocitratase, and Tetrazolium Oxidase were investigated and found unsuitable.

All staining procedures in this experiment were conducted at room temperature. For Peroxidase and Esterase stains refer to Mills and Crowden (1968), for Malate Dehydrogenase stains refer to Brown *et al.* (1978), and for Acid Phosphatase stains refer to Adam and Jolly (1980).

RESULTS AND DISCUSSION

Figure 1-4 are interpretative drawings of isozyme patterns for Peroxidase, Esterase, Malate Dehydrogenase, and Acid Phosphatase of the *Ranunculus* species examined. The diagrams shown represent average band patterns determined after examining 1 number of populations far each species.

Figure 1. Peroxidase isozyme patterns of *Ranunculus* species. A. *R. triplodontus*, B. *R. collinus*, C. *R. decurvus*, D. *R. pimpinellifolius*, E. *R. gunnianus*, F. *R. pascuinus*, G. *R. amphitricus*, H. *R. lappaceus*, I. *R. nanus*.

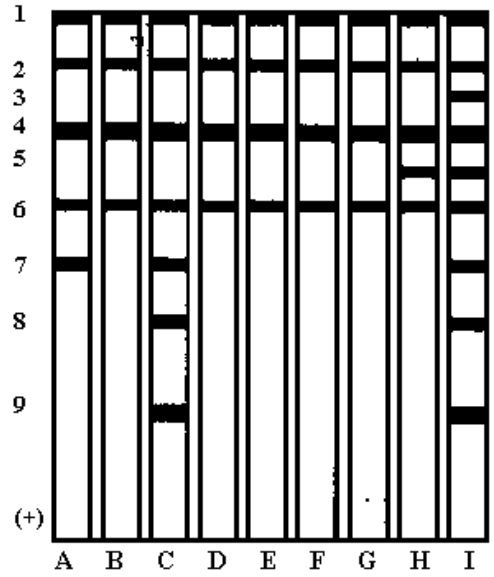


Figure 2. Esterase isozyme patterns of *Ranunculus* species. A. *R. triplodontus*, B. *R. collinus*, C. *R. decurvus*, D. *R. pimpinellifolius*, E. *R. gunianus*, F. *R. pascuinus*, G. *R. amphitricus*, H. *R. lappaceus*, I. *R. nanus*.

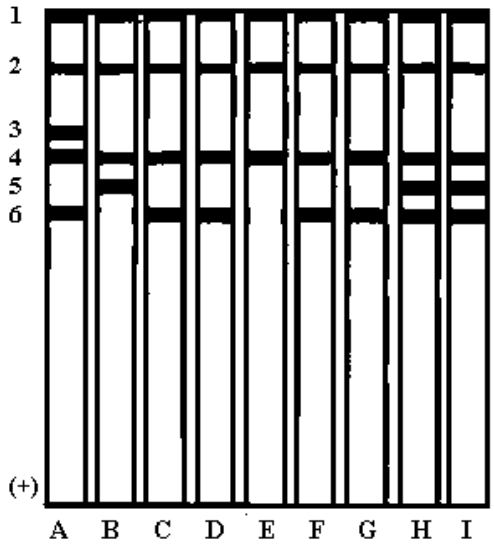


Figure 3. Malate dehydrogenase isozyme patterns of *Ranunculus* species. A. *R. triplodontus*, B. *R. collinus*, C. *R. decurvus*, D. *R. pimpinellifolius*, E. *R. gunnianus*, F. *R. pascuinus*, G. *R. amphitricus*, H. *R. lappaceus*, I. *R. nanus*.

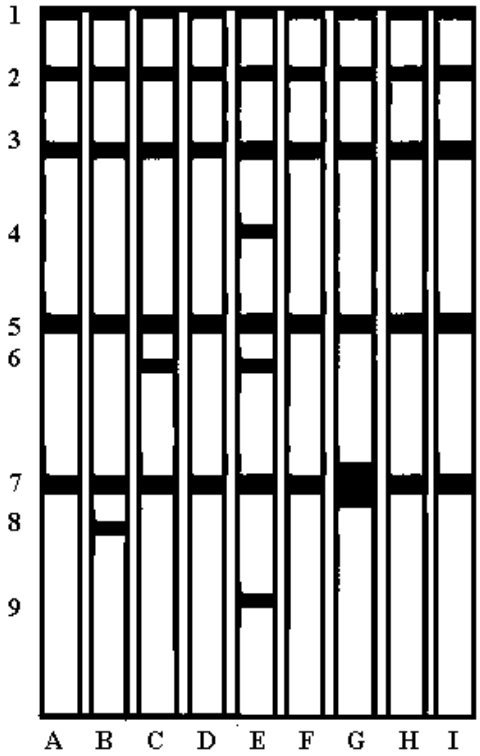


Figure 4. Acid phosphatase isozyme patterns of *Ranunculus* species. A. *R. triplodontus*, B. *R. collinus*, C. *R. decurvus*, D. *R. pimpinellifolius*, E. *R. gunnianus*, F. *R. pascuinus*, G. *R. amphitricus*, H. *R. lappaceus*, I. *R. nanus*.

Peroxidase

Peroxidase showed most variations of the four enzymes tested. The seven isoperoxidases identified arranged in eight distinct patters. Bands 1 and 2 occurred in all species tested. The two species *R. triplodontus* and *R. amphitricus* had the same patterns (5 bands). These are aquatic or semi-aquatic plants. Two other species *R. collinus* and *R. nanus* also had identical peroxidase patterns (6 bands). These species which often co-occur in the same habitat sometimes show overlapping leaf morphology, but they are easily distinguished on floral characters. The other species had distinctive peroxidase patterns.

Esterase

Isozyme bands 1, 2, 4 and 6 appeared in all species. Five species, i.e. *R. collinus. R.*

pimpinellifolius. R. gunnianus R. pascuinus and *R. amphitricus* had the same banding patterns (4 bands). The other 4 species *R. nanus*, *R. decurvus*, *R. lappaceus* and *R. triplodontus* had distinctive patterns. The main variability was in the bands 7, 8 and 9.

Malate Dehydrogenase

Common isozyme bands for all species were numbers 1, 2 and 4. For species *R. decurvus*, *R. pimpinellifolius, R. pascuinus* and *R. amphitricus* had the same bands (4 bands), as did *R. gunnianus* (3 bands). *R. lappaceus*, *R. triplodontus* and *R. nanus* had distinctive patterns. *R. nanus* showed the most complexity of isoenzyme patterns.

Acid Phosphatase

Common isozyme bands in all species tested were bands 1, 2, 3, 5 and 7. Band 5 was absent from *R. amphitricus* while band 7 was very strong in this species. *R. gunnianus* showed a very distinctive pattern, with the unique bands 4 and 9. Six species *R. nanus*. R. *pascuinus*, R. *pimpinellifolius*, *R. gunnianus* and *R. lappaceus* were not resolved.

Each enzyme studied gave a different result, and a different level of species separation. Peroxidase gave the best resolution of the species, compared to the presently accepted taxonomy. However, one would hardly expect all 11 species to be separated on the basis of variation in 1 enzyme system. No sound taxonomic classification will result from examination of a single character, even though that character may have a number of states.

The experimental methods used in this study, of isozyme analysis using polyacrylamide gel electrophoresis, applied to 9 species of *Ranunculus* have supported the general view that such experimental data are useful in delineating in a meaningful (classification) sense. It is almost certainly that the more extensive application of the procedure(s) to include wider range of enzyme tests, examination of more populations, examination of plant organs other than leaves, e.g. seeds, seedling, would broaden the data base, and therefore would gave better result for taxonomists in providing improved taxon delineation

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